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Design, synthesis and evaluation of new methyl piperazine derivatives as anticancer agents

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ABSTRACT (ENGLISH)

Background

To overcome the problem of side effects and toxicity, development of new anticancer agents is needed. Recently, piperidine salicylanilide derivatives with nanomolar epidermal growth factor receptor (EGFR) inhibitory and cytotoxicity activity have been reported. In the present study effect of replacing piperidine in reported piperidine salicylanilide with *N*-methyl piperazine and changing substituent's of phenyl ring at other end on anticancer activity have been explored. A series of sixteen methyl piperazine incorporated phenyl benzamide and phenyl methanone derivatives have been synthesized and tested in a panel of three cancer cell lines (adenocarcinomic human alveolar basal epithelial cells (A-549), human colon carcinoma (HCT-116) and human pancreatic carcinoma (MIAPaCa-2)), using gefitinib as standard. Further, to study the probable mechanism, due to their structural similarity with EGFR inhibitors, docking interactions with EGFR active site were observed using Schrodinger suite.

Result

The results indicated that most of the compounds showed promising activity; out of which, compound A-11 was most active having cytotoxicity much better than that of gefitinib. It showed IC_{50} value of 5.71 μM against A-549 cell line, 4.26 μM against HCT-116 colon cancer line and 31.36 μM against MIAPaCa-2 cell line.

Conclusion

It was found that these compounds fit well in the active site and may be exhibiting anticancer activity via EGFR inhibition.

FULL TEXT

Background

Cancer results from uncontrolled cell growth, and remains one of the most potentially life threatening disease worldwide [1]. As per reports of the International Agency for Research on Cancer, in 2012, 14.1 million new cancer cases were reported, along with 8.2 million cancer deaths worldwide. The global burden of cancer is expected to grow to 21.7 million as new cancer cases and 13 million cancer deaths in 2030 despite the presence of variety of anticancer medicines, development of anticancer agents has received more attention of medicinal chemists, due to occurrence of side effects [2, 3].

EGFR amplification and overexpression are prevalent various cancers including NSCLC, occurring in up to 85% of patients with this type of cancer. Mutations typically arise in exons 18–21, which encompass the kinase domain of

the EGFR gene. The majority, around 90%, of these mutations manifest as either exon 21 L858R point mutations or deletions within exon 21. These genetic alterations lead to heightened EGFR kinase activity, resulting in increased downstream signaling. Conversely, most exon 20 insertion mutations are associated with reduced sensitivity to EGFR TKIs. Hence, in order to address resistance, there is a critical need for the development of novel ligands that function as inhibitors of EGFR [4].

In literature, quinazoline and piperazine containing compounds have been reported to inhibit various types of cancers [4]. Quinazoline derivatives are known for EGFR tyrosine kinase (TK) and other kinase inhibition in non small cell lung cancer (NSCLC) and other carcinomas. Gefitinib is one of the quinazoline derivatives which have been used for anticancer activity, especially in lung cancer. Unfortunately, resistance against quinazoline compounds develops within a short span of use [5].

Similarly, piperazine and benzothiazole piperazine derivatives have been evaluated for their anticancer potential against HCT-116 colon cancer cell line [6–8]. Piperazine substituted adamantanes have been reported for colon and pancreatic cancer, whereas piperazine methanone shows good potency against human breast adenocarcinoma cell line, MCF-7 and mouse embryonic fibroblast cell lines, NIH3T3 [9, 10]. Moreover, various piperazine derivatives have been patented as lung cancer chemotherapy agents [11]. Benzofuran with *N*-aryl piperazine derivatives have also been reported for anticancer activity as hybrid compounds [12]. Recently, phenyl piperazine benzoxazole and benzhydryl piperazine derivatives have been shown to inhibit lung, breast and other carcinomas [13–15].

Hu et al. have described novel piperidine salicylanilide derivatives having nanomolar EGFR TK inhibitory and cytotoxicity activity (Fig. 1) [16]. Using this structure as basis, scaffold I was considered wherein the piperidine was replaced with *N*-methyl piperazine and the phenyl ring on the amidic nitrogen was substituted with various electron donating and withdrawing substituent's, hypothesizing that these changes could enhance the anticancer potential. Further, in scaffold II, the amidic nitrogen was constrained in a piperazine ring and the effect of substituents on the phenyl ring on the other nitrogen was also evaluated.

Fig. 1 [Images not available. See PDF.]

Structures of reported piperidine salicylanilide, scaffolds I and II

Materials

For docking, Glide XP of Schrodinger suite was used and physicochemical properties of the compounds were predicted using Chemaxon Jchem for Excel. The docking protocol was validated to govern the reliability and reproducibility of the docking parameters used for the study. Reaction progress was monitored using analytical grade solvents and pre-coated Silicagel G TLC plates (Kieselgel 60_{F254}, Merck) and visualized in UV light. Melting points were measured with Buchi 530 melting point apparatus and were uncorrected. ^{1}H NMR spectra were recorded on a Bruker Avance-400 MHz system using $CDCl_3$ or DMSO as the solvent. Chemical shifts (d) are reported in parts per millions (ppm) relative to TMS as internal standard. FTIR spectra were performed on IR Prestige 21 Shimadzu using KBr as standard. MS were analyzed on MICROMASS Quattro-II LCMS system (Waters Corporation, Milford, USA). All reagents were obtained from commercial suppliers and used without further purification. Synthesized compounds were tested against A-549 (human lung carcinoma), HCT-116 (human colon cancrcinoma) and MIAPaCa-2 (human pancreatic carcinoma) for anticancer potential.

Method

The X-ray structure of EGFR bound with gefitinib as a cocrystallized ligand (pdb id: 2ITO) was obtained from the brookhaven protein database for docking purposes [17]. Docking of the compounds was performed using Glide 5.9. Running on maestro version 9.4, to investigate their putative binding mode in EGFR binding pocket. The Protein preparation wizard within the Schrödinger suite was employed to prepare the designated protein. The protein underwent distinct preprocessing stages, which involved the removal of the substrate co-factor and water molecules lacking hydrogen bonds, followed by the optimization of hydrogen bonds. Subsequently, a charge was assigned, and the energy was minimized, achieving a Root Mean Square Deviation (RMSD) value of 0.30 Å utilizing the Optimized Potentials for Liquid Simulations-2005 (OPLS-2005) force field. The structures of all compounds were

drawn using ChemSketch and converted into 3D structures through a 3D optimization tool. The ligands, drawn previously, were geometry optimized using the LigPrep 2.6 module, with partial atomic charges computed through the OPLS-2005 force field. The prepared ligands were then subjected to docking with the prepared protein utilizing the Glide 5.9 module, operating in extra precision mode (XP) [18]. The anticancer activity was measured by using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay and Gefitinib was used as positive control.

Synthetic scheme

Total 10 derivatives of 4-(3-(4-methylpiperazin-1-yl)propoxy)-*N*-phenylbenzamide (scaffold I) and 6 derivatives of (4-phenyl)piperazin-1-yl)(4-(3-(4-methylpiperazin-1-yl)propoxy)phenyl)methanone (scaffold II) were synthesized. All the synthesized analogs were subjected to characterization using infrared spectroscopy (IR), proton nuclear magnetic resonance spectroscopy (1H NMR), and mass spectrometry (MS) to determine their molecular weight.
The compounds were synthesized using Scheme 1.

Scheme 1 [Images not available. See PDF.]

Synthesis scheme for piperazinyl phenyl benzamide and phenyl methanone derivatives **a** K_2CO_3, acetone, room temperature, 6 h; **b** K_2CO_3, acetonitrile, reflux, 6 h; **c** NaOH, reflux, 3 h; **d** HOBt, EDC.HCl, triethylamine, tetrahydrofuran, substituted anilines/phenyl piperazines, 0 °C, 12 h

Initially, *N*-methyl piperazine was alkylated using 1-bromo-3-chloropropane. Resultant 1-(3-chloropropyl)-4-methyl piperazine was refluxed with methyl 4-hydroxybenzoate in acetonitrile using potassium carbonate for *O*-alkylation. The methyl 4-[3-(4-methylpiperazin-1-yl) propoxy] benzoate formed was hydrolyzed with base to give 4-[3-(4-methylpiperazin-1-yl)propoxy]benzoic acid, which was then coupled with substituted anilines or phenyl piperazines to give final compounds using hydroxy-*O*-benztriazole (HOBt) and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbidiimide.HCl (EDC.HCl). Purity of the compounds was checked by High Performance Liquid Chromatography (HPLC). All the intermediates and final compounds were characterized by spectroscopic techniques.

Biological evaluation: cell viability/MTT assay

The reduction of tetrazolium salts was used to examine cell proliferation and growth inhibition. Gefitinib was used as positive control and IC_{50} (the concentration which resulted cytotoxicity in 50% cells) values are the mean of three independent experiments [19].

For in vitro evaluation, MTT assays for all compounds against three cell lines viz. A-549 human lung carcinoma, HCT-116 human colon cancer and MIAPaCa-2 human pancreatic carcinoma were performed. The DMEM, RPMI-1640, DMEM-F12, were used as culture mediums for A-549 human lung carcinoma, HCT-116 colon cancer and pancreatic MIAPaCa-2 cell lines, respectively. 10^4 Cells per well were grown in 96-well plates and exposed to different concentrations of various test compounds for 48 h. After 44 h treatment, 20 µl of MTT solution (2.5 mg/ml) was added to each well and incubated at 37 °C for 4 h in a humidified atmosphere containing 5% CO_2. In case of suspension cell lines, the plates were centrifuged at 1500 r.p.m. for 15 min, and the supernatant was discarded while in adherent cell lines, the media was removed without centrifugation. The MTT-formazan crystals were dissolved in 150 µl dimethyl sulfoxide. The absorbance was recorded at a wavelength of 570 nm in the microplate reader and cytotoxicity was calculated as % cell growth inhibition.%cell survival=(At-Ab)/(Ac-Ab)×100where At, Ab and Ac are absorbance of test, blank and control, respectively. Concentrations 1 µM, 10 µM, 20 µM, 30 µM, and 50 µM were used for assay.

Results

Spectral data

1-(3-chloropropyl)-4-methylpiperazine: Liquid, 80%, IR (KBr, cm^{-1}): 2954–2858 (Aliphatic-C-H stretching), 1355 (C-N); ^{1}H NMR (400 MHz, Chloroform-*d*) δ 3.54 (t, *J*=3.9 Hz, 2H), 2.61–2.50 (m, 8H), 2.90–2.570 (m, 2H), 2.32 (s, 3H), 1.98 (tt, *J*=6.4, 4.0 Hz, 2H).

Methyl-4-[3-(4-methylpiperazin-1-yl)propoxy]benzoate: Liquid, 75%, IR (KBr, cm^{-1}): 3217–3004 (Aromatic CH

stretching) 2954–2858 (Aliphatic CH stretching), 1750–1735 (–CO), 1373 (–CN); ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.90–7.84 (m, 2H), 7.05–6.99 (m, 2H), 3.99 (t, *J*=6.1 Hz, 2H), 3.94 (s, 3H), 2.61 (t, *J*=6.4 Hz, 2H), 2.58–2.50 (m, 8H), 2.32 (s, 3H), 1.83 (td, *J*=6.2 Hz, 2H).

Methyl-4-[3-(4-methylpiperazin-1-yl)propoxy]benzoic acid: White solid, 70%, (mp: 180–184 °C), IR (KBr, cm^{-1}), 3210–3010 (Aromatic C–H stretching) 2954–2858 (Aliphatic-CH stretching), 1703 (–CO), 1373 (–CN) 1280–1300 (CO ether); ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.90–7.84 (m, 2H), 7.05–6.99 (m, 2H), 3.99 (t, *J*=6.1 Hz, 2H), 2.61 (t, *J*=6.4 Hz, 2H), 2.58–2.50 (m, 8H), 2.32 (s, 3H), 1.83 (p, *J*=6.2 Hz, 2H).

A-1: 4-[3-(4-Methylpiperazin-1-yl)propoxy]-*N*-phenylbenzamide: White solid, 55%, (mp: 134–136 °C), IR (KBr, cm^{-1}), 3344.57 (–NH stretching) 3010–2980 (Aromatic CH stretching), 2881–2852 (Aliphatic CH stretching) 1633 (–CO amide); ^{1}H NMR (400 MHz, Chloroform-*d*), δ 8.04–7.98 (m, 2H), 7.73–7.67 (m, 2H), 7.36–7.29 (m, 2H), 7.09 (tt, *J*= 7.0, 1.2 Hz, 1H), 7.00–6.94 (m, 2H), 3.99 (t, *J*=6.1 Hz, 2H), 2.61 (t, *J*=6.4 Hz, 2H), 2.54 (d, *J*=1.2 Hz, 8H), 2.32 (s, 3H), 1.83 (td, *J*=6.2 Hz, 2H). ^{13}C-NMR (100 MHz, CDCl3): δ=164.7, 162.6, 137.8, 128.6, 128.9, 128.2, 128.3, 128.2, 125.8, 121.9, 114.5, 114.6, 73.1, 57.2, 57.9, 58.9, 55.4, 55.9, 46.9, 28.6, 27.02. ESI MS: m/z=354.21 (M+1 H)$^{+}$.

A-2: 4-[3-(4-Methylpiperazin-1-yl)propoxy]-*N*-(*p*-tolyl)benzamide: White solid, 58%, (138–140 °C), IR (KBr, cm^{-1}), 3350 (–NH) 3005–2933 (Aromatic CH stretching), 2875–2792 (Aliphatic CH stretching), 1651 (CO); ^{1}H NMR (400 MHz, Chloroform-*d*), δ 8.04–7.90 (m, 2H), 7.44–7.37 (m, 2H), 7.18–7.12 (m, 2H), 7.00–6.90 (m, 2H), 3.96 (t, *J*= 6.1 Hz, 2H), 2.61 (t, *J*=6.4 Hz, 2H), 2.57–2.51 (m, 8H), 2.31 (d, *J*=6.0 Hz, 6H), 1.86 (tt, *J*=6.2 Hz, 2H); ^{13}C-NMR (100 MHz, CDCl3): δ=164.7, 162.6, 137.8, 128.6, 129.9, 128.2, 129.3, 127.2, 125.8, 121.9, 114.5, 114.6, 73.1, 57.2, 57.9, 58.9, 55.4, 55.9, 46.9, 28.6, 27.02, 21.5, ESI MS: m/z=368.2 (M+1 H)$^{+}$.

A-3: 4-[3-(4-Methylpiperazin-1-yl)propoxy]-N-(*m*-tolyl)benzamide: Off white solid, 52%, (mp: 132–134 °C), IR (KBr, cm^{-1}), 3350 (–NH stretching) 3000–2931 (Aromatic CH stretching), 2875–2767 (Aliphatic CH stretching), 1643 (CO amide); ^{1}H NMR (400 MHz, Chloroform-*d*), δ 8.03–7.91 (m, 2H), 7.50 (t, *J*=1.9 Hz, 1H), 7.44 (ddd, *J*=7.7, 1.8, 1.1 Hz, 1H), 7.18 (t, *J*=7.9 Hz, 1H), 7.00–6.90 (m, 3H), 3.99 (t, *J*=6.1 Hz, 2H), 2.61 (t, *J*=6.4 Hz, 2H), 2.57–2.50 (m, 8H), 2.30 (s, 3H), 2.27 (d, *J*=0.7 Hz, 3H), 1.81 (p, *J*=6.2 Hz, 2H): ^{13}C-NMR (100 MHz, CDCl3): δ=164.7, 162.6, 138.8, 128.6, 129.9, 128.2, 129.3, 125.2, 125.8, 121.9, 118.5, 114.6, 114.9, 73.1, 56.2, 57.8, 58.9, 55.4, 55.9, 46.9, 28.6, 21.3; ESI MS: m/z=368.1 (M+1 H)$^{+}$.

A-4: *N*-(2,4-dimethylphenyl)-4-(3-(4-methylpiperazin-1-yl)propoxy)benzamide: White solid, 55%, (mp: 144–146 °C), IR (KBr, cm^{-1}), 3300 (–NH stretching), 3000–2931 (Aromatic CH stretching), 2875–2767 (Aliphatic CH stretching), 1643 (–CO amide); ^{1}H NMR (400 MHz, Chloroform-*d*), δ 8.01–7.92 (m, 2H), 7.71 (d, *J*=8.3 Hz, 1H), 7.03–6.94 (m, 4H), 3.99 (t, *J*=6.1 Hz, 2H), 2.61 (t, *J*=6.4 Hz, 2H), 2.57–2.51 (m, 8H), 2.32 (s, 3H), 2.24–2.17 (m, 6H), 1.81 (p, *J*= 6.2 Hz, 2H); ^{13}C-NMR (100 MHz, CDCl3): δ=164.7, 161.6, 143.3, 134.8, 131.6, 130.9, 128.2, 128.3, 125.8, 121.9, 118.5, 114.5, 114.9, 73.1, 56.2, 57.8, 57.9, 55.4, 55.4, 46.9, 28.6, 21.6, 17.6; ESI MS: m/z=382.18 (M+1 H)$^{+}$.

A-5: *N*-(3,4-dimethylphenyl)-4-(3-(4-methylpiperazin-1-yl)propoxy)benzamide: White solid, 58%, (mp: 138–140 °C), IR (KBr, cm^{-1}), 3300 (–NH stretching), 3010–2931 (Aromatic CH stretching), 2875–2767 (aliphatic CH stretching), 1645 (–CO amide); ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.04–7.98 (m, 2H), 7.55–7.45 (m, 2H), 7.06 (dq, *J*=8.4, 1.0 Hz, 1H), 7.00–6.94 (m, 2H), 3.99 (t, *J*=6.1 Hz, 2H), 2.61 (t, *J*=6.4 Hz, 2H), 2.57–2.50 (m, 8H), 2.32 (s, 3H), 2.21–2.17 (m, 6H), 1.86 (p, *J*=6.2 Hz, 2H).

^{13}C-NMR (100 MHz, CDCl3): δ=^{13}C-NMR (100 MHz, CDCl3): δ=164.7, 162.8, 143.3, 134.6, 135.6, 130.9, 129.2, 129.3, 125.8, 121.9, 117.5, 114.5, 114.9, 73.1, 56.2, 57.8, 57.9, 55.4, 55.4, 46.9, 27.7, 21.2, 17.6; ESI MS: m/z= 382.19 (M+1 H)$^{+}$.

A-6: *N*-(2,5-dimethylphenyl)-4-(3-(4-methylpiperazin-1-yl)propoxy)benzamide: Off-white solid, 52%, (mp: 130–132 °C), IR (KBr, cm^{-1}), 3300.20 (–NH stretching), 3020–2931 (Aromatic CH stretching), 2875–2767 (Aliphatic CH stretching), 1644 (–CO amide); ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.02–7.98 (m, 2H), 7.51 (d, *J*=2.1 Hz, 2H), 7.00–6.94 (m, 2H), 6.90–6.85 (m, 1H), 3.99 (t, *J*=6.1 Hz, 2H), 2.61 (t, *J*=6.4 Hz, 2H), 2.57–2.50 (m, 8H), 2.31 (s, 3H), 2.24 (s, 6H), 1.80 (p, *J*=6.2 Hz, 2H); ^{13}C-NMR (100 MHz, CDCl3): δ=164.7, 161.6, 143.3, 134.8, 131.6, 130.9, 129.2, 129.3, 125.8, 121.9, 118.5, 114.5, 114.9, 73.1, 56.2, 57.8, 57.9, 55.4, 55.4, 46.9, 27.6, 23.0, 21.6; ESI MS: m/z=382.2 (M+1 H)$^{+}$.

A-7: *N*-(4-Methoxyphenyl)-4-(3-(4-methylpiperazin-1-yl)propoxy)benzamide: White solid, 59%, (mp: 140–142 °C), IR (KBr, cm^{-1}), 3304 (–NH stretching), 3000–2931 (Aromatic CH stretching), 2866–2781 (Aliphatic CH stretching), 1643 (–CO amide); ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.04–7.96 (m, 2H), 7.74–7.69 (m, 2H), 7.42–7.35 (m, 2H), 7.00–6.94 (m, 2H), 3.99 (t, *J*=6.1 Hz, 2H), 2.61 (t, *J*=6.4 Hz, 2H), 2.54 (d, *J*=1.1 Hz, 8H), 2.32 (s, 3H), 1.83 (p, *J*= 6.2 Hz, 2H); ^{13}C-NMR (100 MHz, CDCl3): δ=164.7, 162.6, 158, 143.3, 134.8, 131.6, 130.9, 129.2, 129.3, 125.8, 121.9, 118.5, 114.5, 114.9, 73.1, 56.2, 55.8, 57.4, 55.4, 55.4, 46.6, 27.7; ESI MS: m/z=384.1 $(M+1\ H)^{+}$.

A-8: *N*-(4-Chlorophenyl)-4-(3-(4-methylpiperazin-1-yl)propoxy)benzamide: Brown solid, 57%, (mp: 146–148 °C), IR (KBr, cm^{-1}), 3300 (–NH stretching) 3012–2937 (Aromatic CH stretching) 2875–2791 (Aliphatic Ch stretching), 1633 (–CO amide); ^{1}H NMR (400 MHz, Chloroform-*d*) δ, 8.04–7.98 (m, 2H), 7.75–7.69 (m, 2H), 7.42–7.36 (m, 2H), 7.00–6.94 (m, 2H), 3.99 (t, *J*=6.1 Hz, 2H), 2.61 (t, *J*=6.4 Hz, 2H), 2.50 (d, *J*=1.1 Hz, 8H), 2.32 (s, 3H), 1.89 (p, *J*= 6.2 Hz, 2H); ^{13}C-NMR (100 MHz, CDCl3): δ=164.7, 162.8, 136.8, 134.6, 130.9, 128.2, 128.3, 125.8, 121.9, 121.6, 118.5, 114.5, 114.9, 73.1, 56.2, 55.8, 57.4, 55.4, 55.3, 46.6, 27.6; ESI MS: m/z=388.1 $(M+1\ H)^{+}$.

A-9: *N*-(4-Bromophenyl)-4-(3-(4-methylpiperazin-1-yl)propoxy)benzamide: Brown solid, 57%, (mp: 156–158 °C), IR (KBr, cm^{-1}), 3317 (–NH stretching), 3016–2937 (Aromatic CH stretching), 2855–2769 (Aliphatic CH stretching), 1645 (–CO amide); ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.04–7.98 (m, 2H), 7.69–7.63 (m, 2H), 7.53–7.47 (m, 2H), 7.00–6.92 (m, 2H), 3.99 (t, *J*=6.1 Hz, 2H), 2.63 (t, *J*=6.4 Hz, 2H), 2.53 (d, *J*=1.1 Hz, 8H), 2.32 (s, 3H), 1.79 (p, *J*= 6.2 Hz, 2H); ^{13}C-NMR (100 MHz, Chloroform-*d*): δ=164.7, 162.6, 162.2, 136.5, 131.5, 131.5, 126.3, 126.4, 125.8, 115.3, 115.6, 114.5, 114.9, 73.1, 58.2, 55.8, 57.4, 55.4, 55.4, 46.6, 27.7; ESI MS: m/z=432.1 $(M+1\ H)^{+}$.

A-10: *N*-(4-Fluorophenyl)-4-(3-(4-methylpiperazin-1-yl)propoxy)benzamide: Off white solid, 52%, (mp: 142–144 °C), IR (KBr, cm^{-1}), 3300 (–NH stretching) 3000–2975 (Aromatic CH stretching) 2873–2791 (Aliphatic CH stretching), 1656 (–CO amide); ^{1}H NMR (400 MHz, Chloroform-*d*) δ, 8.04–7.93 (m, 2H), 7.62–7.54 (m, 2H), 7.17–7.09 (m, 2H), 7.01–6.94 (m, 2H), 3.99 (t, *J*=6.1 Hz, 2H), 2.64 (t, *J*=6.4 Hz, 2H), 2.54 (d, *J*=1.1 Hz, 8H), 2.32 (s, 3H), 1.81 (p, *J*= 6.2 Hz, 2H); ^{13}C-NMR (100 MHz, Chloroform-*d*): δ=164.7, 162.6, 162.2, 133.5, 128.5, 128.5, 126.3, 126.4, 125.8, 115.3, 115.6, 114.5, 114.9, 73.1, 58.2, 55.8, 57.4, 55.4, 55.4, 46.6, 27.7; ESI MS: m/z=372.1 $(M+1\ H)^{+}$.

A-11: (4-(3-Methoxyphenyl)piperazin-1-yl)(4-(3-(4-methylpiperazin-1-yl)propoxy)phenyl) methanone: Brown solid, 45%, (mp: 166–168 °C), IR (KBr, cm^{-1}), 3050–2956.52 (Aromatic CH stretching), 2858–2762 (Aliphatic CH stretching), 1633 (–CO amide); ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.85–7.78 (m, 2H), 7.12 (t, *J*=7.8 Hz, 1H), 7.00–6.94 (m, 2H), 6.45 (ddd, *J*=7.9, 2.0, 1.3 Hz, 1H), 6.27 (ddd, *J*=7.9, 1.9, 1.2 Hz, 1H), 6.20 (t, *J*=1.9 Hz, 1H), 3.98 (t, *J*=6.1 Hz, 2H), 3.78 (s, 3H), 3.61(t, *J*=5.3 Hz, 4H), 3.36–3.17 (m, 4H), 2.65 (t, *J*=6.4 Hz, 2H), 2.57–2.50 (m, 8H), 2.30 (s, 3H), 1.82 (p, *J*=6.2 Hz, 2H); ^{13}C-NMR (100 MHz, Chloroform-*d*): δ=168.9, 160.2, 161.2, 150.2, 130.2, 127.1, 127.6, 126.2, 114.1, 114.1, 110.5, 106.2, 97.5, 73.1,58.2, 57.6, 57.6, 55.4, 55.4, 55.8, 53.5, 53.0, 50.1, 50.2,46.6, 27.3; ESI MS: m/z=453.2 $(M+1\ H)^{+}$.

A-12: (4-(2-Methoxyphenyl)piperazin-1-yl)(4-(3-(4-methylpiperazin-1-yl)propoxy)phenyl) methanone: White solid, 45%, (mp: 160–162 °C), IR (KBr, cm^{-1}) 3012–2958 (Aromatic CH stretching), 2868–2762 (Aliphatic CH stretching), 1633 (–CO amide); ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.89–7.77 (m, 1H), 7.07–7.01 (m, 1H), 7.00–6.94 (m, 1H), 6.89–6.83 (m, 1H), 3.97 (t, *J*=6.1 Hz, 2H), 3.78 (s, 2H), 3.60 (t, *J*=5.3 Hz, 2H), 3.28–3.15 (m, 2H), 2.61 (t, *J*=6.3 Hz, 1H), 2.54 (d, *J*=1.1 Hz, 4H), 2.31 (s, 2H), 1.82 (p, *J*=6.2 Hz, 1H); ^{13}C-NMR (100 MHz, Chloroform-*d*): δ=168.9, 160.4, 161.2, 150.2, 130.2, 127.8, 127.8, 123.2, 113.1, 114.1, 110.5, 106.2, 97.5, 73.1, 58.2, 57.6, 57.6, 55.8, 55.4, 55.7, 53.5, 53.0, 50.1, 50.2, 46.6, 27.7; ESI MS: m/z=453.2 $(M+1\ H)^{+}$.

A-13: (4-(2-chlorophenyl)piperazin-1-yl)(4-(3-(4-methylpiperazin-1-yl)propoxy)phenyl) methanone: Off white solid, 49%, (mp: 168–170 °C), IR (KBr, cm^{-1}), 3010–2954 (Aromatic CH stretching), 2900–2810 (Aliphatic CH stretching), 1631 (–CO amide); ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.85–7.78 (m, 2H), 7.27 (dd, *J*=7.8, 1.5 Hz, 1H), 7.04 (td, *J* =7.6, 1.5 Hz, 1H), 7.00–6.93 (m, 3H), 6.77 (td, *J*=7.6, 1.6 Hz, 1H), 3.96 (t, *J*=6.1 Hz, 2H), 3.60 (t, *J*=5.3 Hz, 4H), 3.27 (dt, *J*=11.9, 5.3 Hz, 2H), 3.18 (dt, *J*=11.9, 5.3 Hz, 2H), 2.60 (t, *J*=6.4 Hz, 2H), 2.57–2.50 (m, 8H), 2.36 (s, 3H), 1.83 (p, *J*=6.2 Hz, 2H); ^{13}C-NMR (100 MHz, Chloroform-*d*): δ=168.9, 160.4, 161.2, 150.8, 130.1, 129.1, 127.8, 124.2, 113.1, 114.1, 110.5, 106.2, 97.5, 73.1, 58.2, 57.6, 57.6, 55.8, 55.4, 55.7, 53.5, 52.5, 50.1, 50.2, 46.6, 27.7; ESI MS: m/z=458.2 $(M+1\ H)^{+}$.

A-14: [4-(4-chlorophenyl)piperazin-1-yl][(4-(3-(4-methylpiperazin-1-yl)propoxy)phenyl] methanone: Off white solid, 46%, (mp: 174–176 °C), IR (KBr, cm^{-1}) 3000–2954 (Aromatic CH stretching), 2900–2810 (Aliphatic CH stretching), 1643 (–CO amide); ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.86–7.78 (m, 1H), 7.26–7.20 (m, 1H), 7.00–6.94 (m, 1H), 6.77–6.73 (m, 1H), 3.97 (t, *J*=6.1 Hz, 2H), 3.60 (t, *J*=5.3 Hz, 2H), 3.27–3.15 (m, 2H), 2.61 (t, *J*=6.4 Hz, 1H), 2.54 (d, *J*=1.1 Hz, 4H), 2.32 (s, 2H), 1.83 (td, *J*=6.2 Hz, 1H); ^{13}C-NMR (100 MHz, Chloroform-*d*): δ=168.9, 160.4, 149.2, 135.2, 131.8, 127.8, 126.4, 121.2, 118.4, 114.1, 114.5, 110.5, 106.2, 73.1, 58.2, 57.6, 57.6, 55.8, 55.4, 55.7, 53.5, 50.1, 50.2, 46.6, 27.7; ESI MS: m/z=458.2 $(M+2\ H)^{+}$.

A-15: (4-(2,3-dichlorophenyl)piperazin-1-yl)(4-(3-(4-methylpiperazin-1-yl)propoxy)phenyl) methanone: Off white solid, 41%, (mp: 156–158 °C), IR (KBr, cm^{-1}), 3061–2904 (Aromatic CH stretching), 2870–2787 (Aliphatic CH stretching), 1643 (–CO amide); ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.85–7.78 (m, 2H), 7.23–7.14 (m, 2H), 7.00–6.92 (m, 2H), 6.89 (dd, *J*=7.1, 2.0 Hz, 1H), 3.96 (t, *J*=6.1 Hz, 2H), 3.61 (t, *J*=5.3 Hz, 4H), 3.27 (dt, *J*=11.9, 5.3 Hz, 2H), 3.18 (dt, *J*=11.7, 5.3 Hz, 2H), 2.60 (t, *J*=6.4 Hz, 2H), 2.54–2.50 (m, 8H), 2.32 (s, 3H), 1.81 (p, *J*=6.2 Hz, 2H); ^{13}C-NMR (100 MHz, Chloroform-*d*): δ=168.9, 160.4, 150.2, 133.2, 129.8, 127.8, 127.8, 127.8, 126.4, 123.2, 117.6, 114.1, 114.5, 73.1, 58.2, 57.6, 57.6, 55.4, 55.4, 52.1, 52.2, 50.1, 50.2, 46.6, 27.7; ESI MS: m/z=458.2 $(M+2\ H)^{+}$.

A-16: (4-(3-(4-methylpiperazin-1-yl)propoxy)phenyl)(4-(p-tolyl)piperazin-1-yl) methanone: White solid, 42%, (mp: 196–198 °C), IR (KBr, cm^{-1}) 3000–2950 (Aromatic CH stretching), 2880–2778 Aliphatic CH stretching), 1630 (–CO amide); ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.85–7.78 (m, 1H), 7.19–7.12 (m, 1H), 7.00–6.94 (m, 1H), 6.82–6.76 (m, 1H), 3.97 (t, *J*=6.1 Hz, 2H), 3.60 (t, *J*=5.3 Hz, 2H), 3.27–3.14 (m, 2H), 2.61 (t, *J*=6.4 Hz, 1H), 2.57–2.50 (m, 4H), 2.32 (d, *J*=6.0 Hz, 3H), 1.83 (td, *J*=6.2 Hz, 1H); ^{13}C-NMR (100 MHz, Chloroform-*d*): δ=168.9, 160.4, 149.2, 134.2, 134.1, 127.8, 127.8, 126.4, 114.6, 114.1, 93.5, 73.1, 58.2, 57.6, 57.6, 55.4, 55.4, 53.7, 53.7, 50.9, 50.9, 46.6, 34.2, 32.4, 27.7; ESI MS: m/z=436.1 $(M+1\ H)^{+}$.

Cell viability/MTT assay

In adenocarcinomic human alveolar basal epithelial cell line (Lung cancer, A-549) gefitinib showed IC_{50} value of 16.56 μM, in the human colon carcinoma cell line (HCT-116 cell line), it showed an IC_{50} value of 10.51 μM, whereas in human pancreatic carcinoma MIAPaCa-2 cell line, the IC_{50} value was 49.50 μM. Results of MTT assay for all the compounds are given in Table 1.

Table 1. Results of MTT/cell viability assay

Compound code	R	Lung	Colon	Pancreatic
A-549 IC_{50} (μM)	HCT-116 IC_{50} (M)	MIAPaCa-2 IC_{50} (μM)	Scaffold-I	
A-1	H	41.07	6.54	25.01
A-2	4-CH_3	56.26	16.15	27.77
A-3	3-CH_3	>100	>100	>100
A-4	2,4 di-CH_3	44.35	10.11	11.54
A-5	3,4 di-CH_3	35.89	57.65	6.26
A-6	**2,5 di-CH_3**	**7.74**	**18.80**	**14.98**
A-7	4-OCH_3	43.92	35.39	41.77

A-8	4-Cl	>100	>100	>100
A-9	4-Br	>100	>100	48.35
A-10	4-F	43.92	35.39	41.77
Scaffold-II				
A-11	**3-OCH_3**	**5.71**	**4.26**	**31.36**
A-12	4-OCH_3	29.16	18.68	44.42
A-13	2-Cl	>100	>100	>100
A-14	4-Cl	>100	>100	>100
A-15	2,3-diCl	>100	>100	>100
A-16	4-CH_3	14.28	14.42	19.88
	Gefitinib	**16.56**	**10.51**	**49.50**

The most active compounds indicated in bold

In silico studies (docking and physiochemical properties)

The series was prepared keeping similar features to that of piperidine salicylanilide, having EGFR inhibitory activity. Therefore, it was thought to study the in silico docking of active compounds with EGFR to suggest the possible mechanism of action. Glide score and physicochemical propoerties of active compounds are given in Table 2 [20–23].

Table 2. Docking score and physiochemical properties of compounds

Code	R	MW	HBA	HBD	PSA	Log P	GS	DL
Scaffold-I								
A-1	H	353.46	4	1	37.93	2.95	−6.55	2.06
A-2	*p*-CH_3	367.49	4	1	37.93	3.26	−6.53	1.94
A-4	2,4 di-CH_3	381.52	4	1	37.24	3.57	−6.25	1.55
A-5	3,4 di-CH_3	381.52	4	1	37.93	3.57	−6.11	1.42
A-6	2,5 di-CH_3	381.52	4	1	37.93	3.57	−6.79	1.99
A-7	*p*-OCH_3	383.49	5	1	45.48	2.96	−6.07	1.94

A-10	p-F	371.45	4	1	37.93	3.09	−5.84	2.25
Scaffold-II								
A-11	*3*-OCH_3	452.56	6	0	42.30	2.67	−7.07	1.32
A-12	4-OCH_3	452.56	6	0	42.30	2.67	−7.03	1.16
A-16	4-CH_3	436.6	5	0	34.75	2.97	−5.29	1.20

MW: Molecular weight, HBA: hydrogen bond acceptor, HBD: hydrogen bond donar, PSA: polar surface area, GS: glide score, DL: drug likeliness

Discussion

Cell viability/MTT assay

In the A-549 lung cancer cell line, electron-donating substitution on the aniline portion showed better inhibition. Compound A6 (2,5 di-CH_3) with IC_{50} 7.74 μM showed better inhibition as compared to compound A4 (2,4 di-CH_3) and A5 (3,4 di-CH_3). Similarly, among scaffold-II compounds, A11 (3-OCH_3) and A12 (4-OCH_3) showed the IC_{50} value of 5.71 μM, 13.16 μM and A16 (4-CH_3)14.28 μM, respectively. In this cell line, *meta* substitution resulted in favorable activity and compounds with electron withdrawing groups (EWGs) such as, A8 (4-Cl), A9 (4-Br), A10 (4-F) were weakly active.

In the HCT-116 colon cancer line, three compounds showed better activity than gefitinib. Compounds A1 (-H) and A4 (2,4 di-CH_3) displayed IC_{50} value of 6.54 μM and 10.54 μM, respectively while compound A-11 having *meta* methoxy substitution was most active with IC_{50} of 4.26 μM. Again, in this cell line, electron donating substituted compounds exhibited good inhibition and compounds with electron withdrawing groups were inactive.

In the MIAPaCa-2 cell line, dimethyl substituted aniline derivatives showed better results when compared to mono substituted compounds. Compounds A-4 (2,4 di-CH_3), A-5 (3,4 di-CH_3) and A-6 (2,5 di-CH_3) showed IC_{50} values of 11.54, 6.26 and 14.98 μM respectively. From scaffold-II derivatives, compound A-11 (3-OCH_3) and compound A-16 (4-CH_3) showed better inhibition than gefitinib.

Overall, compound A-11 was found to be the most active in two of the three cell lines. Also, by observing the IC_{50} values of all the compounds, it can be said that compounds with electron-donating substituents on the aniline portion exhibited better inhibition as compared to compounds with electron withdrawing groups.

Docking

It was found that the docked ligand superimposed well with reference ligand (gefitinib, co-crystallized ligand) with root mean square deviation value of 0.458 Å. The gefitinib in active site of EGFR displayed hydrogen bonding interaction with MET793 of hinge region, which is essential. Additionally, it shows hydrophobic interactions with CYS797 and LEU792 of hinge region and LEU844 and MET766 in the C-helix with a glide score of − 7.31.

It was observed that compounds with electron-withdrawing substituents did not dock well in the active site and compounds with electron donating substituents fit well. Compound A-10 with *p*-fluoro substituent on scaffold I was the only compound with electron withdrawing substituent and weak in the MTT assay was found to have least activity glide score. Its glide score was less than the unsubstituted compound. It was observed that it does not take part in any hydrogen bonding interaction.

When all the compounds were docked, it was observed that hydrophobic interactions with CYS797, LEU792, MET793 of hinge region, LEU844 and MET766 in the C-helix were similar for all compounds. However, the compounds showed hydrogen bonding interaction with MET793 and those with better score also showed side chain hydrogen bonding with ASP855 in activation loop. This indicates that these compounds do show necessary interactions in active site and hence may exert anticancer activity by inhibiting as EGFR.

Docking interactions of representative compounds are shown in Fig. 2.

Fig. 2 [Images not available. See PDF.]

Representing docking interactions (**A**: 3D interaction diagram of gefitinib, **B**: 2D ligplot of gefitinib, **C**: 3D interaction of compound A-11, **D**: 2D ligplot of compound A-11, **E**: Overlay of all the compunds)

Conclusion

A range of 16 compounds consisting of methyl piperazine-incorporated phenyl benzamide and phenyl methanone derivatives were synthesized and assessed for their potential as anticancer agents against A-549 human lung carcinoma, HCT-116 human colon cancer, and MIAPaCa-2 human pancreatic carcinoma cell lines. It was noted that compounds possessing electron-donating groups exhibited heightened cytotoxicity across the three cell lines. Additionally, a hypothesis was put forward suggesting that the observed anticancer effects may stem from the inhibition of EGFR. Previous literature indicates that hydrogen bond interactions with MET793 have been linked to EGFR inhibition, and the compounds exhibiting superior anticancer activity were found to interact with MET793 within the EGFR binding site. The results suggest that these derivatives function as anticancer agents; nevertheless, further investigations are necessary to ascertain their specificity as EGFR inhibitors.

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Author information

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Author contributions

The contributions of all authors to the manuscript are as follows: MS: design, synthesis, and characterization of the compounds. HRJ: Outlined the study and provided overall guidance. AC: Conducted the anticancer activity assays. PW: Performed the molecular docking studies and prepared the manuscript.

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Competing interests

Author declares no competing interests.

Abbreviations

EGFR
Epidermal growth factor receptor
HCT
Human colon Carcinoma
NSCLC
Non small cell lung cancer
CYS
Cysteine
LEU
Leucine
MET
Methionine

TK
Tyrosine kinase
HOBt
Hydroxy-*O*-benztriazole
EDC.HCl
N-(3-Dimethylaminopropyl)-*N'*-ethylcarbidiimide hydrochloride
MTT
3-(4, 5-Dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
$CDCl_3$
Deuteriated chloroform
DMSO
Dimethyl sulfoxide
TMS
Trimethyl silane

Publisher's Note

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Solid self-nanoemulsifying drug delivery systems of nimodipine: development and evaluation

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ABSTRACT (ENGLISH)

Background

This study aimed to formulate solid self-nanoemulsifying drug delivery systems (SNEDDS) for nimodipine (NIM). The selection of Cremophor RH 40, Lipoxol 300, and PEG 400 as oil, surfactant, and co-surfactant was based on solubility and self-emulsification assessments. A ternary phase diagram determined the optimal oil to Smix (surfactant/co-surfactant) ratio (40:60). By utilizing liquid SNEDDS (NIM-SNEDDS) as an adsorbate and chitosan EDTA microparticles, developed through spray drying (SD-CHEM) and solvent evaporation (SE-CHEM) as adsorbents, the solid SNEDDS were created (NIM-SD-SSNEDDS and NIM-SE-SSNEDDS, respectively).

Results

Both solid formulations exhibited favourable drug loading (NIM-SD-SSNEDDS = 79.67 ± 2.97%, NIM-SE-SSNEDDS = 77.76 ± 4.29%), excellent flowability, and drug amorphization as per XRD and DSC analysis. Scanning electron

microscopy revealed smoothening and filling of adsorbent surfaces by adsorbate (with size range NIM-SD-SSNEDDS = 10–15 µm, NIM-SE-SSNEDDS = 20–25 µm). FTIR confirmed no interaction of drug and excipients. Stability studies demonstrated the physical and thermodynamic stability of reconstituted nanoemulsions with droplet size, PDI, zeta potential, emulsification time, % transmittance and cloud temperature for NIM-SD-SSNEDDS as 247.1 nm, PDI 0.620, 1.353 mV, 38–41 s, 94.64%, 54 °C and for NIM-SE-SSNEDDS as 399.6 nm, PDI 0.821, 1.351 mV, 40–48 s, 92.96%, 49 °C, respectively. FE-SEM images showed globules formed with small sizes, and there was no coalescence evidence, implying the reconstituted nanoemulsions' stability. In vitro dissolution studies revealed a fourfold increase in drug dissolution for NIM-SD-SSNEDDS (84.43%) and NIM-SE-SSNEDDS (76.68%) compared to pure drug (28%). Ex vivo permeation studies indicated almost similar profiles for NIM-SD-SSNEDDS (22.61%) and NIM-SE-SSNEDDS (21.93%) compared to NIM-SNEDDS (25.02%).

Conclusion

NIM-SD-SSNEDDS exhibited superior performance compared to NIM-SE-SSNEDDS, highlighting the efficacy of microparticles developed by the spray drying method (SD-CHEM) as adsorbents for solidification. These results suggest enhanced dissolution and permeation for nimodipine in both the solid SNEDDS.

FULL TEXT

Background

Self-nanoemulsifying drug delivery systems (SNEDDS) showcase a significant capacity for improving the oral bioavailability and biological efficacy of drugs characterized by poor water solubility [1, 2]. Lipid-based systems like SNEDDS have proven effective in addressing the problem of breakdown by enzymes in the gastrointestinal tract during the oral administration of biomolecules [3]. SNEDDS pose various challenges and obstacles, encompassing concerns related to both physical and chemical instability. The fluid nature of SNEDDS introduces several difficulties, such as constraints on dosage manufacturing, restricted choices for dosage forms, reduced drug loading capacity, and intricate issues in handling and storage [4, 5]. This leads the researchers to investigate various approaches to solidify SNEDDS, streamlining a swift and uncomplicated development of solidification of product with the desired properties [6].

There has been a growing emphasis on solid self-nanoemulsifying drug delivery systems (S-SNEDDS). These systems are formulated by integrating a liquid self-nanoemulsifying drug delivery system into a solid dosage form. This approach combines the benefits of SNEDDS with those of a solid dosage form, effectively addressing the limitations associated with liquid formulations [7]. S-SNEDDS offer a multitude of advantages, such as increased surface area leading to improved solubility and bioavailability, enhanced stability, robustness, easy handling, easy scale-up, enhanced drug loading capacity, better flow, minimized drug precipitation, and economical manufacturing [8]. SNEDDS suits BCS (Biopharmaceutical classification system) class II drugs (with low water solubility and high permeability), which have a dissolution-restricted absorption. This restricted absorption can result in a lack of success in therapeutic action because of insufficient drug concentration [9, 10].

Nimodipine (NIM), a BCS class II drug, is a calcium antagonist (dihydropyridine) with poor water solubility (2.30 µg/ml) and high lipophilicity and permeability (log *P* = 3.41) [11]. Because of the high lipophilicity, it can reach the brain and cerebrospinal fluid by crossing the blood–brain barrier [12]. It is highly used in the treatment of delayed ischaemic neurological disorder in patients with subarachnoid haemorrhage and cerebral vasospasm, stroke, senile dementia (due to irreversible loss of neurons) and hemicrania [13–16]. It has a moderate anti-hypertensive effect and is used in sudden sensorineural hearing loss [17].

Its mechanism of action is to block the entry of calcium through specific channels (voltage-dependent) and stop the contraction of vascular smooth muscles, leading to the dilation of the blood vessels [18].

NIM has two polymorphic forms and two enantiomers with different aqueous solubilities [19]. Because of the rapid first-pass metabolism and P-glycoprotein efflux, it has a lower oral bioavailability (around 13%) and requires high dosing (360 mg per day) [20]. Nimodipine was used as a model drug for developing the S-SNEDDS with chitosan-EDTA microparticles developed by spray drying and solvent evaporation techniques.

Materials and methods

Nimodipine was a gift sample from Strides Pharma Science limited (India). Cremophor® RH-40 (Polyoxyl 40 hydrogenated castor oil) was procured from Himedia (India). Lipoxol 300 (PEG 300) was procured from Sasol Chemicals (USA). Polyethylene glycol 400 (PEG 400) was obtained from TCI (India). Caprol® ET (hexaglycerol octasterate), Captex® 200 (propylene glycol dicaprylate), Captex® 300 (glyceryl tricaprylate/tricaprate) were gift samples from Abitech (USA). Labrafac™ PG (propylene glycol dicaprylocaprate) from Gattefossé (Canada) was received as a gift sample. Propylene glycol and Ethylene diamine tetra acetic acid disodium (EDTA disodium) were obtained from CDH (India). Chitosan with 90% deacetylation (DA) was acquired from Marine Hydrocolloids (India). Except for the ones we talked about, we used high-quality chemicals for the research. We used them just as they were, without any changes.

Drug solubility analysis in different excipients

As discussed, NIM is a high-dosing drug, so it becomes vital to obtain maximum drug loading in the SNEDDS. To create and chart the emulsification area, examining how much NIM could dissolve in various excipients was crucial. A surplus of the drug was placed in a sealed container, which was then subjected to a 40 °C water bath for 15 min. The mixture was stirred in an orbital shaker incubator (Remi, India) at 100 revolutions per minute for 72 h [21]. Subsequently, the mixture underwent centrifugation using a Remi RC-8 centrifuge from India at a speed of 4000–5000 rpm for 30 min. The resulting supernatant was filtered through Whatman filter paper with a pore size of 0.45 µm nylon. NIM was quantified at 237 nm using a UV spectrophotometer (UV–VIS spectrophotometer-2371 EI, India). The experimental procedure was repeated three times for accuracy [22].

The capacity for self-emulsification is an important factor when choosing excipients for SNEDDS, in addition to the drug's high solubility in both oil and surfactant. A 10% (w/v) aqueous solution of each surfactant (demonstrating significant high drug solubility) was created to assess this. Subsequently, 10 ml of this solution was titrated with each type of oil, and the volume of oil required to make the emulsion cloudy was recorded.

The combination of oil and surfactant that emulsified a maximum quantity (of oil) was chosen [23]. The selected surfactant (in a 1:1 ratio) with each co-surfactant (which had high drug solubility) was taken to form a mixture (Smix). Using this Smix, various formulations were created with the chosen oil, ranging from 10 to 90% in concentration. Each formulation, consisting of 500 mg, was individually mixed in 500 ml of triple distilled water, and the transparency or appearance of the mixture was then noticed [24].

Plotting of ternary phase diagram

We generated a ternary phase diagram to identify the suitable excipient range for creating nanoemulsion. Ternary mixtures, each totalling 1 g and including equal drug amounts, were prepared with three components. The selected surfactant and co-surfactant were combined in 1:1, 1:2, and 2:1 ratios, forming Smix mixtures. Subsequently, the oil and Smix were combined in nine different weight proportions, ranging from 1:9 to 9:1, in distinct glass vials. The aim was to determine the maximum limits for analysis to outline the phase accuracy boundaries in the diagram. Each formulation underwent titration with 500 ml of triple distilled water to observe nanoemulsion formation. The formation of a transparent/clear solution affirmed nanoemulsion creation. The proportions of oil and Smix were recorded and illustrated in the diagram. Chemix software was utilized for diagram plotting, with ingredients delineating the sides of this representation [25].

Development of self-nanoemulsifying drug delivery system

From the ternary phase diagram experiment, the appropriate proportions of oil and Smix were chosen to create a self-nanoemulsifying drug delivery system. The selected components were Cremophor RH 40 (as the oil), Lipoxol 300 (as the surfactant), and PEG 400 (as the co-surfactant). Subsequently, a liquid self-nanoemulsifying drug delivery system for Nimodipine (NIM-SNEDDS) was formulated. The measured amount of the drug was slowly introduced into the oil in a beaker and stirred at 2000 rpm on a magnetic stirrer until a homogeneous solution was achieved. Dropwise, adding a 1:1 Smix to this solution produced an isotropic mixture under continuous stirring for 30 min. The mixture was left to equilibrate for 48 h at room temperature, and observations were made for any phase separation [26].

Fabrication of SNEDDS to solid self-nanoemulsifying drug delivery system

Our earlier research optimized and created advanced adsorbent microparticles using chitosan-EDTA through spray drying (SD-CHEM) and a solvent evaporation method (SE-CHEM) [27]. These microparticles exhibited heightened abilities to absorb and release oil. Analysis of their surface free energy components revealed increased dispersive features and dynamic advancing contact angles, favourable characteristics for the adsorbent in converting liquid self-nanoemulsifying drug delivery systems (SNEDDS) into Solid SNEDDS (S-SNEDDS).

In the SE-CHEM process, a chitosan-EDTA disodium solution (60:40) underwent solvent evaporation in a Rota evaporator (Micro technologies, India) at a drying temperature of 70 °C for 45–60 min. Subsequently, the resulting dry film was carefully scraped, further dried in an oven for 40–50 min at 70 °C to eliminate residual moisture, and eventually converted into powder using a pestle and mortar. For SD-CHEM, a Chitosan-EDTA disodium solution (50:50) was processed in a Spray dryer (SprayMate JISL, India) with inlet temperature set at 110 °C, aspirator speed at 1000–2000 rpm, atomization pressure at 3 kg/cm^2, and feed pump operating at 15 rpm. In continuation to our research work, we fabricated the developed liquid SNEDDS of NIM with adsorption or solid carrier technique to create a solid self-nanoemulsifying drug delivery system (S-SNEDDS). The microparticles, created through SD-CHEM and SE-CHEM, served as adsorbents for the NIM-SNEDDS (nimodipine self-nanoemulsifying drug delivery system). Sequentially, NIM-SNEDDS was added to SD-CHEM and SE-CHEM individually and thoroughly mixed in a mortar and pestle. This process resulted in the formation of NIM-SD-SSNEDDS (nimodipine solid self-nanoemulsifying drug delivery system with spray-dried microparticles) and NIM-SE-SSNEDDS (nimodipine solid self-nanoemulsifying drug delivery system with solvent-evaporated microparticles) [27, 28]. The ratio of the adsorbate (NIM-SNEDDS) to adsorbent (SD-CHEM &SE-CHEM) was optimized to achieve non-sticky, free-flowing powders, namely NIM-SD-SSNEDDS and NIM-SE-SSNEDDS, respectively.

Different evaluations of the NIM-SNEDDS, NIM-SD-SSNEDDS, and NIM-SE-SSNEDDS

Drug loading efficiency (%)

To assess the drug loading efficiency (%), 100 mg of NIM-SNEDDS, NIM-SD-SSNEDDS, and NIM-SE-SSNEDDS was individually taken in 10 ml of methanol and vortexed in an orbital shaker (Remi, India) for 10 min. The NIM-SNEDDS mixture in methanol was directly analysed after appropriate dilution and examined at 237 nm using a UV spectrophotometer (UV–VIS spectrophotometer-2371 EI, India). The remaining two mixtures (NIM-SD-SSNEDDS and NIM-SE-SSNEDDS) were centrifuged (Remi Rc-8, India) at 4000 rpm for 10 min, and the supernatant obtained was filtered through Whatman filter paper (0.45 µm nylon). Following suitable dilution, these were analysed in a UV spectrophotometer, and the procedure was repeated in triplicate [28]. The drug loading efficiency (%) was calculated using the specified equation.

1

$$\text{Drug loading efficiency}\% = \frac{\text{Actual quantity of NIM present in the known amount of formulation}}{\text{Initial drug NIM load}} \times 100$$

Flowability

For NIM-SD-SSNEDDS and NIM-SE-SSNEDDS, we determined the angle of repose using the fixed funnel method, along with apparent bulk density, tapped density, Carr's Index, and Hausner's ratio, employing standard methods to characterize their flow properties [29].

The angle of repose was determined by placing graph paper on a flat horizontal surface and clamping a funnel above it, maintaining a distance of approximately 7–8 cm between the paper and the funnel top. The powder samples (2 g) were measured and poured into the funnel until the top of the cone-shaped heap just reached the funnel's top. The height (h) and diameter of the cone-shaped heap of powder (D) were measured, and the angle of repose was calculated using the standard formula ($\tan \alpha = 2h/D$). A powder with an angle of repose less than 25° is considered to have excellent flow, while a powder with an angle of repose greater than 40° is considered to have poor flow.

The powder (2 g) was carefully weighed and levelled for bulk density determination without tapping into a graduated glass cylinder. The apparent volume before tapping read as an untapped volume using the USP method was considered for the standard formula (Bulk density = Weight/untapped volume), and values were calculated.

After 500 tapings, the volume of the powder-filled cylinder was measured. Tapping continued until the frequency

difference between the two sets of tapping was less than 0.2 per cent. The final volume was noted, and the tapped density in g/ml was calculated using the standard formula (Tapped density = Weight/tapped volume).
These readings were then utilized to calculate Carr's index (CI) and Hausner's ratio (HR) using the formulas {CI = [(Tapped density − Bulk density/Tapped density) × 100] and HR = Tapped density/Bulk density}. Carr's index values above 25 indicate poor flowability, while values below 15 suggest good flowability. Hausner's ratios below 1.25 signify better flow properties than those above 1.25 [30].

Characterization (solid state)

X-ray diffraction analysis

Following the grinding of each sample (NIM, SD-CHEM, SE-CHEM, NIM-SD-SSNEDDS and NIM-SE-SSNEDDS), the resulting powder was placed and compacted in a sample holder. X-ray diffraction patterns of the samples were then measured using the X-ray diffractometer (XRD Aeris, Malvern Panalytical, UK). The continuous scanning of samples occurred within the range of 10°–50° at a rate of 2° per minute, with 0.02° 2θ increments. The scanning process commenced at 5° and concluded at 50° (2θ), with the scans conducted at 25 °C and the generator configured at 45 kV [28].

Scanning electron microscopy

The surface characteristics of NIM, SD-CHEM, SE-CHEM, NIM-SD-SSNEDDS, and NIM-SE-SSNEDDS were examined using the scanning electron microscope (SEM) (ZEISS Sigma 360, Germany) at 20 kV. The samples were affixed to the SEM stub and coated with a thin layer of gold. Multiple images were captured at various magnifications [30].

FTIR spectroscopy

Different components of the formulations, as well as their physical mixtures, were examined for possible incompatibilities. To create a fine mixture, the prepared samples were dried under vacuum, mixed, and triturated with KBr at a 1:100 ratio. Pellets were formed by pressing this fine mixture in a KBr press. Subsequently, these pellets were placed in a sample cell, and FTIR-ATR analysis was conducted (FTIR Perkin Elmer Spectrum Two, USA) in the 500–4000 cm^{-1} spectral range at room temperature [31].

DSC analysis

Hermetically sealed aluminium pans containing samples (15 mg each) of NIM, NIM-SNEDDS, NIM-SD-SSNEDDS, and NIM-SE-SSNEDDS were utilized. These pans were positioned on the sample pan holder, while empty pans were placed on the reference pan holders in a differential scanning calorimeter (DSC-25 TA, USA). Thermograms for each sample were then recorded within a temperature range of 40–400 °C, employing a heating rate of 10 °C per minute in a nitrogen atmosphere.

Reconstituted nanoemulsion and NIM-SNEDDS evaluations

To assess reconstitution ability, 100 mg of NIM-SD-SSNEDDS and NIM-SE-SSNEDDS was dispersed separately in 100 ml of triple distilled water for 1 h. Subsequently, the dispersions were vortexed in an orbital shaker (Remi, India) for 10 min. The resulting suspension underwent centrifugation (Remi RC-8, India) at 4000 rpm for 10 min to eliminate undissolved particles. The obtained supernatant was reconstituted nanoemulsion and utilized for subsequent investigations. Additionally, a 1:100 w/v dilution of freshly prepared NIM-SNEDDS with triple distilled water was created, dispersed, and used for further analysis.

Droplet size, size distribution, and zeta potential determination

The size of the droplets, polydispersibility index, and zeta potential of the reconstituted nanoemulsions and NIM-SNEDDS were examined using the Zetasizer Nano ZS (at a wavelength of 633 nm) with a scattering angle of 90° at 25 °C. The analysis was conducted in triplicate using equipment from Malvern Panalytical, UK [32, 33].

Self-emulsification time

The NIM-SD-SSNEDDS and NIM-SE-SSNEDDS supernatants and NIM-SNEDDS (1 ml each) were dispersed in 500 ml of triple distilled water and stirred at approximately 100 rpm using a magnetic stirrer. Observations were made to determine the formation of emulsion and the time needed for dispersibility [34].

Per cent transmittance test

When formulating self-nanoemulsifying drug delivery systems for oral administration, drug precipitation is possible upon dilution in the gastrointestinal tract. To assess this, the supernatants of NIM-SNEDDS, NIM-SD-SSNEDDS, and NIM-SE-SSNEDDS were tested for per cent transmittance at 237 nm, with water as the blank, using a UV spectrophotometer (UV–VIS spectrophotometer-2371 EI, India). The test was conducted in triplicate for accuracy [35, 36].

Estimation of cloud point

The supernatants (NIM-SD-SSNEDDS and NIM-SE-SSNEDDS) and NIM-SNEDDS were subjected to a water bath with a gradual temperature increase. The temperature at which immediate turbidity was observed in the samples was recorded [37].

Field emission scanning electron microscopy

The supernatant (50 µl) of both NIM-SD-SSNEDDS and NIM-SE-SSNEDDS was drop-casted onto glass slides that had been cleaned beforehand. The dried samples were then gold-coated using a sputter-coater for 10–15 s under high vacuum conditions. High-resolution images were captured at 15 kV using an accelerating voltage in field emission scanning electron microscopy (FE-SEM) (Quanta 250, Bruker) [38].

Dissolution profile (in vitro)

For assessing the dissolution release of NIM, the pure NIM, NIM-SNEDDS, NIM-SD-SNEDDS, and NIM-SE-SNEDDS, equivalent to 10 mg, were individually filled into hard gelatin capsules. The release study used the USP dissolution apparatus II-paddle at 37 ± 0.5 °C (Electrolab India, India) in pH 4.5 acetate buffer as the dissolution medium, stirring at 50 rpm. At various time intervals, 2 ml of sample aliquot was withdrawn (instantly filtered), and fresh medium was added to maintain sink conditions. After appropriate dilution with pH 4.5 acetate buffer, samples were analysed at 237 nm using a UV spectrophotometer [39]. The experiments were repeated three times to ensure consistent and accurate results.

Ex vivo permeability profile

The ex vivo permeation method was applied, as described by Singh et al. [40]. In summary, the ex vivo permeation method involved utilizing the biological membrane from the porcine small intestine acquired from a local slaughterhouse in Srinagar Garhwal, Uttarakhand, India. The intestine, obtained within one hour of slaughter, was preserved in Kreb's ringer phosphate solution at 4 °C with aeration. A 10–12-cm section of the intestine was dissected, washed with saline, and placed on saline-soaked filter paper. After making a lengthwise cut to flatten it, the serosal membrane faced upward, and the muscle layer was removed using a scalpel. For the permeation study, the intestine member was mounted in a modified Franz diffusion cell with the mucosal layer facing the donor compartment side. A pH 4.5 acetate buffer in the receptor compartment served as the receptor medium, maintained at 37 ± 0.5 °C and stirred at 50 rpm. The donor compartment received pure NIM/NIM-SNEDDS/NIM-SD-SSNEDDS/NIM-SE-SSNEDDS (equivalent to 5 mg of NIM) on the mucosal side of the membrane. At various time intervals, a 1 ml sample was withdrawn and replaced with a fresh 1 ml pH 4.5 acetate buffer in the receptor compartment. The samples were analysed at 237 nm using a UV spectrophotometer after appropriate dilution to determine the amount of NIM diffused through the membrane. The experiments were repeated three times to ensure a consistent and accurate average value.

Stability study

The HDPE bottles (60 ml) containing 40 capsules of each NIM-SD-SSNEDSS and NIM-SE-SSNEDDS (each capsule equivalent to 20 mg of nimodipine) were subjected to accelerated conditions (40 ± 2 °C/75 ± 5% RH) in a stability (humidity) chamber (Newtronic, India) for six months post-sealing. Throughout this period, samples were periodically withdrawn and examined for physical appearance, percentage cumulative drug release (% CDR), and disintegration time [41].

Results

Drug solubility analysis in different excipients

Formulations often encounter the challenge of precipitation before undergoing in situ solubilization. Hence, to verify the stability of the formulation, understanding the drug's solubility in the chosen excipients becomes crucial. High

drug solubility in various formulation excipients is essential for achieving optimal drug loading and bioavailability [42]. For the development of an effective SNEDDS for NIM, it is essential that the drug readily mixes with the selected excipients, minimizing its incorporation into the mixture [43]. Figure 1 illustrates the solubility of NIM in different excipients.

Fig. 1 [Images not available. See PDF.]

Solubility of nimodipine in different excipients (mg/ml)

To achieve effective self-emulsification, it is essential to have the optimal combination of excipients. Analysis of the self-emulsification potential demonstrated that Lipoxol 300, combined with the highest quantity of Cremophor RH 40, resulted in successful emulsification, as illustrated in Fig. 2.

Fig. 2 [Images not available. See PDF.]

Self-emulsification of oils with surfactants ($n=3$)

Upon perceiving this finding, Cremophor RH 40 and Lipoxol 300 were chosen as the oil and surfactant, respectively. Polyethylene glycol 400 (PEG 400) was selected as the co-surfactant based on the broader range of nanoemulsion regions observed compared to propylene glycol (PG), as depicted in Table 1.

Table 1. Nanoemulsion region formation (Smix with different co-surfactants)

Composition		Nanoemulsion region	
Oil (%)	S_{mix} (%)	S_{mix} of Lipoxol 300: PEG 400 (1:1)	S_{mix} of Lipoxol 300: PG (1:1)
10	90	Yes	Yes
20	80	Yes	Yes
30	70	Yes	Yes
40	60	Yes	Yes
50	50	Yes	No
60	40	No	No
70	30	No	No
80	20	No	No
90	10	No	No

PEG 400 polyethylene glycol 400, *PG* propylene glycol

Increased surfactant levels can boost the self-emulsification process. Including a co-surfactant, such as PEG 400, does not compromise the surfactant's ability to decrease interfacial tension around the oily component. Utilizing a co-surfactant allows for a reduction in the overall amount of surfactant in the formulation [44]. In this specific formulation, PEG 400 serves as the co-surfactant.

Plotting of ternary phase diagram

In order to select the right proportion of excipients for self-nanoemulsifying drug delivery systems (SNEDDS) and observe self-emulsification in the nano-range, a ternary phase diagram was constructed with the inclusion of NIM. This diagram aids in comprehending the phase behaviour of nanoemulsions [45].

The water-titration method was employed to create a diagram, incorporating an oily vehicle ranging from 10 to 90% and varying Smix ratios (1:1, 1:2, and 2:1). Transparent regions, indicating nanoemulsion areas were identified, and ternary diagrams were constructed using this information, specifically for three Smix ratios (1:1, 1:2, and 2:1). The shaded portions in Fig. 3a–c represent the transparent nanoemulsion regions with low viscosity. In diagrams, each peak denotes 100% of the corresponding excipient.

Fig. 3 [Images not available. See PDF.]

Pseudo ternary phase diagrams **A** with Smix 1:2, **B** with Smix 2:1, and **C** with Smix 1:1

According to Fig. 3a (for oil and Smix 1:2), when the oil content is less and PEG 400 is more, the shaded nanoemulsion region is observed, and when the oil content is increased and PEG 400 is reduced, the biphasic system can be seen. In the case of Smix 2:1 with oil, the increase in Lipoxol 300 gives a greater nanoemulsion area (in Fig. 3b). As per Fig. 3c, (oil and Smix 1:1) the 30–50% of Cremophor RH 40, and less percentage of Smix is resulting in a nanoemulsion region. When PEG 400 increases, the nanoemulsion region is reduced because of the limited capability of PEG 400 to the interfacial tension. At the same time, Lipoxol 300 has a greater effect of reducing the interfacial tension by making a layer around the oil droplets. The Smix ratio of 1:1, serving as a surfactant/co-surfactant mixture, was identified as suitable.

Development of self-nanoemulsifying drug delivery system and fabrication of SNEDDS to solid self-nanoemulsifying drug delivery system

Based on the findings from the two studies of the ternary phase diagram (Fig. 3c) and nanoemulsion region formation (Table 1), it was clear that a 40:60 ratio of oil (Cremophor RH 40) to Smix (Lipoxol 300: PEG 400 = 1:1; serving as a surfactant/co-surfactant mixture), was producing nanoemulsion. This 40:60 ratio had a significant amount of oil (part), which remains essential for maximum drug solubilization and high drug loading into the formulation. Hence, this was chosen for the development of the SNEDDS development. Following the outlined procedure, the liquid NIM-SNEDDS was created and stored in an airtight container. The ultimate composition of NIM-SNEDDS consisted of 100 mg of NIM, 400 mg of Cremophor RH 40, 300 mg of Lipoxol 300, and 300 mg of PEG 400. The microparticles created through spray drying (SD-CHEM) and the chitosan EDTA microparticles using the solvent evaporation method (SE-CHEM) showcased their capabilities with high oil adsorption and desorption capacities. These microparticles also demonstrated favourable enhancements in surface free energy components and dynamic advancing contact angles. The solid self-nanoemulsifying drug delivery systems (S-SNEDDS) were independently formulated for both SD-CHEM and SE-CHEM through an adsorption or solid carrier technique. Among the two ratios of adsorbate (NIM-SNEDDS) to adsorbent (SD-CHEM), namely 1:1.5 and 1:2.5, the 1:2.5 ratio was identified as yielding a non-sticky, free-flowing powder for NIM-SD-SSNEDDS. For NIM-SE-SSNEDDS, considering two ratios of adsorbate (NIM-SNEDDS) to adsorbent (SE-CHEM), namely 1:2 and 1:2.5, the 1:2.5 ratio was determined to result in a non-sticky, free-flowing powder. This adsorption technique is particularly advantageous for thermolabile drugs as it avoids subjecting the system to heat treatment. The resultant NIM-SD-SSNEDDS, NIM-SE-SSNEDDS, and NIM-SNEDDS (liquid) were subsequently assessed.

Different evaluations of the NIM-SNEDDS, NIM-SD-SSNEDDS, and NIM-SE-SSNEDDS

Drug loading efficiency (%)

As indicated in Table 2, the drug loading efficiency for NIM-SD-SSNEDDS and NIM-SE-SSNEDDS was found to be 79.67 ± 2.97% and 77.76 ± 4.29%, respectively. As anticipated, the drug loading efficiency (%) for NIM-SNEDDS was 82.75 ± 3.56%. Notably, a certain loss in drug loading occurred during the solidification process from NIM-SNEDDS to NIM-SD-SSNEDDS and NIM-SE-SSNEDDS using the adsorption technique.

Table 2. Flow properties, physical characterization, dissolution, and permeation profiles of the formulations

	NIM-SNEDDS	NIM-SD-SSNEDDS	NIM-SE-SSNEDDS
Flow properties			
Bulk density (g/cm^3)	–	0.893±0.209	0.752±0.116
Tapped density (g/cm^3)	–	0.928±0.194	0.861±0.251
Carr's index (%)	–	5.119±0.083	13.588±0.236
Hausner's ratio	–	1.051±0.074	1.135±0.071
Angle of repose(θ)	–	10.752±2.145	9.734±1.921
Flowability	–	Good	Good
Physical characterization			
Droplet size (nm)	245.5±3.745	247.1±3.282	399.6±3.851
PDI	0.715±0.008	0.620±0.003	0.821±0.005
Zeta potential (mV)	1.961±0.426	1.353±0.073	1.351±0.853
Emulsification time (sec)	24–36	38–41	40–48
% transmittance	98.192±0.141	94.645±0.628	92.962±0.932
Cloud temp. (°C)	69	54	49
Dissolution profiles			
Drug loading efficiency (%)	82.757±3.561	79.675±2.973	77.763±4.292
Dissolution efficiency (%)	88.84	84.43	76.68
Permeation profile			
Ex-vivo permeation efficiency (%)	25.026	22.609	21.932
Flux	3.692	3.186	3.305
Permeability constant	0.738	0.637	0.661

Values are presented as mean±standard deviation (S.D.), (n=3)

Flowability

For NIM-SD-SSNEDDS and NIM-SE-SSNEDDS, the resulting flow properties were calculated and are presented in Table 2. The bulk density of NIM-SD-SSNEDDS and NIM-SE-SSNEDDS was 0.893 ± 0.209 g/cm^3 and 0.758 ± 0.116 g/cm^3, indicating the presence of gaps between powder particles, which is crucial in the industry (for efficient capsule filling). The tapped density for NIM-SD-SSNEDDS and NIM-SE-SSNEDDS was 0.928 ± 0.194 g/cm^3 and 0.752 ± 0.116 g/cm^3. The minimal difference between these densities (bulk and tapped), along with Carr's index (NIM-SD-SSNEDDS = 5.119 ± 0.083 and NIM-SE-SSNEDDS = 13.588 ± 0.236), Hausner's ratio (NIM-SD-SSNEDDS = 1.051 ± 0.074 and NIM-SE-SSNEDDS = 1.135 ± 0.071), and angle of repose (NIM-SD-SSNEDDS = 10.752 ± 2.145 and NIM-SE-SSNEDDS = 9.734 ± 1.921), signifies excellent flow properties for the formulations.

Characterization (solid state)

X-ray diffraction analysis

Detecting changes in polymorphism, dissolution rate (solubility), and stability depends on the critical quality attribute of particle crystallinity, as determined by XRD analysis. Figure 4 illustrates the X-ray diffraction (XRD) analysis of NIM, SD-CHEM, SE-CHEM, NIM-SD-SSNEDDS, and NIM-SE-SSNEDDS. NIM exhibited diffraction peaks at 2θ values of 12.53°, 13.06°, 17.54°, 19.89°, 20.51°, 25.11°, and 29.43°. These peaks align with the distinctive crystalline pattern of NIM [19]. The SD-CHEM samples exhibited diffraction peaks at 2θ values of 18.51°, 21.41°, 26.61°, and 29.67°. In the SE-CHEM samples, almost indistinct diffraction peaks at 2θ values of 21.2° and 23° were observed, along with significantly broadened peaks at 26.5° and 29.8°. For NIM-SD-SSNEDDS, diffraction peaks were observed at 2θ values of 18.11°, 20.9°, 22.69°, 26.18°, and 29.28°. NIM-SE-SSNEDDS displayed diffraction peaks at 2θ values of 18.24°, 22.26°, 26.30°, and 29.36°. The absence of distinctive crystalline peaks of NIM in solid SNEDDS suggests the amorphization of the drug in the formulation. This amorphization, coupled with nanometric sizes and the absence of crystallinity, contributes to the anticipated improved dissolution behaviour in self-nanoemulsifying formulations. Importantly, the investigation indicates no signs of NIM precipitation when incorporated into S-SNEDDS.

Fig. 4 [Images not available. See PDF.]

XRD analysis of NIM, SD-CHEM, SE-CHEM, NIM-SD-SSNEDDS, and NIM-SE-SSNEDDS

Scanning electron microscopy

To comprehend the structure and surface characteristics of the solid formulations, scanning electron microscope (SEM) images of NIM, SD-CHEM, SE-CHEM, NIM-SD-SSNEDDS, and NIM-SE-SSNEDDS were captured (as depicted in Fig. 5). In Fig. 5 (a,b), the pure NIM sample is illustrated, and Fig. 5c displays SD-CHEM, exhibiting a spherical shape with uneven surfaces and substantial void spaces, facilitating effective oil adsorption and desorption. Figure 5d showcases SE-CHEM, presenting a flaky appearance with an irregular surface, providing an increased surface area for the adsorption of liquid SNEDDS. Figure 5e depicts NIM-SD-SSNEDDS with a size range of 10–15 µm. The adsorption process with NIM-SNEDDS is evident in these images, as seen by the smoothing of uneven surfaces and void spaces present in SD-CHEM. Likewise, in Fig. 5f, NIM-SE-SSNEDDS exhibits smooth, flaky structures compared to SE-CHEM, with a 20–25 µm size range.

Fig. 5 [Images not available. See PDF.]

Surface morphology of **A** and **B** NIM, **C** SD-CHEM, **D** SE-CHEM, **E** NIM-SD-SSNEDDS, and **F** NIM-SE-SSNEDDS

FTIR spectroscopy

Figure 6 shows all the FTIR spectra. The absorption bands of 3390.4 cm^{-1}, 3371.4 cm^{-1} and 3391 cm^{-1} confirm the O–H stretching, and the absorption bands 2922.1 cm^{-1}, 2875.3 cm^{-1}, and 2873.5 cm^{-1} correspond to the C–H bonds, the absorption peaks, 1249.5 cm^{-1}, 1249.6 cm^{-1} and 1249.4 cm^{-1} confirms the C–O–C stretching (ether) in Cremophor RH 40, Lipoxol 300 and PEG 400, respectively [46–48]. The absorption peak at 1782.5 cm^{-1} confirms the carbonyl group (of ester) in Cremophor RH 40. The absorption bands near 1457 cm^{-1} confirm CH_2 with bending vibrations in Lipoxol 300 and PEG 400, respectively. SE-CHEM and SD-CHEM displayed absorption bands within the range of 1676–1657 cm^{-1} and 1693–1667 cm^{-1}, respectively, indicating the presence of the amide linkage.

Additionally, they exhibited absorption bands between 2378 and 2373 cm^{-1}, suggesting the presence of free acetate moieties. This implies that not all acetate moieties are engaged in the amide linkage. The FTIR spectra of NIM showed all the fingerprint bands of it [49]. The absorption band at 3295.2 cm^{-1} confirms the presence of primary and secondary amines, indicated by N–H stretching vibrations. The absorption band within the 2967.4–2879.5 cm^{-1} range corresponds to alkyl groups, specifically C–H stretching vibrations. The absorption band at 1693.4 cm^{-1} confirms the presence of the ester group, as indicated by C=O stretching vibration. Furthermore, 1645 cm^{-1} and 1620 cm^{-1} absorption bands confirm C=C stretching and C=C aromatic functionalities. Additionally, absorption bands at 1521.4 cm^{-1}, 1381.9 cm^{-1}, and 1133.2 cm^{-1} are attributed to $–NO_2$, $–C–CH_3$, and –C–O– ester, respectively.
Fig. 6 [Images not available. See PDF.]

FTIR spectra of **A** Cremophor RH 40, **B** Lipoxol 300, **C** PEG 400, **D** NIM-SNEDDS, **E** NIM-SD-SSNEDDS, **F** NIM-SE-SSNEDDS, **G** NIM, **H** SD-CHEM, and **I** SE-CHEM

In the spectra of NIM-SNEDDS, NIM-SD-SSNEDDS, and NIM-SE-SSNEDDS, no notable changes were observed concerning the characteristic peaks of NIM. In the NIM-SNEDDS spectra, absorptions were detected at 3440.5 cm^{-1}, 3085.6 cm^{-1}, 2871.2 cm^{-1}, 1647.1 cm^{-1}, 1460 cm^{-1}, and 1108.9 cm^{-1}. The NIM-SD-SSNEDDS spectra exhibited absorptions at 3392.6 cm^{-1}, 2919 cm^{-1}, 2853.5 cm^{-1}, 1629.3 cm^{-1}, 1472 cm^{-1}, and 1106.7 cm^{-1}. Similarly, the NIM-SE-SSNEDDS spectra displayed absorptions at 3424.1 cm^{-1}, 2923.3 cm^{-1}, 1629.3 cm^{-1}, 1459.6 cm^{-1}, and 1107 cm^{-1}. Some peaks exhibited broadening and negligible shifts, potentially attributed to hydrogen bonding between the drug and surfactants [19]. The occurrence of hydrogen bonding could lead to shifts in wavelengths, reducing intensities, or even causing peaks to disappear [50].

DSC analysis

DSC analysis was employed to detect potential polymorphic changes induced by the interaction between the drug and excipients. The thermograms of NIM, NIM-SNEDDS, NIM-SD-SSNEDDS, and NIM-SE-SSNEDDS were obtained and are presented in Fig. 7. The characteristic endothermic peak of NIM, signifying its crystallinity, was observed at 128 °C [51]. In NIM-SNEDDS and NIM-SD-SSNEDDS, this endothermic peak disappeared, while in NIM-SE-SSNEDDS, a broad peak near 128 °C was observed. The disappearance of the characteristic endothermic peak indicates that the NIM is in amorphous form in formulations.
Fig. 7 [Images not available. See PDF.]

DSC curves of **A** NIM, **B** NIM-SNEDDS, **C** NIM-SD-SSNEDDS, and **D** NIM-SE-SSNEDDS

Reconstituted nanoemulsion and NIM-SNEDDS evaluations

Droplet size, size distribution, and zeta potential determination

Determining droplet size is crucial in assessing the self-nanoemulsification characteristics of the system, as it influences drug release and absorption. Smaller droplet sizes in the nanometric range provide a larger interfacial surface area, facilitating better drug absorption. In Table 2, the droplet sizes (and polydispersibility index-PDI) of NIM-SNEDDS, NIM-SD-SSNEDDS, and NIM-SE-SSNEDDS are 245.5 nm (PDI–0.715), 247.1 nm (PDI–0.620), and 399.6 nm (PDI–0.821). The results show that the droplet size of NIM-SD-SSNEDDS is comparable to NIM-SNEDDS, while NIM-SE-SSNEDDS exhibits a significant difference. PDI values for all formulations are closely aligned. Thus, SD-CHEM and SE-CHEM surfaces exhibit small droplet sizes and similar PDI. SD-CHEM (NIM-SD-SSNEDDS), as an adsorbent, outperforms SE-CHEM in producing small droplets and PDI. The zeta potential results for NIM-SNEDDS, NIM-SD-SSNEDDS, and NIM-SE-SSNEDDS are 1.961 mV, 1.353 mV, and 1.351 mV, respectively.

Self-emulsification time

The emulsification times for NIM-SNEDDS, NIM-SD-SSNEDDS, and NIM-SE-SSNEDDS were recorded as 24–36, 38–41, and 40–48 s, respectively, as outlined in Table 2. This indicates that all systems possess the capability to disperse swiftly in aqueous conditions with agitation.

Per cent transmittance test

The outcomes of per cent transmittance for NIM-SNEDDS, NIM-SD-SSNEDDS, and NIM-SE-SSNEDDS, as

depicted in Table 2, exceeded 90%, substantiating the effectiveness of self-emulsification. This transparency further validates the stability of the reconstituted nanoemulsion and eliminates the risk of drug precipitation [52].

Estimation of cloud point

The cloud point temperature signifies the temperature at which the clear appearance of the SNEDDS transforms into a cloudy or turbid state, indicating a change in phase behaviour. This temperature should ideally be higher than the anticipated usage temperature, typically 37 °C. In the case of NIM-SNEDDS, NIM-SD-SSNEDSS, and NIM-SE-SSNEDDS, all recorded cloud point temperatures were above 37 °C, affirming their thermodynamic stability [53].

Field emission scanning electron microscopy

The FE-SEM images in Fig. 8a–c illustrate the reconstituted NIM-SD-SSNEDDS, while Fig. 8d–f displays NIM-SE-SSNEDDS, both captured at various magnifications. The images clearly depict that the reconstituted globules from both solid SNEDDS forms were spherical. Additionally, these images corroborate with the sizes analysed through Zetasizer, as indicated in Table 2. The globules formed exhibited fine sizes, and there was no coalescence evidence, implying the reconstituted nanoemulsions' stability.

Fig. 8 [Images not available. See PDF.]

FE-SEM images (at different magnifications) **A**, **B,** and **C** reconstituted nanoemulsion NIM-SD-SSNEDDS, **C** and **D** reconstituted nanoemulsion NIM-SE-SSNEDDS

Dissolution profile (in vitro)

The in vitro dissolution test investigated the release of pure NIM, NIM-SNEDDS, NIM-SD-SSNEDDS, and NIM-SE-SSNEDDS, with the per cent cumulative drug release (% CDR) graph displayed in Fig. 9. In six hours, the % CDR for pure NIM, NIM-SNEDDS, NIM-SD-SSNEDDS, and NIM-SE-SSNEDDS was 28%, 88.84%, 84.43%, and 76.68%, respectively. Notably, there was a significant increase in drug dissolution for NIM in both NIM-SD-SNEDDS and NIM-SE-SSNEDDS compared to pure NIM. This study has revealed the improvement of dissolution of the drug after formulating into the SNEDDS and S-SNEDDS, this must be due to the loading of the drug into the micelles of the SNEDDS [54].

Fig. 9 [Images not available. See PDF.]

Dissolution profile (% cumulative drug release) of **A** NIM **B** NIM-SE-SSNEDDS, **C** NIM-SD-SSNEDDS, and **D** NIM-SNEDDS

Ex vivo permeability profile

This investigation assessed drug permeability through a biological membrane as a diffusion barrier. A comparative analysis was conducted for pure NIM, NIM-SNEDDS, reconstituted NIM-SD-SSNEDDS, and NIM-SE-SSNEDDS. Figure 10 presents the cumulative amount permeated per cm^2, while Fig. 11 illustrates the flux and permeability constant. Flux, represented as the drug permeated per unit area per unit time ($\mu g/min/cm^2$) $\times 10^{-2}$ (slope of the permeability curve), and permeability constant, defining the ease of drug diffusion from the permeable membrane (flux/drug conc.), were evaluated. The results indicated that the cumulative drug permeability (%) for NIM, NIM-SNEDDS, NIM-SD-SSNEDDS, and NIM-SE-SSNEDDS was 2.93, 25.02, 22.6, and 21.9, respectively. In Fig. 11, higher values of both flux and permeability constant of NIM-SNEDDS, NIM-SD-SSNEDDS, and NIM-SE-SSNEDDS as compared to NIM, indicated the enhancement in permeation across the biological membrane and facilitation of the drug across the membrane by SNEDDS and S-SNEDDS, respectively.

Fig. 10 [Images not available. See PDF.]

Ex vivo permeation profile of **A** NIM **B** NIM-SE-SSNEDDS, **C** NIM-SD-SSNEDDS, and **D** NIM-SNEDDS

Fig. 11 [Images not available. See PDF.]

Ex vivo permeation parameters of NIM, NIM-SE-SSNEDDS, NIM-SD-SSNEDDS, and NIM-SNEDDS. Flux refers to the quantity of drug that permeates a diffusion barrier per unit area and time, expressed as micrograms per minute

per square centimetre (μg/min/cm^2) × 10^{-2} (the slope of the permeability curve). The permeability constant is calculated as the ratio of flux to drug concentration

Stability study

The summarized results of the stability study are presented in Table 3. Analysis of the data reveals that both formulations, NIM-SD-SSNEDDS and NIM-SE-SSNEDDS, remained stable throughout the six-month accelerated stability testing at 40 ± 2 °C/75 ± 5% RH. Therefore, it can be concluded that both NIM-SD-SSNEDDS and NIM-SE-SSNEDDS successfully passed the stability assessment.

Table 3. Stability data of the NIM-SD-SSNEDDS and NIM-SE-SSNEDDS

Formulation	Test parameters	Initial	1 month (40 ± 2 °C/75 ± 5% RH)	3 months (40 ± 2 °C/75 ± 5% RH)	6 months (40 ± 2 °C/75 ± 5% RH)
NIM-SD-SSNEDDS	Description	Yellow opaque capsules with SSNEDDS (No change in appearance)	Yellow opaque capsules with SSNEDDS (No change in appearance)	Yellow opaque capsules with SSNEDDS (No change in appearance)	Yellow opaque capsules with SSNEDDS (No change in appearance)
%CDR	84.43% ± 1.3	83.08% ± 2.1	83.88% ± 1.7	82.59% ± 0.9	Disintegration time (min.)
6 ± 0.3	6 ± 0.8	7 ± 1.4	7 ± 0.9	NIM-SE-SSNEDDS	Description
Yellow opaque capsules with SSNEDDS (No change in appearance)	Yellow opaque capsules with SSNEDDS (No change in appearance)	Yellow opaque capsules with SSNEDDS (No change in appearance)	Yellow opaque capsules with SSNEDDS (No change in appearance)	%CDR	76.68% ± 2.3
76.43% ± 2.5	75.94% ± 1.4	75.13% ± 1.8	Disintegration time (min.)	7 ± 0.5	7 ± 1.1

% CDR percentage of cumulative drug release, values are presented as mean ± standard deviation (S.D.), (n = 3)

Discussion

Because of the number of advantages of solid SNEDDS over SNEDDS, this study aimed to formulate solid self-nanoemulsifying drug delivery systems (S-SNEDDS) for nimodipine (NIM). NIM used as a model drug remains a suitable candidate for SNEDDS due to low hydrophilicity and high lipophilicity. Cremophor RH 40, Lipoxol 300, and PEG 400 were chosen after checking how well they dissolve and form emulsions. A ternary phase diagram determined the optimal oil to Smix (surfactant/co-surfactant) ratio (40:60). The liquid NIM-SNEDDS was used as the material to adsorb on microparticles synthesized from chitosan EDTA by spray drying (SD-CHEM) and solvent evaporation (SE-CHEM) as the absorbents to make solid SNEDDS. Both solid forms had good drug loading (NIM-SD-SSNEDDS = 79.67 ± 2.97%, NIM-SE-SSNEDDS = 77.76 ± 4.29%) and excellent flow properties. XRD analysis revealed drug amorphization as the characteristic diffraction peaks were absent; this offers a great advantage in improvement of drug dissolution.

SEM images revealed smoothening and filling of adsorbent surfaces by adsorbate. For NIM-SD-SSNEDDS, the size was 10–15 μm, and for NIM-SE-SSNEDDS, it was 20–25 μm. The SEM images provide visual evidence of the effective adsorption behaviour of microparticles, validating the excellent adsorption of liquid SNEDDS for both SD-CHEM and SE-CHEM.

FTIR confirmed no interaction of drug and excipients with the broadening of some characteristic drug peaks in the solid formulations because of the hydrogen bonding with surfactants. DSC analysis also confirmed the amorphization of the drug with the disappearance of the characteristic crystalline peaks of NIM.

Stability assessments confirmed the physical and thermodynamic robustness of reconstituted nanoemulsions, considering parameters such as droplet size, polydispersity index (PDI), zeta potential, emulsification time, per cent transmittance, and cloud temperature. For NIM-SD-SSNEDDS, these values were observed as follows: droplet size of 247.1 nm, PDI of 0.620, zeta potential of 1.353 mV, emulsification time ranging from 38 to 41 s, per cent transmittance of 94.64%, and a cloud temperature of 54 °C. On the other hand, for NIM-SE-SSNEDDS, the corresponding values were 399.6 nm, PDI 0.821, zeta potential 1.351 mV, emulsification time ranging from 40 to 48 s, per cent transmittance of 92.96%, and a cloud temperature of 49 °C. These findings suggest that solid SNEDDS closely resemble liquid SNEDDS, indicating the stability of the reconstituted nanoemulsion.

FE-SEM images displayed the formation of droplets characterized by small sizes, with no observable evidence of coalescence, suggesting the stability of the reconstituted nanoemulsions. In vitro dissolution studies indicated a notable fourfold enhancement in drug dissolution for NIM-SD-SSNEDDS (84.43%) and NIM-SE-SSNEDDS (76.68%) when contrasted with the pure drug (28%). The ultimate drug release from liquid SNEDDS and solid SNEDDS exhibited similar patterns, demonstrating comparable release profiles. Ex vivo permeation studies revealed nearly identical profiles for NIM-SD-SSNEDDS (22.61%) and NIM-SE-SSNEDDS (21.93%) when compared to NIM-SNEDDS (25.02%). Stability study of both NIM-SD-SSNEDDS and NIM-SE-SSNEDDS confirmed that the preparations were stable for six months of accelerated stability study. Notably, NIM-SD-SSNEDDS demonstrated superior performance compared to NIM-SE-SSNEDDS, emphasizing the effectiveness of microparticles developed through the spray drying method (SD-CHEM) as adsorbents for solidification. These findings suggest that NIM-SD-SSNEDDS and NIM-SE-SSNEDDS can generate nanosized droplets in nanoemulsion containing the drug, facilitating successful drug diffusion across the biological membrane. These findings indicate improved dissolution and permeation for nimodipine in both solid SNEDDS formulations.

Conclusion

This study aimed to develop effective solid self-nanoemulsifying drug delivery systems (SNEDDS) for nimodipine with Cremophor RH 40, Lipoxol 300, and PEG 400 as the oil, surfactant, and co-surfactant. SNEDDS was formulated with an oil to Smix (surfactant/co-surfactant) ratio of 40:60, determined through a ternary phase diagram. Solid SNEDDS were created using previously developed microparticles (SD-CHEM and SE-CHEM) as adsorbents for NIM-SD-SSNEDDS and NIM-SE-SSNEDDS, respectively, with NIM-SNEDDS as an adsorbate for both. The final adsorbate (liquid NIM-SNEDDS): adsorbent ratio for NIM-SD-SSNEDDS and NIM-SE-SSNEDDS was 1:2.5. Both

formulations exhibited favourable drug loading and flow properties. XRD analysis confirmed drug amorphization in both formulations, and SEM images verified uniform adsorption of liquid SNEDDS over solid microparticles. FTIR analysis indicated no interactions between excipients, while DSC analysis revealed the amorphous form of the drug in both solid SNEDDS. Both formulations showed nanometric globule sizes with minimal polydispersity indices. Stability studies and FE-SEM images confirmed reconstituted nanoemulsions' physical and thermodynamic stability. In vitro dissolution studies demonstrated nearly a fourfold increase in drug dissolution compared to pure drug. Both in vitro and ex vivo permeation studies revealed similar dissolution and permeation profiles for NIM-SD-SSNEDDS and NIM-SE-SSNEDDS compared to NIM-SNEDDS. Notably, NIM-SD-SSNEDDS outperformed NIM-SE-SSNEDDS, indicating the superior performance of microparticles developed by the spray drying method (SD-CHEM) as adsorbents for solidification. Enhanced dissolution and permeation for nimodipine in solid SNEDDS were achieved. Both the formulation systems have proved their efficacy as S-SNEDDS, and the chitosan EDTA microparticle developed by spray drying and solvent evaporation can be utilized in future studies of different drugs SNEDDS to fabricate solid SNEDDS.

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Author contributions

MK carried out formulation and evaluation and wrote the original draft. PAC supported in FTIR, DSC and XRD analysis and interpretations. AF and VC contributed to reviewing, editing, and supervision.

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Declarations

Ethical approval and consent of participate

None.

Consent for publication

None.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

NIM

Nimodipine

BCS

Biopharmaceutical classification system

SNEDDS

Self-nanoemulsifying drug delivery system

GIT

Gastrointestinal tract

S-SNEDDS

Solid self-nanoemulsifying drug delivery systems

NIM-SNEDDS

Liquid self-nanoemulsifying drug delivery system of the nimodipine

SD-CHEM

Spray-dried chitosan-EDTA microparticles

SE-CHEM

Solvent-evaporated chitosan-EDTA microparticles

S-SNEDDS

Solid self-nanoemulsifying drug delivery system
NIM-SD-SSNEDDS
Nimodipine solid self-nanoemulsifying drug delivery system with spray-dried microparticles
NIM-SE-SSNEDDS
Nimodipine solid self-nanoemulsifying drug delivery system with solvent-evaporated microparticles
XRD
X-ray diffraction
SEM
Scanning electron microscopy
FTIR
Fourier-transform infrared spectroscopy analysis
DSC
Differential scanning calorimetry
FE-SEM
Field emission scanning electron microscopy
PDI
Polydispersibility index
%CDR
% Cumulative drug release

Publisher's Note

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Analytical quality by design (AQbD) based optimization of RP-UPLC method for determination of nivolumab and relatlimab in bulk and pharmaceutical dosage forms

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ABSTRACT (ENGLISH)

Background

The Analytical Quality by Design (AQbD) methodology extends the application of Quality by Design (QbD) principles to the management of the analytical procedure life cycle, encompassing method creation, optimization, validation, and continuous improvement. AQbD assists in creating analytical procedures that are robust, reliable, precise, and cost-efficient. Opdualag™ is a combination of Nivolumab and Relatlimab, which are antibodies that block programmed death receptor-1 (PD-1) and lymphocyte-activation gene 3 (LAG-3) receptors, used to treat advanced melanoma. This work aims to develop and validate a reversed-phase ultra-performance liquid chromatography (RP-UPLC) method using AQbD principles to determine NLB and RTB in pharmaceutical products.

Results

A central composite design (CCD) comprising three factors arranged in five distinct levels was implemented via Design-expert® software to optimize the chromatographic conditions. A mathematical model was constructed and the effects of three independent factors namely flow rate (X_1), percentage of methanol in the mobile phase (X_2), and temperature (X_3) on responses including retention time ($Y_{1–2}$), resolution factor (Y_3), theoretical plates ($Y_{4–5}$), and tailing factor ($Y_{6–7}$) were investigated. The software determined the optimal chromatographic conditions for the separation of NLB and RTB, which were as follows: 32.80% methanol in the mobile phase, 0.272 mL/min flow rate, 29.42 °C column temperature, and 260 nm UV detection. The retention time for NLB and RTB were 1.46 and 1.88 min, respectively. The method exhibited linearity across the concentration ranges of 4–24 µg/mL for RTB and 12–72 µg/mL for NLB. The limits of detection (LOD) and limit of quantification (LOQ) for NLB and RTB, respectively,

were 0.89 µg/mL, 2.69 µg/mL and 0.15 µg/mL and 0.46 µg/mL. The percentage relative standard deviation (%RSD) of intraday and interday precision for NLB and RTB was below 2. The recovery percentages for NLB and RTB were determined to be 99.57–100.43% and 99.59–100.61%, respectively. Both drugs were found to be susceptible to oxidative and photolytic degradation in forced degradation studies.

Conclusions

Employing the AQbD-based methodology, a straightforward, fast, accurate, precise, specific, and stability-indicating RP-UPLC method has been established for the quantitative analysis of NLB and its RTB in pharmaceutical formulations.

FULL TEXT

Background

Melanoma is the most aggressive form of skin cancer that develops from melanocytes, the skin pigment-producing cells. Although melanoma only makes up a small fraction of skin malignancies (approximately 1–2%), it is the leading cause of skin cancer-related mortality. The 5-years survival rate for melanoma is 94% when detected early, but it drops dramatically after the cancer has spread to other parts of the body. Melanoma that has progressed beyond its initial skin location to other organs, lymph nodes, or distant tissues is known as metastatic melanoma. Surgical procedures, radiation treatments, chemo, immunotherapy, and targeted therapies are all viable treatment choices [1, 2].

Among all, treatment with immunotherapy drugs such as immune checkpoint inhibitors and interleukin-2 significantly improved the outcome in patients by boosting the body's immune response to selectively target and destroy cancer cells [3]. Recently (March 18, 2022), the United States Food and Drug Administration (FDA) approved a fixed dose combination of two immunotherapy antibodies Nivolumab and Relatlimab (Opdualag™) for adults and paediatric patients (12 years or above) with unresectable and metastatic melanoma [4] (Fig. 1). Both drugs are immune checkpoint inhibitors and act by restoring the T cell's natural ability to target cancer cells by suppressing immune checkpoint proteins, which prevent excessive immune responses. Nivolumab blocks programmed cell death protein 1 (PD-1) in immune cells (T cells) and is widely used to treat melanoma and other cancers. Relatlimab blocks Lymphocyte-activation gene 3 (LAG-3) in immune cells and restores the effector function of exhausted T cells [5]. This combination therapy results in longer progression-free survival and fewer side effects in patients when compared to Nivolumab plus Iipilimumab and Nivolumab alone [6–8]. Opdualag™ is available as an intravenous injection containing 240 mg of NLB and 80 mg of RTB in 20 mL clear to opalescent, colorless to slightly yellow solution in a single-dose vial. Nevertheless, it is not included in any of the official pharmacopoeial monographs. Hence it is of utmost importance to develop an analytical method for estimation of NLB and RTB in commercially available formulations.

Fig. 1 [Images not available. See PDF.]

Structure of Nivolumab (**A**) and Relatlimab (**B**)

The analytical Quality by Design (AQbD) approach is an extension of the quality by design (QbD) concept, used in the pharmaceutical industry to ensure the quality of analytical methods. This approach emphasizes a thorough understanding of the method's critical parameters and their impact on the analytical results, leading to more efficient and effective analytical development and validation processes. AQbD helps in the identification and optimization of significant factors, and their interaction effect and provides appropriate chromatographic conditions for the estimation of analyte. A central composite design is the most predominant experimental design used in response surface methodology for the optimization of chromatographic conditions. These designs provide a great deal of information with a minimum number of experimental trials and are very cost effective [9–11].

The literature study revealed that there are limited analytical methods available for the determination of NLB alone and in combination with different drugs in plasma and pharmaceutical dosage forms. These methods include RP-UPLC [12], LC–MS/MS [13], ELISA [14], LC–MS/HRMS [15], UPLC-MS/MS [16], and the UHPLC/UV-

(HESI/Orbitrap™) MS approach [17]. An RP-UPLC approach was developed to simultaneously determine NLB and RTB in Opdualag™ formulations [18]. However, no analytical method has been reported for the estimation of NLB and RTB using AQbD principles.

The current work focused on the development and optimization of an RP-UPLC method using AQbD principles (central composite design) for the estimation of NLB and RTB in pharmaceutical products.

Methods

Chemicals

Reference standards of NLB (purity 99.80%), and RTB (purity 99.80%) were obtained from Akrivis Pharma Private Limited, Hyderabad. Fisher Scientific (Mumbai, India) provided HPLC-grade methanol and acetic acid. The ultrapure water was acquired from the Millipore Direct-Q®3 UV water purification equipment by Merck Millipore in India. All remaining reagents and chemicals met analytical grade standards.

Instrumentation and chromatographic conditions

The method was developed using a Waters Acquity UPLC system H-class equipped with binary pumps, a tunable UV (TUV) detector, and an autosampler. The Empower 2 software was used for data acquisition and processing. A BEH C18 column (50 × 2.5 mm i.d. particle size of 1.7 µm) was used to accomplish the chromatographic separation. The mobile phase is made up of 32.8:67.2% v/v methanol and 0.01 N phosphate buffer, which is pumped at a flow rate of 0.27 mL/min. The temperature of the column was kept at 29.4 °C, and 260 nm was used for detection. The overall chromatographic run time was 3 min and the injection volume was 5 µL. A 50/50 v/v mixture of methanol and water was utilized as the diluent.

Preparation of standard stock solutions

NLB (24 mg) and RTB (8 mg) were accurately weighed and transferred to a 50 mL clean dry volumetric flask. Add 10 mL of diluent and sonicate for 10 min to completely dissolve them. The volume was increased to 50 mL with diluent to achieve concentrations of 480 µg/mL for NLB and 160 µg/mL for RTB. To create working standard solutions, aliquots of each stock solution were diluted with the diluent to achieve concentrations of 12, 24, 36, 48, 60, and 72 µg/mL for NLB and 4, 8, 12, 16, 20, and 24 µg/mL for RTB.

Preparation of sample solution

Transfer 20 mL of Opdualag™ solution (240 mg NLB and 80 mg RTB) to a 100 mL volumetric flask. Add 50 mL of diluent and sonicate for 25 min. The volume was increased to 100 mL with diluent to achieve concentrations of 2400 µg/mL of NLB and 800 µg/mL of RTB. To acquire the final concentrations of NLB (18 µg/mL) and RTB (16 µg/mL), 0.2 mL of the aforesaid solutions was diluted to 10 mL. The resulting solution was utilized for the assay of NLB and RTB in its marketed formulation.

Method optimization by experimental design

The developed method's chromatographic conditions were optimized using Design-expert® software (Version 11.1.0.1, Stat-Ease Inc., USA) employing a central composite design (CCD) with three factors at five levels (−α, −1, 0, +1, and +α). The mobile phase was chosen based on preliminary investigation using methanol and 0.01N phosphate buffer. The three independent variables chosen were flow rate (X_1),% methanol (X_2), and temperature (X_3), while the dependent variables were retention time of NLB (Y_1), retention time of RTB (Y_2), resolution factor (Y_3), number of theoretical plates of NLB (Y_4), number of theoretical plates of RTB (Y_5), tailing factor of NLB (Y_6), and tailing factor of RTB (Y_7) (Table 1). The Design expert® program proposed twenty runs based on the central composite design. All experiments employed a standard concentration of 48 µg/mL of NLB and 16 µg/mL of RTB.

Table 1. Variables and their levels used in central composite design

Name	−α	−1	0	+1	+α
Independent variables					

X_1: Flow rate (mL/min)	0.2495	0.27	0.30	0.33	0.3505
X_2: % methanol (v/v)	26.59	30.00	35.00	40.00	43.41
X_3: Temperature (°C)	24.95	27.00	30.00	33.00	35.05
Dependent variables					
Y_1: Retention time of NLB					
Y_2: Retention time of RTB					
Y_3: Resolution factor					
Y_4: Number of theoretical plates of NLB					
Y_5: Number of theoretical plates of RTB					
Y_6: Tailing factor of NLB					
Y_7: Tailing factor of RTB					

Method validation

Following ICH Q2 (R1) criteria, the developed RP-UPLC method was validated for system suitability, linearity, LOD, LOQ, precision, accuracy, and robustness [19]. System suitability testing involves injecting six replicates of NLB (48 µg/mL) and RTB (16 µg/mL) standard solutions, evaluating their theoretical plates, tailing factor, resolution, % RSD of retention time and peak area. The linearity of the method was verified by plotting the calibration curve of peak area against concentration for six concentrations of NLB (12–72 µg/mL) and RTB (4–24 µg/mL) working standard solutions. LOD and LOQ were determined using the standard deviation (σ) and slope (S) of the calibration curve: $LOD = 3.3 \times \sigma/S$ and $LOQ = 10 \times \sigma/S$. The method's precision was confirmed by intra-day and inter-day variance investigations. Three concentrations of NLB (12, 36, and 72 µg/mL) and RTB (4, 12, and 24 µg/mL) were tested three times per day for intra-day precision (repeatability), whereas three concentrations on three different days were assessed for inter-day accuracy (intermediate precision). Accuracy was assessed by computing the mean percentage recovery of NLB and RTB standard solutions spiked at different concentration levels (50, 100, and 150%) to the pre-analyzed NLB and RTB samples. The method's specificity was verified by comparing the representative chromatograms of the blank, placebo, and NLB/RTB standard solutions. Robustness was evaluated by purposefully modifying the ideal chromatographic parameters, including flow rate (0.1 and 0.3 mL), methanol percentage (±5% in mobile phase), and column temperature (±5 °C).

Sample solution stability

The stability of the NLB and RTB in the solution was determined by keeping the samples in a volumetric flask at normal ambient laboratory conditions for a period of 24 h. After 24 h, the retention time and peak area of the NLB and RTB were calculated and compared against the initial readings.

Forced degradation studies

Forced degradation studies of NLB and RTB were carried out in various stress conditions. The NLB and RTB solutions were exposed to acid (2N HCl, 60 °C for 30 min), alkaline (2N NaOH, 60 °C for 30 min), dry heat (105 °C for 6 h), oxidizing (60% H_2O_2 60 °C for 30 min), neutral (water at 60 °C for 6 h) and photolytic (UV light for 7 days)

degradation. Following the exposure, the resultant solutions were diluted to obtain 18 µg/mL of NLB and 16 µg/mL of RTB. Five microliters of each solution were then injected into the system, and chromatograms were recorded to evaluate the sample's stability.

Statistical analysis

Data were reported as mean ± SD. The regression coefficient, mean, SD, and % RSD were calculated in Excel. Model and model term significance was determined using Analysis of variance (ANOVA). Model and model terms were significant if the *p*-value was less than 0.05.

Results

Method development studies

The development of the LC method involves the use of complex mobile phases with various solvents (acetonitrile, methanol, ethanol, water and phosphate buffer at a specific pH), different modes of flow (isocratic/gradient), different columns (C_{18}, and C_8) and temperature settings for the separation of compounds. The preliminary trials suggested the use of methanol and 0.01N phosphate buffer as suitable mobile phase for the separation of NLB and RTB with excellent peak shape, shorter retention time and less tailing.

Method optimization by experimental design

The present study employed a central composite design with 20 trials to examine the effect of three independent variables on seven dependent variables. The independent variables include flow rate (X_1), % methanol (X_2), and temperature (X_3). The dependent variables are retention time of NLB (Y_1), retention time of RTB (Y_2), resolution factor (Y_3), number of theoretical plates of NLB (Y_4), number of theoretical plates of RTB (Y_5), tailing factor of NLB (Y_6), and tailing factor of RTB (Y_7). The results are summarized in Table 2. The impact of the independent variables on each dependent variable was examined by fitting the collected responses to different mathematical models and then generating second-order polynomial equations. Analyzing the model and its terms using analysis of variance (ANOVA) allowed us to establish their statistical significance. To better understand the relationship between the dependent and independent variables, 3D-response surface plots and 2D-contour plots were generated. Finally, the numerical optimization technique was used to predict the optimal chromatographic conditions based on the given acceptance criteria.

Table 2. Central composite design along with the observed values for optimization of RP-UPLC method for NLB and RTB

Run no.	X_1:Flow rate (mL/min)	X_2:% MeOH (%/v/v)	X_3:Tem (°C)	Y_1:Retention time NLB (min)	Y_2: Retention time RTB (min)	Y_3: Resolution factor	Y_4: Theoretical plates NLB	Y_5:Theoretical plates RTB	Y_6:Tailing factor NLB	Y_7: Tailing factor RTB
1	0.33	40	27	1.382	1.715	2.8	3088.50	4164.70	1.39	1.21
2	0.30	35	30	1.334	1.652	3.3	3436	4305	1.30	1.28
3	0.30	35	30	1.335	1.652	3.3	3445	4359	1.30	1.28
4	0.27	40	27	1.705	2.305	3.6	4578.90	6318	1.38	1.12
5	0.27	30	27	1.654	2.207	4.4	4398.90	6053.50	1.30	1.36
6	0.33	30	33	1.118	1.676	3.4	1998	2268.60	1.25	1.29

7	0.30	26.591	30	1.414	1.764	3.8	3330.50	4364.80	1.41	1.32
8	0.30	35	35.0454	1.108	1.567	3.3	3076.90	3209.50	1.23	1.30
9	0.30	35	24.9546	1.716	2.130	4.0	4279.70	5601.70	1.26	1.22
10	0.33	40	33	1.011	1.382	2.5	2232.30	3000.40	1.21	1.40
11	0.2495	35	30	1.618	2.282	4.1	4234.70	6378.60	1.39	1.26
12	0.30	43.409	30	1.237	1.542	2.4	3817	5071.90	1.45	1.19
13	0.33	30	27	1.375	1.751	3.6	3468.90	4257.90	1.36	1.27
14	0.30	35	30	1.335	1.653	3.3	3454	4314	1.29	1.28
15	0.27	40	33	1.282	1.758	2.9	4663.50	5506.60	1.45	1.20
16	0.3505	35	30	1.055	1.451	2.8	2012.40	2540.40	1.24	1.33
17	0.30	35	30	1.334	1.652	3.3	3406	4328	1.30	1.28
18	0.30	35	30	1.335	1.652	3.4	3418	4378	1.30	1.28
19	0.27	30	33	1.453	1.880	3.9	3680.20	4815.50	1.40	1.26
20	0.30	35	30	1.334	1.652	3.2	3412	4310	1.30	1.27

Fitting of responses to the model

The best-fit model was determined by fitting the observed responses from all 20 runs to each of the mathematical models using the Design-Expert program. All models' values of SD, correlation coefficient (R^2), adjusted and predicted R^2s, coefficient of variation (CV), and predicted residual sums of squares (PRESS) are displayed in Table 3. High R^2 values, low SD, CV, and PRESS, and close proximity between adjusted and predicted R^2 values were the criteria for selecting the best-fitting model across all responses. For all the responses, (Y_1–Y_7), the quadratic model was found to be the best fit.

Table 3. Regression analysis for different responses Y_1 to Y_7 for fitting to different polynomial models

Models	SD	R^2	Adjusted R^2	Predicted R^2	PRESS	CV (%)	Remark
Response (Y_1): Retention time of NLB (min)							

Linear	0.0464	0.9555	0.9472	0.9205	0.0615	3.42	Suggested
2FI	0.0394	0.9738	0.9618	0.9119	0.0681	2.91	Quadratic
0.0263	0.9910	0.9829	0.9289	0.0550	1.94	Cubic	0.0096
0.9993	0.9978	0.8443	0.1203	0.70	Response (Y_2): Retention time of RTB (min)		
Linear	0.1250	0.8108	0.7754	0.6936	0.4050	7.08	Suggested
2FI	0.1185	0.8618	0.7981	0.6034	0.5243	6.71	Quadratic
0.0354	0.9905	0.9820	0.9278	0.0954	2.01	Cubic	0.0267
0.9968	0.9898	0.2868	0.9429	1.51	Response (Y_3): Resolution factor		
Linear	0.1698	0.9110	0.8943	0.8439	0.8097	5.05	Suggested
2FI	0.1727	0.9252	0.8907	0.8548	0.7528	5.13	Quadratic
0.0649	0.9919	0.9846	0.9618	0.1983	1.93	Cubic	0.0593
0.9959	0.9871	0.9477	0.2714	1.76	Response (Y_4): Theoretical plates of NLB		
Linear	283.54	0.8839	0.8621	0.7854	2.377E+06	8.17	Suggested
2FI	188.60	0.9583	0.9390	0.8961	1.150E+06	5.43	Quadratic
98.79	0.9912	0.9833	0.9335	7.365E+05	2.85	Cubic	42.70

0.9990	0.9969	0.8192	2.002E+06	1.23	Response (Y_5): Theoretical plates of RTB		
Linear	207.95	0.9723	0.9671	0.9524	1.189E+06	4.64	Suggested
2FI	159.59	0.9867	0.9806	0.9636	9.087E+05	3.56	Quadratic
61.61	0.9985	0.9971	0.9883	2.925E+05	1.38	Cubic	27.77
0.9998	0.9994	0.9977	56,896.44	0.62	Response (Y_6): Tailing factor of NLB		
Linear	0.0673	0.2836	0.1493	− 0.2870	0.1301	5.08	Suggested
2FI	0.0570	0.5819	0.3889	0.1164	0.0893	4.30	Quadratic
0.0093	0.9914	0.9837	0.9371	0.0064	0.70	Cubic	0.0086
0.9956	0.9862	0.2194	0.0789	0.64	Response (Y_7): Tailing factor of RTB		
Linear	0.0502	0.4472	0.3436	− 0.0293	0.0751	3.95	Suggested
2FI	0.0155	0.9573	0.9376	0.8868	0.0083	1.22	Quadratic
0.0077	0.9919	0.9846	0.9453	0.0040	0.60	Cubic	0.0056

Effect of independent variables on the Retention time of NLB (Y_1)

The following quadratic equation describes the relationship between the independent variables and the retention time of NLB (Y_1).

1

Y1RetentiontimeofNLB=1.33-0.15X1-0.03X2-0.16X3+0.002X1X2-0.0005X1X3-0.042X2X3+0.0035X12-0.0004X22+0.03X32

The equation shows a negative relationship between the flow rate (X_1), percentage of methanol (X_2), and temperature (X_3) with the retention time of NLB. This indicates that when the flow rate, methanol percentage, and temperature increase, the retention time decreases. The high coefficient value of X_3 indicates that, among the three variables, temperature has the most impact on the retention time of NLB. The combined interaction terms X_1X_2, X_1X_3 and X_2X_3 have a positive effect on the retention time of NLB. Table 4 shows the results of the ANOVA for the NLB

retention time data. An F-value of 122.69 indicates statistical significance for the model. The likelihood of an F-value this high being caused by random chance is 0.01%. The significance of model terms is indicated by *p*-values below 0.0500. Significant model terms here are X_1, X_2, X_3, X_2X_3, and X_3^2. There is a reasonable match between the predicted R^2 of 0.9289 and the corrected R^2 of 0.9829. The signal-to-noise ratio is a good indicator of adequate precision. Optimal ratios are greater than 4. The obtained ratio of 39.438 suggested an adequate signal. It is possible to navigate the design space using this model. The 3D response surface plots and the associated 2D contour plots in Fig. 2A, B illustrate how independent variables affect the retention time of NLB (Y_1). It was observed from the plots that an increase in flow rate, % methanol, and temperature decreased the retention time.

Table 4. Analysis of variance (ANOVA) test results and adequate precision for various responses

Source	Responses												
Y_1		Y_2		Y_3		Y_4		Y_5		Y_6		Y_7		F-value
p-value	F-value	*p*-value	F-value	*p*-value	F-value	*p*-value	F-value	*p*-value	F-value	*p*-value	F-value	*p*-value	Model	122.69
<0.0001	115.89	<0.0001	135.89	<0.0001	125.00	<0.0001	730.29	<0.0001	128.71	<0.0001	136.02	<0.0001	X_1	490.19
<0.0001	533.21	<0.0001	382.36	<0.0001	791.53	<0.0001	4608.82	<0.0001	277.14	<0.0001	149.68	<0.0001	X_2	28.29
0.0003	30.86	0.0002	596.75	<0.0001	25.27	0.0005	149.45	<0.0001	29.68	0.0003	271.87	<0.0001	X_3	546.15
<0.0001	289.75	<0.0001	144.13	<0.0001	186.37	<0.0001	1642.03	<0.0001	24.59	0.0006	130.39	<0.0001	X_1X_2	0.0721
0.7938	9.32	0.0122	0.2972	0.5976	21.96	0.0009	3.31	0.0989	28.31	0.0003	258.88	<0.0001	X_1X_3	0.0029
0.9582	21.62	0.0009	14.56	0.0034	36.71	0.0001	40.15	<0.0001	305.68	<0.0001	111.79	<0.0001	X_2X_3	20.35
0.0011	22.75	0.0008	2.67	0.1330	25.75	0.0005	51.59	<0.0001	14.45	0.0035	258.88	<0.0001	X_1^2	0.2496

0.6282	80.04	<0.0001	8.27	0.0165	13.77	0.0040	8.30	0.0164	10.99	0.0078	5.34	0.0435	X_2^2	0.0037
0.9526	0.7378	0.4105	19.08	0.0014	5.79	0.0370	72.58	<0.0001	396.36	<0.0001	21.81	0.0009	X_3^2	18.90
0.0014	68.30	<0.0001	49.20	<0.0001	14.64	0.0033	2.91	0.1188	46.03	<0.0001	14.42	0.0035	Adequate precision	39.43

X_1, X_2 and X_3 coded levels of independent variables; X_1X_2, X_1X_3 and X_2X_3 are interaction terms; X_1^2, X_2^2 and X_3^2 quadratic terms

Fig. 2 [Images not available. See PDF.]

The 3D response surface plots (**A**) and the associated 2D contour plots (**B**) illustrating the effect of independent variables on the retention time of NLB (Y_1)

Effect of independent variables on the retention time of RTB (Y_2)

The following quadratic equation can be used to illustrate how the independent variables affect the retention time of RTB (Y_2).

2

Y2RetentiontimeofRTB=1.65-0.22X1-0.053X2-0.16X3-0.038X1X2+0.058X1X3-0.059X2X3+0.083X12-0.008X22+0.077X32

The equation shows that flow rate (X_1), % methanol (X_2), and temperature (X_3) have a negative effect on the RTB retention time. As flow rate, methanol percentage, and temperature increase, the retention time of RTB decreases. The high coefficient value of X_1 shows that flow rate affects RTB retention time more than % methanol and temperature. X_1X_2 and X_2X_3 negatively affect RTB retention time, while X_1X_3 positively affects it. The ANOVA results for the data of the retention time of RTB are demonstrated in Table 4. The F-value of 115.89 indicates the significance of the model. In this case X_1, X_2, X_3, X_1X_2, X_2X_3, X_1^2, and X_3^2 are significant model terms. The predicted R^2 of 0.9278 matches the adjusted R^2 of 0.9820. Precision measures signal-to-noise ratio. A ratio over 4 is ideal. The obtained ratio of 38.524 suggests a good signal. This model can be used to navigate the design space. The 3D response surface plots and 2D contour plots in Fig. 3A, B show how independent variables affect the retention time of RTB. It was observed from the plots that an increase in flow rate, % methanol, and temperature decreased the retention time.

Fig. 3 [Images not available. See PDF.]

The 3D response surface plots (**A**) and the associated 2D contour plots (**B**) illustrating the effect of independent variables on the retention time of RTB (Y_2)

Effect of independent variables on the resolution factor (Y_3)

The quadratic equation below explains how independent factors affect the resolution factor (Y_3).

3

Y3(ResolutionFactor)=3.30-0.34X1-0.42X2-0.21X3+0.012X1X2+0.087X1X3-0.037X2X3+0.049X12-0.074X22+0.119X32

The equation displays that the flow rate (X_1), % methanol (X_2) and temperature (X_3) have a negative effect on the resolution factor. This means that the resolution factor decreases with an increase in the flow rate, % methanol, and

temperature. The high coefficient value of X_2 shows that % methanol affects the resolution factor more than the flow rate and temperature. The combined interaction term X_1X_2, and X_1X_3 has a positive effect and X_2X_3 has a negative effect on the resolution factor. The ANOVA results for the data of the resolution factor are demonstrated in Table 4. The F-value of 135.89 indicates the significant model. Model terms with *p*-values under 0.050 are significant. Here, X_1, X_2, X_3, X_1X_3, X_1^2, X_2^2, and X_3^2 are significant model terms. *p*-values less than 0.0500 indicate model terms are significant. The predicted R^2 0.9618 matches well with the adjusted R^2 0.9846. A precision ratio of 45.170 suggests a good signal. Hence the quadratic model can navigate design space. The 3D response surface plots and 2D contour plots in Fig. 4A, B show how independent variables affect the resolution factor. It was observed from the plots that an increase in flow rate, % methanol, and temperature decreased the resolution factor between NLB and RTB.

Fig. 4 [Images not available. See PDF.]

The 3D response surface plots (**A**) and the associated 2D contour plots (**B**) showing the effect of independent variables on the resolution factor (Y_3)

Effect of independent variables on the number of theoretical plates of NLB (Y_4)

The quadratic equation below explains how independent factors affect the theoretical plate of NLB (Y_4).

4

Y4TheoreticalPlatesofNLB=3426.78-752.09X1+134.39X2-364.95X3-163.68X1X2-211.63X1X3+177.25X2X3-96.57X12+62.60X22+99.56X32

The equation displays that the flow rate (X_1), and temperature (X_3) have a negative effect whereas % methanol (X_2) has a positive effect on the theoretical plates of NLB. This means that the number of theoretical plates of NLB decreases with an increase in the flow rate and temperature and increases with an increase in % methanol. The large coefficient value of X_1 shows that flow rate affects the theoretical plate of NLB more than other variables. The interaction terms X_1X_2 and X_1X_3 negatively affect NLB theoretical plates, while X_2X_3 positively affects them. Table 4 shows the ANOVA findings of the obtained data. The F-value of 125.00 indicates the model is significant. Model terms with *p*-values under 0.05 are significant. In this case X_1, X_2, X_3, X_1X_2, X_1X_3, X_2X_3, X_1^2, X_2^2, and X_3^2 are important model terms. The predicted R^2 of 0.9833 is in fair agreement with the adjusted R^2 value of 0.9335. Adequate precision measures signal-to-noise ratio. A ratio over 4 is ideal. The obtained ratio of 38.332 suggests a good signal. Hence the quadratic model can be used to navigate the design space. The 3D response surface plots and 2D contour plots in Fig. 5A, B show how independent variables affect the theoretical plates of NLB. It was observed from the plots that an increase in flow rate, and temperature decreased the theoretical plates of NLB whereas an increase in % methanol increased the theoretical plates of NLB.

Fig. 5 [Images not available. See PDF.]

The 3D response surface plots (**A**) and the associated 2D contour plots (**B**) showing the effect of independent variables on the theoretical plates of NLB (Y_4)

Effect of independent variables on the number of theoretical plates of RTB (Y_5)

The following quadratic equation explains how independent variables affect the theoretical plate of RTB.

5

Y5TheoreticalplatesofRTB=4332.06-1131.82X1+203.81X2-675.57X3-39.63X1X2-138.03X1X3+156.45X2X3+46.75X12+138.27X22+27.69X32

The equation shows that flow rate (X_1) and temperature (X_3) negatively affect RTB theoretical plates, whereas % methanol (X_2) positively affects them. This implies that theoretical plates of RTB decrease with flow rate and temperature and increase with % methanol. The flow rate affects the theoretical plates of RTB more than other variables, as shown by the large coefficient value of X_1. The interaction terms X_1X_2 and X_1X_3 negatively affect the theoretical plates of RTB, while X_2X_3 positively affects them. ANOVA findings for obtained data are in Table 4. The model F-value of 730.29 suggests the model is significant. The *p*-values (<0.05) imply X_1, X_2, X_3, X_1X_3, X_2X_3, X_1^2,

and X_2^2 are significant terms. The predicted R^2 of 0.997 is consistent with the adjusted R^2 of 0.9883. The adequate precision ratio of 93.860 indicates a good signal. So, the quadratic model can navigate the design space.
The 3D response surface plots and 2D contour plots in Fig. 6A, B show how independent variables affect the theoretical plates of RTB. It was observed from the plots that an increase in flow rate, and temperature decreased the theoretical plates of RTB whereas an increase in % methanol increased the theoretical plates of RTB.
Fig. 6 [Images not available. See PDF.]

The 3D response surface plots (**A**) and the associated 2D contour plots (**B**) showing the effect of independent variables on the theoretical plates of RTB (Y_5)

Effect of independent variables on the tailing factor of NLB (Y_6)

The following quadratic equation explains how the independent variables affect the tailing factor of NLB.

6

Y6Tailingfactorof;NLB=1.30-0.0419X1+0.0137X2-0.0125X3-0.0175X1X2-0.0575X1X3-0.0125X2X3+0.0081X12+0.0488X22-0.0166X32

According to the equation, the tailing factor of NLB is positively affected by % methanol (X_2) and negatively affected by flow rate (X_1) and temperature (X_3). This means that the tailing factor of NLB decreases with an increase in the flow rate and temperature and increases with an increase in % methanol. A higher coefficient value for X_1 indicates that, relative to other variables, the flow rate significantly affects the tailing factor of NLB. The interaction terms X_1X_2, X_1X_3, and X_2X_3 have a detrimental impact on the tailing factor of NLB. The results of the ANOVA for the collected data are shown in Table 4. An F-value of 128.71 indicates statistical significance for the model. Model terms are considered significant when the *p*-value is less than 0.0500. Here, important model terms include X_1, X_2, X_3, X_1X_2, X_1X_3, X_2X_3, X_1^2, X_2^2, and X_3^3. The predicted R^2 of 0.9837 is reasonably close to the adjusted R^2 of 0.9371. The adequate precision' ratio of 37.845 indicates a good signal. Therefore, the quadratic model serves as a useful tool for exploring the design space. Figure 7A, B show the 3D response surface plots and 2D contour plots, respectively, that illustrate the effect of the independent variables on the NLB tailing factor. It was observed from the plots that an increase in flow rate, and temperature decreased the tailing factor of NLB whereas an increase in % methanol increased the tailing factor of NLB.
Fig. 7 [Images not available. See PDF.]

The 3D response surface plots (**A**) and the associated 2D contour plots (**B**) showing the effect of independent variables on the tailing factor of NLB (Y_6)

Effect of independent variables on the tailing factor of RTB (Y_7)

The impact of the independent variables on the tailing factor of RTB can be elucidated by the subsequent quadratic equation.

7

Y7TailingfactorofRTB=1.28+0.025X1-0.034X2+0.023X3+0.043X1X2+0.028X1X3+0.043X2X3+0.004X12-0.009X22-0.007X32

The equation indicates that the flow rate (X_1) and temperature (X_3) positively influence the tailing factor of RTB, whereas the percentage of methanol (X_2) has a negative impact. This means that the tailing factor of RTB increases with an increase in the flow rate and temperature and decreases with an increase in % methanol. The large coefficient value of X_2 shows that % methanol affects the RTB tailing factor more than other factors. The tailing factor of RTB is positively affected by the combined interaction term X_1X_2, X_1X_3 and X_2X_3. Table 4 displays the ANOVA results for the collected data. An F-value of 136.02 indicates a significant model. Model terms are considered significant when the *p*-value is less than 0.05. X_1, X_2, X_3, X_1X_2, X_1X_3, X_2X_3, X_1^2, X_2^2, and X_3^2 are significant terms in this model. Both the predicted R^2 of 0.9453 and adjusted R^2 of 0.9846 are within reasonable range. The adequate precision' ratio of 50.283 specifies an adequate signal. Hence the quadratic model useful tools for exploring design space. Figure 8A, B shows the 3D response surface plots and their related 2D contour plots that

demonstrate the impact of independent factors on the tailing factor of RTB, respectively. According to the graphs, the tailing factor of RTB increased with increasing temperatures and flow rates, while it reduced with increasing methanol concentrations.

Fig. 8 [Images not available. See PDF.]

The 3D response surface plots (**A**) and the associated 2D contour plots (**B**) showing the effect of independent variables on the on the tailing factor of RTB (Y_7)

Selection of optimized chromatographic condition

To choose the best chromatographic parameters, the Design expert® software's numerical optimization method was employed. The program gave the optimal chromatographic parameters for the separation of NLB and RTB, which included a mobile phase consisting of 32.80% methanol, a flow rate of 0.272 mL/min, a column temperature of 29.42 °C, and ultraviolet detection at 260 nm. A standard concentration of 48 µg/mL of NLB and 16 µg/mL of RTB were injected into the UPLC system with optimized chromatographic conditions and their responses were recorded. Table 5 displays the anticipated and observed values for the responses, as well as their percentage residual values. The residual values ranged from −0.320 to 2.53, indicating that the QbD design used for selecting optimum chromatographic conditions for separating NLB and RTB was valid. The validation of the developed method was conducted using the optimized chromatographic conditions. The validity of the central composite design used was confirmed by the low % residual error values obtained for the observed values.

Table 5. The predicted and observed values of the responses obtained from the optimized chromatographic conditions

Response	Predicted value	Observed value	Residual values (%)
Retention time NLB (min)	1.53	1.50	1.96
Retention time RTB (min)	1.97	1.92	2.53
Resolution factor	3.90	3.90	0.00
Theoretical plates NLB	3980	3993	−0.32
Theoretical plates RTB	5468	5451	0.31
Tailing factor NLB	1.33	1.33	0.00
Tailing factor RTB	1.29	1.29	0.00

Method validation

Table 6 displays the system suitability parameters and their corresponding acceptance criteria. The number of theoretical plates (3956 ± 34 for NLB and 5436 ± 19 for RTB), tailing factor (1.33 for NLB and 1.28 for RTB), resolution factor (3.90) and % RSD for the retention time and peak area (< 1 for NLB and RTB) meet the specified reference values (theoretical plates > 2000, tailing factor < 2, resolution factor < 2, and % RSD < 2). The standard calibration curves were linear within the concentration ranges of 12–72 µg/mL for NLB and 4–24 µg/mL for RTB, as depicted in Fig. 9. The regression coefficient (r^2) values for NLB were found to be 0.9998 with its regression equation $y = 22997x + 2505.9$. In the case of RTB, the regression coefficient (r^2) was found to be 0.9997 with regression equation $y = 21594 + 1344.2$. The LOD and LOQ values were determined to be 0.89 µg/mL and 2.69 µg/mL for NLB and 0.15 µg/mL and 0.46 µg/mL for RTB respectively. The % RSD of intraday and interday precision for NLB and

RTB was found to be less than <2 (Table 7). The % recovery was found to be in the range of 99.57–100.43% for NLB and 99.59–100.61% for RTB (Table 8). The specificity of the developed method is shown in Fig. 10. No co-eluting interfering peaks from the formulation excipients were found at the retention time of NLB and RTB in the placebo and blank samples indicating the specificity of the established method. The results of the robustness data are shown in Table 9. The % RSD values for peak area and retention time were both below 2%, indicating minimal variance. System suitability characteristics including theoretical plates, tailing factor and resolution factor showed no significant variation. The percentage assay of drug content was found to be 99.46 for NLB and 99.98 for RTB in the marketed formulation. The negligible changes in the peak areas of the NLB and RTB before and after keeping the solutions at room temperature for 24 h indicate the solution stability of both drugs.

Table 6. System suitability test parameters (n = 6)

Parameter*	NLB (48 µg mL^{-1})	RTB (16 µg mL^{-1})	Acceptance criteria
Retention time (t_R, min)	1.46 ± 0.005	1.88 ± 0.005	–
RSD % of retention time	0.35	0.27	<1 for n ≥ 5
Peak area	1,110,380 ± 3844	346,204 ± 1968	–
RSD % of peak area	0.34	0.56	<1 for n ≥ 5
Theoretical plates (N)	3956 ± 34	5436 ± 19	>2000
Tailing factor (T)	1.33 ± 0.004	1.28 ± 0.005	≤ 2.0
Resolution (R_s)		3.90 ± 0.001	>2.0

Fig. 9 [Images not available. See PDF.]

Calibration curves of Nivolumab (**A**) and Relatlimab (**B**)

Table 7. Recovery studies of NLB and RTB

Compound	Contents (µg)	Quantity added (µg)	Recovered amount (µg)	Recovery (%)	% RSD
NLB	48	24	23.89	99.57	0.43
48	48	48.13	100.28	0.54	48
72	72.31	100.43	0.44	RTB	16
8	7.96	99.59	0.39	16	16
16.09	100.61	0.26	16	24	23.93

Table 8. Intraday and interday precision of NLB and RTB

Compound	Content (µg/mL)	Intraday (n=6)		Inter day (n=3)					
Day 1		Day 2		Day 3		Found (µg/mL)	% RSD	Found (µg/mL)	% RSD
Found (µg/mL)	% RSD	Found (µg/mL)	% RSD	NLB	12.00	11.60	0.69	11.56	0.58
11.55	0.69	11.69	0.59	36.00	36.22	0.58	36.00	0.64	36.42
0.97	36.25	0.12	72.00	71.74	0.46	71.71	1.09	72.08	0.78
71.42	0.73	RTB	4.00	3.94	0.46	3.92	0.63	3.95	0.85
3.95	0.74	12.00	12.33	0.97	12.42	1.10	12.19	0.98	12.38
0.86	24.00	23.82	0.13	23.79	1.12	23.82	0.40	23.85	0.75

n, number of replicates; RSD, relative standard deviation

Fig. 10 [Images not available. See PDF.]

Chromatograms of Blank (**A**), placebo (**B**) and standard NLB and RTB (**C**) showing the specificity of the established UPLC method

Table 9. Results of robustness study (n=6)

Parameter		Modification		Observed value					
% RSD of peak area		Theoretical plate(N)		Tailing factor (T)		Resolution factor		NLB	RTB
NLB	RTB	NLB	RTB	NLB	RTB	Flow rate	0.1 mL/min	1	0.7

3920	5506	1.33	1.28	–	3.33	Optimized	0.35	0.27	3993
5451	1.33	1.28	–	3.9	0.3 mL/min	1.8	1	3923	5546
1.32	1.28	–	3.8	% methanol in mobile phase	31.16% MeOH	1	0.9	3961	5523
1.33	1.28	–	3.78	Optimized	0.35	0.27	3993	5451	1.33
1.28	–	3.9	34.44% MeOH	1.3	0.8	3922	5266	1.33	1.28
–	3.84	Column temperature	27.95 °C	1.8	0.4	4488	5309	1.32	1.28
–	3.74	Optimized	0.35	0.27	3993	5451	1.33	1.28	–
3.9	30.89 °C	1.3	0.6	4063	5408	1.31	1.27	–	3.8

n, number of replicates; RSD, relative standard deviation

Forced degradation studies

The results of the forced degradation studies of NLB and RTB under different stress conditions are shown in Fig. 11 and Table 10. Both NLB and RTB undergo a negligible extent of degradation (less than 2%) under the specified acid, alkali, thermal and neutral conditions. Under oxidative degradation, NLB showed 5.81% degradation whereas RTB showed 6.19% degradation with one degradation peak at 1.71 min. In the case of photolytic degradation, both NLB and RTB showed a degradation of 8.79 and 7.27% with three degradation peaks at 1.25, 2.21 and 2.67 min. Further, it was observed that there was no interference from the degradation peaks.

Fig. 11 [Images not available. See PDF.]

Chromatograms showing the degradation profile of NLB and RTB under different stress conditions acid (**A**), alkali (**B**), oxidative (**C**), dry heat (**D**), neutral (**E**), and photolytic degradation (**F**)

Table 10. Stability studies of NLB and RTB

Stress condition	Treatment	% Degradation	
NLB	RTB	Acid hydrolysis	2N HCl, 60 °C for 30 min
0.43	0.59	Alkaline hydrolysis	2N NaOH, 60 °C for 30 min
1.72	1.87	Oxidative degradation	60% H_2O_2 60 °C for 30 min
5.81	6.19	Thermal degradation	105 °C for 6 h
0.93	0.48	Photolytic degradation	UV light for 7 days
8.79	7.27	Neutral hydrolysis	Water at 60 °C for 6 h

Discussion

The current research utilized AQbD to develop an RP-UPLC method for estimating NLB and RTB. A central composite design (CCD) with three components at five levels was employed to optimize chromatographic conditions for the developed method utilizing Design-expert® software (Stat-Ease Inc., USA). Polynomial equations, 3D response plots, and 2D contour plots were created to analyze the impact of three independent factors (flow rate, % methanol, and temperature) on dependent variables (retention time, resolution factor, number of theoretical plates, and tailing factor). ANOVA was employed to assess the statistical significance of the model and its terms. Subsequently, a numerical optimization technique was utilized to predict the ideal chromatographic conditions. According to the polynomial equations and 3D-response plot, temperature and flow rate were the most significant factors affecting the retention time of NLB and RTB respectively. The resolution factor was highly influenced by % methanol in the mobile phase in comparison to flow rate and temperature. The flow rate was found to have a more pronounced effect on the number of theoretical plates for both drugs. In the case of the tailing factor, flow rate was found to be the more influential factor for NLB whereas % methanol was the more decisive factor for RTB. The QbD model effectively selected optimum chromatographic conditions for estimating NLB and RTB, as shown by the minimal residual error and close agreement between actual and anticipated values.

Analytical methods are validated to confirm their reliability and suitability for their purpose. The developed RP-UPLC method was validated as per ICH Q2 R1 guidelines. An essential component of method development is the system suitability test, which has been used to guarantee that the selected chromatographic system is operating properly throughout the analysis. All evaluated parameters such as theoretical plates, tailing factor, resolution factor and %RSD for peak area and retention time were well within the suggested ranges proving the suitability of the chromatographic system. The correlation coefficient (r^2) values>0.999 for both drugs indicate a good correlation and excellent linearity over the proposed concentration ranges. Low LOD and LOQ values showed that the proposed method was very sensitive for estimating RTB and NLB in pharmaceutical dosage forms. Analytical precision refers

to the degree of agreement between measurements taken from multiple samples under similar conditions. The % RSD values for both intraday and interday precision studied at three different levels were less than 2% demonstrating the good precision of the developed method. Recovery studies assess how closely the experimental result matches the true value. The % recovery values for NLB and RTB ranged from 99.57 to 100.43% and 99.59 to 100.61% respectively demonstrating minimal variability and high agreement between experimental and actual values. An analytical method's specificity is defined as its ability to assess the target analyte even when interferences from contaminants, degradation agents, and excipients are present in commercially available formulations. The developed method is specific for the quantification of NLB and RTB from pharmaceutical dosage forms, as there are no co-eluting interference peaks at the retention time of both drugs. Robustness refers to a method's ability to remain consistent despite small changes in chromatographic parameters, demonstrating the method's reliability in everyday use. The low % RSD values, and lack of significant changes in system suitability parameters following deliberate changes in experimental conditions demonstrated good robustness of the developed method. The percentage assay results (99.46–99.98% for label claim) indicate the applicability of the developed RP-UPLC method for the estimation of NLB and RTB in marketed formulations. The stability investigation confirmed that NLB and RTB remained stable in solution at room temperature for 24 h.

Stability studies are crucial for evaluating the quality of pure drugs and drug products. The method's ability to evaluate the stability of NLB and RTB is demonstrated by assessing their degradation following exposure to different stress situations. Both drugs were unstable under oxidative and photolytic conditions but exhibited great stability under acidic, alkaline, thermal, and neutral conditions. These findings indicate the stability indicating the nature of the developed method.

Conclusions

An analytical quality by design methodology, specifically central composite design, was effectively utilized to optimize the RP-UPLC method for quantifying NLB and RTB. The optimized chromatographic conditions for the separation of NLB and RTB consisted of 32.80% v/v methanol in the mobile phase, 0.272 mL/min flow rate, column temperature of 29.42 °C, and UV detection at 260 nm. The method showed well-resolved peaks at the retention time of 1.46 and 1.88 min for NLB and RTB respectively. The method was verified according to ICH criteria and demonstrated linearity, accuracy, precision, sensitivity, specificity, and robustness. The forced degradation studies showed well-resolved peaks of NLB and RTB along with degradation peaks. The findings of the present study conclude that AQbD is an effective methodology for method optimization and the established method is suitable for the accurate determination of NLB and RTB in pharmaceutical dosage forms.

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Author contributions

MVN carried out the experimental work and prepared the manuscript. RS interpreted the analytical data and helped in handling of Design expert software. AKT designed and supervised the experimental work. All authors read and approved the final manuscript.

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Availability of data and materials

The data that support the findings of the present study are available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate

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Consent for publication

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Human and animal rights

Studies involving plants must include a statement specifying the local, national or international guidelines and

legislation and the required or appropriate permissions and/or licences for the study: Not applicable.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

AQbD
Analytical quality by design
QbD
Quality by design
PD-1
Programmed death receptor-1
NLB
Nivolumab
LAG-3
Lymphocyte-activation gene 3
RTB
Relatlimab
RP-UPLC
Reversed-phase ultra performance liquid chromatography
CCD
Central composite design
LOD
Limit of detection
LOQ
Limit of quantification
FDA
Food and drug administration
LC–MS/MS
Liquid chromatography tandem mass spectrometry
ELISA
Enzyme linked immunosorbent assay
LC–MS/HRMS
Liquid chromatography–mass spectrometry/High resolution mass spectrometry
UPLC-MS/MS
Ultra-performance liquid chromatography-tandem mass spectrometry
UHPLC/UV-HESI
Ultra-high-performance liquid chromatography/ultraviolet-heated electro spray ionization
UV
Ultra-violet
TUV
Tunable ultra-violet
BEH
Bridged ethylene hybrid
HCl
Hydrochloric acid
NaOH
Sodium hydroxide
H_2O_2

Hydrogen peroxide
SD
Standard deviation
RSD
Relative standard deviation
ANOVA
Analysis of variance
3D
Three dimensional
CV
Coefficient of variation
PRESS
Predicted residual error sum of squares

Publisher's Note

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Expanding telmisartan's therapeutic horizon: exploring its multifaceted mechanisms beyond cardiovascular disorders

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ABSTRACT (ENGLISH)

Background

Telmisartan, a potent angiotensin II type-1 receptor blocker as well as partial PPAR–gamma agonist, has emerged as a versatile therapeutic agent with diverse pharmacological actions beyond its primary indication for essential hypertension. This review explores the complex mechanisms of action of telmisartan and clarifies its effectiveness in an inflammation, cancer, metabolic, and CNS disorders.

Main body

Telmisartan inhibits many biochemical processes involved in the control of the cardiovascular system, such as vascular smooth muscle contraction, aldosterone production, and sympathetic tone modulation, by specifically targeting the angiotensin II type-1 receptor. Its distinct partial agonist action toward peroxisome proliferator-activated receptor gamma also imparts anti-inflammatory, antiproliferative, and antioxidant activities, making it a viable treatment for various diabetic patients who have atherosclerosis and myocardial infarction.

Conclusion

Telmisartan's diverse pharmacological actions, encompassing anti-inflammatory, neuroprotective, nephroprotective, anticancer, and anti-anxiety properties, position it as a promising treatment option for a broad spectrum of medical conditions.

FULL TEXT

Background

Telmisartan, a nonpeptide antagonist directed at the angiotensin II type-1 (AT1) receptor, has surfaced as a versatile therapeutic option, demonstrating notable effectiveness and longevity in managing essential hypertension [1]. Its unique mode of action entails the targeted and enduring inhibition of the AT1 receptor's reactivity to angiotensin II, while preserving the functionality of other receptor systems implicated in cardiovascular control [2]. This selectivity

not only forms the basis of its powerful antihypertensive action but also lays the groundwork for its diverse pharmacological characteristics [3].

In addition to its role as an angiotensin receptor blocker (ARB), telmisartan exhibits partial agonist activity toward the peroxisome proliferator-activated receptor gamma (PPAR-γ) [4]. This unique dual activity confers a myriad of additional benefits, including antioxidative, anti-inflammatory, and antiproliferative effects, particularly in conditions such as atherosclerosis [5]. Such pleiotropic effects position telmisartan as a promising therapeutic option for diabetic patients with myocardial infarction, offering a comprehensive approach to cardiovascular management beyond blood pressure control [6].

Additionally, telmisartan's capacity to mitigate fibrotic alterations linked to diabetes-related cardiac fibrosis by activating endogenous PPARδ [4] and enhancing STAT3 [7, 8] expression highlights its potential in addressing diverse pathological pathways [9]. The activation of PPARδ not only maintains a balance in metabolism and inflammation [10] but also contributes to cardiac protection, thereby broadening telmisartan's therapeutic scope beyond hypertension [11].

Furthermore, telmisartan's influence extends beyond cardiovascular well-being, encompassing a range of advantageous effects on metabolic syndrome, neuroprotection [12], and nephroprotection. Its anti-inflammatory [13], antioxidative [14], antineoplastic [15], and nephroprotective [16] attributes further emphasize its potential as a valuable therapeutic agent across various diseases and disorders [17].

In this extensive review, we explore the multifaceted mechanisms of action of telmisartan, shedding light on its diverse pleiotropic effects and underscoring its potential as a fundamental component in the management of cardiovascular conditions and beyond. In this study, we examined over 323 research papers, with 199 of them being thoroughly included in our analysis. By meticulously analyzing the available literature, our objective is to offer valuable insights into the wide-ranging therapeutic capabilities of telmisartan, facilitating its exploration across diverse clinical scenarios.

Methodology

The scope of the review article on telmisartan will encompass its pharmacological properties, therapeutic indications, and emerging research trends for treatment of other than cardiovascular disorders. The objectives include evaluating the efficacy of telmisartan in the management of various CNS, cancer, diabetes, and its complications, exploring its potential mechanisms of action, and discussing its comparative effectiveness. For comprehensive coverage of literature on telmisartan, relevant databases such as PubMed, Web of Science, Scopus, and Google Scholar were utilized. A comprehensive search strategy was developed using keywords such as “telmisartan plus neurological disorders,” “telmisartan + metabolic syndrome,” “telmisartan and Diabetes,” Cancer and telmisartan, etc., and search filters will be employed to refine the search and capture relevant literature on telmisartan's pharmacology. Search results were screened based on relevance to the review's objectives. Titles and abstracts were reviewed to assess their suitability for inclusion in the review. Studies focusing on telmisartan's pharmacological properties were prioritized for further evaluation. Inclusion criteria include studies published in English, observational studies, meta-analyses investigating telmisartan's efficacy for other than cardiovascular disorders in preclinical studies. Exclusion criteria comprise clinical trials, case reports, and studies with insufficient data or irrelevant outcomes. During the study, we examined over 323 research papers, with 199 of them being thoroughly included in our analysis. Full-text articles of potentially relevant studies were retrieved through institutional subscriptions, interlibrary access, or direct contact with authors. Selected studies were critically evaluated for their quality and validity. Factors including study design, sample size, methodology, and risk of bias were considered in the assessment.

Relevant data from selected studies were extracted using a standardized data extraction form. Key information including study characteristics, type of animal models, intervention details, outcomes, and conclusions was systematically recorded for creation of review. Findings from the selected studies were synthesized to address the review's research questions and objectives. Synthesized information was organized and presented in a tabular form for different disorders and diseases.

The review process involved continuous literation, with ongoing refinement of search strategies and inclusion/exclusion criteria to ensure comprehensive coverage of literature and alignment with the review's objectives. Additionally, updates were made to incorporate new findings and emerging research trends on telmisartan's therapeutic applications beyond cardiovascular disorders. (Methodology section was included to provide clarity and reproducibility).

Neuroinflammation and telmisartan

Angiotensin receptor II induces inflammation and oxidative stress via ROS production through the NADPH oxidase complex [18, 19]. Toll-like receptors (TLRs) and PPARγ receptors in the CNS play vital roles in neuroinflammation [20]. Excessive RAS activation, especially through AT1 receptors, contributes to brain inflammation [21, 22]. IL-1β, generated by microglia, has diverse roles and is implicated in neurodegenerative disorders [23, 24].

Telmisartan induces PPARγ activation independently of AT1R, preventing NFκB-mediated inflammatory cascades [13]. PPARγ activation leads to a dose-dependent increase in SARM expression, a negative regulator of pro-inflammatory cytokines [25].

Telmisartan reduces LPS-induced inflammation in neuronal cells via SARM activation through TLR4 signaling independently of AT1R [13]. TLR4 activation triggers NFκB, AP1, and IRF3, with a focus on MyD88-mediated pro-inflammatory cytokine mechanisms [26].

Telmisartan reduces IL-1β-induced COX-2 expression, PGE2 release, and ROS production. Telmisartan mitigates IL-1β-induced upregulation of IL-1R1 receptor and NOX-4 mRNA expression [27]. Telmisartan attenuates hydrogen peroxide-induced COX-2 gene expression and reduces JNK and c-Jun activation. Telmisartan's neuroprotective effects are independent of PPARγ activation, as confirmed in primary rat cortical neurons [28, 29].

In conclusion, telmisartan demonstrates a multifaceted approach to neuroprotection by modulating specific pathways associated with inflammation and oxidative stress. These findings highlight its potential therapeutic role in neurodegenerative diseases.

Ocular inflammation and telmisartan

Endotoxin-induced uveitis (EIU) serves as an animal model for acute ocular inflammation induced by lipopolysaccharide (LPS) [30, 31]. Severe vision impairment complications include retinal vasculitis, retinal detachment, and glaucoma. Angiotensin II, a key renin-angiotensin system effector, interacts with AT_1 and AT_2 receptor [32, 33]. Recent studies reveal diverse biological roles of angiotensin II, including modulation of angiogenesis, vascular remodeling, and inflammation [34]. Angiotensin II enhances vascular permeability [35], induces chemokines and adhesion molecules, and influences inflammatory cell proliferation and differentiation. AT1R blockade, including telmisartan, effectively attenuates these inflammatory processes [36–38]. Upregulation of AT1R is associated with ocular inflammation in EIU [39]. Telmisartan effectively attenuates inflammatory parameters, including ICAM-1-mediated leukocyte adhesion and infiltration in EIU eyes. LPS stimulation leads to the upregulation of inflammatory mediators contributing to EIU development. ICAM-1 plays a pivotal role in leukocyte adhesion; its upregulation is inhibited by telmisartan. Telmisartan suppresses retinal ICAM-1 upregulation and mitigates various EIU-induced cytokines [40, 41]. The anti-inflammatory effects are associated with downregulation of NF-κB-induced molecules [42]. Insights into the RAS highlight its involvement in various inflammatory conditions, such as atherosclerosis, cerebral infarction, and pancreatitis [36]. Telmisartan substantially reduces anterior-chamber cell infiltration but shows limited impact on protein leakage [43].

Anti-inflammatory effects of telmisartan in Experimental Autoimmune Uveitis (EIU), a model for studying ocular inflammation. Telmisartan is noted for its ability to target important inflammatory mediators such as ICAM-1 (intercellular adhesion molecule-1), various cytokines, and molecules induced by NF-κB (nuclear factor-kappa B), a key transcription factor involved in inflammation. The reference to ICAM-1 suggests that telmisartan may inhibit the adhesion of immune cells to vascular endothelial cells, thereby reducing inflammation. Additionally, by modulating cytokines, which are signaling molecules involved in the immune response, telmisartan likely attenuates the inflammatory cascade in EIU. NF-κB-induced molecules further emphasize the drug's ability to interfere with transcriptional processes that promote inflammation.

Diabetes-induced vascular inflammation and telmisartan

Individuals with diabetes experience exposure of the endothelium to uncontrolled high glucose levels [44]. Endothelial dysfunction is the fundamental pathophysiology in diabetic macrovascular complications [45, 46]. Hyperglycemia triggers inflammatory responses crucial in the development of diabetic macrovascular diseases, including atherosclerotic coronary artery and cerebrovascular diseases [39]. Adhesion of inflammatory leukocytes to the vascular endothelium is a pivotal step in atherosclerosis development [47]. Adhesion molecules like VCAM-1, intercellular adhesion molecule-1, and endothelial-leukocyte adhesion molecule-1 play a role in leukocyte adhesion, leading to vascular inflammation [48]. Angiotensin II type-1 receptor blockers (ARBs), including telmisartan, are prescribed for hypertensive patients with diabetes mellitus [49]. Early reduction of inflammatory leukocyte homing and attachment to the endothelium is considered an effective therapeutic strategy. Telmisartan, an ARB, protects against vascular inflammation induced by hyperglycemia. Reports suggest that telmisartan reduces vascular inflammation by inhibiting the expression of IKKβ in endothelial cells [1]. Telmisartan induces GSK3β-Ser9 phosphorylation in endothelial cells. GSK3β-Ser9 phosphorylation decreases hyperglycemia-induced NFκB p65-Ser536 phosphorylation, VCAM-1 expression, and adhesion of THP-1 monocytes. GSK3β-S9A, a constitutively active mutant of GSK3β, restores the inhibition of NFκB p65-Ser536 phosphorylation, VCAM-1 expression, and THP-1 monocyte adhesion by telmisartan [1].

Telmisartan inhibits IKKβ expression in a GSK3β-Ser9 phosphorylation-dependent manner. Among various ARBs, only telmisartan demonstrates an increase in GSK3β-Ser9 phosphorylation. Telmisartan treatment mitigates HFD-induced upregulation of NFκB p65-Ser536 phosphorylation, VCAM-1 expression, and IKKβ expression in aortic tissues. Telmisartan alleviates hyperglycemia-exacerbated vascular inflammation by inducing GSK3β-Ser9 phosphorylation, inhibiting IKKβ expression, NFκB p65-Ser536 phosphorylation, and VCAM-1 expression in a PPARγ-independent manner. Telmisartan operates within diabetes by engaging with crucial signaling molecules implicated in inflammation and vascular irregularities, including GSK3β, IKKβ, NF-κB, and VCAM-1. Through its influence on these pathways, telmisartan can alleviate the inflammatory reactions linked to diabetes and its accompanying complications, offering promising therapeutic possibilities for individuals with diabetes.

Chronic inflammation

The renin–angiotensin system produces angiotensin II, activating the AT1 receptor, leading to oxidative stress and inflammation [50]. Telmisartan, an AT1 receptor antagonist, also acts as a partial agonist on peroxisome proliferator-activated receptor-γ (PPAR-γ), providing antioxidative and anti-inflammatory effects [51]. Recent studies highlight telmisartan's additional PPAR-γ partial agonist activity, impacting metabolic and inflammatory pathways, improving left ventricular functions, and showing benefits in post-infarct ventricular remodeling [51, 52]. Telmisartan's dose–response relationship in animal models of chronic inflammation suggests antiproliferative and anti-arthritic activities, inhibiting inflammatory reactions [53]. Tissue injury triggers pro-inflammatory cytokine release, but telmisartan's PPAR-γ activation decreases hypertrophic prostanoid production, potentially modulating inflammation [54, 55]. Telmisartan's antioxidant and anti-inflammatory effects involve preventing nuclear factor-κB (NF-κB) signaling pathway activation [56].

Telmisartan exhibits pleiotropic effects, including anti-inflammatory, antioxidative, and antiproliferative actions in atherosclerosis and myocardial infarction.

It also demonstrates a protective effect against gastric mucosal lesions induced by stress and indomethacin [56, 57]. Telmisartan functions as a partial agonist at PPAR-γ, inducing catalase gene expression and inhibiting NF-κB. These actions collectively combat oxidative stress and downregulated a majority of pro-inflammatory responses [51, 58].

The anti-inflammatory impact of telmisartan is attributed to its PPAR-γ agonist activity. The modulation of PPAR-γ expression observed during various inflammatory disorders provides a robust foundation for utilizing potent PPAR-γ ligands, like telmisartan, to attenuate or modulate the progression of inflammation. This finding underscores the potential of PPAR-γ as a therapeutic target in inflammatory conditions, given its altered expression in several inflammatory disorders [59].

This sequence outlines the key mechanisms of action of telmisartan, emphasizing its dual role as an AT1 receptor antagonist and a partial agonist at PPAR-γ, contributing to its multifaceted effects on inflammation, oxidative stress, and related pathways.

Ulcerative colitis and telmisartan

There is a global rise in inflammatory bowel diseases (IBDs), particularly UC. Anti-TNF antibodies are commonly used for UC treatment. Telmisartan is a promising therapeutic candidate with anti-inflammatory properties. Telmisartan suppresses TNF-α-induced activation of nuclear factor-kB (NF-κB) in vascular endothelial cells [60]. Varying doses of telmisartan lead to decreased tissue levels of TNF-α and increased anti-inflammatory activity. Telmisartan's benefits extend to modulating colonic inflammation, oxidative stress, and apoptosis in inflammatory bowel disease [61].

Telmisartan administration mitigates pathological changes induced by arachidonic acid (AA)-induced colitis model, including oxidative stress, alterations in colonic weight, ulceration, tissue necrosis, and inflammatory infiltrate [62]. Telmisartan treatment accelerates the shift from the acute to the chronic phase of inflammation in UC [63]. Effectively it reduces neutrophil infiltration, as indicated by diminished myeloperoxidase (MPO) levels [64]. Telmisartan suppresses the expression of TNF-α and intervenes in the AA-induced colitis model by reducing malondialdehyde (MDA) levels, showcasing its protective role against oxidative cellular injury. Telmisartan increases levels of interleukin-10 (IL-10), known for its anti-inflammatory properties [63].

Telmisartan offers diverse anti-inflammatory effects in treating ulcerative colitis. By inhibiting NF-κB activation triggered by TNF-α, it reduces tissue TNF-α levels and enhances overall anti-inflammatory activity. Telmisartan also combats oxidative stress and apoptosis, common in colonic inflammation, and shields against AA-induced colitis. It expedites the shift from acute to chronic inflammation, curtails neutrophil infiltration, and dampens TNF-α expression. Furthermore, it lowers malondialdehyde levels, indicating defense against oxidative cellular harm, and boosts IL-10 levels, reinforcing its anti-inflammatory prowess. These combined actions highlight telmisartan's promise as a therapeutic option for ulcerative colitis.

Crohn’s disease and telmisartan

Crohn’s disease (CD) patients are often underweight, and there's a significant increase in the ratio of intra-abdominal adipose tissue to total abdominal fat in these individuals [65]. Mesenteric fat serves as a crucial indicator of intestinal inflammation in CD [66].

MAT in CD patients exhibits notable inflammatory infiltrate and altered adipocyte morphology compared to healthy subjects [67]. MAT comprises various cell types, including adipocytes, preadipocytes, macrophages, endothelial cells, fibroblasts, and leukocytes. Telmisartan administration has a positive impact on mesenteric adipocytes in a mouse model of spontaneous colitis, restoring morphological changes and increasing adipocyte diameter. Mesenteric adipocytes contribute to C-reactive protein production in CD [68]. Leptin and adiponectin, hormones produced by adipose tissue, play roles in IBD pathogenesis [69].

Telmisartan treatment significantly alters the production of leptin and adiponectin in MAT, potentially influencing inflammatory processes. The neurotensin/miR-155 signaling pathway, involved in adipose inflammation and adipocyte differentiation, is modulated by telmisartan [70, 71].

Telmisartan treatment inhibits this pathway in MAT, suggesting a potential therapeutic contribution to attenuate MAT alteration and gut inflammation in CD. The renin-angiotensin system plays a role in the pathophysiology of colitis. Angiotensin receptor antagonists, including telmisartan, demonstrate effectiveness in preventing experimental colitis. Telmisartan exhibits a multifaceted impact on visceral adipose tissues, reducing leptin expression, increasing adiponectin levels, and attenuating MAT inflammatory parameters [72]. Telmisartan administration has a beneficial effect in an animal model of spontaneous colitis, reducing MAT inflammation, cytokine production, and ameliorating mesenteric adipose tissue alterations[73].

In conclusion, Telmisartan shows promise as a therapeutic option for managing inflammatory bowel diseases, particularly Crohn's disease, by influencing various aspects of adipose tissue composition, hormone regulation, and inflammatory pathways. The diverse actions of Telmisartan suggest its potential as a comprehensive approach to

address the complex mechanisms associated with CD (Table 1 and Fig. 1).

Table 1. Role of telmisartan in inflammation

Type of study	Route of administration	Treatment duration	Target	Effect	References
a) Colitis					
3 mg/kg/day IL-10¯/¯mice	Oral	12 weeks	IFN-g and TNF-a, IL-6 and IL-17, leptin Adipoleptin	Increased adiponectin decrease in levels of leptin, IL-6, and IL-17	Li et al. [196]
b) Ulcerative colitis					
5 mg/kg (Wistar rats)	Oral	3 day before and 2h &24 h after induction of disease	TNF-alpha, MPO, and MDA IL-10	Increase level of TNF-alpha, MPO, and MDA IL-10	Guerra et al. [2]
c) Neuronal Inflammation					
10 µmol/l (SK-N-SH human neuroblasts and primary rat cortical neurons)	Incubation of cells	Cells were incubated for 3 h	NADPH oxidase-4 (NOX-4) mRNA expression, NADPH oxidase activity, ROS generation, hydrogen peroxide-induced COX-2 gene expression, c-Jun N-terminal kinase (JNK), and c-Jun activation	Decreases in the IL-1β, mRNA expression, NADPH, hydrogen peroxide-induced COX-2 expression,	Pang et al. [27, 28]
d) Vasculitis					
5 mg/kg (Nephrectomized rats (Nx))	Intraperitoneal		PPAR-γ, NADPH, VCAM-1, Osteopontin,	Increase PPAR-γ, decrease NADPH oxidase, osteopontin, VCAM-1	Toba et al. [113]
e) Skin inflammation (psoriasis)					

40 mg/kg (K14-IL-17A ind/+and IL-17A ind/+mice)	Oral	4 weeks	IL-17A, AT II, PASI,	Increase in IL-17A, erythema, scaling, skin thickness, the affected area, and cumulative psoriasis area and severity index (PASI), decrease systolic blood pressure	Wild *et* al. [197]
f) Neuroinflammation					
100 µg/ml (Mouse Neuro2A neural cells)	Incubation of cells	Cells incubated for 24 h	PPAR-γ β-Actin, NF-κB, MyD88, cytokines IL-10	NF-κB, MyD88 activation, cytokines, motor co-ordination, cognitive functions, and activated SARM and PPAR-γ protein levels β-Actin, Increase secretion of IL-10	Balaji et al. [198]
g) Vascular Inflammation					
5 mg/kg (Male C57BL/6 mice)	Oral	13 weeks	GSK3b Ser^{9} phosphorylation, NF-kB p65	Inhibited hyperglycemia-induced NF-kB p65, inhibit GSK3b activity	Song et al. [1],
h) Acute colitis					
0.01 and 5 mg/kg (Female C57Bl/6 J mice)	Rectal route	6 days	Tumor necrosis factor (TNF)-a, transforming growth factor (TGF)-b1, interleukin-1b and monocyte chemoattractant protein (MCP)-1, caspase-3 and -7	Increase anti-apoptotic protein Bcl-2, Decrease in mRNA levels of pro-inflammatory cytokines such as tumor necrosis factor a, interleukin-1b, interleukin-6 and monocyte chemoattractant protein 1 as well as cellular DNA damage, caspase-3 and -7	Arumugam et al. [199]
i) Chronic inflammation					

(0.1, 0.2. 0.4, 0.6, 1.5, 3 mg/kg) (Wistar rats)	Oral	6 days	Nuclear factor-jB signaling pathway, PPAR-c	Decreases paw edema, reactive oxygen species and pro-inflammatory mediators, PPAR-c	Walid et al. [200]
j) Endothelial Inflammation					
Human umbilical vein endothelial cell (HUVEC)s	Incubation of cells	48 h	Vascular (VCAM-1) and intercellular (ICAM-1)	Decrease in TNF-α-stimulated VCAM-1	Cianchetti et al. [207]
k) Retinal inflammation					
20 mg/kg (C57BL/6 mice)	Intraperitoneal	2 days	AT1R and AT2R	Increase in angiotensin II expression, decrease in synaptophysin and rhodopsin expression	Kurihara et al. [208]

Fig. 1 [Images not available. See PDF.]

Target effectors of telmisartan in inflammation. VCAM-1: Vascular cell adhesion molecule-1, RANK: receptor activator of nuclear factor-kappa B, MPO: myeloperoxidase, TNF-ά: tumor necrosis factor alpha, MDA: malondialdehyde, TLR4: toll-like receptors, IkB: inhibitor nuclear factor-kappa B

Telmisartan in CNS disorders

Depression, characterized by anhedonia and persistent sadness, poses significant threats to life and cognitive functions [74, 75]. Stress plays a crucial role in depression development and associated memory issues. Ethical concerns limit direct research on depression causes and treatments in affected individuals. Chronic stress contributes to oxidative stress, leading to the generation of reactive oxygen species and compromised central nervous system functioning [76].

Chronic unpredictable mild stress (CUMS) rat model, introduced by Willner, simulates daily stressors and is instrumental in exploring depression origins and testing antidepressant interventions [77]. Telmisartan, a commonly used angiotensin receptor blocker (ARB), easily crosses the blood–brain barrier, inducing central AT1 receptor blockade [78].

Telmisartan holds promise as a potential oral antidepressant, possessing neuroprotective properties and mitigating cognitive impairments induced by chronic stress in rats [79].

Chronic stress results in decreased locomotor activity, sucrose preference, and impaired novel object recognition [80]. Telmisartan, especially at 1 mg/kg/day, significantly improves the impaired ability of novel object recognition, suggesting a potential antidepressant effect. AT1 receptor blockers, including telmisartan, gain attention for potential antidepressant effects [81].

The renin-angiotensin system (RAS) plays a crucial role in the body's response to stress [82].

Resting-state functional magnetic resonance imaging (r-fMRI), measuring intrinsic neural activity, is valuable in neuropsychiatric disorder research [83]. Telmisartan's impact on depression explored using ALFF and ReHo methods in a rat model was the first study of its kind. Telmisartan's effects on stress-induced alterations in brain regions were explored using ALFF and ReHo methods. Telmisartan at 1 mg/kg showed potential in reversing or attenuating stress-induced alterations in brain regions. Telmisartan demonstrated potential in decreasing hypercoordination of neural activity, particularly in the thalamus. Increased ReHo in limbic system regions and

altered connectivity in various cerebral regions suggested neuroimaging markers for depression [81].
Telmisartan alleviated depressive behaviors induced by unpredictable chronic mild stress in BALB/c mice.
Telmisartan's antidepressant effects were linked to its impact on serotonin transporter expression through PPARδ activation [84, 85].
Telmisartan's neuroprotective and antidepressant properties were associated with its impact on oxidative stress and pro-inflammatory mediators [86].
Its partial PPARδ agonistic property was considered crucial for reducing cytokine levels and improving cognitive decline [87].
Depression is a significant contributor to morbidity and mortality, requiring a multifaceted approach for accurate diagnosis and treatment [88, 89].
Telmisartan's dual action as an AT1 receptor blocker and PPAR-gamma agonist provides neuroprotection against various brain disorders, including depression [17, 90].
Telmisartan shows promise as a depression treatment through multiple mechanisms. It protects neurons, enhances cognition, and influences the body's stress response by modulating the renin-angiotensin system. Telmisartan also restores neural activity in stressed brain regions, impacting serotonin transporter expression via PPARδ activation. Its anti-inflammatory and antioxidant properties, partially through PPARδ agonism, alleviate depressive symptoms. Acting as both an AT1 receptor blocker and PPAR-gamma agonist, telmisartan offers comprehensive neuroprotection against depression.

Telmisartan in epilepsy

Epilepsies involve sudden, abnormal, and excessive neuronal activity in the brain, affecting 5–10% of the population. Long-term therapy challenges and medication side effects contribute to issues with compliance. Epilepsy may be associated with comorbid conditions like hypertension, diabetes, and renal disorders. Research suggests that drugs addressing these disorders, including ACE inhibitors and AT II receptor antagonists, may have a role in preventing seizures. The brain's RAS influences various functions, including regulating cerebral blood flow, stress, depression, seizures, and memory consolidation. Angiotensin II, a RAS component, acts as a neurotransmitter in the central nervous system, influencing the release of other neurotransmitters [91]. Angiotensin II inhibits GABAergic synaptic transmission by activating presynaptic AT1 receptors. Drugs like ACE inhibitors and AT1 receptor antagonists, including telmisartan, have the potential to enhance GABAergic transmission, beneficial in preventing seizures. Telmisartan, an AT1 receptor antagonist, improves the anticonvulsant effects of medications like valproate, lamotrigine, and topiramate in mouse models.
Telmisartan's unique properties, including potential depression-like effects, contribute to its anticonvulsant properties. Telmisartan exhibits neuroprotective effects by reducing local angiotensin II expression, blocking AT1 receptors, and promoting the relative upregulation of AT2 receptor function. In a rat model, ACE and AT1 receptors were upregulated in the brain after repetitive seizures. Telmisartan, especially at a 10 mg/kg dose, significantly decreases hind limb extension duration in the maximal electroshock (MES) model. Telmisartan exhibits substantial seizure inhibition and protection in the pentylenetetrazol (PTZ) test, suggesting dose-dependent antiepileptic activity. Telmisartan's slow dissociation from receptors, penetration of the blood–brain barrier, and increased potency at brain AT1 receptors contribute to its effectiveness. Telmisartan modulates the renin-angiotensin system, affecting glutamate/GABA release, decreasing glutamate levels, increasing GABA levels, and facilitating seizure prevention. AT1 receptor blockers, including telmisartan, contribute to decreased glutamate levels, increased GABA levels, and potential seizure prevention [92].
Telmisartan's higher lipophilicity and potency at brain AT1 receptors make it effective in modulating these neurological functions [93]. Angiotensin affects ion channels, including voltage-dependent potassium and calcium currents, influencing neuronal excitability and seizures [94].
Stress-induced changes in cortical BZ1 receptor expression are regulated by AT1 receptor activity. AT1 receptor antagonists may protect against seizures induced by inflammatory cytokines in chronic inflammatory disorders [95]. Seizure generation involves the activation of inflammatory cytokines, and AT1 receptor antagonists can be

considered for epilepsy treatment [96].
Telmisartan, with its AT1 receptor antagonistic properties, demonstrates promising antiepileptic effects. Understanding its mechanisms involving GABAnergic transmission, modulation of RAS, and impact on ion channels provides insights for potential therapeutic applications in epilepsy treatment. Together, these mechanisms underscore telmisartan's potential as an antiepileptic agent, providing neuroprotection, seizure management, modulation of neurotransmitter balance, and regulation of inflammatory pathways.

Telmisartan traumatic brain injury and cerebral edema

Cerebral edema is a serious complication of TBI, leading to elevated intracranial pressure and unfavorable clinical outcomes [97, 98].
The RAS is implicated in neuroinflammation and neurodegenerative disorders [99, 100].
Angiotensin receptor blockers (ARBs), especially telmisartan, effectively inhibit angiotensin II, offering anti-inflammatory and neuroprotective effects [101, 102]. Telmisartan, with its high lipid solubility, effectively penetrates brain tissue. Recognized for neuroprotective effects through angiotensin II receptor type-1 (AT1R) blockade [103]. Scientists investigated the anti-edemic effect of telmisartan through single oral gavage administration. Reduction in cerebral edema observed at 12 and 24 h post-TBI. The anti-edemic effect of telmisartan was not strictly dose-dependent [104]. Telmisartan demonstrated sustained inhibitory effects on AT1R, presenting an extended window for pharmacological intervention. IL-1β, a pro-inflammatory cytokine elevated in TBI, is implicated in cerebral edema [105, 106].
Telmisartan has shown efficacy in mitigating IL-1β-induced inflammatory responses in various brain injury models. Telmisartan improved neurological function, reduced lesion volume, and exhibited neuroprotective effects in the TBI model. Investigation into telmisartan's impact on the pro-inflammatory cytokine IL-1β. Telmisartan was found to inhibit the assembly and activation of the NLRP3 inflammasome [105, 107]. This inhibition provides a mechanism for telmisartan's anti-edemic role in TBI. The study confirmed the role of NLRP3 inflammasome-regulated IL-1β in traumatic cerebral edema. Telmisartan demonstrated potential in sustaining BBB integrity, reducing edema, and improving neurological function through NLRP3 inflammasome modulation [108].
Telmisartan, an angiotensin receptor blocker, offers a versatile approach to managing brain injury and cerebral edema. By inhibiting angiotensin II activity, it provides anti-inflammatory and neuroprotective benefits. Its lipid solubility enables direct brain tissue access for targeted action, primarily inhibiting AT1R to reduce inflammation and enhance neurological function. Telmisartan also disrupts NLRP3 inflammasome activation, reducing IL-1β-induced inflammation. Its sustained AT1R inhibition diminishes cerebral edema post-TBI, while also maintaining blood–brain barrier integrity. Telmisartan shows promise as a therapeutic agent for brain injury and cerebral edema management.

Anxiolytic effect of telmisartan

Anxiety disorders, affecting 7–30% of the world's population, represent a significant mental health challenge, contributing to economic burdens and health concerns [109]. Neurotransmitters like serotonin and GABA are implicated in anxiety pathophysiology, with selective serotonin reuptake inhibitors (SSRIs) recommended as first-line drugs. Emerging evidence suggests the involvement of the brain renin-angiotensin system in anxiety states, where angiotensin modulates neurotransmitter release [110]. Angiotensin receptor blockers (ARBs), used for cardiovascular conditions, have been associated with increased anxiety prevalence, particularly due to the blockade of AT1>AT2 receptors in the brain [111]. Telmisartan, crossing the blood–brain barrier, exhibits significant anti-anxiety effects, possibly through AT1 receptor blockade in circumventricular organs and potential cerebral AT receptor blockade. The mechanism of telmisartan's anti-anxiety effects involves hypothesized upregulation of angiotensin levels and receptors in the brain during anxiety, influencing neurotransmitters like noradrenaline and serotonin [112]. Telmisartan's additional activities via PPAR-gamma and NADPH oxidase may contribute to its role in oxidative stress management, providing added benefits. [113].
In summary, telmisartan, through its action on the RAS and neurotransmitter modulation, emerges as a promising agent in managing anxiety, offering potential avenues for novel treatments targeting neurogenesis, plasticity, and

cell survival (Table 2).

Table 2. Role of telmisartan in CNS disorders

Type of study	Route of administration	Treatment duration	Target	Effect	References
a) Stress-induced depression					
1 mg/kg (male Sprague–Dawley rats)	Oral	5 weeks	ReHo in the motor cortex, pons, thalamus, visual cortex, midbrain, cerebellum, hippocampus, hypothalamus, and olfactory cortex, B-A/B + A in the ORT	Decreased ReHo in the motor cortex and pons, increased ReHo in the thalamus, visual cortex, midbrain, cerebellum, hippocampus, hypothalamus, and olfactory cortex, Improved B-A/B + A in the ORT	Li et al. [81]
1 mg/kg (male BALB/c mice), (Rat-derived hippocampus H19-7)	Oral	6–7 weeks	PPAR-δ and 5-HTT	Reduced level of PPARδ and 5-HTT in hippocampus, Increased level of PPARδ and 5-HTT in H19-7 cell lines	Li et al. [89]
2 mg/kg (male albino mice)	Oral	2 weeks (15 days)	PPAR-gamma	Increased in PPAR-gamma, decrease in the immobility time	Brattiya and Sivaraman [90]
b) Epilepsy					
5 mg/kg, 10 mg/kg (Swiss albino mice)	Oral	1 h after test and standard drug administration	AT1, AT2	Blockade of AT1 receptors, increase in activity of AT2 receptors, Decease in hindlimb extensor phase, tonic hind limb extension	Pushpa et al. [92]
c) Cerebral edema					

5, 10, and 20 mg/kg (male C57BL/6 mice)	Oral	72 h	NLRP3, IL-1b, IL-18,	Decrease in NLRP3, IL-1b, IL-18, mRNA, and protein levels	Wei et al. [104]
d) Anxiety					
5 and 10mg/kg (Swiss albino mice	Oral	2 weeks	AT1	Stimulation of AT1, increase time spent in social interaction test, increase time spent in open arms	Swetha et al. [201]

Telmisartan in *cancer*

Endometrial *cancer*

Endometrial cancers are prevalent malignant tumors affecting the female genital tract, with a rising incidence [114, 115]. Despite the increasing incidence, effective agents for advanced and recurrent cases are lacking [115]. Peroxisome proliferator-activated receptor gamma and its ligands are known to induce apoptosis in various cancer types, including endometrial cancer [116, 117].

PPAR-gamma belongs to a nuclear hormone receptor family linked to endometrial carcinoma risk factors like obesity, excess estrogen, type II diabetes, and hypertension [118, 119].

Telmisartan emerges as a therapeutic option, inducing DNA damage and apoptosis in endometrial cancer cells. Telmisartan, acting as a partial agonist of PPAR-gamma, activates the receptor independently through AT1R interaction [51].

Telmisartan induces DNA double-strand breaks (DSBs) before triggering apoptosis in endometrial cancer cells. Among ARBs, only telmisartan inhibits cell viability in endometrial cancer, and this effect is diminished by a PPAR-gamma inhibitor (GW9662) [120].

PPAR-gamma immunoreactivity is detected in endometrial carcinoma tissue, and PPAR-gamma ligands show antiproliferative activity [116].

In vivo experiments using a nude mouse model demonstrate apoptosis within the tumor area in mice treated with telmisartan. Telmisartan's antitumor activity without major side effects suggests potential effectiveness in individuals with minimal residual disease post-surgery, chemotherapy, or radiotherapy [120].

In summary, telmisartan exhibits promise in endometrial cancer treatment through its involvement in PPAR-gamma mediated pathways, highlighting its potential as a therapeutic agent for individuals with minimal residual disease.

Telmisartan in colon *cancer*

Telmisartan, an angiotensin II receptor blocker (ARB) for hypertension, has been found to activate PPARγ in colon cancer cells, suppressing malignancy through differentiation and apoptosis [121].

Telmisartan effectively reduces cell viability, inhibits proliferation, and induces apoptosis in selected colon cancer cell lines [15].

The addition of GW9662, a PPARγ blocker, did not hinder telmisartan's inhibition of cell proliferation and viability, suggesting a PPARγ-independent pathway. Combined use of telmisartan and GW9662 intensified the reduction in cell viability and antiproliferative effects, particularly in SW-480 and SW-620 cells. Telmisartan demonstrated an apoptotic effect similar to pioglitazone, and this effect was not hindered by GW9662, indicating PPARγ-independent apoptosis induction [122]. Ligand-dependent activation of PPARγ resulted in the downregulation of PPARγ mRNA and upregulation of target genes, including CSTA. Telmisartan, in a dose-dependent manner, affected relative PPARγ mRNA expression and upregulated CSTA [123].

Addition of GW9662 led to significant downregulation in relative PPARγ mRNA expression, suggesting a PPARγ-independent pathway. Telmisartan's effects were superior to pioglitazone in certain cells, especially in the presence

of the PPARγ blocker GW9662 [122].
Telmisartan's apoptotic effect was comparable to the full PPARγ agonist pioglitazone, and this action appeared to be independent of PPARγ [124, 125].
Examination of gene expression shed light on the influence of GW9662 on PPARγ and CSTA mRNA expression, providing valuable insights into the molecular mechanisms underlying telmisartan's effects on colon cancer cells [123].
In summary, telmisartan exhibits notable effects on colon cancer cells through a PPARγ-independent pathway, with enhanced efficacy in combination with GW9662. The study provides crucial molecular insights into telmisartan's actions on colon cancer cells, emphasizing its potential as a therapeutic agent.

Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the primary liver malignancy and a significant cause of cancer-related deaths globally [126, 127]. Telmisartan's impact on proliferation was assessed in various HCC cells, including HLF, HLE, HepG2, HuH-7, and PLC/PRF/5. Telmisartan effectively inhibited the proliferation of poorly differentiated HCC cells (HLF, HLE, and HepG2) while showing reduced sensitivity in well-differentiated HCC cells (HuH-7 and PLC/PRF/5) [128].
Telmisartan induced G0/G1 cell cycle arrest in HLF cells by impeding the G0-to-G1 transition [129].
The observed cell cycle arrest was accompanied by a significant reduction in the levels of cyclin D1, cyclin E, and other cell cycle-related proteins [130].
Telmisartan increased the activity of the AMP-activated protein kinase (AMPK) pathway and concurrently inhibited the mammalian target of rapamycin (mTOR) pathway [131, 132].
Telmisartan contributed to apoptosis in HLF cells, as indicated by an increase in caspase-cleaved cytokeratin 18 (cCK18) levels and a reduction in the phosphorylation of ErbB3. The study identified 163 differentially expressed miRNAs in response to telmisartan, emphasizing its inhibitory effects, particularly in poorly differentiated HCC cells.
Telmisartan's mechanisms involve cell cycle regulation, apoptosis induction, and modulation of key signaling pathways like AMPK/mTOR [14].
Telmisartan exhibits potent antiproliferative effects against hepatocellular carcinoma (HCC), especially in poorly differentiated cells. It arrests the cell cycle at G0/G1 phase, reduces cyclin D1 and cyclin E expression, and activates the AMPK pathway while inhibiting mTOR, hampering HCC proliferation. Telmisartan also induces apoptosis by increasing cCK18 levels and decreasing ErbB3 phosphorylation in HLF cells. Altered miRNA expression underscores its efficacy, particularly in poorly differentiated HCC. Overall, telmisartan regulates the cell cycle, induces apoptosis, and modulates key signaling pathways like AMPK/mTOR, making it a promising therapy for HCC.

Ovarian *cancer* and telmisartan

Ovarian cancer, ranking third in incidence among female reproductive system malignancies, poses a significant health challenge with epithelial ovarian cancer having the highest mortality rate among gynecological tumors [133]. Challenges at advanced stages highlight the critical need for early detection strategies [134].
Peroxisome proliferator-activated receptor gamma (PPARγ), a key player in various cancers, emerges as a potential avenue for cancer intervention [135]. Telmisartan, primarily an antihypertensive agent, gains attention for activating PPARγ. In HEY cells, telmisartan inhibits growth in a time- and dose-dependent manner, inducing apoptosis and reducing caspase-3 activity, consistent with outcomes in other cancer cells [136].
PPARγ's role takes center stage in regulating physiological processes, including ovarian tissue functions crucial for reproduction [137, 138]. Telmisartan's ability to elevate PPARγ expression reinforces its potential as a multifaceted therapeutic. Telmisartan's impact on MMP-9 expression, linked to cancer progression, further supports its potential in ovarian cancer treatment [139].
PPARγ agonists, including telmisartan, exhibit positive effects in contexts like renal fibrosis and early pregnancy chorionic villi, influencing trophoblast cell invasion [140]. Telmisartan's reduction in MMP-9 expression in HEY cells aligns with potential therapeutic effects observed in periodontitis and acute myocardial infarction [141, 142].

Telmisartan's mechanism of action in ovarian cancer encompasses PPARγ activation, suppressing cancer cell growth, promoting apoptosis, regulating MMP-9 expression, and offering potential multifaceted therapeutic effects (Fig. 2 and Table 3).

Fig. 2 [Images not available. See PDF.]

Telmisartan in cancer. ICAM-1: intercellular adhesion molecule-1, MMP-9: matrix metalloprotease 9, ROS: reactive oxygen species, DR5: death receptor 5

Table 3. Role of telmisartan in cancer

Type of study	Route of administration	Treatment duration	Target	Effect	References
a) Human endometrial cancer cells					
1–100 mM (HHUA human endometrial cancer cell line, immunodeficient mice)	Incubation of cells	Cells are incubated for 48 h	Annexin V+/PI2, HHUA cells, caspase-3 and -7, PPAR-c, Bcl-2, Bcl-x	Increase annexin V+/PI2 fraction (early apoptotic) and annexin V+/PI+(late apoptotic) subpopulations, cleaved PARP in HHUA cells, caspase-3 and -7 Decrease in expression of Bcl-2 and Bcl-x	Koyama et al. [120]
b) Human colon cancer cells					
0.2–5 μM (Human colon cancer cells) (HT-29, SW-480, and SW-620)	Incubation of cells	Incubation of cells for 24 h with drug	PPARγ	Reduced cell survival in the HT-29, SW-480 and SW-620 cell, PPARγ mRNA expression, cell viability	Lee et al. [122]
c) Human Lung Adenocarcinoma					
10–100 μM (A549 cells)	Incubation of cells	Incubation of cells for 24–48 h with drug	PPARγ, mRNA, protetn,	Increase in mRNA and protetn expression of PPARγ, DNA binding activity of PPARγ, decrease in mRNA and protetn expression of ICAM-1 and MMP-9 survival rates and cell viabilities of A549 cells	Li et al. [196]
d) Colorectal cancer					

0.2mM (The human primary tumor (SW-480), metastatic (SW-620) colon cancer cell lines, and human immortal keratinocyte (HaCaT)	Incubation of cells	Incubation of cells for 24 h with drug	PPAR-gamma, HEY cells	Increase the expression of PPARγ, Inhibit growth of HEY cells	Puta et al. [202]
e) Lung cancer					
40 μM (A549, HCC-15)	Incubation of cells	Incubation of cells for 24 h with drug	ROS, DR5	Increase ROS production, Increase DR5 upregulation	Rashed uzzaman et al. [203]
f) Ovarian cancer					
10 and 100 μM (HEY human ovarian cancer cell)	Incubation of cells	Incubation of cells for 48 and 72 h with drug	PPARγ, MMP-9 protein	upregulating PPARγ, Increase Apoptosis, cell viability Decrease in MMP-9 protein expression,	Pu et al. [204]

Telmisartan in diabetes

Telmisartan, recognized as an angiotensin II receptor antagonist specifically targeting the angiotensin II type-1 receptor, has become a widely utilized medication for the management of hypertension. In recent times, the focus on telmisartan's impact on peroxisome proliferator-activated receptors (PPARs) has expanded [10]. PPARs, belonging to the nuclear hormone receptor superfamily, are ligand-activated transcription factors. Telmisartan's distinctive feature lies in its reported partial PPARγ-agonistic effect, avoiding safety concerns associated with full PPARγ agonists [10].

Renowned for its partial PPARγ-agonistic effect, telmisartan exhibits capabilities in the regulation of glucose and lipid metabolism, improvement of insulin resistance, and potential effectiveness in addressing metabolic syndrome [10, 143].

Telmisartan's ability to lower glucose levels is linked to its capacity to inhibit reactive oxygen species and its agonistic influence on PPARγ [10]. The medication has demonstrated a reduction in oxidative stress, elevation of antioxidant levels, and enhancement of insulin sensitivity in individuals with diabetes [144]. The dual impact of telmisartan, involving partial activation of PPARγ and angiotensin type-1 receptor blockade, holds significance in preventing and managing type 2 diabetes mellitus (T2DM), metabolic syndrome, and atherosclerotic cardiovascular disease [10]. In hypertensive patients with T2DM, telmisartan exhibits favorable effects on insulin sensitivity, blood pressure, glucose and lipid metabolism, and endothelial function. Its cardioprotective attributes encompass the reduction of cardiac fibrosis and hypertrophy, prevention of adverse cardiac remodeling, and potential mitigation of diabetic cardiomyopathy [145]. Telmisartan's dual action on angiotensin type-1 receptor and PPARγ positions it as a valuable therapeutic option for hyperlipidemia, insulin resistance, hypertension, and stroke [146]. Additionally, the medication has displayed efficacy in improving the lipid profile, with notable reductions in triglycerides and increases in high-density lipoprotein cholesterol levels. Its potential hepatic partial PPARα agonist activity further contributes to its anti-dyslipidemic effects [147].

Telmisartan emerges as a promising therapeutic agent for T2DM, offering a comprehensive approach by addressing glucose regulation, lipid metabolism, insulin resistance, and providing cardiovascular and renal protection [10]. Telmisartan, known for its action as an angiotensin II receptor antagonist, also exhibits partial agonistic effects on PPARγ, distinct from full agonists. This feature enables telmisartan to regulate glucose and lipid metabolism and improve insulin sensitivity, making it valuable in managing type 2 diabetes mellitus (T2DM) and metabolic syndrome. By inhibiting reactive oxygen species and enhancing antioxidant levels through PPARγ activation, telmisartan reduces oxidative stress and enhances insulin sensitivity in diabetic individuals. Its dual action on PPARγ and angiotensin type-1 receptors provides a multifaceted approach to managing T2DM and metabolic syndrome.

Diabetic nephropathy

The renin-angiotensin system, vital for cardiovascular and renal functions, involves Angiotensin II (Ang II) binding to AT1 and AT2 receptors. Ang II, through AT1 receptor activation, induces vasoconstriction, sodium reabsorption, and various cellular processes in the kidney. Reactive oxygen species (ROS) [148] play a role in Ang II signaling, influencing critical events like transactivation of the epidermal growth factor receptor [149, 150].

In diabetic nephropathy (DN), PKC-α activation varies across renal structures, with implications for Na+-K+-ATPase inhibition and albumin uptake [151, 152]. Increased PKC-α expression correlates with TGF-β1 and VEGF levels, contributing to DN pathogenesis [153]. Telmisartan, an AT1R blocker, attenuates PKC-α and VEGF expression, suggesting nephroprotective effects via PKC-α in the RAS-PKC signaling cascade [154].

In vitro, telmisartan suppresses ROS generation induced by high glucose levels, protecting against cellular damage [155]. In diabetic mice, telmisartan reduces albuminuria, mesangial expansion, and inflammation-associated markers, demonstrating protective effects [156]. Telmisartan decreases oxidative stress markers (8-OHdG, Nox4), apoptosis (Bax), and improves kidney function and structure in diabetic rats. It regulates mitochondria-related pathways, inhibiting oxidative stress [157, 158].

Telmisartan's dual action as a PPAR-gamma agonist and AT1 receptor inhibitor contributes to renal protection It downregulates gene expression in the oxidative phosphorylation pathway, potentially inhibiting excessive mitochondrial ROS production [159, 160]. The upregulation of nephrin and podocin, crucial components of the slit diaphragm, signifies protective effects against diabetic nephropathy [161, 162].

Telmisartan exhibits multifaceted renoprotective effects, addressing oxidative stress, inflammation, and apoptosis in diabetic nephropathy. Its actions on PKC-α and various pathways provide valuable insights, suggesting its therapeutic potential in managing diabetic kidney disease (Fig. 3).

Fig. 3 [Images not available. See PDF.]

Telmisartan in diabetic nephropathy. TGF-b: Transforming growth factor beta, 8-OHdG: 8-hydroxydeoxyguanosine, (MCP)-1: monocyte chemoattractant protein, CD68: cluster differentiation, TNF-α: tumor necrosis factor alpha, PKC-α: protein kinase C alpha, Nox4: NADPH oxidase-4, NADPH: nicotinamide adenine dinucleotide phosphate

Diabetic neuropathy

Nerve healing is influenced by various factors, including the time between trauma and surgery, severity of trauma, type of damaged nerve, patient's age, and surgeon's experience [163]. Peripheral nerve regeneration is closely tied to apoptosis, and inhibiting the renin-angiotensin system (RAS) receptors, especially AT1 receptors, reduces apoptosis, inflammation, and oxidative stress [102]. Telmisartan, with its high affinity for AT1 receptors, demonstrates anti-inflammatory effects, supporting positive effects on nerve healing [102, 164].

The PPAR-γ ligand in telmisartan mediates its positive effects by inhibiting post-ischemic inflammation, neuronal degeneration, and apoptosis regeneration [165]. Telmisartan attenuates hemorrhage expansion, perihematomal edema formation, and neuropathic pain, attributed to its anti-inflammatory properties [102, 166]. It prevents nerve cells from injury by decreasing the apoptotic pathway, inhibiting caspase-3 activity, and reducing inflammatory cytokines [167, 168]. In conditions like chronic constriction injury (CCI), RAAS overactivation is associated with increased inflammatory mediators, oxidative stress, and pain-related markers [169, 170]. Telmisartan, by modulating RAAS components and downregulating signaling pathways like JAK2/STAT3 and P38-MAPK, [171] exerts beneficial

effects in neuropathic pain modulation. ACE-Is are superior to ARBs in neuroprotective and antioxidant effects [172]. Telmisartan administration in diabetic rats prevents the progression of diabetic neuropathy (DN) and enhances thermal and mechanical analgesia [12]. Its anti-inflammatory and neuroprotective properties, [27] attributed to PPAR-γ activation, alleviate hyperglycemia-associated alterations and reduce pro-inflammatory biomarkers [12]. Telmisartan suppresses the production of inflammatory cytokines and inhibits thermal hyperalgesia development and progression. It attenuates nerve growth factor degeneration and behavioral abnormalities in diabetic animals [173].

In summary, telmisartan's multifaceted actions, involving anti-inflammatory, neuroprotective, and antioxidant effects, position it as a potential therapeutic candidate in nerve healing and diabetic neuropathy. Its modulation of RAAS components and downstream signaling pathways contributes to its beneficial effects on nerve regeneration and pain modulation (Fig. 4).

Fig. 4 [Images not available. See PDF.]

Telmisartan in diabetic neuropathy. PPAR-G: peroxisome proliferator-activated receptor gamma, RAAS: renin–angiotensin–aldosterone system, STAT3: signal transducers and activators of transcription, NF-kB: nuclear factor-kappa B, NGF: transforming growth factor

Diabetic retinopathy

The activation of the renin-angiotensin system, particularly through the angiotensin II type-1 receptor (AT1R), has been identified as a potential mechanism for damaging retinal neurons in individuals with diabetes [174]. TNFα, a key inflammatory factor, can disrupt the blood-retinal barrier (BRB), leading to leukocyte accumulation in the retina and promoting cell death [175]. Caspase, indicators of cell death, are activated early in the retina of individuals with diabetes. Telmisartan effectively reduces caspase-3 activity in the diabetic retina, providing protection against neuronal damage [174]. The AT1R blocker effect extends to modulating levels of brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), tyrosine hydroxylase (TH), glutathione (GSH), and caspase activity in the retina [174]. Telmisartan, by inhibiting AT1R activation, addresses imbalances in these factors contributing to neurodegeneration in diabetic retinopathy. Telmisartan's ability to inhibit AT1R, which is stimulated by angiotensin II, presents a promising approach for treating diabetic retinopathy (DR) [176]. Telmisartan demonstrates a multifaceted protective role in diabetic retinopathy (DR). By inhibiting the activation of the angiotensin II type-1 receptor (AT1R) [177], telmisartan intervenes in the renin-angiotensin system (RAS), a potential mechanism implicated in damaging retinal neurons in diabetes [178].

The drug effectively reduces caspase-3 activity, providing protection against early cell death in the diabetic retina. Telmisartan's impact extends to the modulation of various factors crucial for neurodegeneration in diabetic retinopathy. It addresses imbalances in brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), tyrosine hydroxylase (TH), and glutathione (GSH), offering a promising approach for treating DR [179]. Its high affinity for AT1 receptor subtypes allows telmisartan to effectively block the RAS, leading to a sustained protective effect and slowing down the progression of diabetic retinopathy [180, 181]. The neuroprotective effects of telmisartan are further highlighted by its ability to elevate BDNF and GSH levels in both blood and the retina, responding to oxidative stress. By blocking AT1R activation induced by diabetes [182], telmisartan may deactivate NADPH oxidase, reducing oxidative stress and potentially increasing BDNF levels. The preservation of neurons, indicated by increased levels of TH, reinforces the protective impact of Telmisartan, particularly on dopaminergic amacrine cell functionality [183]. In addition, telmisartan effectively suppresses the elevated expression of VEGF-A, RAGE, and TNF-α in the retina, contributing to the mitigation of retinal complications [184]. Its specific binding to intraocular angiotensin receptor 1 (ATR 1) inhibits excessive activation of the Ang II-mediated RAS system, postponing the breakdown of the blood-retinal barrier (BRB) [184]. Telmisartan's actions encompass inhibition of AT1R, modulation of neuroprotective factors, reduction of oxidative stress, and suppression of pro-inflammatory mediators [185].

Telmisartan exerts a multifaceted protective effect against retinal damage in diabetic retinopathy by inhibiting AT1R,

modulating neuroprotective factors, reducing oxidative stress, suppressing pro-inflammatory mediators, and preserving the blood-retinal barrier (Fig. 5).

Fig. 5 [Images not available. See PDF.]

Telmisartan in diabetic retinopathy. BRB: Blood retina barrier, VEGF: vascular endothelial growth factor, RAGE: receptor for advanced glycation end products, TNF-α: tumor necrosis factor alpha, RAAS: renin–angiotensin–aldosterone system

Telmisartan and diabetic ulceration

Patients diagnosed with type 2 diabetes mellitus frequently experience a high incidence of acute gastric inflammation and ulcer disease, and this occurrence is notably linked to the duration of diabetes. Moreover, peptic ulcers associated with diabetes mellitus tend to be more severe, exhibiting a delayed healing rate and a higher propensity for complications, including gastrointestinal bleeding [186, 187].

The heightened vulnerability of gastric mucosa in diabetic animals to damage involves a multifaceted mechanism. This includes changes in gastric motility [188] compromised duodenal bicarbonate secretion, and diminished angiogenesis, along with dysfunction in capsaicin-sensitive neurons crucial for safeguarding the gastric mucosa [189]. Telmisartan, a medication utilized for various conditions, exhibits antioxidant and anti-inflammatory properties attributed to its inhibition of the nuclear factor-κB (NF-κB) signaling pathway. This pathway plays a pivotal role in the transcription of genes involved in oxidative stress and inflammation, including NADPH oxidase, tumor necrosis factor-α (TNF-α), and inducible nitric oxide synthase (iNOS). Angiotensin II, a known inducer of oxidative stress, activates NADPH oxidase, leading to the production of reactive oxygen species (ROS) like superoxide anion, hydrogen peroxide, and hydroxyl radicals [190]. Moreover, it triggers inflammatory pathways, promoting the synthesis of TNF-α, contributing to gastric mucosa damage. Telmisartan acts as a partial agonist for the nuclear peroxisome proliferator-activated receptor-γ (PPAR-γ). Activation of PPAR-γ stimulates the expression of the catalase gene and inhibits NF-κB activity, thereby mitigating oxidative stress and reducing pro-inflammatory reactions [191, 192]. Stimulation of PPAR-γ also enhances the production of leptin protein, known for its ability to reduce gastric acid secretion [193]. The drug's impact on gastric health extends to blocking the activation of caspase-3 in the stomach lining, preventing the cascade of events leading to cell apoptosis [194]. Pro-inflammatory cytokines, especially TNF-α, and oxygen-derived free radicals are triggers of cell death by activating caspases. Telmisartan's ability to inhibit apoptosis is likely due to its capacity to reduce reactive oxygen species production and inhibit the synthesis of TNF-α and excessive nitric oxide [102, 195].

In summary, Telmisartan's mechanism of action involves inhibiting NF-κB signaling, reducing oxidative stress, suppressing inflammatory responses, and preventing apoptosis, collectively contributing to its potential therapeutic role in protecting against gastric ulcer formation (Fig. 6 and Table 4).

Fig. 6 [Images not available. See PDF.]

Telmisartan in diabetic ulcer. SOD: Superoxide dismutases, NF-kB: Nuclear factor-kappa B, PPAR-G: peroxisome

Table 4. Role of telmisartan in diabetes

a) Diabetic Nephropathy

5mg/kg/day Telmi [Male db/db and db/m mice]	Intraperitoneal	9 weeks	CD68, MCP-1, TGF-β osteopontin 8-OHdG Nox4 Bax, ROS	Reducing oxidative stress, decreased expression of 8-OHdG and Nox4, increased expression levels of the macrophage marker CD68, the chemokine MCP-1	Sato-Horiguchi et al. [156]
9 mg/kg/day—Telmi, [100 mg/kg-STZ (i.p.), Male C57BL/6 mice]	Orally	6 weeks	PKC-α pathway, ARB	Increased expressions of TGF-β1 and VEGF, decreased activation of PKC-α	Yao et al. [205]
5 and 10 mg/kg—Telmi 60mg/kg-STZ (i.p.), [Male Sprague–Dawley rats]	Orally	12 weeks	Analyses of individual genes, pathway enrichment, GO terms annotation, oxidative phosphorylation pathway, PPAR-g pathway, slit diaphragm	Increased glucose, H2O2, decreased MDA, inhibit overproduction in superoxide and ROS, suppressed AT1R expression	Zhang et al. [162]
b) Diabetic Neuropathy					
10 mg/kg-Telmi, [Male Wistar rats] Traumatization of sciatic nerves	Orally	4 weeks	(IL-1β) gene expression, TNF-alpha and interferon-gamma (IFN-γ), caspase-3 mRNA expression, PPAR-γ pathway	Increase mRNA expression, decrease caspase-3,	Yuksel et al. [167]
5 mg/kg-Telmi [Male albino rats] CCI of the sciatic nerve	Orally	2 weeks	NFκB signaling pathway TNF-α, NADPH oxidase and catalase, STAT3 activation, P38-MAPK pathway ACE, AT1R, AT2R, ACE, ARBs	Increased GFAP expression, COX-2, PGE2 level, decreased MBP expression,	Hegazy et al. [7, 172]
5 and 10 mg/kg/day-Telmi, (55 mg/kg)-STZ (i.p.) [male Wistar albino rats]	Orally	4 weeks	Tumor necrosis factor-α, interleukin-1β, and interleukin-6, Nerve growth factor (NGF), COX-2, MMP-2, and MMP-9, PPAR-γ activation pathway	Increase in pro-inflammatory biomarkers, inhibited NGF levels, decrease in glucose levels, mean body weights	Al-Rejaie et al. [12]

c) Diabetic Retinopathy					
10 mg/kg/day-Telmi, 55 mg/kg-STZ (i.p.), [Male Wistar rats]	Orally	9–10 weeks	RAS receptors, AT1R blockers, NADPH oxidase	Increases the GSH level, improving the protein expression level of BDNF, CNTF, and TH, decreases the caspase-3	Ola et al. [174]
10mg-Telmi, 50mg/kg-STZ (i.p.), [C57BL/6J mice]	Intravitreal	6 months	ATR1, RAAS system, VEGF, RAGE, TNF-ά, NF-κB and PKC pathways	Increase endothelial vascular growth inducer, decrease in tight junction proteins	Cao et al. [184]
d) Diabetic ulceration					
1 mg/kg/day-Telmi, 60 mg/kg-STZ (i.p.), [Male Sprague–Dawley rats]	Orally	1 week	Tumor necrosis factor-α, nuclear factor-κB, Caspase-3 PPARγ pathway	Increased lipid peroxidation, decreased the vulnerability of the gastric mucosa	Fouad et al. [206]

Conclusion

Telmisartan demonstrates neuroprotective effects by targeting inflammation and oxidative stress pathways, potentially useful in neurodegenerative diseases. It also exhibits significant anti-inflammatory properties, particularly in mitigating acute ocular inflammation associated with endotoxin-induced uveitis (EIU). It shows promise in mitigating diabetes-induced vascular inflammation by reducing adhesion molecule expression and inflammatory leukocyte attachment. It also exhibits nephroprotective benefits by modulating PKC-α and VEGF expression. Telmisartan, alleviate neuropathic pain by modulating RAAS components and suppressing JAK2/STAT3 and P38-MAPK signaling pathways. Telmisartan, acting as an AT1 receptor antagonist and PPAR-γ partial agonist, displays antioxidative and anti-inflammatory effects, impacting metabolic and inflammatory pathways. It suppresses TNF-α-induced NF-κB activation, reducing neutrophil infiltration in ulcerative colitis.

Additionally, its ACE inhibitor and AT1 receptor antagonist properties may augment GABAergic transmission, potentially benefiting seizure prevention and reducing edema in traumatic brain injury. Telmisartan shows anti-anxiety effects by blocking AT1 receptors in the brain and may inhibit cerebral AT receptors. It also demonstrates potential as a therapy for endometrial and colon cancer by inducing DNA damage and apoptosis through PPAR-gamma activation. Telmisartan inhibits the proliferation of poorly differentiated hepatocellular carcinoma cells and reduces MMP-9 expression in ovarian cancer cells.

Limitations

Our findings are limited by our main focus on preclinical studies, which means we did not review the effects of telmisartan in clinical studies. Moreover, our attention was directed toward assessing telmisartan's impact on common diseases and disorders, rather than those that are rare.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable for this work.

Consent for publication

The authors declare no conflict of interest.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

ARB
Angiotensin receptor blocker
AT1
Angiotensin II type-1
CD
Crohn’s disease
EIU
Endotoxin-induced uveitis
GSK3β
Glycogen synthase kinase-3 beta
IBDs
Inflammatory bowel diseases
ICAM-1
Intercellular adhesion molecule
IFN-g
Interferon gamma
TNF
Tumor necrosis factor
IKKβ
Inhibition if nuclear factor-kB (IkB) kinase beta
IL
Interleukin
JNK
Jun N-terminal kinase
LPS
Lipopolysaccharide
MAT
Medication-assisted treatment
MCP
Monocyte chemoattractant protein -1
MDA
Malondialdehyde
MPO
Myeloperoxidase levels
PASI
Psoriasis area and severity index

PPAR-γ
Peroxisome proliferator-activated receptor
RANKL/RANK
Signaling pathway
SARM
Selective androgen receptor modulator
TLRs
Toll-like receptors
VCAM-1
Vascular cell adhesion molecule-1
CUMS
Chronic unpredictable mild stress
r-fMRI
Resting-state functional magnetic resonance imaging maximal electroshock (MES) model
PTZ
Pentylenetetrazol test
SSRIs
Selective serotonin reuptake inhibitors
5-HTT
Serotonin
DSBs
DNA double-strand breaks
HCC
Hepatocellular carcinoma
AMP
Activated protein kinase (AMPK) pathway
(mTOR)
Mammalian target of rapamycin pathway
cCK18
Caspase-cleaved cytokeratin 18
MMP-9
Matrix metalloprotease 9,
ROS
Reactive oxygen species,
DR5
Death receptor 5
TGF-b
Transforming growth factor beta
8-OHdG
8-Hydroxydeoxyguanosine
MCP-1
Monocyte chemoattractant protein
CD68
Cluster differentiation
TNF-α
Tumor necrosis factor alpha
PKC-α

Protein kinase C alpha

CCI

Chronic constriction injury

Publisher's Note

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Cyclodextrin inclusion complex and amorphous solid dispersions as formulation approaches for enhancement of curcumin's solubility and nasal

epithelial membrane permeation

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ABSTRACT (ENGLISH)

Background

Curcumin is a compound that occurs in the rhizomes of the turmeric plant (*Curcuma longa*) and has shown potential for the treatment of illnesses including certain neurodegenerative diseases. The bioavailability of curcumin is hindered by its extremely poor aqueous solubility.

Results

This study aimed to apply formulation strategies such as inclusion complex formation with hydroxypropyl-β-cyclodextrin (HPβCD), as well as amorphous solid dispersion (ASD) formation with poly(vinylpyrrolidone-*co*-vinyl acetate) (PVP VA64) and hydroxypropyl methylcellulose (HPMC) to increase curcumin's solubility and thereby its nasal epithelial membrane permeation. The curcumin formulations were evaluated by means of DSC, TGA, FT-IR, XRPD, microscopic imaging, aqueous solubility and membrane permeation across nasal respiratory and olfactory epithelial membranes. The solubility of curcumin was substantially increased by the formulations from 8.4 µg/ml for the curcumin raw material to 79.0 µg/ml for the HPβCD inclusion complex, 256.4 µg/ml for the HPMC ASD and 314.9 µg/ml for the PVP VA64 ASD. The HPMC ASD only slightly changed the membrane permeation of curcumin, while the PVP VA64 ASD decreased the membrane permeation of curcumin. The HPβCD inclusion complex enhanced the nasal epithelial membrane permeation of curcumin statistically significantly across the olfactory epithelial tissue and extensively across the respiratory epithelial tissue.

Conclusion

Complexation of curcumin with HPβCD enhanced the solubility of curcumin and thereby also increased its permeation across excised nasal respiratory and olfactory epithelial tissue. This indicated high potential of the curcumin-HPβCD complex for nose-to-brain delivery of curcumin for treatment of neurodegenerative diseases by means of intranasal administration.

FULL TEXT

Background

Turmeric, a spice that originates from the rhizomes of the *Curcuma longa* plant, has been used for centuries in Asia and India as an alternative herbal medicine for many acute and chronic diseases (e.g., certain cancers, bacterial infections, inflammatory conditions and oxidative conditions) [1–6]. The biologically active compound found in turmeric is a natural polyphenol identified as curcumin (1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dion) [2, 4, 7]. Curcumin extracted from *C. longa* is accompanied by two other closely related curcuminoids, namely demethoxycurcumin and bis-demethoxycurcumin [1]. Curcumin has been proven to be safe and well-tolerated by many studies, even at doses as high as 8 g/day [3, 4, 6]. In a review by Gupta et al. [3], curcumin's clinical relevance is made prominent with reference to the different diseases that can be treated with this compound, which includes neurodegenerative diseases. Although the exact pharmacological mechanism of action for the many therapeutic actions of curcumin is not yet fully understood, previous studies indicated the possibility of biochemical pathway modulation to suppress inflammation and various other cellular activities [4, 8].

Curcumin's therapeutic potential is hindered by its extremely low solubility, and in addition, it also exhibits relatively low membrane permeability and is susceptible to rapid metabolism [4]. Consequently, curcumin has very low oral bioavailability, which means that large oral doses are required to achieve the desired therapeutic outcomes. This

necessitates the consideration of using a different route of administration than the oral route. The nasal route of drug administration is a favorable alternative as the nasal cavity is highly vascularized and bypasses first-pass metabolism [9]. This route of drug administration also has the unique capability of bypassing the blood–brain barrier for improved drug delivery into the brain [10]. The respiratory region of the nasal cavity contains the trigeminal nerve that is linked to the pons and cerebrum [9–11], through which molecules such as curcumin can reach the brain. More importantly, the olfactory region in the nasal cavity has been associated with direct nose-to-brain drug delivery [9–11]. This region is innervated by the olfactory nerves that directly connect the nasal cavity to the brain through the olfactory epithelium [9, 12]. Drug molecules can move to the brain from the olfactory region via different mechanisms including passive diffusion across the olfactory epithelium, along the olfactory neuron's axon or through the gaps between the olfactory nerves and ensheathing cells [9].

The concentration gradient required to drive the passive diffusion process can be aided by enhancing curcumin's solubility [13], which can be done by utilizing formulation strategies such as preparation of inclusion complexes with hydroxypropyl-β-cyclodextrin (HPβCD) as well as the formation of amorphous solid dispersions (ASD) with polymers. HPβCD is a cyclic oligosaccharide with a cone-shaped conformation that has a hydrophobic core and hydrophilic outer surface, making it an ideal carrier for hydrophobic molecules such as curcumin to enhance its solubility [14]. ASDs are formulations where the active compound is dispersed within the matrix of a hydrophilic polymer, thereby altering the crystalline packing of the molecules, resulting in the stable amorphous state with increased solubility [15].

Methods and materials

Materials

The curcumin (98%) raw material used in this study was obtained from Toronto Research Chemicals Inc., Toronto, Canada. The purity of the curcumin raw material was provided on a Certificate of Analysis by the supplier and also determined by comparing the concentration of the same quantity of a curcumin reference standard by means of HPLC analysis. The hydroxypropyl-β-cyclodextrin (HPβCD), poly(vinylpyrrolidone-*co*-vinyl acetate) (PVP VA64) and hydroxypropyl methylcellulose (HPMC) were sourced from DB Fine Chemicals, Johannesburg, South Africa. All solvents used in the preparation of the formulations, including ethanol and methanol, were purchased from ACE Chemicals, Johannesburg, South Africa. The ingredients to produce Krebs-Ringer Bicarbonate Buffer (KRB) used in the permeation studies and Lucifer yellow (LY) were acquired from Sigma-Aldrich, Johannesburg, South Africa. Consumables used, which include 100 µl crimp cells, were purchased from Mettler Toledo, Greifensee, Switzerland; 1-µm Acrodisc® Glass Fiber membrane filters were sourced from Pall Corporation, Bengaluru; Costar® 96-well plates were obtained from The Scientific Group, Randburg, South Africa; hydrochloric acid was sourced from Merck, Johannesburg, South Africa; acetic acid glacial and *n*-hexane were sourced from ACE Chemicals, Johannesburg, South Africa, while dimethylsulfoxide was purchased from Merck Chemicals (Pty) Ltd, Johannesburg, South Africa. The dialysis membranes (Membra-Cel™, 34 mm, 14 k Da) used in the compound release studies were sourced from Viskase® Companies, Inc, Illinois, USA.

Methods

Analytical methods

Ultraviolet (UV) spectroscopic analytical method

All samples were quantified for curcumin content by means of UV absorbance spectroscopy at a wavelength of 432 nm using a Spectramax® Paradigm Multi-Mode microplate reader (Molecular Devices, San José, California, USA). The UV absorbance analytical method was validated in terms of linearity, repeatability (intra-day precision), intermediate precision (inter-day precision), specificity and accuracy [16]. The limit of detection (LOD) and the limit of quantification (LOQ) were also determined. The stock solution of curcumin (42 µg/ml) used for the serial dilution was prepared in 40% *v/v* methanol (MeOH) in ultrapure water (UPH_2O).

Fluorescence spectrophotometric analytical method

Lucifer yellow containing samples collected during the membrane integrity experiment were analyzed using fluorescence spectroscopy with a Spectramax® Paradigm Multi-Mode microplate reader with the excitation

wavelength set to 485 nm and emission wavelength set at 525 nm [17]. The fluorescence analytical method was also validated in terms of linearity, repeatability (intra-day precision), intermediate precision (inter-day precision) and accuracy, and the LOD and LOQ were also determined.

Curcumin–cyclodextrin inclusion complex preparation

The inclusion complex prepared between curcumin and HPβCD in this study was conducted according to a previously reported solvent evaporation method by Yadav et al. [18]. In order to obtain a stoichiometric ratio of 2:1 for HPβCD:curcumin in solution, a quantity of 1.00 g curcumin was added to 400 ml of methanol, which was stirred until a clear solution was obtained and then a quantity of 6.12 g HPβCD was added to the curcumin solution and stirred until everything was dissolved. The solution was left at room temperature on a magnetic stirrer in a fume hood for 72 h to evaporate the solvent. The dry powder (i.e., curcumin-HPβCD inclusion complex) obtained was then passed through a sieve (aperture size: 850 μm), and the product was stored in an amber bottle in a desiccator.

Curcumin-polymer amorphous solid dispersion (ASD) preparation

The curcumin ASDs were prepared based on a solvent evaporation method published by Wegiel et al. [19]. Two ASDs were prepared, each with a different polymer by employing a 1:4 weight ratio of curcumin:polymer.

For preparation of the curcumin ASD with PVP VA64, a quantity of 1.00 g curcumin was added to 400 ml ethanol, which was stirred until a clear solution was obtained. A quantity of 4.00 g PVP VA64 was then added to the curcumin solution and stirred until a clear solution was obtained. The solution was placed in a round-bottom flask and placed on a rotary evaporator (Rotavapor R-210 and heating bath B-491, Buchi Flawil, Switzerland) at 40 °C for 1 h. The residue was emptied into a glass baking dish that was placed in the fume hood (at room temperature) and left to evaporate all the remaining solvent overnight. The crystals obtained were ground into a fine powder with a mortar and pestle.

To prepare the ASD consisting of curcumin and HPMC, the following method was employed. A mixture of water and an organic solvent (ethanol) was used to ensure sufficient solubility of both curcumin and the polymer [13]. A quantity of 1.99 g curcumin was allowed to dissolve in 85% v/v ethanol in water solution. When fully dissolved, a quantity of 8.00 g HPMC was gradually added, while stirring until no solid particles were visible. The solution was then transferred to a round-bottom flask and placed on a rotary evaporator (Rotavapor R-210 and heating bath B-491, Buchi Flawil, Switzerland) for 2 h at 40 °C. The residue was decanted into a glass baking dish and left overnight in a fume hood to evaporate all the solvent at room temperature. The powder (i.e., ASD) obtained after evaporation was ground into a fine powder with a mortar and pestle.

Characterization of curcumin–cyclodextrin inclusion complex and amorphous solid dispersions

Fourier transform infrared spectrometry (FT-IR)

Fourier transform infrared spectrometry (FT-IR) was employed to identify if complex formation occurred between curcumin and HPβCD, as well as the formation of ASDs with the two selected polymers. FT-IR spectra were recorded for the curcumin-HPβCD inclusion complex, the curcumin-PVP VA64 ASD, the curcumin-HPMC ASD, simple physical mixtures of all the combinations and each of the raw materials individually, with a Shimadzu IR Tracer-100 spectrophotometer (Shimadzu, Kyoto, Japan), utilizing a spectral range of 750–4000 cm^{-1}, 64 scans and a 4 cm^{-1} resolution.

X-ray powder diffraction (XRPD)

X-ray powder diffraction (XRPD) is an analytical technique commonly used to identify the presence of crystalline material within a sample of solid material. The sample is exposed to the radiation of X-rays, and the diffractometer then measures the intensity and the scatter provided by the sample. The diffractogram of a specific material is unique to its crystal structure [20]. An XRPD pattern or diffractogram was generated using a PANalytical Empyrean diffractometer (PANalytical, Almelo, Netherlands) for each of the following: the curcumin-HPβCD-complex, the curcumin PVP VA64 ASD, the curcumin-HPMC ASD, physical mixtures of all the combinations and each raw material. The diffractograms were recorded under the following conditions: target, copper; voltage, 40 kV; current, 30 mA; divergence slit, 2 mm; anti-scatter slit; 0.6 mm; detector slit, 0.2 mm; monochromator; scanning speed, 2°/min (stepsize 0.025°; step time, 1.0 s).

Simultaneous thermal analysis (DSC/TGA)

To observe any heat flow endotherms and weight changes, simultaneous differential scanning calorimetry (DSC) and thermogravimetrically analysis (TGA) were performed. Thermograms of the curcumin-HPβCD inclusion complex, curcumin-PVP VA64 ASD, curcumin-HPMC ASD, physical mixtures of all the combinations and each raw material were recorded using a Mettler DSC 3+(Mettler Toledo, Greifensee, Switzerland) connected to a computer equipped with Mettler STARe Default DB V14.00 software (V16.30a). A quantity of 5–6 mg of each material was transferred to crimp cells (100 µl) and placed in an automatic sampler, which transferred the cells into the furnace. The samples were heated to 195 °C at a heating rate of 10 °C/min and purged with nitrogen gas at a rate of 5 ml/min. An empty 100 µl crimp cell was implemented as the reference.

Visual inspection with polar and light microscopy

Microscopic inspection of the samples was carried out using a Nikon Eclipse E4000 microscope equipped with a Nikon DS-Fi1 camera (Nikon, Japan). A small quantity of each material was centrally placed on a microscopic slide and inspected with the microscope at a magnification of 4× and 10×. The first comparison was drawn between the curcumin-HPβCD complex and a physical mixture of curcumin and HPβCD in the 1:2 molar ratio without the polarity filter. The second comparison was between the ASD and their physical mixtures, each containing 25% curcumin, assessed with the polarity filter.

Aqueous solubility

Solubility studies were executed similar to a method published by Mangolim et al. [21]. The solubility was determined over a period of 3 h in accordance with the time of exposure of each material in solution to the excised nasal epithelial membranes during the ex vivo permeation studies. For the aqueous solubility studies, a sufficient quantity of curcumin-HPβCD inclusion complex, curcumin-PVP VA64 ASD, curcumin-HPMC ASD, physical mixtures of all the combinations and curcumin raw material was added to 10 ml of ultrapure water in amber glass tubes to provide supersaturated solutions (5 mg/ml). The solubility studies were conducted in triplicate ($n=3$). A water bath was preheated to 37 °C (±2 °C) and the samples were then placed onto a rotary axis within the water bath. The tubes were rotated for 3 h whereafter it was removed, contents filtered (1-µm glass fiber membrane filter) and analyzed for curcumin concentration using UV–visible spectroscopy (wavelength=432 nm).

Curcumin inclusion efficiency in hydroxypropyl-β-cyclodextrin complex

A method previously published by Mendes et al. [22] was used to determine the quantity of curcumin included in the complex formed with HPβCD. This method is based on the practical insolubility of cyclodextrin and its complexes in *n*-hexane [22]. Approximately 5 mg of the complex was accurately weighed to which 0.1 ml acetic acid was added and it was then made up to 5 ml with *n*-hexane to remove the free curcumin molecules not included in the cyclodextrin complex. The supernatant was then removed and analyzed for curcumin content, which represented the free curcumin. The residue (curcumin-HPβCD complex) was dried at 35 °C for 20 min or until it was dry. After drying, 400 µl DMSO and 400 µl 2% *v/v* acetic acid were added to the powder. The mixture was allowed to precipitate over a period of 12 h. Thereafter, it was centrifuged at 3000 rpm for 10 min. The supernatant was diluted with MeOH (ratio: 1:1) and analyzed for curcumin content, which represented the curcumin included in the cyclodextrin complex. Analysis was performed using the validated UV absorbance analysis method on a Spectramax® paradigm plate reader at a wavelength of 426 nm. The percentage curcumin included into the cyclodextrin complex (i.e., % inclusion efficiency) was calculated with the following equation:

1

$$\%\,\text{inclusion efficiency} = \frac{\text{Complexed curcumin}}{\text{Free curcumin} + \text{Complexed curcumin}} \times 100$$

Ex vivo permeation studies

Preparation of nasal epithelial tissue

The sheep nasal epithelial tissue was excised from animals slaughtered at an abattoir and prepared for the ex vivo permeation studies as previously published by Haasbroek-Pheiffer et al. [17] and Gerber et al. [23]. In brief, a reciprocating saw was used to remove the snout from a slaughtered sheep's head at an abattoir by a horizontal cut anterior to the eyes. The snout was then immersed in cold KRB (±4 °C) and taken to the laboratory. At the

laboratory, the skin was removed via blunt dissection and the snout was separated along the septal medial line using a bandsaw, allowing the abstraction of the septum and exposing the respiratory tissue (ventral nasal conchae). First, a vertical incision across the most distal part of the ventral conchae was made slightly proximal to its round edge, followed by a second horizontal incision across the inferior portion of the ventral conchae parallel to the hard palate. This allowed the separation of the respiratory epithelial tissue from the underlying cartilage, providing a sheet of epithelial tissue. The olfactory epithelial tissue was removed from ethmoid conchae in a similar way by carefully detaching it from the cartilage. The dissected epithelial tissue was then cut into strips (approximately 1 cm × 2.5 cm) that were mounted onto the half-cells of a Sweetana–Grass diffusion chamber. After assembly of the half-cells of the Sweetana–Grass diffusion apparatus, a volume of 7 ml pre-heated KRB was added to each chamber, then placed into a heating block and connected to a carbogen source, containing 95% oxygen and 5% carbon dioxide. The mounted tissue was equilibrated at 34 °C for 30 min before the commencement of the permeation study.

Determining the integrity of the mounted nasal epithelial tissue

Lucifer yellow (LY) was used as an exclusion marker molecule to provide evidence that the nasal epithelial tissues (i.e., respiratory and olfactory) mounted in between the Sweetana–Grass half-cells remained intact for the duration of the permeation study. LY permeation was determined as previously published by Haasbroek-Pheiffer et al. [17] and Gerber et al. [23]. In brief, a LY stock solution (50 µg/ml) was prepared in pre-heated (34 °C) KRB and 7 ml of this solution was placed into each of the six apical chambers of the Sweetana–Grass diffusion chamber apparatus. The basolateral chamber contained pre-heated KRB with 5% *v/v* ethanol. Samples (180 µl) were withdrawn from the basolateral chamber at 20-min intervals over a period of 2 h. The withdrawn volume (180 µl) was replaced with an equal amount of pre-heated KRB containing 5% *v/v* ethanol. The samples were collected in a Costar 96-well plate, and the samples were analyzed for LY content by means of fluorescence spectroscopy (excitation: 485 nm, emission: 525 nm) using a Spectramax® Paradigm Multi-Mode microplate reader as described above.

Curcumin permeation across nasal olfactory and respiratory epithelial tissue

For the curcumin permeation studies done in the absorptive direction, experimental solutions were prepared for each curcumin material (i.e., curcumin raw material alone, curcumin-HPβCD complex, curcumin-PVP VA64 ASD and curcumin-HPMC ASD) in pre-heated KRB (34 °C) at a concentration of 3 mg/ml curcumin. A volume of 7 ml of each experimental solution was placed into each apical chamber, while a volume of 7 ml pre-heated (34 °C) KRB containing 5% *v/v* ethanol was placed into each basolateral chamber. At pre-determined time intervals (i.e., 20, 40, 60, 80, 100 and 120 min) samples, 1000 µl) was withdrawn from the basolateral chamber, which was immediately replaced with the equal quantity of pre-heated (34 °C) KRB containing 5% *v/v* ethanol. The samples were collected in a transparent Costar 96-well plate and assessed for curcumin content. This was done by measuring UV absorbance at a wavelength of 432 nm, utilizing a Spectramax® Paradigm Multi-Mode microplate reader from Molecular Devices in San José, California.

Curcumin entrapment in nasal olfactory and respiratory epithelial tissue

At the end of the permeation study (i.e., at 120 min), the nasal epithelial tissue was removed from the chamber and cut into small pieces with dissection scissors and placed in 1 ml methanol, after which it was ultrasonicated for 10 min, centrifuged and a sample was withdrawn from the supernatant to determine the quantity of curcumin retained in the tissue. These samples were collected in a clear Costar 96-well plate and analyzed for curcumin concentration with UV absorbance at a wavelength of 432 nm using a Spectramax® Paradigm Multi-Mode microplate reader (Molecular Devices, San José, California).

The permeation studies were conducted across respiratory and olfactory nasal tissues with six independent repeats (n=6) for each experimental solution.

Curcumin release and permeation across synthetic dialysis membrane

To assess curcumin release from the cyclodextrin inclusion complex and ASD formulations (i.e., from the curcumin-HPβCD complex, curcumin PVP VA64 ASD and curcumin HPMC ASD), permeation studies were conducted across a synthetic dialysis membrane (Membra-Cel™, 34 mm, 14 k Da). Segments of the synthetic dialysis membrane,

approximately 1 cm × 2.5 cm in size, were prepared and mounted on the half-cell of the Sweetana–Grass diffusion chamber apparatus. The experimental solutions were prepared following the same procedure for the permeation studies across the nasal epithelial tissue. The apical chamber was filled with the experimental solutions (n = 3), after which 180 µl samples were extracted from the basolateral chamber every 20 min for a duration of 2 h. These samples were then analyzed for curcumin content utilizing the validated UV absorbance method on a Spectramax® Paradigm Multi-Mode microplate reader.

Data processing

The percentage permeation of curcumin across nasal epithelial tissue

To calculate the percentage of curcumin permeated across the excised nasal epithelial tissue at each withdrawal time point of each experimental group, Eq. 2 was employed.

2

%Curcuminpermeation=CurcuminconcentrationattimeintervalCurcuminconcentrationapplied×100

Furthermore, the apparent permeability coefficient (P_{app}) values for each experimental group have been calculated employing Eq. 3 [17].

3

P_app=(dc)/(dt)(1/(A×60×C_0))where dcdt is the permeability rate (concentration/min, slope of the graph of percentage permeation as a function of time), A is the area of the tissue across which diffusion takes place (cm^2), and C_0 is the initial concentration (3 mg/ml) of curcumin applied to the apical chamber.

Statistical analysis

Statistical analysis of the permeation data was performed utilizing a one-way ANOVA (analysis of variance). Subsequently, the data were subjected to measurements of normality and homogeneity after which the post hoc tests were conducted using the Tukey adjustment method.

Results

Analytical method validation

The validation results for the ultraviolet (UV) spectroscopic (for analysis of curcumin samples) and fluorescence spectrophotometric (for analysis of Lucifer yellow samples) analytical methods are portrayed in Table 1.

Table 1. Validation results for the ultraviolet (UV) spectroscopic and fluorescence spectrophotometric analytical methods

Validation parameter	Ultraviolet (UV) spectroscopic method	Fluorescence spectrophotometric method
Linearity (R^2)	0.9993	0.9993
Intra-day precision (%RSD)	1.78	0.86
Inter-day precision (%RSD)	0.78	0.29
Accuracy (%)	99.84	101.46
Limit of detection (µg/ml)	1.91	2.22
Limit of quantification (µg/ml)	5.80	6.74
Selectivity	Yes	N/A (single compound)

The validation results for both the UV spectroscopic and fluorescence spectrophotometric methods complied with previously published criteria for validation of these analytical methods (i.e., linearity: R^2>0.998, precision: %RSD <2% and accuracy: 98–102%) [16, 24].

Characterization of inclusion complex and amorphous solid dispersions

Fourier transform infrared spectrometry (FT-IR)

The FT-IR spectra for curcumin raw material (Cur RM), HPβCD raw material (CD RM), a physical mixture thereof (Cur-CD PM) and the prepared curcumin-HPβCD inclusion complex (Cur-CD complex) are presented in Fig. 1.

Fig. 1 [Images not available. See PDF.]

FT-IR spectra of curcumin raw material (Cur RM), HPβCD raw material (CD RM), a physical mixture of curcumin and HPβCD (Cur-CD PM), and the prepared inclusion complex between curcumin and HPβCD (Cur-CD Complex)

The first characteristic peak of the Cur RM spectra showcases the presence of free phenolic O–H stretching vibration with an absorption band located at 3505 cm^{-1} [25–27]. Additionally, two absorption bands appear at 2967 cm^{-1} and 2841 cm^{-1}, resembling the asymmetric vibrations associated with the methyl group and methoxy group, respectively [27]. Furthermore, the stretching seen at 1627 cm^{-1} is evidence of the keto-enol [17] located on the curcumin molecule and vibrations at 1510 cm^{-1} reveal C=O and C=C on the curcumin molecule. In the CD RM spectrum two absorption bands can be seen, which resemble the O–H and the $C–H_2$ molecules of the HPβCD, located at 3406 cm^{-1} and 2927 cm^{-1}, respectively [25]. Another characteristic band on the CD RM spectra is assigned to the C–O–C bond of this molecule and exhibits stretching at 1033 cm^{-1} [25–27]. In the spectrum for the physical mixture of curcumin and HPβCD, the absorption peaks of each of them are evident. On the other hand, the spectral data of the curcumin-HPβCD complex differ from those of the individual components.

The FT-IR results obtained for the curcumin-polymer ASDs are displayed in Fig. 2. The FT-IR spectrum for HPMC raw material exhibits a broad absorption band located at 3458 cm^{-1}. The same spectrum shows characteristic peaks of HPMC, at 1497 cm^{-1} and at 950 cm^{-1}. The physical mixture, containing curcumin and HPMC, showed a combination of the two component's spectra. A noteworthy peak broadening and shift from 1602 to 1587 cm^{-1} showcase a bond between the HPMC and curcumin.

Fig. 2 [Images not available. See PDF.]

FT-IR spectra of **a** curcumin raw material (Cur RM), HPMC raw material (HPMC RM), a physical mixture of curcumin and HPMC (Cur HPMC PM) and the prepared curcumin-HPMC ASD (Cur HPMC ASD) and **b** curcumin raw material (Cur RM), PVP VA64 raw material (PVP VA64 RM), a physical mixture of curcumin and PVP VA64 (Cur PVP VA64 PM) and the curcumin-PVP VA64 ASD (Cur PVP VA64 ASD)

The FT-IR spectrum of PVP VA64 presented with characteristic peaks at 1668 cm^{-1} and 1737 cm^{-1}. The physical mixture consisting of curcumin and PVP VA64 exhibited a combination of the peaks on the spectra of each individual material. When observing the FT-IR spectrum of curcumin-PVP VA64 ASD (Fig. 2b), an amine bending vibration can be seen at 1584 cm^{-2}.

X-ray powder diffraction (XRPD)

The XRPD diffractogram of the respective raw materials and physical mixtures, the inclusion complex between curcumin and HPβCD (Cur CD complex) and the ASDs formed between curcumin and the two selected polymers, namely PVP VA64 (Cur PVP VA64 ASD) and HPMC (Cur HPMC ASD), are shown in Fig. 3.

Fig. 3 [Images not available. See PDF.]

XRPD diffractogram **a** of curcumin raw material (Cur RM), HPβCD raw material (CD RM), a physical mixture of curcumin and HPβCD (Cur-CD PM), the prepared curcumin-HPβCD inclusion complex (Cur-CD Complex), diffractogram **b** ASD prepared with curcumin and HPMC (Cur HPMC ASD), relevant raw materials and physical mixture, and, diffragtogram **c** with PVP VA64 (Cur PVP VA64 ASD), relevant raw materials and physical mixture

In Fig. 3a, curcumin raw material exists in a crystalline state presenting a diffractogram with high-intensity peaks,

whereas HPβCD produced a diffractogram showcasing its amorphous form [25]. As expected, the simple physical mixture produced a diffractogram with a combination of the peaks in both its components, where these peaks were less potent and may be due to the dilution effect of HPβCD and curcumin. The prepared Cur-CD complex on the other hand produced the loss of some peaks that are present in the abovementioned materials such as 9.07°, 14.67°, 17.36°, 18.28° (2Θ). Furthermore, additional peaks appeared for the inclusion complex (14.2°, 26.01° and 27.14° (2Θ)) that did not previously exist in the other materials.

A striking difference can be observed in the X-ray powder diffractogram of the HPMC ASD prepared with HPMC and curcumin (Fig. 3b), and the same phenomenon can be observed when comparing the curcumin diffractogram to that of the curcumin PVP VA 64 ASD (Fig. 3c).

Differential scanning calorimetry (DSC)

The DSC thermograms of the relevant raw materials, their physical mixture and the Cur-CD complex are shown in Fig. 4a. Curcumin exhibited an intensive endothermic peak at 182.24 °C. In contrast to this, the DSC thermogram of the CD RM did not exhibit any endotherm in the scanned temperature region. The DSC thermogram of the physical mixture showcased a combination of the individual components with a melting point of 182.24 °C, corresponding with that of curcumin. The Cur-CD complex exhibited a shift in the endothermic peak at 173.73 °C.

Fig. 4 [Images not available. See PDF.]

DSC thermograms of curcumin raw material (Cur RM) including that of **a** HPβCD raw material, physical mixture (PM) as well as inclusion complex (Cur-CD complex), **b** HPMC raw material, physical mixture (PM) and ASD, c) PVP VA64 raw material, physical mixture (PM) and ASD

Figure 4b and c shows the DSC thermograms for the ASDs of curcumin prepared with PVP VA64 and HPMC, respectively. The polymers (HPMC and PVP VA64) exhibited no endotherm, characteristic of the amorphous state thereof. The physical mixture of the above-mentioned polymers and curcumin produced a thermogram that had a subtle curcumin melting peak, possibly due to the dilution effect of curcumin by the polymer. The curcumin-HPMC and -PVP VA64 ASDs, on the other hand, showed no endotherms, therefore indicating the amorphous state of these ASDs [28].

Thermogravimetric analysis (TGA)

The TGA thermograms of curcumin raw material, HPβCD, PVP VA64, HPMC, their physical mixtures, the curcumin-HPβCD inclusion complex, curcumin-PVP VA64 ASD and curcumin-HPMC ASD are shown in Fig. 5.

Fig. 5 [Images not available. See PDF.]

TGA thermograms of the prepared formulations prepared with curcumin (Cur RM), and different polymers (HPMC RM and PVP VA64 RM). **a** TGA of curcumin-HPβCD, **b** TGA of curcumin HPMC ASD and **c** TGA of curcumin PVP VA64 ASD

In the TGA thermogram Fig. 5a, curcumin did not show any weight loss at the temperatures scanned and the Cur-CD physical mixture had a 3.45% weight loss. The CD RM produced a 4.73% weight loss, whereas the Cur-CD complex produced a noteworthy difference with a 9.02% weight loss.

Displayed in Fig. 5b and c are the TGA thermograms of the prepared curcumin-HPMC ASD and curcumin-PVP VA64 ASD formulations, respectively. The total weight loss of the HPMC sample was 3.5% between 40 and 90 °C. The curcumin-HPMC ASD, PVP VA64 raw material and curcumin-PVP VA64 ASD presented with an 5.3%, 8.5% and 7.6%, weight loss, respectively.

Visual inspection with polar and light microscopy

In Fig. 6, the visual differences between the particles of the prepared Cur-CD inclusion complex powder and the physical mixture between curcumin and HPβCD can be seen. The HPβCD in the physical mixture appears as spherically shaped transparent particles and the curcumin appears as bright orange particles. The Cur-CD inclusion complex (Fig. 4b) appears as light, yellow-colored particles with uniformity in texture and no distinction could be made between the two components.

Fig. 6 [Images not available. See PDF.]

Light microscope photographic images of **a** a physical mixture of curcumin and HPβCD and **b** a curcumin-HPβCD (Cur-CD) inclusion complex (magnification ×10)

Figure 7a displays the polarized microscopic image of a physical mixture of curcumin and HPMC, while Fig. 7b displays the polarized microscopic image of the Cur-HPMC ASD. It is evident that in the physical mixture, crystalline curcumin particles are visible in the microscopic image, whereas the microscopic image of the curcumin-HPMC ASD particles indicates it exists in the amorphous state. The second ASD formulation showcases the curcumin PVP VA64 ASD (Fig. 7d) and its physical mixture (Fig. 7c), and the same birefringence can be seen for the crystalline curcumin inside the physical mixture with none in the ASD.

Fig. 7 [Images not available. See PDF.]

Polarized microscope images of **a** physical mixture of curcumin and HPMC (magnification: ×4). **b** Cur-HPMC ASD (magnification: ×10). **c** The physical mixture containing curcumin and PVP VA64 (magnification: ×4). **d** The Cur-PVP VA64 ASD (magnification: ×10)

Cyclodextrin inclusion efficiency

An inclusion efficiency of 98.86% was achieved by the curcumin-HPβCD complex prepared in this study.

Aqueous solubility

Different values have previously been reported for the solubility of curcumin in aqueous media with values ranging from 0.60 µg/ml [29], 0.98 µg/ml [30], 0.011 µg/ml [31] to 40.00 µg/ml [32].

In this study, the solubility of curcumin raw material was found to be 8.4 µg/ml at 3 h in water at 37 °C. The aqueous solubility of curcumin was substantially enhanced by the formation of the Cur-CD inclusion complex (79.0 µg/ml), Cur-HPMC ASD (256.4 µg/ml) and Cur-PVP VA64 ASD (314.9 µg/ml).

Curcumin permeation across nasal olfactory and respiratory epithelial tissue as well as the dialysis membrane

The ex vivo permeation results (P_{app} values) for curcumin across excised nasal respiratory and olfactory epithelial tissues after the application of curcumin raw material (RM) as well as the CD inclusion complex and two ASDs are presented in Fig. 8.

Fig. 8 [Images not available. See PDF.]

Apparent permeability (P_{app}) values of curcumin raw material (CURCUMIN RM), curcumin-HPβCD complex (CD COMPLEX) and the ASD formulation with curcumin and HPMC (HPMC ASD) as well as curcumin and PVP VA64 (PVP VA64 ASD), respectively, across excised sheep nasal mucosa as well as the dialysis membrane. *The curcumin-HPβCD complex group showed a statistically significant ($p<0.05$) difference when compared to the curcumin raw material (control) across the olfactory nasal tissue. #The curcumin-PVP VA64 ASD also resulted in a statistically significant ($p<0.01$) difference when compared to the control across both tissue types

The permeation of curcumin raw material (Fig. 8) was extremely low across the excised sheep nasal respiratory epithelial tissue ($P_{app} = 7.1 \times 10^{-8}$ cm/s) as well as across the excised sheep nasal olfactory epithelial tissue ($P_{app} = 6.1 \times 10^{-8}$ cm/s). The permeation of curcumin, when applied as the HPMC ASD, was lower than that of curcumin raw material ($P_{app} = 6.2 \times 10^{-8}$ cm/s) across the respiratory epithelial tissue. On the other hand, it was slightly higher than curcumin raw material across the olfactory epithelial tissue ($P_{app} = 6.6 \times 10^{-8}$ cm/s). The permeation of curcumin when applied as the PVP VA64 ASD was significantly lower across both the respiratory epithelial tissue ($P_{app} = 2.7 \times 10^{-8}$ cm/s) and olfactory epithelial tissue ($P_{app} = 2.4 \times 10^{-8}$ cm/s) as compared to that of curcumin raw material. Curcumin released from the HPMC ASD ($P_{app} = 3.03 \times 10^{-8}$ cm/s) across the dialysis membrane was slightly higher than that of the PVP VA64 ASD, but it was still relatively low (Fig. 8).

Curcumin permeation from of the Cur-CD inclusion complex was significantly higher across the olfactory epithelial tissue ($P_{app} = 8.9 \times 10^{-8}$ cm/s) and markedly higher across the respiratory epithelial tissue ($P_{app} = 8.2 \times 10^{-8}$ cm/s) as compared to that of curcumin raw material. In addition, the HPβCD inclusion complex demonstrated the highest

permeation (P_{app} = 4.9 × 10^{-8} cm/s) of the three formulations across the dialysis membrane.

Curcumin entrapment in nasal olfactory and respiratory epithelial tissue

A comparison of the percentage of curcumin entrapped in the nasal olfactory and respiratory epithelial tissue at the end of the permeation experiment (120 min) for each experimental group can be seen in Fig. 9.

Fig. 9 [Images not available. See PDF.]

Percentage entrapment values of curcumin alone (CURCUMIN RM), curcumin HPβCD complex (CD COMPLEX) and the two ASD formulations with curcumin and HPMC (HPMC ASD) as well as curcumin and PVP VA64 (PVP VA64 ASD), respectively, across excised sheep nasal mucosa. #The curcumin-HPβCD complex showed a statistically significant (p<0.01) difference when compared to both nasal tissue types. ##The curcumin PVP VA64 ASD also resulted in a statistically significant (p<0.01) difference when compared to the control across the olfactory tissue. *The HPMC ASD resulted in a statistically significant (p<0.05) difference compared to curcumin across the olfactory epithelia

Curcumin PVP VA64 ASD produced a statistically significantly lower entrapment percentage of curcumin in the olfactory tissue (0.22%) when compared to that of the curcumin RM (1.26%). The curcumin-HPMC ASD presented with a significantly higher quantity of curcumin entrapped in the olfactory tissue (2.38%) when compared with the curcumin RM. The curcumin entrapped in the respiratory tissue (1.13%) was markedly higher than that of curcumin raw material (0.79%).

In line with the membrane permeation results obtained, the curcumin-HPβCD complex showed the highest membrane entrapment values, which were significantly higher than that of curcumin raw material in both nasal epithelial tissue types with 3.09% in the olfactory epithelial tissue and 2.51% in the respiratory tissue.

Discussion

The FT-IR spectral data of the curcumin–cyclodextrin inclusion complex and raw materials can be seen in Fig. 1. Cur RM spectra exhibited characteristic resemblances with previously published results [25, 33]. Seeing that the Cur RM did not exhibit a carbonyl stretch at ± 1700 cm^{-1} (characteristic of its di-keto form), it can be concluded that the keto-enol form of curcumin was used. The FT-IR spectrum of the CD RM also displayed similar characteristics as previously obtained [3, 25]. When referring to the physical mixture of curcumin and HPβCD, the absorption peaks of each of them are evident. On the other hand, the spectral data of the curcumin-HPβCD complex differ from those of the individual components. The overpowering absorption peaks seen are allocated to the CD RM, disguising the characteristic peaks associated with curcumin. The unique C-O and C–C stretching vibration seen at 1516 cm^{-1} confirms the conformation of a Cur-CD complex [25].

The FT-IR spectral data of the curcumin-polymer ASDs and raw materials can be seen in Fig. 2. The broad absorption band located at 3458 cm^{-1} in the HPMC raw material spectrum (Fig. 2a) is indicative of the O–H group found in HPMC [34]. Characteristic peaks of HPMC are visible, at 1497 cm^{-1}, indicating vibration of the O–H group, and the peak at 950 cm^{-1} reveals a C–O stretching [29], which is in accordance with previously published literature [34]. The physical mixture of curcumin and HPMC presented with a combination of the two component's spectra. The fingerprint zone found between 1500 and 750 cm^{-1} has a unique pattern for each compound normally consisting of bending vibrations [35]. A noteworthy peak broadening and shift from 1602 to 1587 cm^{-1} showcase a bond between the HPMC and curcumin. This corresponds with a similar bond identified by Chai, Isa [36]. The intermolecular interactions observed on the FT-IR spectrum of the prepared curcumin-HPMC ASD material and the disappearance of some crystalline peaks of curcumin are suggestive thereof that an ASD was indeed formed between curcumin and HPMC as polymer.

A recent article published by Dong et al. [37] noted that there are two characteristic peaks on the FT-IR spectrum of PVP VA64, namely the C=O located on the pyrrolidone ring at 1668 cm^{-1} and the other being the vinyl acetate at 1737 cm^{-1}. The physical mixture of curcumin and PVP VA64 presented with a combination of the spectra of each individual material. The PVP VA64 polymer within the curcumin-PVP VA64 ASD conceals most of the curcumin peaks except the amine bending vibration at 1584 cm^{-2}. From the FT-IR spectra, it can be concluded that the

curcumin-PVP VA64 ASD has been formed.

The formation of an inclusion complex between curcumin and cyclodextrin has been confirmed by the XRPD diffractogram (Fig. 3), These findings align with results published in a previous study by Mangolim et al. [21]. ASD formation with both HPMC and curcumin and PVP VA 64 and curcumin was confirmed by its characteristic amorphous state. The difference between the ASD diffractograms and the curcumin RM is due to a halo-pattern characteristic of the amorphous state of the ASD, which has been described in previously published work as a confirmation of ASD formation [38–40]. A possible restricting factor of XRPD analysis is its inability to differentiate between the amorphous phases. The distinguishing of these phases is beneficial to identify phase separation (drug-rich or polymer-rich phases) in ASDs and the possibility of subsequent recrystallization [41]. When DSC analysis is performed in conjunction with XRPD, this problem can be overcome [41].

DSC thermograms (Fig. 4) confirmed the endothermic peak for curcumin raw material at 182.24 C which correlates with the melting point of this compound [42]. The CD RM did not exhibit an endotherm reaction, while the physical mixture presented with a melting point of 182.24 °C. A shift in the endothermic peak was observed at 173.73 °C by the Cur-CD complex, indicating an interaction between curcumin and HPβCD. This phenomenon was recently reported by Low et al. [43] as confirmation of the inclusion of curcumin into an HPβCD complex. It was not possible to identify the glass transition temperature (Tg) of the ASDs at the temperature range scanned. This aligns with the findings of Li et al. [44], who reported that when the curcumin content is below 25%, the ASD has no recrystallization or melting point and is therefore regarded as amorphous and protected from recrystallization at high temperatures. Weight loss determined by the thermogravimetric analysis (TGA) of the physical mixture of Cur-CD can be ascribed to the loss of water [42]. The weight loss presented by the HPMC RM can be attributed to sorbed water in the polymer matrix McPhillips et al. [45]. In addition, water was used as a solvent in the preparation of the HPMC ASD. It is well-established in the literature that PVP molecules exhibit a pronounced affinity for water absorption [46]; therefore, the TGA thermogram in Fig. 5c presented a greater weight loss for the PVP VA64 raw material, which may be due to curcumin molecules replacing water molecules.

Light microscope images of a physical mixture of curcumin and HPβCD and a curcumin-HPβCD (Cur-CD) inclusion complex align with results previously published by Benucci et al. [47], who concluded that the lighter-colored powder particles were inclusion complexes that enhanced the solubility of curcumin. Polarized light microscopy is a technique used to confirm the crystallinity of a compound in solid state. As discussed by Chasse et al. [48], it utilizes a polarizing filter that results in crystalline materials exhibiting polarization colors also known as birefringence and the amorphous state of a drug will appear dark.

The solubility of curcumin was substantially enhanced by the formation of the Cur-CD inclusion complex, Cur-HPMC ASD and Cur-PVP VA64 ASD. When curcumin is entrapped in the hydrophilic cavity of HPβCD, it causes a solubilization effect and thereby increases the apparent solubility of curcumin [49]. Prepared ASDs have potential energy, which was released during the dissolution process, enabling curcumin to reach a supersaturated state [50]. The increased solubility of curcumin could provide a higher concentration gradient across the membrane, which could act as a thermodynamic driving force to propel the curcumin molecules across the membrane [13]. Another advantage described for ASD formulations is the ability to form liquid–liquid phase separation, which can produce small curcumin-rich droplets in solution that will serve as a reservoir and replace the curcumin in the solution that permeates across the membrane [51].

Finally, the curcumin RM, CD inclusion complex, Cur-HPMC ASD and Cur-PVP VA64 ASD were evaluated in terms of permeability across excised ovine respiratory, olfactory and dialysis membrane. Permeation from the HPMC ASD was lower than that of curcumin raw material across the respiratory epithelial tissue and only slightly higher across olfactory epithelial tissue, while permeation from the PVP VA64 ASD was significantly lower than the curcumin RM across both respiratory and olfactory tissue.

The permeation of curcumin from the HPMC ASD showcased a higher permeation when compared to the PVP VA64 ASD across both epithelial tissue types, which can possibly be explained by an interaction between the charged chains of HPMC and the phospholipid bilayer of the epithelial membrane. This may have contributed to the

increased membrane permeability, due to the phospholipid bilayer disruption [52, 53]. Although the HPMC ASD showcased an increased permeation when compared to that of the PVP VA64 ASD, it did not increase the permeation of curcumin when compared to the raw material (control), probably due to the relatively slow release of the curcumin molecules as observed in the results obtained from the dialysis membrane permeation study.
Yu et al. [54] explained that the in vitro release of the compound from the ASD is directly proportional to the compound/polymer ratio, where a higher compound/polymer ratio (e.g., 1:30) resulted in a slower drug release rate. In the PVP VA64 ASD, a 1:25 curcumin:polymer ratio was used and it is hypothesized that this has caused a slow release rate of curcumin from the polymer matrix in the ASD, which restricted the membrane permeation of the curcumin molecules. Maincent, Williams [50] mentioned that the diffusion pathway of compound molecules from the polymer matrix in ASDs decreases as the polymer swells, thereby restricting the diffusion of compound molecules from the polymer matrix. Another possibility was proposed by Schittny et al. [55] namely that the polymer formed a gel-like layer on its outer surface through which the compound will have to diffuse before it is released in order to become available to permeate across the membrane. This explanation was substantiated by the results obtained from the curcumin release study conducted across the dialysis membrane from the PVP VA64 ASD (incorporated in Fig. 8). Given that the combined molecular size of the ASD containing curcumin and the PVP VA64 polymer exceeded the 14 kDa cutoff limit, curcumin molecules that were not released into the solution were unable to traverse the membrane, which resulted in the relatively low P_{app} value of 2.64×10^{-8} cm/s. It can therefore be concluded that the poor release of curcumin from the PVP VA64 polymer could have contributed to the poor membrane permeation.
The increase in permeation was possibly due to the increased concentration gradient as a result of the solubility increase of curcumin when formulated as a cyclodextrin inclusion complex [56]. Another characteristic of the curcumin-HPβCD complex is that the compound is directly deposited onto the cell membrane after dissociation from the complex [57], resulting in an additional factor to increase the concentration gradient and therefore contributing to a higher passive diffusion rate [56].
From all the formulations investigated, the curcumin molecules were therefore released at the highest rate from the HPβCD complex. Although the permeation of curcumin across the dialysis membrane (without a phospholipid bilayer) was not as high as across the nasal epithelial tissue from the HPβCD complex, it can possibly be attributed to the interaction of cyclodextrin with the cholesterol composition of the biological membrane that increased the permeation of curcumin. According to dos Santos et al. [58] and López et al. [59], HPβCD can increase biological membrane fluidity by disruption (removal of cholesterol molecules) and this can increase drug permeation across the membrane.
In general, the permeation of curcumin was higher across the excised olfactory epithelium than across the respiratory epithelium. This is in agreement with previously published work by Fransén et al. [60]. They proposed that it was possibly due to transport along the olfactory nerves, the gaps between the nerves and other diffusion mechanisms. Furthermore, Du et al. [61] explained that the differences in the morphology and thickness of the epithelial tissue types from the different nasal regions may contribute to the difference in their permeability.
Similar to the permeation results, the curcumin PVP VA64 ASD produced a significantly lower entrapment percentage of curcumin within the olfactory tissue than the curcumin RM, and as previously discussed, it could likely be due to the curcumin molecules being released at a low rate and extent from the PVP VA64 polymer matrix [50]. The higher percentage curcumin entrapped within the respective nasal tissues after administration of the curcumin-HPMC ASD could be due to the higher concentration gradient combined with mucoadhesion [48, 54] and potential membrane disturbance [17, 54], can be caused by the charged HPMC polymer chains, which might have been responsible for the increase in the percentage of curcumin delivered into the membrane. Due to the highly lipophilic properties of the curcumin molecules, they remained inside the membrane and did not partition into the aqueous environment on the basolateral side. The significant membrane entrapment of curcumin from the curcumin-HPβCD complex is ascribed to the increased concentration gradient across the membrane providing the driving force to increase the diffusion of curcumin molecules into the membranes. However, due to the highly lipophilic nature of

curcumin, it did not partition out into the aqueous environment on the basolateral side [56].

Conclusion

The curcumin–cyclodextrin (HPβCD) inclusion complex elevated the solubility and nasal epithelial membrane permeation of curcumin substantially across the respiratory epithelial tissue and statistically significantly across the olfactory epithelial tissue. The HPβCD inclusion complex therefore showed potential as a formulation approach to enhance intranasal delivery of curcumin into the systemic circulation (across respiratory epithelial tissue) and into the brain (olfactory epithelial tissue). Enhanced solubility and intranasal delivery of curcumin by means of HPβCD inclusion complex formation means that lower doses can be administered to reach effective concentrations at the site of action. This has the additional advantage of decreasing side effects. Both the ASD formulations (HPMC and PVP VA64) increased the solubility of curcumin noticeably. However, the HPMC ASD only slightly influenced the curcumin permeation as compared to that of curcumin raw material, while the PVP VA64 ASD decreased the permeation significantly probably due to slow release of the curcumin molecules. The ASDs formulated for curcumin with the selected polymers (HPMC and PVP VA64) therefore did not show potential as a formulation approach to enhance intranasal delivery of curcumin.

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Author contributions

C.S. helped in methodology, validation, investigation and formal analysis, writing of original draft. S.v.N. was involved in review and editing of writing. J.H. contributed to conceptualization, methodology, resources, supervision, review and editing of writing, funding. W.L. helped in conceptualization, methodology, resources, supervision, review and editing of writing. All authors have agreed to publish this version of the manuscript.

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Availability of data and material

Data, materials and protocols will be made available on reasonable request to the corresponding author.

Declarations

Ethical approval and consent to participate

The use of excised sheep nasal epithelial tissue in the ex vivo permeation studies was approved by the Animal Ethics Committee of the North-West University (NWU-AnimCare REC with ethical approval number: NWU-00765-22-A5). This study used tissues excised from animals slaughtered at an abattoir for meat production purposes without study-related animal welfare implications.

Consent for publication

Not applicable, as no personal data were used in this current study.

Competing interests

The authors of this study have no conflict of interest to disclose.

Abbreviations

ANOVA

Analysis of variance

ASD

Amorphous solid dispersion

DSC

Differential scanning calorimetry

FT-IR

Fourier transform infrared spectrometry

HPMC

Hydroxypropyl methylcellulose

HPβCD

Hydroxypropyl-β-cyclodextrin

KRB

Krebs-Ringer bicarbonate buffer

LOD

Limit of detection

LOQ

Limit of quantification

LY

Lucifer yellow

P_{app}

Apparent permeability

PVP VA64

Poly(vinylpyrrolidone-*co*-vinyl acetate)

RM

Raw material

TGA

Thermogravimetric analysis

UV

Ultraviolet

XRPD

X-ray powder diffraction

Publisher's Note

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Recent application of green analytical chemistry: eco-friendly approaches for pharmaceutical analysis

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ABSTRACT (ENGLISH)

Background

The substantially operated analytical instruments dealing in the area of analytical chemistry are traditional methods like high-performance liquid chromatography (HPLC) and gas chromatography (GC). Since they use solvents, produce trash, and require energy, these methods seriously compromise the natural milieu. The excessive consumption of an enormous number of organic solvents, along with the trash created from it, can contaminate the environment. As a result, researchers are now creating novel Green Analytical Chemistry approaches to address these environmental problems and create an ecologically preferable replacement.

Main body of the abstract

Ecologically preferable replacement can be accomplished by using a green solvent, such as ethanol or water, in place of harmful solvents. Additionally, the need for solvent can be decreased by omitting the sample preparation stage wherever possible or by utilising alternate green extraction methods. Adoption of compact methods like ultra-high-performance liquid chromatography (UHPLC) may also result in a decrease in the amount of energy used and trash produced during analysis.

Short conclusion

This review features information on using sustainable practises in analytical chemistry as well as details on using green solvents and sample preparation methods such as Solid Phase Extraction (SPE), Qucheers. It also provides information related of application of green analytical techniques such UHPLC, High-Performance Thin Layer Chromatography (HPTLC), and Thin Layer Chromatography (TLC).

FULL TEXT

Background

Implementing green chemistry and placing a focus on it in the laboratory are two themes that are becoming more and more popular in the chemical world. Chemists all across the world are striving to adapt more sustainable, energy-efficient processes and reactions for conventional ones. Green chemistry can take many different shapes, such as redesigning experiments with more environmentally friendly ingredients or actively managing waste [1]. Anastas initially defined "green chemistry" as a field that aims to eliminate or significantly reduce the production of dangerous compounds during any type of chemical process [2]. Although Anastas coined the phrase “green chemistry,” Badami, Nameroff, and Keltz are responsible for starting and maintaining the notion [3, 4]. The first steps in the exertion of green analytical chemistry (GAC) in the analytical chemistry field were taken in 1995 when Anastas highlighted the need to create GAC ideologies in an editorial that was published in a special issue of the Analyst journal that was devoted to the miniaturisation, containment, and reagent replacement in analytical methods [5].

To compare the efficiency of green chemistry approaches to conventional methods, many systems have been devised. This tactic is based on the twelve guiding Principles of Green Analytical Chemistry, which are listed in Table 1 [6–10] and may be remembered by the term significance.

Table 1. 12 Principles of green analytical chemistry

Principle	Green analytical chemistry
1	Use direct analysis wherever feasible
2	Reduce sample size and quantity as much as possible
3	Analysis ought to be done on-site wherever possible
4	Process integration between operations and analysis is necessary to conserve resources
5	Analyses ought to be automated if feasible
6	Refrain from derivatizing
7	Waste output should be kept to a minimum, and good waste management is crucial
8	To reduce the quantity of testing necessary, the technique should, if feasible, be optimised for usage on several analytes
9	Energy usage should be kept to a minimum
10	It is preferable to use renewable and sustainable reagents
11	Less dangerous, poisonous, and safer chemicals are recommended
12	Operator security is essential

One instance area that is making an effort to incorporate green chemistry ideas into equipment and analytical procedures is analytical chemistry. Creating more effective and resilient sample preparation protocols is one of the biggest concerns in analytical chemistry [11–13]. Therefore, as shown in Table 1, the first principle specifies that introducing the sample with minimal or no preparation is the most viable approach to decrease waste. However, most analytes require matrix extraction prior to analysis. The second, fifth, seventh, ninth, tenth, eleventh, and twelve principles may and should be used in situations when sample preparation is required. Techniques for preparing samples should be improved to the greatest extent feasible to decrease the amount of energy needed, solvent usage, waste output, and employee contact. This may be done in an assortment of manners, such as by limiting the amount and quantity of representative, using utile sample extraction equipment, and staying away from organic solvents that are hazardous [14]. Chromatographic strategies, which are used for distinguishing between the components of complicated mixtures in diverse matrices, are one of the topics of chemical analysis that GAC is passionate about. In the procedure of chromatographic analysis, the detrimental effects of harmful solvents to the health of humans and the environment must be ignored. The trash produced by a traditional chromatographic process, that demands a lot of organic solvent and can produce 1–1.5 L of scrap per day, must be dismissed of [15]. Since the majority of utilised organic solvents are volatile, they can quickly disperse and damage the surroundings. Thus, it is the duty of analytical scientists to reduce the detrimental impact produced by their work and to prevent, or at the at least, minimise it.

This systemic analysis review will give information on implementation of sustainable practises in analytical chemistry as well as details on using green solvents and sample preparation methods. Additionally, we have described the

execution of GAC principles for the validation and development of analytical methods utilising HPLC, UHPLC, Ultraviolet–Visible spectroscopy (Uv–Visible Spectroscopy), HPTLC, and TLC.

Main text

Outline of sample preparation approaches and the effects they have on analytical green techniques

Since the processes shown in Fig. 1 may entail the employ of hazardous substances and/or volatile solvents, the sample preparation stage tends to be the analytical method that causes the greatest pollution [Grob, 1984].

Fig. 1 [Images not available. See PDF.]

Analytical methods for measuring an analyte in matrix sample

The use of direct chromatographic methods in the absence of sample preparation

Based on the perspective of the GAC, direct analytical techniques—i.e. those that do not need sample preparation—are especially preferred. It is vital to adopt direct chromatographic procedures as the vast majority of chromatographic studies performed and the likelihood to produce residues during sample preparation. Direct injection of both liquid &solid samples may be effectively carried out by GC or liquid chromatography (LC) analysis, according to Tobiszewski and Namiesnik's 2009 statement [16]. It used to be discouraged to add the sample straight into the chromatographic capillary column, which may be water or a water–ethanol combination. A thick, nonpolar column stationary phase is utilised because water is thought to promote column bleeding, specifically when polar stationary phases are used. Significant improvements in column stationary phase quality and state-of-the-art cross-linking tactics have boosted resistance to deterioration triggered by water. When environmental water is injected into a column, notably when the water carries an immense amount of salt, performance issues with the column and decreased detector sensitivity can arise. In order to keep organic non-volatile &inorganic salts substances out of the analytical column, deactivated pre-columns must be positioned in front of it. The initial on-column injection techniques were the primary aim of the direct gas chromatographic approaches Grob et al. developed in 1978 [17]. In order to identify halogenated chemicals in sample of water by direct aqueous injection, Grob et al. developed an amalgamation of on-column injection and electron capture detection in 1984 [18]. A different approach was created using direct aqueous injection-gas chromatography and mass spectrometric detection to identify polar and nonpolar volatile chemical molecules in sample of water [19]. High-boiling volatile organic chemicals in surface water have recently been identified using greater volume direct aqueous injection-gas chromatography with flame ionisation detection, electron capture detection, and flame photometric detection [20]. The main drawback of these approaches is that they are restricted to the sample with generally pure matrices free of suspended debris [21]. The degradation of the chromatographic column might happen quickly if the analysed sample is not sufficiently clean due to an accumulation of particulates or non-volatiles that cannot release out of the column. Spirits &petroleum portions are illustrative of clean matrix that may be infused onto chromatography columns without any preparation. Since they merely call for straightforward sample preparation methods like filtering, dilution, or centrifugation, several approaches may be categorised as almost direct [22–24]. Thus, there are two ways that direct chromatographic procedures connect to green chemistry. They adhere to the principles of waste reduction and decreased usage of solvents, auxiliaries, and energy by skipping the sample preparation stage.

Green sample preparation

1. Solid Phase Extraction (SPE)

The substantially operated methods for preparing samples is SPE. In SPE, solutes are adsorbed onto a short column of a compatible solid sorbent after an aqueous sample is run over it. The analysed substances are retrieved from the absorbent in minuscule amounts of highly eluting organic solvents, which results in their enrichment [25]. Small quantities of solvent are used during solid phase extraction, which also produces minimal waste. It is therefore regarded as an environmentally beneficial method. SPE may be mechanised with the use of basic, low-cost apparatus, increasing laboratory throughput while increasing accuracy and precision. Despite this method's

advantages, there are certain possible drawbacks that should be taken into account to prevent ineffective analyte extraction. One of the problems is the unevenness of the packing material's bed, that might result in a reduction in efficiency. A secure solution to this issue is through the utilisation of commercial cartridges. Insufficient retention of extremely polar molecules may also result from certain traditional sorbents' low selectivity. Another problem is the conflict over retention between analytes and sample matrix, which has a significant negative influence on analyte recovery. As a result, rigorous process optimisation is necessary to guarantee successful analyte extraction [26].

1. QuEChERS Extraction Methodology

Popular extraction technique QuEChERS is known for being rapid, simple, inexpensive, efficient, robust, and safe. Anastassiades et al. [27] established the comprehensive QuEChERS technique in 2002. This method is regarded as a green extraction method since it only adopts a tiny number of organic solvents in comparison with other extraction means. Two essential phases are included in QuEChERS procedures: the extraction of solvents and sample clean-up. The sample is vigorously shaken throughout the solvent extraction process together with buffer (to safeguard base-sensitive analytical agents inside the sample), anhydrous magnesium sulphate, sodium chloride (to salt out). In order to get rid of interfering matrix components such carbohydrates &fatty acids, the sample cleansing phase is essential. Implementing magnesium sulphate (to soak any remaining water) and a weak anion exchanger known as "primary secondary amine" (PSA) absorbent as the dispersive solid-phase extraction agent, rapid dispersive SPE is enhancing purity of the extract [28]. Several applications used QuEChERS for extracting analytes of interest from blood specimens, including the eradicating a variety of pollutants from human specimens of blood [29], and removing amphetamine, opiate, and cocaine from blood through tandem mass spectroscopy and liquid chromatography [30], and the recently completed extraction of tetrahydrocannabinol (THC) [31].

1. Solid Phase Microextraction (SPME)

The process of extraction &enrichment might be combined using SPME, resulting in solvent-free sample preparation. The initial version of this approach was created in 1990 by Arthur and Pawliszyn. This method makes use of silica fibre that has been coated with the appropriate adsorbent phase. Direct extraction of the analyte from the solution and concentration on the fibre layer are both used [32]. In the SPME approach, the effectiveness of the analyte preliminary concentration might vary depending on a no. of facets, along with the fibre type, sample stirring, extraction duration, etc. [33]. By using SPME in conjunction with, high-performance liquid chromatography (HPLC), GC/mass spectrometry (MS), GC, and LC–MS, substances in food samples could be isolated. Minimal expenditure, ease of use, the removal of solvent disposal expenses, quick sample duration of preparing samples, dependability, susceptivity, and perceptiveness are certain main benefits of SPME. However, there are some shortcomings that need to be identified to prevent adverse outcomes. They include the fibre's brittleness, which makes it potentially breakable when handled, the risk of coating damage from recurrent Competitive absorption when sorbent-type coatings are put to use, the profound impact of mass &temperature transmit factors on equalisation, matrix effects, and so on. [34].

1. Stir-Bar Sorptive Extraction (SBSE)

As a substitution to SPME, SBSE is an environment friendly, free of solvent extraction method that was launched in 1999. The isolation and purification of volatile analytes from the water-based matrix was the original purpose of SBSE. Later, its uses were expanded to include non-volatile analytes in conjunction with HPLC and the examination of the headspace over liquid/ solid samples, or gaseous samples, and other samples. SBSE uses sorptive extraction, just as SPME. Analytical substances get absorbed into a polymer (such as polydimethylsiloxane) matrix

wrapped around a magnetic stir rod in this instance as opposed to a fibre, however. The substantially higher amount of the sorptive phase employed in SBSE compared to SPME is the primary distinction between the two. Better sensitivity is the outcome, particularly when utilising high sample volumes or substances with low partition coefficients. This approach is entirely without solvent in this instance since the coating's ability to capture volatile analytes allows them to be thermally desorbed right into a GC. Non-volatile analytical substance is desorbed using tiny amounts of a diluent, upholding the method's environmentally friendly nature [35].

1. Dispersive Liquid–Liquid Microextraction (DLLME)

The method uses three-phase solution that includes an aqueous sample, an extracting solvent that is immiscible with water, &some dispersive fluid which is solvable with in all the phases. The two distinct solvents are combined, &resulting solutions quickly introduced in the samples to create an extremely fine emulsion. As a result, the analytes may be transferred in the dispersed extraction solution extremely quickly. After the emulsion is created, it is centrifuged. The phase of increasing extraction density is retrieved by using a micro syringe and added to a preferred analytical device. DLLME has a number of benefits, including a small sample volume, little solvent consumption, high enrichment factor, excellent reproducibility, and high recovery. Enormous areas of proximity among the watery part &the solvent mixture are produced by the emulsion created following adding it to the fluid amalgam. As a result, the extraction is effective and the equilibration happens quickly [36].

1. Pressurised fluid extraction (PFE)

This methodology, referred as accelerated solvent extraction (ASE), involves doing the extracting using high pressures &temperatures that are beyond the solvent's boiling point at atmospheric pressure. The analytes’ solubility and diffusion rate rise at the higher temperature, while the solvent’s viscosity and surface tension decrease. Increased pressure makes it easier for the extractant to enter the matrix pores. PFE is regarded as a green technology since it uses less solvent, enables utilizing eco-friendly solvents like ethanol, methanol, and achieves quick and effective extraction in a lesser amount of period with less energy [36].

1. Microwave-Assisted Extraction (MAE)

Microwave radiation is employed in MAE to speed up the extraction procedure. The MAE principle relies on heating the system as a result of polar molecules absorbing microwaves, which heats the solvent that interact with the material to enable the extraction of analytes into a solvent or a water-based solution. It is possible to use the entire range of the microwave frequency, which ranges from 300 MHz to 100 GHz; traditional ovens should only function at 2.45 GHz. The major benefits of the MAE are its quick heating, elevated temperatures, and simplicity of use. Its one drawback is the restricted heating up investigated solvent caused by the dielectric constant [37].

1. Ultrasound-Assisted Extraction (UAE)

Since there are several drawbacks associated with traditional or other newer procedures, such as costly capital investment, the use of energy, and the use of harmful organic compounds for extraction, the use of ultrasound for extraction has grown over the past ten years. Ultrasound-assisted extraction (UAE) is a technique that uses ultrasound during the preapplication processes, and it might be favoured since it is a clean and ecologically friendly extraction approach [38]. As a result, the usage of UAE is now acknowledged as a safe and practical alternative to traditional food production methods. Therefore, when compared to other extraction procedures, ultrasound is a simple to use, multidirectional, versatile, and little investment required technology. Application of ultrasound broadens the range of commonly regarded as safe solvents.

1. Supercritical Fluid Extraction (SFE)

Supercritical fluid extraction (SFE) is an environmentally friendly, simple, and fully automated analytical technique. The term “environmentally benign sample preparation method” (e.g. “typical SFE”) refers to a technique that employs water or other non-toxic solvents. Short extraction times, lower pressures, and temperatures, together with the ability to preserve the integrity of food's functional components, allow SFE to almost minimise the possibility of activity loss [39]. The benefits of SFE include clean extract since less solvent is used and the extraction process takes less time. The extraction of the analytes is completed with no additional clean-up procedures. The sample pretreatment step of this approach lends itself to the most widespread usage of nontoxic and non-polluting extraction fluids [40]. Carbon dioxide, nitrous oxide, ethane, propane, n-pentane, ammonia, and sulphur hexafluoride are some of the most popular supercritical fluids. Due to its lack of abrasive and explosive properties, CO_2 is the most often used gas. Quantitative, straightforward, quick, selective, and ecologically friendly are the SFE's key benefits [41] (Table 2).

Table 2. Green sample preparation technique

	Organic solvent consumption	Energy consumption	Reusability	Laboratory waste	Automation
SPE	0	0	0	1	0
QUECHERS	0	0	0	1	0
SPME	0	0	0	0	0
DLLME	1	1	1	0	1
PFE	0	1	1	0	0
UAE	1	1	1	0	1
SFE	0	1	0	0	1

0-Low/Yes

1-High/No

Greener organic solvents in analytical chemistry

The most ecologically conscious and long-lasting solvent that might be used in analytical chemistry is unquestionably with zero solvent. But in order to make samples liquid and make it easy to separate useful components, both sample preparation and analytical procedures need solvent. In order to alter traditional organic solvents, which are recognized due to their great volatility, combustibility, and toxicity, current developments in embracing the concepts of green chemistry into analytical procedures leads to the invention and use of novel solvents [42]. Solvent selection guides (SSGs) are instruments that help in the choosing of green organic solvents [43]. A replacement to traditional organic solvents, emphasising the three most significant green solvent types: amphiphilic solvents (encompassing alcohols, carboxylic acids and surfactants), ionic liquids (ILs) and analogues [44], and deep eutectic solvents (DESs) [45]. All of these solvents have been applied to the preparation of analytical

samples (as extraction solvents) as well as to liquid chromatography (LC) (mostly as mobile phase enhancers or in the form of pseudo stationary phases).

1. Alternative solvents for sample preparation

Water is the most ecologically friendly, economically viable, ecologically sound, and harmless solvent. Maceration is the most basic and widely employed method for doing extraction with water as the solvent. Specific additives can be used to subtly alter the characteristics of water. Water plus an additional substance—a surfactant or a hydrotrope, respectively—are necessary for both micellar and hydrotropic extractions. By producing micelles and aggregates, respectively, these amphiphilic molecules can increase the dissolution of water hating substances in aqueous conditions [46, 47]. Micellar extraction mimics a method of two-liquid-phase but hydrotropic extraction simply includes a continuous liquid phase. Additionally, nonpolar molecules can be extracted with water using subcritical water extraction (SWE).

Natural Deep Eutectic Solvents (NADESs) are combinations of naturally occurring solids with melting points that are far less than any of their separate constituents. The primary cause of this specificity is the formation of between molecules hydrogen bonds connecting hydrogen bond donors &acceptors. Since NADESs are solvent that are ecologically beneficial and easily recyclable [48, 49].

According to the agro-sector from where they were created, the "bio-based solvents" collect an enormous number of biomolecules that may be splited into three groups: wood, oleo-proteaginous, and cereal/sugar. Representatives of bio-based solvents include ethanol, ethyl acetate, lactic acid esters, succinic acid derivatives, and furfural and its derivatives, such as 2-methyl-tetrahydrofuran, cyclopentyl methyl ether (CPME), and others [50, 51].

Gases that may liquefy in its vapour pressure inside of a pressurised vessel at a pressure (1–100 bar) are referred to be liquefied gases. Due to their volatility, they make it simple to separate them from the extracts and have the benefits of dissolving natural ingredients at temperatures that are comparatively low, protecting delicate components from degradation. Increasing numbers of research document, the usage of liquefied gases as eco-friendly solvents for extraction such as dimethyl ether, n-propane &n-butane [52]

Substances at temperatures and pressures over their vital points are referred to as supercritical fluids (SFs). The major benefit of an SFs is that it can be tailored for density by adjusting temperature and/or pressure, allowing for selective extraction of specific analytes. Additionally, SFs have virtually zero surface tension, which enables them to enter microporous surfaces and leave essentially little solvent residue in the final extract. The most often used SF is carbon dioxide (CO_2) because of its modest significant pressure and temperature, which are 31 °C and 74 bar, respectively. Additionally, this fluid is tasteless, odourless, affordable, not combust ecologically friendly, and inert [53].

1. Using more sustainable solvents as mobile phase

High-purity organic solvents are needed in quite enormous quantities as the mobile phase in chromatography. The transition to reversed phase chromatography from normal phase, which used nonpolar, poisonous solvents, was the initial step towards environmentally friendly chromatography [54]. Methanol (MeOH), Ethanol (EtOH), Acetonitrile (ACN), acetone, ethyl acetate, or their combinations, or their mixes with water, are often used in reversed phase liquid chromatography. Among these solvents, EtOH, acetone, and ethyl acetate are preferred in terms of environmental friendliness, and some attempts are made to replace the more hazardous acetonitrile and methanol with them [55]. The disadvantage of ethanol compared to acetonitrile is that it has a somewhat high viscosity [56]. Ethanol is slightly harmful organic mobile phase ingredients. While the separation efficiency is statistically equivalent, acetone is another more environmentally friendly alternative to acetonitrile [57]. With water containing

0.04% triethylamine as the mobile phase, the use of polyethylene glycol as the stationary phase enabled successful partition of all 4 basic and acidic chemicals [58]. Only 0.85 mL EtOH was used per chromatographic run when a water-based mobile phase was used in extreme temperature liquid chromatography [59, 60].

Normal phase systems are used to determine some samples which are nonpolar and non-volatile (such as lipids), hence it's important to focus on applying more environmentally conscious solvents in this field. As mobile phase ingredients, cyclopentyl methyl ether, hexamethyldisiloxane, isopentyl acetate &2-methyltetrahydrofuran, have all been employed satisfactorily [61, 62] (Figs. 2, 3 and Table 3).

Fig. 2 [Images not available. See PDF.]

Methodological introduction of green analytical chemistry principles

Fig. 3 [Images not available. See PDF.]

Green Analytical Techniques

Table 3. A few instances of alternate analysis solvent use [63–71]

Solvent	Polarity	Technology
EtOH/Water (H_2O)	Polar	HPLC (High-performance liquid chromatography)
Liquid Water	Polar	Micellar and submicellar liquid chromatography
Milli-Q Water	Polar	Subcritical water chromatography
$CHCl_3$ and Ethylene glycol	Polar	Modified micellar chromatography
Supercritical CO_2	Nonpolar	Supercritical fluid chromatography
Liquid CO_2	Nonpolar	Flash chromatography

Recent applications of green technology

By using green solvents in HPLC

Elsheikh et al. have developed and optimised straightforward, accurate, and reliable stability-indicating chromatographic methods, such as the HPLC method supported with the Central Composite Design, to achieve an environmentally conscious time- as well as money-saving approach. He attempted to employ benign solvents here to detect zonisamide (ZNS) even in the presence of its breakdown product. By using a Kromasil MS C18 (150 mm, 4.6 mm, i.d. 5 µm) column at 35 °C with a green and safe mobile phase (MP) containing EtOH: H_2O (30:70%v/v), at (1 mL/min), and for detection, a photodiode array detector was used at 280 nm, ZNS was chromatographically separated from its degradation product. ZNS 50 g/mL HPLC chromatogram (retention time, Rt-2.806) and its degraded form 10 g/mL (Rt-1.553). Reproducible results were obtained in the range of (2–10 µg/band). The newly developed HPLC procedures have ECO Scale scores of 91 with modest green colours. The comparison of the results with the official USP procedure (81 eco scale score) demonstrates that the recommended methods were more environmentally friendly [72].

Kokilambigai et al. had optimised the HPLC technique in evaluation of atorvastatin calcium using analytical Quality

by design (AQbD) and more environmentally conscious solvent approaches. The Central Composite Design was employed to filter out factors that could have an impact on method development. The analysis was done using a Zorbax Eclipse plus C18 column (150×4.6 mm, 5 µm). Since neat EtOH along water as mobile phase produces broad peaks with unnecessary fronting and asymmetry, the best chromatographic analysis was accomplished utilising 0.5% v/v aqueous acetic acid: EtOH (42.5: 57.5%v/v) with a flow of 0.91 mL/min. Detection was performed at 246 nm using photodiode array. For atorvastatin, the Rt was 6.27 min, while the run-time was 12 min. There was good linearity between 10 and 150 µg/mL, with a correlation coefficient (R^2>0.9999). According to a forced degradation research, atorvastatin is more vulnerable to degradation when exposed to acidic stress. The created approach received a total score of 90, indicating that it adhered to all green standards and was therefore ecologically benign [73].

By using a Quality by Design-based HPLC approach, Perumal et al. discussed a rapid, simple, specific, accurate, reproducible, and environmentally friendly approach for determining the existence of escitalopram (ESC) &etizolam (ETZ) in the formulation. Utilising Phenomenex column C18 with EtOH and phosphate buffer (60:40%v/v), with a flow rate maintained at 1 mL/min, and detected at 254 nm using a dual absorption detector, is the best method for separating ESC and ETZ and the products of their deterioration. It was discovered that the ESC and ETZ had retention durations of 3.5 and 6.5 min, respectively. 99.55 and 99.94 percent, respectively, were found to be the percentage recovered for ESC &ETZ. This technique was tested and shown linearity ranges from 5 to 30 µg/mL for ESC, 2–12 µg/mL for ETZ, precision, repeatability, and selectivity with R^2 values of 0.9975 and 0.9987 for ESC and ETZ, respectively. In the forced degradation trials, ESC deteriorated significantly in an acidic environment, then most under photodegradation stress. In alkaline condition, ETZ's % recovery decreased, indicating the drug's susceptible to alkali. According to the analytical greenness metric approach (AGREE), the method's greenness was assessed, and the result was a score of 0.78 [74].

In order to quantify zidovudine (ZDV), lamivudine (3TC), and nevirapine (NVP), Vieira-Sellai et al. devised an environmentally sustainable HPLC approach. This method employs ethanol as the sample preparation solvent and as the MP for the analysis of the drugs. The analysis was carried by lowering the prepared solution's solvent volume and analysis time, as well as the column's diameter (3 mm as rather than 4.6 mm). Utilizing C18 column (ARV4 5 µm 250×3.0 mm, Interchim), the analysis was done in a gradient mode by injecting volume of 10 µL, with a flow rate 0.4 mL/min, while detection is done at 270 nm on a photodiode array (PDA) detector. Linearity was evaluated by preparing five standard solutions in the range from 80 to 120% of nominal concentration (37.5 µg/mL for 3TC, 75.0 µg/mL for ZDV and 50.0 µg/mL for NVP). As the mean recoveries fall between the range of 99.95% and 100.27%, the approach assures good accuracy. This technique obtains a count of 75 on the ECO Scale, indicating that it is environmentally friendly [75]

Dharuman et al. show the novel inclusion of analytical quality by design with GAC in the establishment of analytical approach for measurement of Benidipine hydrochloride (BEN) &Chlorthalidone (CTD) in bulk and formulations. For optimisation, a central composite design was employed. Using a MP of ratio 40:60%v/v of EtOH &potassium dihydrogen orthophosphate analysis was accomplished utilising an Agilent Eclipse Plus (C18, 250 mm 4.6 mm i.d, 5 µm) at 1 ml/min, and was detected via a diode array detector at 230 nm. The buffer solution (pH adjusted to 3.5) was chosen because it elutes analytes from the stationary phase more quickly, needs fewer time, and yields the best retention period with excellent peak symmetry, little tailing, and theoretical plates CTD and BEN had retention times of 3.1 and 5.1 min, respectively. Linearity range of 3.2–4.8 µg/mL for BEN and 5.0–7.5 µg/mL for CTD was obtained. The values for LOD and LOQ were obtained to be 1.812 and 5.493 µg/ml, respectively. The medication CTD was more likely to decompose under acidic and alkaline circumstances, whereas BEN was more vulnerable to

hydrogen peroxide, according to the forced degradation trials. The developed techniques' ECO Scale score is 93, with mild green colours [76].

Sukumar et al. proposed an easy, effective, and reproducible approach to analyse Atorvastatin (ATO), Ezetimibe (EZB), and Fenofibrate (FF), utilising stress studies and RP-HPLC green analytical chemistry principles. The buffer (0.1% triethanolamine in water) and EtOH was used to separate these medicines in the ratio of 90:10 on MP, whereas stationary phase (SP) used is Inertsil ODS 3 (250 mm × 4.6 mm), 5 µm column, employing a photodiode array detector (PDA) detector at a wavelength of 256 nm. The forced degradation investigations revealed that EZB decay greater than 15% at alkali hydrolysis, while the ATR has >5% deterioration in acid, peroxide, and heat deterioration. The Rt of ATO, EZB, and FF are 2.86, 6.723, and 11.13, respectively. ECO Scale of the developed methods show a score of 91 with faint green colours [77].

By combining UV spectrophotometric &HPLC techniques, Perumal et al. devised an analytical quality by design approach that is quick, affordable, accurate, precise, and environmentally friendly. Analysis of Molnupiravir and its breakdown products was done on C18 column having a MP of EtOH and phosphate buffer in ratio of 42.5:57.5% v/v, at flow rate of 0.9 mL/min, and detected at 235 nm. Utilizing the UV and HPLC techniques, the molnupiravir linearity ranges were 4–9 and 10–150 g/mL; also, the R^2 values showed 0.9999 and 0.9999. For molnupiravir, the recovery rate ranged from 98 to 102%. The proposed strategy received a 94 on the ECO Scale, signifying that it is beneficial to the environment [78].

By combining analytical quality design with green analytical chemistry concepts, Kannaiah et al. devised a green, straightforward, and trustworthy approach for estimation of betamethasone dipropionate (BD) and calcipotriene (Cal) through a UHPLC method. The Central Composite Design (CCD) was utilised to filter out factors that could have an impact on method development. Dikma Endeversil C18 ODS (2.1 × 50 mm, 1.8 µm) column was used for separation. For best chromatographic separation, EtOH and potassium dihydrogen phosphate (3.0 pH) buffer were mixed 51:49 (%v/v), at a flow of 0.31 mL/min, and detected at 254 nm. Betamethasone and Calcipotriene had linear detector responses at 125–750 and 12.5–75 µg/mL, with detection limits and quantifications of 12.484, 37.831 and 3.229, 9.785 µg/mL, respectively. The recovery percentage was shown to be within tolerances of less than 1.5%. BD showed a degrades of more than 15% in the alkali, while degradation in Cal was nearly 15% in oxidation, according to a forced degradation research. AGREE metrics software program shows the total score of 0.89, greenest in all respects [79].

A single reversed-phase HPLC technique devised by Vijaykumar et al. for the estimation of apremilast, its enantiomer and its seven impurities in the medicinal material. Apremilast chromatographic separation from its enantiomer product was achieved by utilising immobilised chiral SP having a chiral selector "tris (3,5-dimethyl phenyl carbamate) derived from amylose-Chiralpak IA-3 (250 mm × 4.6 mm, 3 µm) column at 25 °C with green MP consists of buffer (0.01 M NH_4HCO_3, PH 8.0) and ACN in equal ratio at (0.4 mL/min). Two contaminants, impurity-2 (open ring acid impurity) and impurity-5 (deacetylated impurity), were discovered through forced degradation research. The impurity recoveries range from 96.1 to 102.1%. It was discovered that the suggested method's AGREE matric score was 0.66 as its consumption of ACN is comparatively less than previous established methods [80].

Kowtharapu et al. devised a linear, exact, accurate, particular, robust, environmentally friendly and sustainable LC method for determining olopatadine hydrochloride. A Boston eco-friendly C8 column (150 × 4.6 mm, 5 µm i.d.) was used to optimise and validate the isocratic chromatography procedure. With flow rate of 1.0 mL/min and a column temperature of 30 °C, a MP of sodium dihydrogen phosphate buffer of pH 3.5 and ACN in the ratio of 75:25 (%v/v) was utilised. The detection was carried out at 299 nm. The accuracy outcomes varied from 99.9 to 100.7%, the relative standard deviation (RSD) from the precision was 0.5, and the correlation coefficient from the linearity

experiment was more than 0.999. Analytical eco scale tool was used which gave an outstanding analytical eco-score of 77 was discovered [81].

Kowtharapu et al.; optimised the High-Performance Liquid Chromatographic technique for evaluating ondansetron hydrochloride utilising AQbD design approaches. The devised approach is straightforward, considerate and beneficial to the environment. A Discovery (250 × 4.6) mm, 5 µm column using phosphate buffer pH 5.7 and acetonitrile as the MP, at flow rate fixed at 0.9 mL/min, and detected at 216 nm were used to achieve separation. The relative standard deviation for the accuracy findings ranged between 0.55 and 2.72% for all contaminants. The linearity experiment yielded correlation coefficients greater than 0.995 for both contaminants and analyte. The accuracy ranged from 88.4 to 113.0% for all impurities. Results for accuracy varied from 99.9 to 100.7% [82].

For the simultaneous analysis of the innovative combination of metformin hydrochloride (MTF), pioglitazone hydrochloride (Pio), and glibenclamide (GBC) and also with MTF toxic impurities, Fawzy et al. proposed a reliable, specific, eclectic, and environmentally conscious HPLC–UV technique. With a short run time and less environmental risks, the suggested approach is green. On a VDSpher Pur 100 C18-E (250 mm × 4.6 mm, 5 m) column, contaminants and antihyperglycemic medications were separated and quantified using gradient elution with a mobile phase made up of 0.1 M heptane sulfonic acid having a pH 2.2 and ACN under a flow of 1.5 mL/min and detection done at 225 nm using photodiode array detector (PDA). Retention intervals for MTF = 3.640, Pio = 5.062, and GBC = 7.788 min, respectively. Analytical AGREE tool reveal the score of 0.76 suggesting it as green [83].

The four medications, tamsulosin HCl (TAM), tadalafil, alfuzosin HCl, and solifenacin succinate (SOL), were examined by Abdel-moety et al. utilising a mix of GAC and AQbD techniques using HPLC–DAD methodologies. Derringer's desirability function and CCD was utilised to identify and optimise key technique limits. Optimal chromatographic separation was achieved using EtOH and phosphate buffer (pH 4.0) in a ratio of 40:60 (%v/v) for mixture I and in equal ratio for mixture II flowing at 2.3 mL/min, and detected through UV detector at 210 nm. Separation was performed on Agilent HPLC–DAD system C18 column (100 mm × 4.6 mm). The suggested approaches also make effective utilization of monolithic-based columns by enabling high throughput analysis with just a little under 4 millilitres of ethanol and a roughly 3-min run time. The percentage recoveries ranged between 98 and 102%. Linear concentration was in a range of 3–70 µg/mL for TAM, 5–110 µg/mL for tadalafil and 5–90 µg/mL for both alfuzosin &SOL. The Analytical Eco Scale was employed to measure the method's greenness, and the result was a score of 94[84].

For the determination of ketoconazole along with beclomethasone utilising RP-HPLC &multi-analytical UV spectrophotometric approach, Kannaiah et al. developed a straightforward, dependable, exact, effective, and ecologically friendly technique. The absorption ratio, first-order the absorption ratio, and area under curve approaches are the three environmentally friendly approaches covered by the spectroscopy technique for estimate, which is the initial approach. The second method employed an eco-friendly rotatable CCD analytical quality via a design-based RP-HPLC technique and an ODS reversed-phase column (250 × 4.6 mm, 5 µm). The best chromatographic separation was obtained through a MP of EtOH: 0.1 M potassium dihydrogen phosphate buffer (pH 2.5) 33: 67%v/v with a flow of 1.0 mL/min. Analytical eco scale, green analytical procedure index (GAPI), and AGREE were used to examine the technique's greenness, and it was determined that the approach is green [85].

Badr-Eldin et al. suggested a simple, recursive, quick, accurate, exact, repeatable, resilient, specific, and stability-indicating RP-HPLC technique for identifying clonazepam and its associated compounds. With an ODS column and a mobile phase made up of 2% sodium dodecyl sulphate, 0.05 M sodium acetate buffer pH 3.5, and isopropanol in the ratio (25:55:20%v/v/v), with a flow of 1.5 mL/min the chromatography process was optimised and verified. Detected at 254 nm using a DAD detector. The recovered percentage ranged from 101.33 to 99.40%. Linearity for

clonazepam and its associated compounds was between the range of 4–140 µg/mL and 4–64 µg/mL, respectively. Clonazepam and its related compound have LOD of 0.024 µg/mL and 0.380 µg/mL while LOQ of 0.0799 µg/mL and 1.2870 µg/mL for, respectively. It was discovered that clonazepam breaks down more quickly in oxidative environments. The approach is also straightforward in terms of susceptibility, utilisation of an ecologically friendly MP, straightforward extraction techniques, considerably shorter Rt [86].

For the simultaneous testing of a combination of sofosbuvir and ledipasvir, El-Shorbagy et al. proposed a straightforward, quick, prudent, linear, exact, accurate, and environmentally conscious RP-HPLC–UV-Fluorescence approach. On C18 (250 mm × 4.6 mm, 5 µm in particle size), isocratic elution at a flow rate of 1.2 mL/min was used at room temperature. The UV and fluorescence detectors are both installed in the column. The MP contained 35: 35: 30 (%v/v/v) acetonitrile, methanol, and 0.01% triethylamine that had been pH-adjusted to 3. The UV detector was operated at 261 nm until the SOF was eluted, at which point it switched to 333 nm (the LED maximum) for five minutes. By using a UV detector, it was discovered that the mean % recovery of sofosbuvir and ledipasvir was 100.33 and 100.06, respectively. Linearity for sofosbuvir was between the range of 1 and 40 µg/mL while for ledipasvir 0.4–20 µg/mL. LOD for sofosbuvir was 0.012 µg/mL while for ledipasvir was 0.011 µg/mL. LOQ were 0.040 µg/mL and 0.036 µg/mL for sofosbuvir and ledipasvir, respectively [87].

Subhadip et al. devised an ecologically sound, resilient, quick, accurate, specific, linear, and exact RP-HPLC technique for predicting favipiravir in its medicinal dosage form, and compared it using ANOVA and in-vitro dissolution studies. With a C18 column (4.6 mm × 150 mm, 3 µm spherical particles), MeOH, EtOH, and H_2O (25:35:40%v/v/v) as the MP, with a flow of 0.80 mL/min, a Rt of 7.216 min, at temp of 25 °C, and detected at 236 nm, in isocratic mode, with a run-time of 10 min, separation was accomplished. Oxidative stress was shown to cause the greatest deterioration percentage. The average recovery rate was more than 98%. The created procedures have an eco scale score of 92, which means they are more considerably secure as well as environmentally friendly [88].

A green and environmentally friendly HPLC technique was created by Khaled et al. to measure vitamin D_3 and calcium levels in atorvastatin. In less than 10 min, the two medicines were separated using a gradient of 0.1% orthophosphoric acid (OPA) having pH = 2.16 and EtOH as the MP &a photodiode array detector. The column was Symmetry column C18 (100 × 4.6 mm, 3.5 µm). The column temperature had been set 40 °C while the MP was introduced at a rate of 1 ml/min. Atorvastatin calcium &vitamin D_3 were detected at wavelengths of 246 and 264 nm, respectively. The average recovery for atorvastatin calcium &vitamin D_3 was determined to be 102.87% &101.35%, respectively. Linearity of the method was verified in the range from 5 to 40 µg/mL for atorvastatin calcium and 1–8 µg/mL for vitamin D_3 while LOD are 0.475 µg/mL and 0.041 µg/mL for atorvastatin calcium and vitamin D_3. They evaluated the greenness of our suggested procedure such as AGREE &GAPI tools. The developed approach has a score of 0.75 and is a semi-automatic, miniature method [89].

Using a Teknokroma C18 (150 mm × 4.6 mm, 5 µm particle size) reversed-phase guard column, Sepideh et al. devised a straightforward, quick, economical, and environmentally friendly HPLC test for estimating capecitabine in plasma. An oral 5-fluorouracil (5-FU) prodrug called capecitabine is frequently administered to people with colorectal and breast cancer. Formic acid solution (pH = 3): EtOH (55:45%v/v) flowing in a flow rate of 1.0 mL/min along with UV detection at 310 nm was used as the MP for the extraction. The temperature inside the column was fixed at 50 °C. Protein was precipitated from the sample using a zinc sulphate-ethanol solution. This approach yields a high capecitabine recovery in human plasma, which can vary from 95.98 to 102.50% [90].

For determining domperidone aspirin and in bulk or formulation using HPLC, Sneha et al. devised an analytical approach that is affordable, quick (short retention time), straightforward, precise, reliable, and sustainable. On a

Prontosil C-18 column (4.6×250 mm, 5 µ particle size), separation was carried out isocratically using a MP of 10 mM KH_2PO_4: ACN (20:80%v/v) pH 3.5. Chromatograms were taken at 231 nm by a UV–visible detector [91].

Nazrul et al. devised a simple, rapid, reliable, precise, robust, specific, environmentally friendly &stability determining technique for quick measurement of rosuvastatin calcium in conventional medicines utilising HPLC–UV. As a sustainability-conscious green eluent, a mixture of EtOH, MeOH, and ethyl acetate (6:3:1%v/v/v) at a rate of 1.0 mL/min was chosen to produce a quick and straightforward assay with a reasonable run time (5 min), which was the most effective to detect rosuvastatin calcium &its separation. A NUCLEODUR 150 mm 4.6 mm RP C8 column filled with 5 m filler as the SP was used to identify rosuvastatin calcium, with detection taking place at 254 nm. The estimated Rt for rosuvastatin calcium is 1.52 min. Linear concentration was found in range of 0.1–100 µg/mL with correlation coefficient of 0.996. The % recovery was found to be 99.99%. According to investigations on forced degradation, rosuvastatin calcium deteriorated under oxidative stress in the presence of H_2O_2 and in 0.1 M NaOH [92].

For the measurement of clorsulon, albendazole, triclabendazole, and ivermectin utilising monolithic columns, Rashed et al. devised a straightforward, quick, environmentally friendly, and highly sensitive micellar liquid chromatographic approach. With a MP made up of 120 mM sodium dodecyl sulphate, 15% propanol, &15 mM phosphate buffer (pH 5.5), separation was carried out on an Anonyx monolithic C18 column (100×4.6 mm) at 40 °C using UV detection at 225 nm. The average recoveries varied from 99.2 to 101.2%. Linearity of the method was verified in the range from 0.625 to 25 µg/mL for clorsulon, albendazole, triclabendazole and 30–300 µg/mL for ivermectin. LOD are 0.16, 0.18, 0.18 and 6.15 µg/mL and LOQ are 0.54, 0.60, 0.60 and 20.50 µg/mL for clorsulon, albendazole, triclabendazole and ivermectin, respectively. The recommended method's analytical Eco Scale value was determined, and it received a score of 75, which is very near to being an exceptional green analysis [93].

Magdy et al. devised a unique and environmentally friendly reversed-phase HPLC technique with fluorescence detection, as well as an analytical QBD approach, for the simultaneous quantification of lesinurad &febuxostat (FBX) with diflunisal (DIF). On the Hypersil BDS C18 column, the separation via chromatography is carried out using isocratic elution at 40 °C. ACN: potassium phosphate buffer (30.0 mM; pH 5.5, 32.2:67.8% v/v), pumped at 1.0 mL/min with an injection volume of 20.0 µL, was the MP. Fluorescence detector (FLD) detection was carried out at 280/370 nm. The ternary mixture may be separated using the suggested procedure in about 10 min. The Rt was determined to be 6.599, 7.641, and 9.155 min for DIF, lesinurad, and FBX, respectively. Linear concentration was found in range of 50–500 ng/mL, 50–700 ng/mL and 20–700 ng/mL, respectively, for DIF, lesinurad and FBX. The recoveries for DIF, lesinurad, and FBX were 99.32%, 98.8%, and 99.65%, respectively. The relative standard deviation was found to be less than 2. The recommended method's analytical Eco Scale value was computed, and a score of 87 was discovered [94].

By combining the concepts of GAC, Saroj et al. established a reliable &environmentally friendly RP-HPLC technique for determination of fenoverine. With a mobile phase of methanol and ammonium acetate buffer 20 mM (81:19% v/v), on a flow rate of 1.0 mL/min, and a column oven temperature of 33ºC, while employing UV detection at 262 nm, separation was accomplished on a Spherisorb C18 column (150×4.6 mm, 3 µm). The peak of fenoverine in the unstressed condition was seen in the chromatogram at 7.8 min. According to a forced degradation research, considerable degradation occurs under 10% H_2O_2 and hydrolytic conditions. Linear concentration was found in range of 0.5–160 µg/mL with a limit of detection and limit of quantitation of 0.1 and 0.3 µg/mL, respectively. The % recovery was found to be 99.7%. The developed HPLC method had an efficiency analysis trees (EAT) value of 41.82, while the literature method had a score of 44.01, which concluded that the approach that was created had a smaller effect on the nature because it was developed entirely without using ACN [95].

A brand-new, straightforward liquid chromatographic method for brivaracetam detection together with piracetam and carbamazepine was devised and validated by Mansour et al. On a Promosil C18 column (100 mm × 4.6 mm, 5 µm particle size), separation was accomplished using a MP of ACN: H_2O containing 0.1% triethylamine at a ratio of 30:70%v/v at pH 6.5 corrected with orthophosphoric acid with a column temperature of 25 °C. The flow rate is 0.6 mL/min, the run period was around 9 min, and the UV detector used a 215 nm wavelength for detection. Piracetam, brivaracetam, and carbamazepine were shown to have retention times of 1.4, 4 and 8 min, respectively. The recovery rates were found to be between 94.8 and 101.05%. Values of the limit of quantification were 3.7, 2.3 and 1.8 µg/mL for the piracetam, the brivaracetam and the carbamazepine, respectively. The recommended method's analytical Eco-Score was discovered to be 85[96].

For the simultaneous detection of amlodipine (AML) &metoprolol (MET), Mabrouk et al. devised and validated a rapid, sensitive, and accurate green micellar liquid chromatographic technique. The XBridge ODS column (1150 × 4.6 mm, 5 µm particle size) was used for separation. The mobile phase was composed of SDS and sodium dihydrogen phosphate in water, while the pH was adjusted to pH 3.0 using phosphoric acid. The injection volume was 50 µL, and the flow rate was 1.5 mL/min at 40 °C. For MET and AML, the fluorescence detector was adjusted to emit light at 275/303 nm and excite light at 364/455 nm, respectively. The MET Rt was 3.46 min, and AML was at 6.35 min. Linearity of the method was verified in the range from 0.1 to 10 µg/mL for MET and 0.2–2 µg/mL for AML. The recovery was close to 100.156%. RSD values were below 2%, which demonstrates adequate precision. LOD was 20 ng/mL and 50 ng/mL for MET and AML while LOQ was 61 ng/mL and 81 ng/mL for MET and AML. The developed method was found to have an analytical Eco Scale value of 80 indicating an excellent green HPLC method [97].

A quick, secure, and ecologically conscious analytical technique for determining daptomycin in lyophilized powder was developed by Totoli et al. Using a MP of EtOH-H_2O (55:45%v/v) around pH 4.5, pumped at a flow rate of 0.6 mL/min for an 8-min run period, and UV detection at 221 nm with a DAD detector, separation was accomplished on an Agilent Zorbax C18 analytical column (4.6 × 150 mm, 5 µm). Daptomycin had a retention period of 5.8 min. Linearity of the method was verified in the range from 20.0 to 70.0 µg/mL. The recovery was close to 100%. RSD values were below 2%, which demonstrates adequate precision. LOD was 1.87 µg/mL and the LOQ was 5.68 µg/mL. According to a study on force degradation, the sample was less stable under basic circumstances [98].

Bhukya et al., had developed and validated stability indicating HPLC method for separation and identification of Panobinostat and its degradant product. The analysis was done using a column Waters X bridge C18 3.0 µm (50 × 4.6 mm) was used with mobile phase A as 10 mM NH_4COOH buffer adjusted to pH 3.0 with HCOOH and Ethanol as mobile phase B with gradient elution mode. The mobile phase flow rate is 0.5 mL/min, injection volume are 3 µL and at 277 nm as UV wavelength. The method found linear from 12 to 300 µg/mL with $R^2 \geq 0.998$. The % recovery for analyte was in between 99.28 and 100.36 with 0.60% RSD. The %RSD for the analyte in Method precision and intermediate precision is below 1.0. According to the AGREE, the method's greenness was assessed, and the result was a score of 0.74 [99].

Hammad et al., had developed a solvent free, robust, sensitive, selective and fast chromatographic method for determination of metronidazole (MTR) and ciprofloxacin (CIP). The analysis was done using a X-Bridge (150 × 4.6 mm, 5 µm) column. The separation was achieve using 0.13 M sodium dodecyl sulphate and 0.02 M polyoxyethylene lauryl ether (Brij-35) solution to pH 2.5 using phosphoric acid at a flow rate of 1.3 mL/min and 280 nm wavelength in UV detector. The method was said to be greener as it eliminates the use of harmful organic solvent as well as it minimises the run time (8 min) to reduce cost and energy consumption. The method found linear from 0.5 to 50 µg/mL with $R^2 \geq 0.9998$ for MTR and 0.9997 for CIP. The % recovery for MTR and CIP was to be

99.45 and 100.13%, respectively. The %RSD for the analyte in Method precision and intermediate precision is below 2.0. The AGREE software was employed to evaluate the technique's greenness, and the resultant score of 0.81 [100].

Michael et al., had developed green, accurate, selective and stability-indicating method for determination of levetiracetam using HPLC. The analysis was done using Lichrosorb RP-18 (250×4.6 mm i.d., 5 µm particle size) column. The separation was achieve using phosphate buffer (pH 3.1) and acetonitrile (87:13%v/v) mobile phase with the flow rate of 1.0 mL/min, and UV detection was carried out at 210 nm. The Rt of drug was found to be at 5.6 min. The AGREE software was employed to evaluate the technique's greenness, and the resultant score of 0.82 [101].

Yabre, M., et al., developed an easy, green, and stable RP-HPLC methodology for measuring contaminants in artesunate and amodiaquine using a Quality by Design (QbD) methodology. It effectively handled the difficulty of evaluating nine compounds under a single chromatographic condition by using a gradient elution with EtOH, resulting in a 95% confidence level for identifying the method's operational domain region. To achieve greenness, EtOH and 10 mM acetic acid were used as the mobile phase, which was tested using accuracy profiles and successfully used to raw materials and fixed-dose combination tablets. The technique used an Xbridge BEH C18 column (150 mm×3 mm, 5 µm) with a flow rate of 0.4 mL/min. [102]

Megahed et al.; described an experimental approach to establish a green, gradient chromatographic technique for simultaneously analysing metronidazole (MTR) and spiramycin (SPR). The optimal chromatographic conditions involved a mobile phase of ethanol and 20 mM sodium dihydrogen phosphate solution (pH adjusted to 2.5) in a 2:98 (%v/v) ratio for 2 min, followed by a change to 30:70 (%v/v). The flow rate was 1.3 mL/min, and the separation and analysis were done on an X-bridge C18 column (150 mm×4.6 mm×3.5 µm). The detection wavelength was 230 nm, and the column oven temperature was set at 40 °C. The approach showed a linear response for both drugs in the range of 5–125 µg/mL. [103]

Duan et al.; developed and validated a green HPLC technique for the simultaneous measurement of nine sulphonamides utilizing EtOH as the green alternative solvent, and it was effectively applied to beef and milk samples. The Venusil XBP C18 column (Lanzhou Acetch Technologies Co., Ltd., Lanzhou, China) consisting of the following features: 250 mm, 4.6 mm in diameter, 5 µm particle size was used. The pH of the mobile phase was around 3. Furthermore, no more than 30 mL of trash was created each sample. The four quadrants of the greenness profile are represented by the following criteria: persistent, bio accumulative and toxic (PBT), hazardous, corrosive, and waste. The score of the analytical Eco Scale was 86, indicating an excellent value for the proposed method [104].

Habib et al.; used quality by design principles and green analytical chemistry which aided in the creation of a green micellar HPLC technique for assessing atorvastatin calcium and amlodipine besylate in binary combinations and tablet dosage forms in 8 min. The optimal conditions were a mobile phase of 0.17 M sodium dodecyl sulphate solution (pH 2.9) with 10%v/v n-butanol, a flow rate of 1.5 mL/min, and a column temperature of 45 °C utilizing an X-Bridge™ (150 mm×4.6 mm, 5 µm). Fluorescence detection was set at 276/378 nm for atorvastatin and 366/442 nm for amlodipine. The approach showed linear responses for both medicines in the range of 0.2–25 µg/mL and was verified for tablets. [105]

Elmansi et al.; developed the method for separation of three BDZs (diazepam, clonazepam, and bromazepam) utilizing a green micellar mobile phase. Brij-35, a non-ionic surfactant, substitutes organic solvents, resulted in much shorter retention times and more ecologically friendly separation. The procedure, was carried out on a monolithic C18 column, uses a 50:50% v/v mixture of sodium dodecyl sulphate (SDS) and Brij-35 as the mobile phase. Using UV detection at 240 nm and a flow rate of 1.0 mL/min, separation was accomplished in less than ten minutes, even

without an organic modifier. Furthermore, the method's greenness was evaluated using the analytical eco scale, demonstrating its suitability for testing the three medications. It was found that the developed micellar HPLC method, had an Eco scale value of 95 [106].

Kokilambigai et al.; used analytical quality by design (AQbD) and greener solvent technologies to improve a High-Performance Liquid Chromatographic (HPLC) method for detecting atorvastatin calcium in bulk and pharmaceutical dose forms. A rotating central composite design was used, with EtOH volume and flow rate as essential method variables that influenced analytical characteristics. Using a Zorbax Eclipse plus C18 column (150×4.6 mm, 5 µm), optimum separation was achieved using a mobile phase of 0.5% v/v aqueous acetic acid: EtOH (42.5:57.5%v/v) at 0.91 mL/min. Atorvastatin was detected at 246 nm, resulting in a retention time of 6.27 min and a total run duration of 12 min. The technique demonstrated linearity from 10 to 150 µg/mL, and validation trials produced good findings. The method's greenness and ecological effect were assessed using a variety of indicators, which validated its great environmental profile. When evaluated using the analytical eco scale, the recommended HPLC method received a score of 90 [73].

Stability-indicating techniques for modafinil (MDF) and the by-product of its acid-induced deterioration have been devised and confirmed by Elsheikh et al. The procedure was an environmentally conscious (HPLC) procedure that used an XTERRA MS C-18 column (100 mm×4.6 mm, 5 µm id), EtOH-H_2O (30:70, %v/v) as the MP at 40 °C at a flow of 1 mL/min, and UV scanning at 220 nm. With a lowest time to retention of 3.6 min, the HPLC chromatogram of MDF at 50 g/mL has a Rt of 3.628 while that of its degradation product at 10 g/mL has a Rt of 1.129. Linearity was in ranges of 2–10 µg/mL. Accuracy was found to be 100.01%. LOD and LOQ was 0.127 and 0.384 µg/mL. The created HPLC Eco Scale procedures yield scores of 90 [119] (Table 4).

Table 4. Summary of applications of green solvent in HPLC

Drug	Approach	Greenness	ECO Scale/AGREE Score	References
Zonisamide	HPLC	EtOH: H_2O MP	91 and 84	[72]
Atorvastatin calcium	HPLC	0.5% v/v aqueous acetic acid: EtOH MP	90	[73]
Escitalopram and Etizolam	HPLC	EtOH and phosphate buffer MP	0.78 (AGREE)	[74]
Lamivudine, Zidovudine, and Nevirapine	HPLC	EtOH as MP, reducing column diameter to3mm	75	[75]
Benidipine hydrochloride &Chlorthalidone	HPLC	EtOH and potassium dihydrogen orthophosphate MP	93	[76]
Atorvastatin, Ezetimibe, and Fenofibrate	RP-HPLC	Buffer (0.1% triethanolamine in H_2O) and EtOH MP	91	[77]

Molnupiravir and its breakdown products	UV spectrophotometric and HPLC	EtOH and phosphate buffer MP	94	[78]
Betamethasone and Calcipotriene	RP-HPLC	EtOH and phosphate buffer MP	0.89(AGREE)	[79]
Apremilast, its enantiomer and its seven impurities	RP-HPLC	Green mobile phase buffer (0.01 M NH_4HCO_3, PH 8.0) along with ACN	0.66(AGREE)	[80]
Olopatadine hydrochloride	LC method	Boston green C8 column along with sodium dihydrogen phosphate buffer (pH 3.5) with ACN as MP	77	[81]
Ondansetron hydrochloride	HPLC	Phosphate buffer pH 5.7 and ACN MP	–	[82]
Metformin hydrochloride, Pioglitazone hydrochloride, and Glibenclamide	HPLC–UV	Short run time and less environmental risks	0.76(AGREE)	[83]
Tamsulosin HCl, Tadalafil, Alfuzosin HCl, and Solifenacin succinate	HPLC–DAD	EtOH and phosphate buffer MP and effective use of monolithic-based columns by enable analysis with just 4 millilitres of EtOH	94	[84]
Ketoconazole and Beclomethasone	RP-HPLC &UV spectrophotometric	EtOH: 0.1 M potassium dihydrogen phosphate buffer MP	–	[85]
Clonazepam and its associated compounds	RP-HPLC	2% sodium dodecyl sulphate, 0.05 M sodium acetate buffer pH 3.5, and isopropanol as MP	–	[86]
Sofosbuvir and Ledipasvir	RP-HPLC–UV-Fluorescence	–	–	[87]
Favipiravir	RP-HPLC	MeOH, EtOH, and water MP	92	[88]
Vitamin D3 and calcium levels in atorvastatin	HPLC	0.1% orthophosphoric acid and EtOH	0.75(AGREE)	[89]
5-fluorouracil (5-FU)	HPLC	Formic acid solution and EtOH MP	–	[90]
Aspirin and Domperidone	HPLC	KH_2PO_4: Acetonitrile	–	[91]
Rosuvastatin calcium	HPLC	MeOH, EtOH and ethyl acetate MP	–	[92]

Clorsulon, Albendazole, Triclabendazole, and Ivermectin	Micellar liquid chromatographic	Sodium dodecyl sulphate, 15% propanol, and 15 mM phosphate buffer	75	[93]
Lesinurad and Febuxostat	RP-HPLC	ACN: potassium phosphate buffer MP	87	[94]
Fenoverine	Reversed-phase liquid chromatographic	MeOH and ammonium acetate buffer 20 mM	41.82(EAT value)	[95]
Brivaracetam detection together with Piracetam and Carbamazepine	LC	ACN: H_2O containing 0.1% triethylamine	85	[96]
Metoprolol and Amlodipine	Micellar liquid chromatographic	SDS and sodium dihydrogen phosphate in H_2O MP and technique	80	[97]
Daptomycin	HPLC	EtOH- H2O MP	–	[98]
Panobinostat	HPLC	NH_4COOH buffer-EtOH	0.74 (AGREE)	[99]
Metronidazole and Ciprofloxacin	HPLC	0.13 M sodium dodecyl sulphate and 0.02 M Brij-35 solution	0.81 (AGREE)	[100]
Levetiracetam	HPLC	Phosphate buffer and ACN (87:13%v/v)	0.82 (AGREE)	[101]
Artesunate and Amodiaquine	RP-HPLC	Solvent EtOH and 10 mM CH_3COOH	–	[102]
Metronidazole (MTR) and Spiramycin (SPR)	HPLC	EtOH and 20 mM sodium dihydrogen phosphate solution	–	[103]
Nine sulphonamides	HPLC	EtOH	86	[104]
Atorvastatin calcium and Amlodipine	HPLC	0.17 M sodium dodecyl sulphate solution (pH 2.9) with 10%v/v n-butanol	–	[105]
Three BDZs (diazepam, clonazepam, and bromazepam)	HPLC	50:50% v/v mixture of sodium dodecyl sulphate (SDS) and polyoxyethylene lauryl ether (Brij-35)	95	[106]
Atorvastatin	HPLC	EtOH	90	[73]

Modafinil	HPLC	EtOH-H_2O	90	[119]

By minimising solvent consumption

1. UHPLC (Ultra high-performance liquid chromatography)

A QbD-compliant simple, quick, robust, sensitive, efficient, economical, and ecologically conscious UHPLC approach for chrysin quantification has been developed by Sharma et al. Utilising Box Behnken Design, essential parameter optimisation was completed. The best technique conditions are a C8 column with an ACN and H_2O mobile phase that flow at a rate 0.5 mL/min by injecting volume of 5 µL at a maximum wavelength of 267 nm via a PDA detector. Here, ethanol is not used as the mobile phase because it produces high pressure, which can be reduced by raising the column temperature, but this was avoided because doing so would increase energy requirement, which is in contradiction of the GAC principles, so ACN along with water were chosen as the mobile phases instead. Additionally, the UHPLC method is referred to as a “greener” method because it minimised the amount of waste generated and operator and ecological safety risks associated with exposure to organic solvents. Other benefits of the method include increased method effective; decreased back pressure, and low solvent consumption. Chrysin had a retention time of 4.802 min. The accuracy testing revealed that the percent recovery ranged from 98.34 to 104.80%, proving that the procedure is very accurate. The method linearity for chrysin concentration ranging from 0.1 to 50 µg/mL. The method showed 0.028 µg/mL as LOD and 0.075 µg/mL as LOQ. The Analytical Eco Scale employed to evaluate the technique's greenness, and the resultant score of 81 [107].
An environmentally friendly AQbD based UHPLC technique has been proposed by Chanduluru et al. for the environmentally friendly measurement of isosorbide dinitrate (ISDN) and hydralazine hydrochloride (HHC). On a Phenomenex C18 (50×2.1 mm, 2 µm) column with EtOH and 0.1% trifluoroacetic acid (60:40% v/v) at a flow rate of 0.5 mL/min, separation was carried out. The devised approach discovered a runtime of just 3.5 min and an excellent resolution of 5.4 between the two medications. The recovery rate falls between 98.4 and 101.9%. Linearity ranges from 10–60 µg/mL and 18.75–112.5 µg/mL, with R^2 of 0.9998 and 0.9992 for ISDN and HHC, respectively. LOD was found to be 0.923 µg/mL and 0.549 µg/mL while LOQ was found to be 2.797 µg/mL and 1.666 µg/mL for ISDN and HHC, respectively. When the technique's greenness was evaluated through the ECO Scale tool, the score was discovered to be 96, which is very excellent [108].
Kannaiah et al. suggested an environmentally conscious, simple, and reliable technique for estimating Crotamiton (CTM) and Hydrocortisone (HCT) by combining GAC principles with an AQbD-rotatable CCD methodology employing RP-UHPLC. The Waters X-Bridge (C18 50×2.5 mm i.d, 1.7 µm) column were utilisd for estimation, and the MP composition was ethanol: 0.01 M ammonium acetate buffer (pH 3.0) 30:70%v/v with the flow of 0.3 ml/min. Linearity concentration was made in range of 70–130 µg/mL for CTM and 1.75–3.75 µg/mL for HCT. Here, UHPLC was chosen over HPLC because it requires less energy as well as solvent, and analytical time. Using the AGREE tool, the method's greenness was evaluated, and the analytical score came out to be 0.83[109].
The UHPLC technique was proposed by Chanduluru et al. to measure pitavastatin and ezetimibe (EZB). Rotatable central composite design, which is modelled after the Ishikawa fishbone diagram, was utilised for optimisation. The satisfactory chromatographic estimation was done on Agilent UHPLC-PDA system (1290 infinity II LC system), alongside Kinetex phenyl hexyl (50×4.6 mm, 2.6 µm) column and MP of 72: 28% v/v EtOH and 0.1% orthophosphoric acid (pH 3.5), at a 0.31 mL/min flow rate with detection on 240 nm wavelength through photodiode array (PDA) detector. Rt for pitavastatin was 0.85 and for EZB was 1.91 min. Recovery for pitavastatin and EZB was determined to be 99.1%-100.94% and 99.9%-101.52%, respectively. Linearity ranging from 2–30 and 10–150 µg/mL

with an R^2 of 0.9999 and 0.9997, respectively, for pitavastatin and EZB. LOQ for pitavastatin and EZB was 0.0584 µg/mL and 0.0233 µg/mL, respectively, while LOD was 0.0192 µg/mL and 0.0076 µg/mL, respectively. Studies on forced deterioration have revealed that whereas EZB was hydrolysed under basic conditions, pitavastatin was vulnerable to acid hydrolysis. The result of the ECO Scale was found to be 97 [110].

Muzaffar et al. presented a UHPLC-MS/MS approach for fast measurement of umifenovir in plasma samples and measured greenness. Using the ACQUITY UPLC BEH C18 column (2.1 × 100; 1.7 m) and a MP made up of 15 mM ammonium acetate and ACN in an 80:20 (%v/v) ratio while being pumped at 0.3 mL/min, umifenovir and the IS were separated in under 2.5 min. The auto-sampler and column oven were each set to a temperature of 10 and 40 °C, respectively. The material was ionised using positive electrospray ionisation. The developed method showed excellent linearity in the concentration range of 1.32–625 ng/mL. Accuracy was in the ranges of 90.5–105.8%. The score, which ranges from 0.75 to 1.00 and indicates an environmentally favourable procedure, was determined to be 0.77 [111].

A unique UHPLC-MS/MS assay was created by Muzaffar et al. for the quick measurement of delafloxacin. Delafloxacin (DFX) and the internal standard (losartan) were separated on a UHPLC BEH C18 column (50 × 2.1 mm; 1.7 m) using gradient programming of an MP containing 0.1% formic acid in acetonitrile and 0.1% formic acid in water, with a flow rate of 0.3 mL/min, injection volume of 5 µL (partial loop mode), column oven temperature at 35 °C. A positive mode triple quadrupole mass detector was used for the quantification at an electrospray ionisation interface. The Rt of DFX and IS were, respectively, 1.72 and 1.79 min. Linearity was between concentration ranges of 2.92–6666 ng/mL with an acceptable correlation coefficient of $R^2 \geq 0.995$. The accuracy values ranged between 94.4 and 106.1%. The approach's greenness evaluation was evaluated by using AGREE software having a score of 0.78, confirming excellent greenness of the approach [112].

Kamal et al.; developed method using The Waters Acquity UHPLC BEH C18 column (100 × 2.1 mm, 1.7 µm) which efficiently removed Meropenem MPM, contaminants, and peaks from the sample matrix. Mobile phase A was a 25 mM monobasic potassium phosphate buffer adjusted to pH 4.05 using orthophosphoric acid (OPA) solution, while mobile phase B was a 30:70 (%v/v) combination of water and ACN. A simple binary gradient program (0.0–12.0 min, 87%→0% A, 13%→100% B) with a flow rate of 0.4 mL/min allowed for component separation in 15 min. The method's greenness was assessed with GAPI, AGREE, and analytical eco scale methods. Each sample preparation used 40.0 mL of ACN, resulting in a total consumption of <50 mL per sample analysis and a penalty point score of 15. The approach achieves an eco scale score of 85, indicating exceptional greenness. [113]

Muchakayala et al. devised a green, robust, and speedy stability indicating chromatographic approach for the simultaneous measurement of fluorescein sodium, benoxinate hydrochloride, and their degradation products within 4 min. Fractional factorial and Box-Behnken designs were used for screening and optimization, respectively. The ideal chromatographic conditions included a mobile phase of isopropanol and 20 mM potassium dihydrogen phosphate solution (pH 3.0) in a 27:73%v/v, a flow rate of 1.5 mL/min, and a column oven temperature of 40 °C. The analysis was done on an Eclipse plus C18 column (100 mm × 4.6 mm × 3.5 µm) with a DAD detector tuned to 220 nm. Linear responses were obtained between 2.5–60 µg/mL for benoxinate and 1–50 µg/mL for fluorescein. The approach proved successful in quantifying pharmaceuticals in ophthalmic solution, with mean %recovery ± SD of 99.21 ± 0.74 for benoxinate and 99.88 ± 0.58 for fluorescein. Compared to previously described approaches, the suggested method is quicker and more environmentally friendly. Its greenness was proven by a high analytical eco scale score of 90 and a low EAT score of 4.21 [114] (Table 5).

Table 5. Summary of green technique by reducing solvent consumptions using UHPLC

Drug	Approach	Greenness	ECO Scale/AGREE Score	References
Chrysin	UHPLC	ACN with water MP and UHPLC technique minimised the amount of waste generated	81	[107]
Isosorbide dinitrate and hydralazine hydrochloride	UHPLC	EtOH and 0.1% trifluoroacetic acid MP	96	[108]
Crotamiton and Hydrocortisone	RP-UHPLC	EtOH: 0.01 M ammonium acetate buffer	0.83(AGREE)	[109]
Pitavastatin and Ezetimibe	UHPLC	EtOH and 0.1% orthophosphoric acid MP	97	[110]
Umifenovir	UHPLC-MS/MS	Technique as well as 15 mM ammonium acetate and acetonitrile MP	0.77(AGREE)	[111]
Delafloxacin	UHPLC-MS/MS	Technique as well as 0.1% formic acid in acetonitrile and 0.1% formic acid in H_2O MP	0.78(AGREE)	[112]
MPM	UHPLC	Potassium phosphate buffer	85	[113]
Fluorescein sodium &Benoxinate hydrochloride	UHPLC	Isopropanol and 20 mM potassium dihydrogen phosphate) in a 27:73%v/v	90 &4.21(EAT)	[114]

1. HPTLC (High-performance thin layer chromatography)

Using the AQbD methodology, Prajapati et al. developed a reliable and ecological conscious HPTLC approach for the measurement of thiocolchicoside (THC) in its medicinal dose forms. Utilising Box Behnken Design, essential parameter optimisation has been accomplished. In contrast to HPLC, HPTLC uses a little quantity of MP to analyse several samples at once, decreasing the quantity of time and money required for each analysis. Chromatographic separation was carried out using toluene-acetone–water (1.5:7.5:1.0, %v/v/v) as the MP and 10 cm × 10 cm aluminium plates pre-coated using 250 µm of silica gel 60 F254 at 25 °C, 35% relative humidity, 15 min chamber saturation time, and 90 mm displacement distance. According to the degradation research, thiocolchicoside is more susceptible to oxidative degradation and acidic-alkaline hydrolysis, but less susceptible to photolysis, dry-heat, and water hydrolysis. thiocolchicoside, acidic deteriorated product DP1, and acidic deteriorated product DP2 have spots at retardation factor, R_f = 0.53, 0.65, and 0.80, respectively. The recovery rate falls between 99.39 and 101.65%. Linearity ranges from 100 to 500 ng/spot with R^2 of 0.9979. LOD was found to be 4.03 µg/band while LOQ was

found to be 12.21 µg/band [115].

In order to determine a novel blend of mebendazole (MBZ) &quinfamide formulations, Ibrahim et al. devised a simple, quick, inexpensive, sensitive, selectivity, broader linearity ranges, ecological, and short analytical time technique. The first approach is HPTLC, in which at 254 nm, distinct bands were scanned. After being separated employing silica gel HPTLC F254 plates &a simple MP made of MeOH: toluene (2:6, %v/v). It was discovered that the values of the R_f for MEB and quinfamide were 0.45 and 0.75, respectively. Linearity was in ranges of 0.2–2.5 and 0.1–2 µg/band for quinfamide and MEB, respectively. Accuracy was found to be 99.62% and 100.10% for QF and MBZ, respectively. LOD and LOQ for quinfamide was 0.055 and 0.168 µg/band, for MBZ was 0.031 and 0.094 µg/band. The second technique used RP-HPLC and involved isocratic separation of both pharmaceuticals on a Phenomenex C18 column with a green MP made of double distilled H_2O: MeOH (30:70%v/v) with a flow rate of 0.8 mL/min and a run duration of 4 min and UV detection at 254 nm. The volume of trash was reduced to 3.2 mL/run using a short column (100 mm) having tiny particle size (3.5 µm), which is thought to be a significant indicator of greenness of process. MBZ and quinfamide had retention times of 2.69 and 3.43, respectively, Linearity was in ranges of 1–60 and 2–80 µg/band for QF and MBZ, respectively. Accuracy was found to be 100.04% and 99.87% for QF and MBZ, respectively. LOD and LOQ for quinfamide was 0.323 and 0.979 µg/band, for MBZ was 0.643 and 1.948 µg/band [116].

A novel technique for the estimation of furosemide (FRS), spironolactone (SPL), &canrenone (CAN) is being researched by Ibrahim et al. Alumina plates (20 × 20 cm) coated by 0.25 mm silica gel 60 F254 plates were utilised for Method 1's HPTLC separation, with ethyl acetate, triethylamine, and acetic acid (9: 0.7: 0.5, by volume) serving as the forming system &UV detection at 254 nm serving as the detection wavelength. For the three distinct components, FRS, SPL, and CAN have the Rf values of 0.320.02, 0.640.02, and 0.790.02, respectively. Linearity was in ranges of 0.2–2, 0.05–2.6 and 0.05–2 µg/band for FRS, SPL and CAN, respectively. Accuracy was found to be 100.11%, 99.65% and 99.98% for FR, SP and CN, respectively. LOD and LOQ for FRS was 0.059 µg/band and 0.195 µg/band, for SPL was 0.015 µg/band and 0.048 µg/band and for CAN was 0.014 and 0.045. Using a ZOBRAX Eclipse Plus C18 (4.6 × 100 mm) column and a MP made up of EtOH and deionized water (45:55%v/v), Method 2 was developed that uses green isocratic RP-HPLC with UV estimation at 254 nm. The flow rate was adjusted to 1 ml/min and the pH to 3.5 using glacial acetic acid. Within 5 min, the three chemicals had been successfully eluted with satisfactory separation. The duration of retention values for SPL, FRS, and CAN were determined to be 3.36, 1.64, and 4.42 min, respectively. Linearity was in ranges of 5–60, 2–60 and 2–60 µg/mL for FRS, SPL and CAN, respectively. Accuracy was found to be 100.10%, 100.03% and 100.01% for FRS, SPL and CAN, respectively. LOD and LOQ for FRS was 1.333 µg/mL and 4.00 µg/mL, for SPL was 0.485 µg/mL and 1.60 µg/mL and for CAN was 0.60 µg/mL and 1.80 µg/mL [117].

Bang et al., had developed and validated green HPTLC technique for estimation of Lenvatinib. He had developed two green methods, i.e. RP-HPTLC and NP-HPTLC, mobile phase used here are ethanol and water (60:40, %v/v), and 50:50%v/v of ethanol and ethyl acetate, respectively. The detection was carried out at 243 nm. Analytical greenness (AGREE) scores were estimated as 0.88 and 0.82, respectively, for the RP and NP [118] (Table 6).

Table 6. Summary of green technique by reducing solvent consumptions using HPTLC

Drug	Approach	Greenness	ECO Scale/AGREE Score	References

Thiocolchicoside	HPTLC	HPTLC uses a minimal amount of mobile phase to analyse	–	[115]
Quinfamide and Mebendazole	HPTLC and RP-HPLC	Short analytical time technique and water: methanol	–	[116]
Furosemide, Spirolactone, and Canrenone	HPTLC and RP-HPLC	Short analytical time technique and EtOH and deionized H_2O MP	–	[117]
Lenvatinib	RP-HPTLC, NP-HPTLC	ethanol and water (60:40, %v/v), and 50:50%v/v of ethanol and ethyl acetate	0.88 &0.82 (AGREE)	[118]

1. TLC (Thin layer chromatography)

Stability-indicating techniques for modafinil and the by-product of its acid-induced deterioration have been devised and confirmed by Elsheikh et al. using TLC-Densitometric, which uses plates that are precoated with silica gel G60 F254 and are 10 × 20 cm in size and 0.25 mm thick. Dichloromethane-methanol (90:10, %v/v) was employed as the growing solution, and the chromatograms are scanned at 254 nm afterwards. Modafinil had a R_f of 0.48 and its degradation product had an R_f of 0.77. Linearity was in ranges of 1–10 µg/band. Accuracy was found to be 100.11%. LOD and LOQ was 0.110 and 0.335 µg/band. The created TLC-densitometry analytical Eco Scale procedures yield scores of 80 [119].

Elsheikh et al. have developed and optimised straightforward, accurate, and reliable stability-indicating TLC-densitometric technique for zonisamide (ZNS) in the presence of its breakdown product, which was carried out using aluminium plates covered with silica gel 60F254 as a SP, while chloroform, MeOH, and acetic acid (80:15:5%v/v/v) as a growing solution, and detection at 240 nm under a UV lamp. ZNS had an R_f value of 0.76 and its degradation product had an R_f value of 0.11. The newly developed TLC-densitometry procedures have ECO Scale scores of 84, with modest green colours which shows that the method is eco-friendly [72].

Green spectroscopy methods

An eco-sustainable spectrophotometric simultaneous equation approach was suggested by Kokilambigai et al. for the measurement of paracetamol (PCM), aceclofenac (ACF), and thiocolchicoside (THC) pharmaceuticals in their combined dose form. Instead of methanol, the diluent phosphate buffer with pH 7.8 was employed as an ecological solvent since it has no effect on the medicines' UV spectral properties. PCM, ACF, and THC all had maximum absorptions (λ_{max}) at wavelengths of 243, 274, and 259 nm, respectively. The devised procedure was accurate since the recovery amount varied from 99.56 to 100.99% w/w. The linear range was established between 5 and 15 µg/mL for paracetamol, 1–5 µg/mL for aceclofenac, and 1–5 µg/mL thiocolchicoside. The LOD values for PCM, ACF and THC were in the ranges of 0.24 µg/mL, 0.33 µg/mL and 0.32 µg/mL, respectively. The LOQ value were in the ranges of 0.78 µg/mL, 1.01 µg/mL and 0.95 µg/mL, respectively. The ECO Scale tool was used to assess the greenness profile score, and it revealed a greenness score of 100 [120].

For the simultaneous quantification of Paracetamol (PCM), Aceclofenac (ACF), and Eperisone Hydrochloride (ES) Rathinam et al. have devised an environmentally safe, user-friendly, quick, sensitive, and affordable UV spectrophotometric approach. The diluent phosphate buffer pH 7.8 is employed as a green solvent since it has no effect on the medicines' UV spectral properties. When the wavelengths of the PCM, ACF, and ES were scanned across 200–400 nm with phosphate buffer pH 7.80 serving as the reference, their λ_{max} were 243, 272, and 262 nm,

respectively. Linearity concentration ranges between 12 and 18 µg/mL for paracetamol, 3.69–5.53 µg/mL for Aceclofenac, and 2.76–4.15 µg/mL Eperisone hydrochloride, and the LOD values were in the ranges of 0.48 µg/mL, 0.20 µg/mL and 0.13 µg/mL, respectively. The LOQ value were in the ranges of 1.44 µg/mL, 0.61 µg/mL and 0.38 µg/mL, respectively. The %recovery for PCM, ACF and ES was 100.48%, 100.33 and 100.07%. Software called ECO Scale determined that the simultaneous equation score was 96 [121].

Using Ultra-Violet (UV) spectrophotometric techniques, Chanduluru et al. have presented a straightforward, distinctive, economical, environmentally conscious, stability-indicating test approach to evaluate Chlorthalidone (CTD) and Cilnidipine (CIL). This approach uses propylene carbonate rather than methanol as a green solvent for CIL and CTD analyses because propylene carbonate is harmless in terms of influence on water, air, health risks, combustibility, stability. According to area under curve approaches, the absorbance ranges for CTD and CIL were 218–227 nm and 224–232 nm, respectively. Linearity concentration range of 7–13 µg/mL for CTD and 8.75–16.25 µg/mL for CIL. According to the agree programme, the simultaneous equation score was 0.91[122].

By utilising spectrophotometric techniques, Fawzy et al. produced accurate, eclectic, affordable, simple, and sensitive spectrophotometric tactics for the simultaneous estimation of saxagliptin hydrochloride and dapagliflozin propanediol monohydrate. The initial strategy, known as the induced dual-wavelength approach (IDW), mainly depended upon employment of substitute equality factors (*F*) to exclude the influence of dapagliflozin propanediol monohydrate while determining saxagliptin hydrochloride and vice versa. The second approach, known as the ratio difference method (RDM), determined the amplitude difference on the ratio spectrum of saxagliptin hydrochloride and DAG, respectively, using divisors of 25 µg/mL of dapagliflozin propanediol monohydrate and 20 µg/mL of saxagliptin hydrochloride. The third approach, plateau subtraction followed by multiplication by the division of dapagliflozin propanediol monohydrate 25 µg/mL, was used to compute SAG at λ_{max} 221 nm. The ECO Scale score for given technique was 88[123].

Fawzy et al. develop ecologically sustainable, easy, and reliable spectrophotometric and chemometric approaches for the simultaneous quantification of Fluticasone propionate and Azelastine. Although methanol is used as a diluent, the procedure is ecologically favourable in terms of a number of other aspects, including the utilization of instruments, chemicals, and disposal of waste. For both medications, the absorbance is at 258 nm (λiso). This approach received an ECO Scale score of 88[124].

For the measurement of ramipril as well as temisartan both coupled with hydrochoryhiazide (HCTZ), Elsonbaty et al. have presented a specific, quick, green, exact, and simple analytical methodology employing a reconstituted FSD aided UV spectrophotometric technique. HCTZ is measured at 276 nm, which is the zero-crossing point for temisartan, whereas temisartan was measured at 257 nm, which was the no contribution point of HCTZ. HCTZ was concurrently measured at 244 nm and ramipril was calculated at 231 nm for ramipril /HCTZ, which is the zero-crossing point of both HCTZ and ramipril. The linear ranges for telmisartan, ramipril, and hydrochlorothiazide were 1–25 µg/mL, 5–35 µg/mL, and 1–10 µg/mL, respectively, and the LOD values were in the ranges of 0.067–0.747 µg/mL. The %recovery for about drugs was 100.82, 99.10, 99.55, respectively. The ease of use, speed, lack of demand for complex software, and increased sensitivity of the new technology over the conventional deconvolution-curve fitting are its advantages. The analytical eco scale's overall score is 88, which is very good [125].

Lofty et al. devised a straightforward, inexpensive, quick, efficient, and environmentally friendly approach for evaluating single-tablet regimens of atenolol (Ate), amiloride (AMD), and chlortalidone (CTD). Zero order spectra, which serve as spectral profiles or fingerprints for pharmaceuticals, were recovered using the Parent Spectrum Restoration Technique (PSRT). This approach used three computing approaches: the well-known smart method

spectrum subtraction (SS), the derivative ratio transformation utilising normalised spectra or any other divisor, and the dual amplitude difference (DAD) through factorised spectra. Utilising methanol as both a blank and a solvent, the λ_{max} for Ate, AMD, and CTD were discovered to be 226 nm, 362 nm, and 230 nm, respectively. Linearity for each drug was ranging between 4.0 and 40.0 µg/mL for Ate, 3.0–20.0 µg/mL for both AMD and CTD. The %recovery for AMD, CTD, Ate was 99.61%, 99.92%, and 99.82%, respectively. The UV-methods obtained a better score for greenness 89 due to their use of less energy and solvent than the HPLC described approach, which received a middling score of 84[126].

Kannaiah et al. suggested a simple, sustainable, inexpensive, sturdy, specific, and successful extraction technique, as well as a multivariant green analytical approach, for estimating ketoconazole using UV spectroscopy. The diluent is made by combining water and ethanol (99.9%) in 80:20%v/v. The drug's λ_{max} at 226 nm over a diluent in blank solution was discovered. The linearity was found at concentration of 12.8–19.2 µg/mL with R^2 value of 0.9992. LOD and LOQ was found to be 0.338 and 1.026 µg/mL, respectively. The %recovery was 100.31%. Through the ECO Scale tool, the analytical value was discovered to be 96 [127].

Salicylic acid (SA) &Mometasone furoate (MF) were analysed concurrently using a green capillary zone electrophoretic technique (CZE) that was developed &validated by Amira et al. Agilent CE instrument 7100 series along with a Diode Array Detector was utilised for separation. Agilent Technologies provided a capillary made of fused silica with an effective length of 50 cm and an internal diameter (id) of 50 m. Measurements were made in the hydrodynamic mode at a temperature of 25 °C. MF and SA showed good linearity in the series of 0.2–40 µg/mL and 0.1–80 µg/mL, respectively. The detection and quantification limits for the assay of MF were 0.04 and 0.13 µg/mL, and of SA were 0.03 µg/mL and 0.1 µg/mL, respectively. The% recovery was 99.60% and 99.35%, respectively. The discovered approach has various benefits, including little solvent use, low operating expenses, minimal sample time for analysis requirements, and no need for sample preparation. The recommended method's analytical Eco Scale value was computed, and its score of 86 was discovered [128].

For the estimation of remogliflozin (REM) &metformin (MET), Attimarad et al. devised and validated straightforward, precise, sensitive, affordable, and green UV spectrophotometric techniques. The diluent is ethanol. For REM and MET, the peak amplitudes were at 233.0 nm &252.2 nm, respectively. The assay was found to be in the range of 98.16–101.37% for REM and 98.10–101.76% for MET. REM and MET showed good linearity in the series of 1–20 µg/mL and 2.5–35 µg/mL, respectively, with correlation coefficient of 0.998. The %RSD for the analyte in Method precision and intermediate precision is below 1.0. The recovery rate was shown to be between 98.4 and 101.5%. The new spectroscopic techniques scored 94 on the eco scale, showing their greenness [129].

Megahed et al., had developed a simple, rapid, robust, selective, green and sensitive spectrofluorimetric method for determination of favipiravir. The stock solution of FAV was prepared in distilled water then transfer FAV stock solution followed by 2.5 mL of 0.2 M borate buffer pH 8.0 to make a range of 40–280 ng/mL. The solutions were measured at 432 nm after excitation at 361 nm. The% recovery was found to be 100%. The %RSD for the analyte in Method precision and intermediate precision is below 1.0. The eco scale was found to be 92 [130].

Abdelazim et al., had developed an eco-friendly spectrophotometric method for the determination of Molnupiravir. A standard stock solution of molnupiravir was prepared in ethanol then 0.5 mL of hydrochloric acid [0.5 M] and 0.5 mL sodium nitrite was added for diazotization of molnupiravir. After that 1 mL of 8-hydroxyquinoline [0.35%] was added. Then 0.5 mL sodium hydroxide [2 M] was added and the prepared solutions were allowed to stand for 5 min. Finally, the volume was made with ethanol. The absorbance of the red-coloured azo dye complexes was measured at 515 nm against a blank. The method found linear from 1.0 to 12.0 µg/mL with $R^2 \geq 0.9996$. The %recovery was 99.82%. The %RSD for the analyte in Method precision and intermediate precision is below 1.0. The AGREE

software was employed to evaluate the technique's greenness, and the resultant score of 0.81 [131] (Table 7).

Table 7. Summary of Green Spectroscopy techniques

Drug	Approach	Greenness	ECO Scale/AGREE Score	References
Paracetamol, Aceclofenac, and Thiocolchicoside	Spectrophotometric	Diluent phosphate buffer pH 7.8	100	[120]
Paracetamol, Aceclofenac, and Eperisone Hydrochloride	UV spectrophotometric	Diluent phosphate buffer pH 7.8	96	[121]
Chlorthalidone and Cilnidipine	Ultra-Violet (UV) spectrophotometric	Propylene carbonate as diluent	0.91(AGREE)	[122]
Saxagliptin hydrochloride and Dapagliflozin propanediol monohydrate	Spectrophotometric	Favourable use of instruments, reagents, and the disposal of waste	88	[123]
Fluticasone propionate and Azelastine	Ultra-Violet (UV) spectrophotometric	Favourable use of instruments, reagents, and the disposal of waste	88	[124]
Ramipril as well as Temisartan coupled with Hydrochoryhiazide	FSD aided UV spectrophotometric	Technique	88	[125]
Atenolol, Amiloride, and Chlortalidone	UV-methods	Less solvent consumption	89	[126]
Ketoconazole	UV-methods	$H_2O$and EtOH diluent	96	[127]
Mometasone furoate and salicylic acid	Capillary zone electrophoretic	Technique itself	86	[128]
Metformin and Remogliflozin	UV spectrophotometric	Technique	94	[129]
Favipiravir	Spectrofluorimetric	0.2 M borate buffer	92	[130]
Molnupiravir	UV spectrophotometric	EtOH	0.81(AGREE)	[131]

Bold value indicates score that has been obtained from analytical eco-scale's tool that suggests that the method is greener compared to traditional method

Conclusion

The insights provided in this review article on green analytical techniques incorporating examples of GAC using

HPLC, HPTLC, UHPLC, and TLC lay the groundwork for several future advancements and applications. Firstly, it serves as a comprehensive reference for researchers, analysts, and industry professionals seeking to adopt more sustainable, novel, cost-effective, efficient, and ecologically practices in their analytical workflows. By highlighting the benefits and feasibility of green analytical techniques, this review facilitates the widespread adoption of environmentally friendly methodologies across various sectors, including pharmaceuticals, food analysis, environmental monitoring, and beyond.

Liquid chromatographic equipment is extensively used in pharmaceutical quality control globally, often consuming large quantities of organic solvents and leading to unsustainable waste generation. This has driven considerable interest in greening liquid chromatographic techniques, aiming to protect both analysts and the environment. Solvent use is a significant contributor to waste and poses toxicity concerns, which can be addressed by integrating green analytical chemistry principles into method development. Various tools for assessing the environmental impact of liquid chromatography methods play a crucial role in developing environmentally friendly methods with minimal adverse effects. It's advisable to incorporate suitable green evaluation tools into method design to ensure better environmental consideration. Common aspects considered across assessment tools include evaluating a method's environmental impact in terms of solvents, energy consumption, and waste generation. Incorporating green solvents like Sodium dodecyl sulphate and propylene carbonate into methods can help address environmental concerns. Additionally, adopting miniaturised techniques like UHPLC may reduce energy consumption and waste generation during analysis. This shift towards greener practices in pharmaceutical analysis is essential for minimising adverse impacts while maintaining analytical performance standards.

Additionally, this review fosters ongoing innovation in the field of analytical chemistry by identifying areas for further research and development. It encourages the exploration of new green solvents, alternative separation techniques, and novel instrumentation to enhance the eco-efficiency and analytical performance of green analytical methods. Moreover, the examples provided offer inspiration for the design and implementation of customized green analytical protocols tailored to specific analytical challenges and applications.

Furthermore, this review contributes to the global sustainability agenda by promoting awareness and advocacy for greener practices within the scientific community and beyond. By emphasizing the societal and environmental benefits of adopting green analytical techniques, it encourages policymakers, regulatory agencies, and industry stakeholders to prioritise sustainability in chemical analysis and decision-making processes.

In essence, the future usefulness of this review lies in its ability to catalyse positive change, inspire innovation, and promote collaboration towards a more sustainable and environmentally conscious approach to analytical chemistry. This review includes developing methodologies, unique technology, and creative approaches that have not garnered much attention. Green extraction technologies, solid-phase microextraction, and supercritical fluid extraction are all revolutionary procedures that utilise fewer organic solvents and generate less waste. The review also examines the use of sophisticated technologies such as microfluidics, nanotechnology, and spectroscopic approaches to create more efficient and environmentally friendly analytical procedures.

Our evaluation provides a unique viewpoint or focus on certain parts of green analytical methodologies. We have prioritised conversations about the application of green chemistry concepts, the use of greener solvents, and the assessment of environmental effect using diverse methodologies. We have included the most recent advances in green analytical approaches, including data from recent research publications.

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develop a novel, cost-effective, efficient, and ecologically sustainable approach. Also, vast research is going on to creating novel Green Analytical Chemistry approaches to address these environmental problems and create an ecologically preferable replacement.

Author contributions

Authors have collected all the information, analysed that and prepared the manuscript. Authors approve the final manuscript. Here, all authors approve the manuscript for submission.

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Abbreviations

GAC

Green analytical chemistry

HPLC

High-performance liquid chromatography

UPLC/UHPLC

Ultraperformance liquid chromatography

UV

Uv-visible

HPTLC

High-performance thin layer chromatography

TLC

Thin layer chromatography

GC

Gas chromatography

LC

Liquid chromatography

MS

Mass spectroscopy

MeOH

Methanol

ACN

Acetonitrile

H_2O

Water

$CHCl_3$

Chloroform

MP

Mobile phase

R_f

Retardation factor

AQbD

Analytical quality by design

Rt

Retention time

PDA

Photodiode array

RP

Reverse phase

SP

Stationary phase

Publisher's Note

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Short-cut route validated for monitoring fentanyl and its metabolite in urine using LC–MS/MS, in a wide concentration range

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ABSTRACT (ENGLISH)

Background

Fentanyl is a highly potent analgesic, used in surgery, frequently abused or used in drug-facilitated crimes (DFC) and in military activities. It is also increasingly used in the treatment of chronic pain (especially in cancer patients). The improper use of transdermal patch forms can cause toxicity and deaths, related to overdose or combined use with other drug substances. Methods are needed for fast, reliable and inexpensive fentanyl detection and we aimed to develop such a method in urine using LC–MS/MS, especially for toxic and fatal concentrations which lack in the literature.

Results

An LC–MS/MS method has been presented for the co-determination of fentanyl and its main metabolite, norfentanyl in urine. The recoveries of the extraction method were 95(±6)% and 70(±9)% for fentanyl and norfentanyl, respectively. LOD and LOQ values are 1.7 and 14.0 ng/mL for fentanyl, while they were 20.6 ng/mL and 42.0 ng/mL for norfentanyl.

Conclusion

A rapid, sensitive, very practical, inexpensive and a high-recovery analysis method is developed and validated. This is the only fentanyl monitoring LC–MS/MS method in urine having a linearity over a wide range up to 500.0 ng/mL and its success is demonstrated on real samples in the therapeutic drug monitoring of fentanyl and is expected to contribute to clarify intoxications/deaths related to its use.

FULL TEXT

Background

Fentanyl is a potent, synthetic, narcotic, analgesic opioid with an analgesic activity that is approximately 80 times more effective than morphine and 500 times more potent than meperidine, with fewer side effects [1, 2]. 85% of the fentanyl taken into the body is excreted in the first 72 h; 7% is excreted unchanged (6% urine, 1% feces), 78% is metabolized in the liver (70% urine, 8% feces) [3]. Its main metabolite is norfentanyl. Studies conducted in countries where polydrug use is common, show that a significant group of these individuals who lost their lives due to overdose, combined one or more drugs with the active substance fentanyl [4, 5]. The transdermal route is superior to both oral and parenteral routes in terms of ease of administration in therapy [6]. Dose-related poisoning occurs frequently, as a result of simultaneous use of several transdermal fentanyl bands in home treatment to increase analgesic effect, or in misuse [7]. There are many case reports regarding inappropriate use or misuse of fentanyl skin patch content via intravenous, oral, rectal and inhalation [8, 9]. Defective patches are one of the causes of intoxication [10].

Fentanyl has forensic importance as a drug of abuse, which can also be used for suicidal and criminal purposes,

besides having toxicological and clinical importance in environmental and occupational exposure, regarding healthcare personnel while administering [11]. Environmental exposure might occur via inhalation of powders/aerosols, ingestion, fentanyl patches or items contaminated with fentanyl (which may be present in powder, tablet or liquid forms). Its production and consumption is increasing day by day [12]. In recent years, deaths due to fentanyl overdose have been detected among patients hospitalized for chronic pain [6].

In cases of polymorphisms seen in the CYP3A4 gene which metabolizes fentanyl, serious toxicological effects may occur related to fentanyl use [13, 14]. Therefore, drug monitoring is important for fentanyl. To monitor the levels of fentanyl and its metabolite norfentanyl or to clarify the cases of toxicity/death due to the use of fentanyl transdermal patches in people who use or misuse the patch or in their relatives or health personnel who are exposed to them; a simple and rapid method in urine, with low detection limit, higher recovery, having a wide linearity range, was developed using LC–MS/MS. Although the extraction method was modified from study of Coopman et al. [15], differently here, steps were reduced using undiluted urine; recovery for norfentanyl was increased twice; linearity range was increased up to a very high amount as 500 ng/mL; and a 2.5 times enrichment was performed, while a lower injection volume was used. Papaverine hydrochloride was chosen as the internal standard (IS) and a 2.5-min ultra-fast LC–MS/MS analysis was performed using C_{18} poroshell column. The whole validated method is for quantitative purposes with given LOD and LOQ values. This method is the first short-cut fentanyl monitoring method in the literature with the widest concentration range, requiring no dilution at toxic concentrations.

Material and methods

Reagents and chemicals

Acetonitrile (HPLC-Grade) and formic acid (99–100%) were used in mobile phase (Merck, Germany). Ethylacetate, *n*-hexane and potassium carbonate were of analytical grade (Sigma-Aldrich, UK). Fentanyl and norfentanyl standard (both %99.9) were purchased from Lipomed (Germany). Preliminary investigations were performed using fentanyl from Janssen-Clag (Bulgaria). Papaverine hydrochloride was provided from Sopharma (Bulgaria). All standard solutions were prepared using HPLC grade methanol (Merck, Germany).

Instrumentation

A QQQ Tandem Gold ESI-LC–MS/MS (Zivak, Turkey) was used. Chromatographic elution was performed at 60 °C, using Poroshell 120 2.7 µm, $C_{18,}$ 100 × 3.0 mm analytical column (Agilent, USA) fitted to a guard cartridge of 4.0 mm × 2.0 mm (Phenomenex, USA). Autosampler temperature was 4 °C. Multiple reaction monitoring (MRM) channels for the analytes and IS were determined after the optimization of the capillary voltage and collision energies (Table 1). During the optimization, 0.1% formic acid (pH: 2.6)/acetonitrile (50:50, v/v) mobile phase composition was used. MS parameters for needle, shield voltage, nebulizer gas pressure and drying gas pressure, drying gas temperature were: + 5500.0 V, + 600.0 V, 55.0 psi, 30.0 psi and 400 °C, respectively. Single ion monitoring (SIM) width was 1.5 amu. Atmospheric pressure ionization (API) housing was kept at 65.0 °C and argon pressure at 2.40 mTorr. An accurate analysis was performed in an ultra-short elution time (2.5 min) with an isocratic elution via 0.1% formic acid/acetonitrile (50:50, v/v) composition (flow rate: 0.3 mL/min). Injection volumes were 10.0 µL.

Table 1. MRM table of the instrument

ANALYTE	Q_1	Q_3	Capillary voltage (V)	Collision energy (V)	Dwell time (s)
Fentanyl	337.0	188.0	+ 90.0	20.0	0.15
Norfentanyl	233.2	150.1	+ 60.0	13.0	0.15
Papaverine HCl (IS)	340.1	202.0	+ 92.0	22.5	0.15

Sample collection

Urine samples were collected from five healthy individuals (between ages 24 and 36 years old) with no drug use and

with no known chronic disease, and mixed in a pot to form a blank urine pool for method development and validation. To test the method in real cases, approximately 50 mL of urine samples was obtained from three inpatients (aged between 54 and 66), two of whom were female, in cooperation with Istanbul University-Cerrahpasa Medical Faculty, Department of Algology, for analysis. Two of the participants had diagnosed with servix cancer (body weight: 42 and 65 kg) and one of them with soft tissue cancer (body weight: 73 kg) in the last 2 years and all were treated with transdermal fentanyl patches to overcome violent pain. None had tobacco or alcohol use and all were unrelated from the point of kinship and did not use any other medication. The daily doses of the amount of fentanyl used by patients varied between 50 and 100 µg/h. All collected urine samples were placed in sterile, plastic urine containers and were stored at + 4 °C until analysis. Standard solutions were spiked to these urine samples, extracted and analyzed.

Preparation of the standard solutions and urine samples

Analyte and IS solutions were prepared using methanol at final concentrations of 500.0, 5000.0, 12,500.0, 50,000.0 ng/mL and added freshly to blind urine samples in predetermined volumes, not exceeding 20.0 µL. A 20.0 µL IS solution of 20.0 µg/mL was also added. Finally, methanol in varying amounts was added to each sample in order to standardize the spiking volume as 40.0 µL. Blind urine samples were also studied. All stock and test solutions were prepared freshly.

Optimization of the extraction method

The extraction method was modified from the liquid–liquid extraction (LLE) method of Coopman et al. [15] and optimized. In the modification and optimization studies; the effect of mixing time, sample volume and the number of extraction steps on the recovery efficiency were investigated. Unlike the former study, undiluted urine was used for extraction. The effect of salt on extraction efficiency was investigated through testing NaF, Na_2SO_4 and K_2CO_3 in the extraction with 7.00 mL of n-hexane/ethylacetate (7:3, v:v). When NaF and Na_2SO_4 were used, the phase separation was not clear, and K_2CO_3 provided the best phase separation. Since fentanyl and norfentanyl were weak bases, the nonionized fractions of the analytes and the recovery were increased after the added urine was alkalized (to pH = 11.50–12.00) with K_2CO_3 solution. The effect of mixing time on the recovery of fentanyl in LLE was also investigated; trying 3, 5 and 7 min extraction times. 7 min was observed to give much higher recovery than the Coopman's method. Sample volumes of 1.00 and 2.00 mL were tried and the best recovery was obtained with 1.00 mL sample volume. Also a two-step extraction method was tried with a total of 7.00 mL extraction solvent. Since no significant difference was observed between the one-step and two-step extraction methods, a single-step extraction was preferred. Unlike the former study, a mild block temperature of 35 °C was chosen for evaporation under nitrogen, in order not to degrade the analytes.

The optimized sample preparation method below had higher precision and higher analytically acceptable recoveries than the unmodified form:

1.00 mL of urine was placed in a polypropylene test tube, 0.363 M K_2CO_3 was added till a pH between 11.5 and 12.0 was obtained. After 30 s vortexing, 7.00 mL of n-hexane/ethylacetate (7:3, v:v) mixture was added and vortexed for 7 min. After 5 min centrifugation at 5000 rpm, the upper phase was evaporated to dryness at 35 °C under nitrogen, reconstituted in 400.0 µL methanol and transferred to vials for analysis in LC–MS/MS.

All measurements were performed, wherever possible, at low temperatures (autosampler: at 4 °C, evaporation: at 35 °C) and in a short period of time.

Validation

The chromatograms of solvent, extracted blank and spiked samples ($n \geq 6$) were compared to determine the specificity of the method. It was checked whether there was any interference from the solvent and the matrix, at the ion channels and retention times of the peaks of fentanyl and norfentanyl. In addition, in order to provide the selectivity, characteristic MRM ion channels specific to each of the analytes and IS were created. Each standard was injected one by one to monitor whether their peaks interfere with each other's ion channels to demonstrate the selectivity. Data evaluation and calculations of validation parameters were performed using Microsoft Excel. A matrix-matched calibration technique was used for the compensation of matrix effects: Spiked matrix samples were

prepared in increasing concentrations between 1.0 and 1250.0 ng/mL for construction of the calibration curves. Least squares method was used to create the calibration curves. Standard deviations obtained from at least two injections of each of three samples ($n \geq 6$) for each concentration level were calculated. The relative standard deviation (RSD%) values for each calibration level were used in LOQ estimation via Eurachem method [16], which is the most strict method utilizing the reasonable and a gradual decrease of the repeatabilities in the y-axis of the Eurachem graph versus increasing concentration and regarding 15% RSD as the LOQ. This was estimated through plotting RSD% values versus concentrations of the spiked samples analyzed during the matrix-matched calibration. LOD was calculated using Eq. 1 [17]:

1

LOD=3.3sslopeofthecalibrationcurves: standard deviations of the analyte area/IS area ratios.

Recovery studies were performed at low, medium and high concentrations of the linear range of fentanyl and norfentanyl for urine samples, each with at least six replicates. Average recovery rates along with their precision values were calculated from Eq. 2 [18]:

2

RE%=pre - extractionspikepost - extractionspike×100

The matrix effect was evaluated by comparing spiked matrices with the standard solutions in methanol ($n \geq 6$) and was studied for full concentration scale in the calibration graph (Eq. 3) [19]:

3

MatrixEffect,%=slopeanalyticalcurvestandardinmatrix-slopeanalyticalcurvestandardinsolventslopeanalyticalcurve standardinsolvent×100

Results

In this study, a fast and simple LC–MS/MS method for fentanyl and its metabolite norfentanyl in urine is developed and validated. The sample preparation method was modified from Coopman et al. [15] and optimized. In the method developed for screening and confirmation, the analytes were determined in 1.00 mL urine sample, with a very fast (2.5 min) isocratic elution in LC–MS/MS, following a 30–35 min LLE. LLE was performed at a pH between 11.5 and 12.0 (K_2CO_3) using 7.00 mL of n-hexane/ethylacetate solution, in 7 min and the organic phase was enriched 2.5 times for analysis in LC–MS/MS.

The retention times for fentanyl and norfentanyl were 1.73(±0.00) and 1.38(±0.01) minutes. MRM transitions were 337.0>188.0 m/*z* for fentanyl and 233.2>150.1 m/*z* for norfentanyl. The retention time for IS was 1.73(±0.00) minutes, while its MRM transition was 340.1>202.0 m/*z*. The validation was performed by means of specificity, selectivity, linearity, linear range, recovery, LOD, LOQ and matrix effect.

Validation of the optimized method

In the validation of the optimized method, the selectivity was confirmed by the determination of the analytes in their characteristic ion channels. As a result of the comparison of the chromatograms of the analytes in urine extracts, with the methanol and blank urine chromatograms; it was observed that no interference from the solvent and the blank urine existed in the retention times of the analytes, so the specificity was also confirmed (Fig. 1).

Fig. 1 [Images not available. See PDF.]

MRM Chromatograms of **a** fentanyl and **b** norfentanyl, added to the blind urine matrix before extraction at concentrations of 250.0 ng/mL and **c** Papaverine (IS) at 5.0 ng/mL

Calibration of the whole method, including sample preparation and instrumental method, was performed with matrix-matched calibration technique at five different concentrations. The equations of the calibration curves, linear ranges, regression limit value, LOD and LOQ values of the instrumental method regarding standard fentanyl and norfentanyl in solutions and in urine matrix are shown in Table 2. Calibration curves of the standard solutions as well as the matrix-matched calibration curves for fentanyl and norfentanyl, along with their r^2 values are demonstrated in Fig. 2. Matrix effect calculated for each analyte ($n \geq 6$) was found as 6.25% for fentanyl showing an ion enhancement and -20% for norfentanyl indicating anion suppression. The validation results obtained in standard solutions and sample

extracts fit for the purpose for analysis in tablet and injectable solutions and drug monitoring/toxicological analysis in urine, respectively.

Table 2. Linearity, linear ranges, calibration equations, LOD and LOQ values for fentanyl and norfentanyl regarding the whole method

Analyte	Calibration with standard solution				Matrix-matched calibration			
Linear Range[a]	Equation	LOD[a]	LOQ [a]	Linear Range[a]	Equation	LOD[a]	LOQ [a]	Fentanyl
2.5–1250.0	$y=0.0032x-0.0130$	0.7	2.5	14.0–500.0	$y=0.0034x+0.0354$	1.7	14.0	Norfentanyl

r^2>0.99; [a]ng/mL, number of analyses n≥6, RSD%<15

Fig. 2 [Images not available. See PDF.]

Calibration curves of fentanyl (**a**), norfentanyl in neat solutions (**b**) and matrix-matched calibration curves for fentanyl (**c**) and norfentanyl (**d**) in urine

An average recovery of 95(±6) % was obtained for fentanyl and 70(±9) % for norfentanyl, regarding low, mid and high concentrations (Table 3). Recovery findings were found to be within acceptable limits.

Table 3. Recovery and precision values for fentanyl and norfentanyl regarding the whole method at low, mid and high concentrations

Analyte	Spiked concentration (ng/mL)	Recovery%	%RSD
	10.00	92	6
	50.0	98	6
Fentanyl	250.0	94	8
	500.0	96	5
	50.0	69	16
Norfentanyl	250.0	71	4
	500.0	70	8

Number of analyses n≥6

Real examples

The method was applied to urine samples of three patients treated with fentanyl in Istanbul University-Cerrahpasa Medical Faculty, Department of Algology. The analysis results obtained using LC–MS/MS are shown in Table 4. Since the amount of substance in the urine of the patients is variable, blank methanol was analyzed between

samples, in order to prevent contamination. The chromatograms obtained after each blank methanol analysis were examined and it was seen that there was no carry over between the analyses. Fentanyl and norfentanyl concentrations were calculated using calibration graphs. The lowest amount of fentanyl detected was 32.6 ng/mL and the highest amount was 111.3 ng/mL.

Table 4. Concentrations of fentanyl and its metabolite found in the urine of the patients after the given doses ($n \geq 3$)

Sample No	1	2	3
Given dose for fentanyl (μg/h)	100 μg/h	100 μg/h	50 μg/h
Last fentanyl intake (before urine collection)	5 days before	2 days before	1 day before
Fentanyl concentration found (ng/mL)	32.6 (±0.5)[a]	111.3 (±4.4)[a]	44.9 (±2.7)[a]
Norfentanyl concentration found (ng/mL)	267.2 (±28.1)[b]	183.0 (±19.5)[b]	151.3 (±18.9)[b]

[a]RSD % for fentanyl ≤ 6

[b]RSD % for norfentanyl ≤ 12

Discussion

In the literature, deaths due to fentanyl absorption in high doses have been detected in patients treated with fentanyl transdermal patches for chronic pain [6]. Various LC–MS/MS methods have been developed for the determination of fentanyl and norfentanyl, in urine and other biological materials, as well as in formulations [15, 20]. Although our basic extraction method was modified from Coopman's work [15], the recovery obtained here is almost doubled for norfentanyl (raised from 40 to 70%). Wider linearity range up to 500 ng/mL was provided (so that no dilution will be required in higher concentrations), which does not exist in any of the studies encountered. Since it is important to see higher concentrations in serious toxicities, wider linearity range will provide immediate results without dilution steps and without any change of matrix effect. Also, in our method, urine was used without dilution (number of steps were decreased), mixing time, sample volume and elution and drying temperatures were optimized, papaverine hydrochloride was chosen as the IS, and a 2.5 times enrichment was performed, while a lower injection volume was used. In this study, a quantitative method was validated by calculating LOQ values in standard solutions and in urine. The effect of different salts was also examined. Furthermore, this method is very fast compared to the literature methods, especially when the 2.5 min chromatographic elution is considered. An average of 95(±6)% recovery was obtained for fentanyl and all RSD% values were <15.0%.

Also, our modified extraction method is simpler, faster [21–23] and 2.5 min LC–MS/MS method is shorter than all the equivalent literature methods regarding fentanyl and norfentanyl, except the method of Mahlke et al., where fentanyl was eluted in 1.68 min (1.73 in our study) and norfentanyl was eluted in 1.56 min (1.38 in our study). However, the linear range in most of the studies including Mahlke et al. were in maximum up to 10.0 or 50.0 ng/mL, while our method can detect higher and toxic concentrations without any dilution [10, 23–27].

Verplaetse and Tytgat [17] have used solid phase extraction (SPE) for determination of fentanyl and norfentanyl in urine and have given a linearity up to 5 ng/mL and 10 ng/mL, respectively [11]. They also developed another SPE method with excellent recoveries for nine narcotic analgesics and metabolites including fentanyl and norfentanyl, with a 7.2 min elution time for fentanyl and 10 and 40 ng/mL as the highest levels of given linearity range, for the drug and its metabolite [12]. Cunha et al. [28] have performed a microextraction by packed sorbent, with a chromatographic run of 10.3 min. Recovery was at least 27% for all analytes and 13% for norfentanyl. Eckart et al. [29] have developed an automated SPE method for a wide range of opioids with a highest calibration level 10 ng/mL, where the recovery for fentanyl and norfentanyl was between 62 and 78% and the elution times were 21.3 min and 10 min. As a result, the calibration range of our study is wider than the published equivalent studies,

and our method is faster, easier, besides presenting high recoveries.

Fentanyl test strips are also used for testing the presence of fentanyls in illicit drug products. However, most fentanyl test strips are based on competitive lateral flow immunoassays on simple paper-based devices [30] and there is a considerable lack of knowledge about the selectivity and sensitivity of testing with urine fentanyl strips [31]. Since the test results are highly concentration-dependent, detection of lower concentrations can be problematic [30]. The fentanyl test strips do not measure the exact fentanyl concentration and do not provide results that differentiate between or among fentanyl and any of the analogs present in a sample [32]. Most of the strips give only qualitative results, but with a validated fast chromatographic method, exact concentration can be determined quantitatively and sensitively. An additive effect would occur in case of presence of acetylfentanyl, acrylfentanyl, furanylfentanyl and butyrfentanyl at low concentrations of fentanyl (10 ng/mL), thus false positive results may be obtained. It was only demonstrated on the Nal van Minden test, but this additive effect is expected to be a general principle for all test strip brands (false positive). Four brands of fentanyl test strips (Rapid Response, One Step, Nal van Minden and Rapid Self Test) were examined using single-component drug solutions [30]. 21–24 of the 28 fentanyl analogues were tested. The effect of co-presence of heroin or ascorbic acid on test results was also examined and one of the test strip brands gave false positive results in the presence of ascorbic acid. With strip, fentanyl and norfentanyl cannot be distinguished from other fentanyl analogues, but a chromatographic analysis can selectively determine fentanyl. Since a clean-up step is applied in this method, the selectivity is much more higher than testing with fentanyl strips.

Different amounts of fentanyl and norfentanyl found with this method in the urine samples of patients who were treated with patches containing the same dose, arouse the conviction that individual characteristics (gender, age, enzyme polymorphism, foodstuffs, other drugs taken together with fentanyl, urine concentration, etc.) or periodical/non-periodical applications might be the reasons. Besides these inter-individual differences, from time to time, differences within individuals are reported during the treatment [33]. The patient with the highest value has received the last fentanyl dose (100 µg/hour) two days before the urine sample was taken, and the drug administration was continued at 72-h intervals. The lowest amount of norfentanyl detected was 151.3 ng/mL and the highest amount was 267.2 ng/mL. According to the information of the patient with the lowest level of norfentanyl; a dose of 50 µg/hour was administered, and it was not repeated at 72-h intervals due to the patient's disrupting the medication.

Conclusion

Considering the risk of intoxication/death that may be encountered during treatment or because of misuse of fentanyl, a fast (30–35 min sample preparation + 5 min elution time in total), validated, sensitive LC–MS/MS analysis method with high recovery is presented here for the simultaneous determination of fentanyl and its metabolite in urine; to benefit in therapeutic drug monitoring, emergency toxicology and in forensic cases as toxicity/death caused by this drug and in supporting the presence of polymorphism of CYP3A4 gene (metabolizing fentanyl). The recovery obtained by the modified extraction method is almost doubled for norfentanyl and the upper linearity range was increased to at least10 times that of the former methods. Since this method has the widest linearity range in the literature, it is employable in a wide concentration range and no dilution will be required in higher concentrations. Especially, overdilution of the matrices will change the matrix intensity and errors may arise in the results. We suggest that this analysis method should be tested in future studies for the determination of new and more potent fentanyl derivatives in the market. Since it is important to determine higher concentrations in serious toxicities, wider linearity range will be useful in reaching the results immediately. This study fills this gap along with its high accuracy, speed and simplicity.

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Munevver Acikkol is retired.

Author contributions

F.C.Y. and B.A. designed the research; F.C.Y., B.A. performed research; B.A. and F.C.Y. analyzed the data, wrote the paper and revised; F.C.Y. collected the samples, F.C.Y. and B.A. provided the materials for experiments from their projects, M.A have contributed in the conception and supervised the study.

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Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the authors on reasonable request.

Declarations

Ethics approval and consent to participate

This study was performed in line with the principles of the Declaration of Helsinki, with the consent of the participants. Approval was granted by the Ethics Committee of Istanbul University-Cerrahpasa Cerrahpasa Medical Faculty (No:8954, date: 09.03.2011).

Consent for publication

Consent for publication is obtained from each of the owners of the real samples.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Abbreviations

API
Atmospheric pressure ionization
DFC
Drug-facilitated crimes
ESI-LC–MS/MS
Electrospray ionization liquid chromatography mass spectrometer
IS
Internal standard
LLE
Liquid–liquid extraction
LOD
Limit of detection
LOQ
Limit of quantification
MRM
Multiple reaction monitoring
QQQ
Quadrupole–quadrupole–quadrupole
RSD
Relative standard deviation
SIM
Single ion monitoring
SPE
Solid phase extraction

Publisher's Note

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Comparative study of UV spectroscopy, RP-HPLC and HPTLC methods for quantification of antiviral drug lamivudine in tablet formulation

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ABSTRACT (ENGLISH)

Background

In the current study, estimation of lamivudine (LMU) by UV spectroscopy, reverse-phase HPLC (RP-HPLC) and HPTLC methods in tablet formulation was developed, and comparative studies between the methods were investigated by analytical results and statistical test analysis of variance (ANOVA) to find out best method. In the UV spectral method, LMU was quantified at 271 nm absorption maxima using methanol as the solvent. In the RP-HPLC method, the Shimadzu C18 column (250 mm × 4.6 mm i.d., 5 µm particle size) was employed for chromatographic separation. The mobile phase used consists of methanol: water (70:30 v/v) in an isocratic mode with a 1.0 mL/min flow rate. In the HPTLC method, the chromatogram was developed on a pre-coated plate of silica gel 60 F254 with a mobile phase composition of chloroform: methanol (8:2 v/v). The quantification was performed at an absorbance mode of 271 nm by densitometry. The methods were validated according to the International Conference on Harmonization (ICH) guideline Q2 (R1). The degradation conditions were employed as per ICH guidelines Q1A(R2) and Q1B which include acid, alkaline, neutral, thermal and photostability to determine the intrinsic stability of the drug in varied environmental conditions.

Results

LMU absorption maxima was found to be 271 nm. The retention time of LMU was 3.125 min, and the total analysis time was 5 min. The R_f value of LMU was 0.49–0.62. The methods were linear within 2–12 µg/mL range. The correlation coefficient (r^2) for UV, HPLC and HPTLC was 0.9980, 0.9993 and 0.9988, and percent recoveries were calculated as 98.40–100.52%, 99.27–101.18% and 98.01–100.30%, respectively, with percentage relative standard deviation (RSD) less than 2% showing that methods were precise and accurate.

Conclusion

Developed UV, RP-HPLC and HPTLC methods are free from intervention caused by excipients present in tablets and thus can be used for regular quantitative analysis of LMU in tablet formulation. Based on analytical results and statistical tests, ANOVA, it is inferred that the HPLC method is best for LMU quantification tablet formulation due to its high reproducibility, good retention time and sensitivity; it has a higher percent recovery and has less analysis time, i.e., 5 min. The degradation peaks were well separated from the LMU peak indicating stability of the HPLC method.

FULL TEXT

Background

Lamivudine, Fig. 1, (4-amino-1-[(2R, 5S)-2-(hydroxyethyl)-1, 3-oxathiolan-5-yl] pyrimidin-2-one) is a reverse transcriptase inhibitor. It is used for HIV (human immunodeficiency virus) and hepatitis B infections. It is used for both HIV-1 and HIV-2. LMU is a synthetic nucleoside analogue of cytidine. Lamivudine triphosphate (L-TP) was formed by going through intracellular phosphorylation. An active 5′-triphosphate metabolite that competes with it is incorporated into the DNA of the virus. They impede reverse transcriptase enzymes competitively. The inserted nucleoside analogue has a 3′-OH group missing which functions as a chain breaker of DNA synthesis, which is necessary for the production of the 5′ to 3′ phosphodiester linkage needed for DNA chain extension. LMU is marketed under the brand name Lamivir 150-mg film-coated tablet [1–3].

Fig. 1 [Images not available. See PDF.]

LMU Chemical Structure

An extensive literature survey disclosed that LMU has been determined independently or in combination with other drugs by UV spectroscopy [4, 5], RP-HPLC [6–13] and HPTLC [14–16]; however, there was not a single research work that has been done reporting that LMU individually was determined simultaneously by all three methods, i.e., UV spectrophotometry, RP-HPLC and HPTLC, and investigating the best method among them. Further to carry out

stability indicating study of the selected superior method for separating the active analyte present in the pharmaceutical dosage is carried out, which makes the present research work unique and novel. Hence, we have strived to develop precise, accurate, sensitive and inexpensive methods and compare them based on analytical results such as sensitivity, % recovery and % assay of the drug. Validation of the method was performed according to Q2 (R1) ICH guidelines [17].

Methods

Materials

LMU was procured from *Cipla Ltd, Kurkumbh*, Maharashtra, India, as a gift sample. The marketed pharmaceutical tablets of Lamivir 150 mg (manufactured by Cipla Ltd) were purchased from a nearby pharmacy. Double distilled water was obtained from the Millipore unit. HPLC-grade chloroform and methanol were acquired from *Sisco Research Laboratories Pvt. Ltd, Mumbai, India*.

Instrumentation

A double-beam UV-1800 Shimadzu UV spectrophotometer along with a pair of matched quartz cells 10 mm is used. The HPLC system was a Shimadzu model no. DGU-20A5R which consists of a PDA detector. HPTLC was carried out on a pre-coated silica gel 60 F254 TLC Merck plate using a sample syringe of Camag 100 µl with an applicator of Linomat 5 and a twin-trough chamber; densitometry was executed with a CAMAG TLC Scanner 3 with Visioncats software. Shimadzu ATX224 digital analytical balance and PCi analytics ultrasonic bath were employed for weighing the samples and for sonication, respectively.

Sample preparation for UV, RP-HPLC and HPTLC method development

Standard solution preparation

Five milligrams of LMU was precisely weighed and poured into a 50-mL volumetric flask. Methanol was used as a solvent to make up the volume. 1 mL of stock solution was poured into a 10-mL volumetric flask, and the volume was raised to have a final concentration of 10 µg/mL.

Sample solution preparation

Approximately 20 tablets were weighed, and an average weight was determined for each tablet. A powder equal to five milligrams was weighed and poured into a 50-mL volumetric flask, consequently adding 15 mL of methanol and sonicating for 30 min. Later on, the volume was raised to the mark and filtered from the Whatman filter paper No. 41. Appropriately the solution was diluted to get a concentration of 10 µg/mL.

UV method development

Determination of LMU maximum absorbance (λmax)

The standard solution of LMU in the region of 200–400 nm is scanned. An absorption maximum was determined to be 271 nm, which was selected as the analytical wavelength for further analysis. The spectrum was recorded as shown in Fig. 2.

Fig. 2 [Images not available. See PDF.]

UV spectrum of LMU standard solution. The λmax was determined to be 271 nm

HPLC method development

Optimization of HPLC method

To achieve optimized chromatographic conditions, the below parameters were modified in each trial. The trial runs are shown in Table 1 (Additional file 1).

Table 1. Trial run for optimization of chromatographic conditions

Mobile phase	Flow rate (mL/min)	Retention time (min)	Comment

Methanol:Water (50:50)	0.8	4.135	Sharp peak appears, but retention time was more
Methanol:Water (60:40)	0.8	3.795	Sharp peak appears, but retention time was more
Methanol:Water (60:40)	1.0	3.534	Sharp peak appears, but retention time was more
Methanol:Water (65:35)	1.0	3.351	Sharp peak appears, but retention time was more
Methanol:Water (70:30)	1.0	3.125	Sharp peaks appear with less retention time

From the trial, the best possible chromatographic condition was selected based on peak shape that is sharp evaluated by theoretical plates and tailing factor which were within specified limit, and retention time is 3.125 min which is much less. Therefore, separation of LMU was performed on a Shimadzu C_{18} (250 mm × 4.6 mm i.d., 5 µm particle size) consisting of methanol: water (70:30) as a mobile phase; by using a membrane filter it was filtered and degassed. The flow rate was retained at 1.0 mL/min. The injection volume was kept at 10 µl at a column oven temperature of 30 °C, and effluents were checked at 271 nm. The mode of separation was isocratic. The chromatogram of LMU and its 3D image are shown in Figs. 3 and 4, respectively.

Fig. 3 [Images not available. See PDF.]

Chromatogram showing the separation of LMU

Fig. 4 [Images not available. See PDF.]

3D image of LMU chromatogram

Peak purity

The peak purity of the LMU peak was examined in a degradation solution using a photodiode array detector. Peak purity for each solution was passed at the threshold level. The peak purity report is depicted in Table 2, and peak purity spectra and profile are depicted in Figs. 5 and 6.

Fig. 5 [Images not available. See PDF.]

Peak purity spectra of LMU

Fig. 6 [Images not available. See PDF.]

Peak profile of LMU

Table 2. Peak purity description

Impurity	Not detected
Peak purity index	0.284

Single-point threshold	1.00

HPTLC method development

Optimization of mobile phase by TLC

Different compositions of the mobile phase were tried first on the TLC plate. The trial runs are presented in Table 3. From the trial data, chloroform: methanol (8:2) is selected as a suitable mobile phase since it shows a detectable significant spot of LMU (Additional file 1). This optimized mobile phase is used for HPTLC method development.

Table 3. Trial run for optimization of mobile phase for HPTLC

Trial run	Volume of toluene (mL)	The volume of ethyl acetic acid (mL)	Volume of methanol (mL)	Volume of glacial acetic acid (mL)	Volume of chloroform (mL)	Rf value	Comment
1	4	4	1.5	0.5	–	0.46	Spot shows tailing
2	6	2	2	–	–	0.71	Spot shows tailing
3	4	2	4	–	–	–	No spot appears
4	5	2	3	–	–	–	No spot appears
5	–	–	2	–	8	0.60	Significant spot appears without tailing

The LMU standard solution of 2 µl was employed as spot bands of 4 mm to the HPTLC plates under the stream of nitrogen using LINOMAT V. Application locations were at least 15 mm from the edges and 10 mm from the foot of the plate. The development chamber was kept for saturation with chloroform: methanol (8:2 v/v) before each run for 20 min. Development of the plate was performed to migrate a distance of 7 cm by the ascending technique. The analyses were performed in a temperature-controlled laboratory (20–24 °C). Densitometry scanning was carried out using a deuterium lamp in absorbance mode at 271 nm. The chromatogram is depicted in Fig. 7.

Fig. 7 [Images not available. See PDF.]

HPTLC chromatogram of LMU. Rf value 0.49–0.62

Forced degradation studies by the HPLC method

Forced degradation includes the degradation of active substances and drug products which results in degradation products that are studied to evaluate the intrinsic stability of the molecule. Degradation conditions such as acidic, alkaline, thermal, neutral and photostability were represented by ICH guidelines Q1A, Q1B [18, 19] and Q2 (R2). In a stability-indicating method, the acceptable degradation percentage should not exceed 20% .

Results

Method validation

System suitability parameters

After equilibrating the column with the mobile phase, the standard solution was autoinjected five times and the chromatograms were noted. The data are presented in Table 4.

Table 4. System suitability results (RP-HPLC)

Sr. No	Peak area	Tailing factor	Theoretical plates
1	2,413,422	1.162	2860
2	2,431,570	1.147	2908
3	2,459,066	1.142	2846
4	2,467,969	1.140	2884
5	2,431,480	1.146	2870
% RSD	0.91	0.75	0.83
Limit	NMT 2%	NMT 1.5	NLT 2000

Linearity

The standard stock solution of LMU was serially diluted to yield 6 distinct concentrations, i.e., 2, 4, 6, 8, 10 and 12 µg/mL. At 271 nm, their absorbance was measured against a blank. For HPLC, similar dilutions were performed and these solutions were autoinjected with optimized chromatographic conditions. For HPTLC, a volume of 2 µl of each serially diluted solution was employed on the HPTLC plate to carry 2, 4, 6, 8, 10 and 12 µg/mL of LMU per spot. The UV, HPLC and HPTLC methods confirmed linearity in the 2–12 µg/mL range, and the linearity equations were $y = 0.0421x - 0.0016$, $y = 33177x - 534$ and $y = 0.001x + 0.0018$ with r^2 of 0.9980, 0.9993 and 0.9988, respectively. Table 5 shows the results. The calibration plots for UV, HPLC and HPTLC are depicted in Figs. 8, 9 and 10, respectively.

Fig. 8 [Images not available. See PDF.]

Calibration curve of LMU by UV

Fig. 9 [Images not available. See PDF.]

Calibration curve of LMU by HPLC

Fig. 10 [Images not available. See PDF.]

Calibration curve of LMU by HPTLC

Table 5. Calibration curve data by UV, HPLC and HPTLC

UV		HPLC		HPTLC	
Conc (µg/mL)	Absorbance	Conc (µg/mL)	Area	Conc (µg/mL)	Area
2	0.090	2	66,582	2	0.00377

4	0.158	4	133,162	4	0.0055
6	0.251	6	199,746	6	0.00775
8	0.338	8	258,328	8	0.00966
10	0.411	10	332,908	10	0.01145
12	0.510	12	399,492	12	0.01331
r^2	0.9980	r^2	0.9993	r^2	0.9988

Accuracy

Accuracy was measured at 50%, 100% and 150% by spiking a standard solution of LMU (0.5, 1.0, 1.5 µg/mL) into the sample solution. The recoveries were ascertained in the range of 98.40–100.52%, 99.27–101.18% and 98.01–100.30% by UV, HPLC and HPTLC, respectively. The % RSD was found to be less than 2, indicating that there was no interference of the excipients while determining LMU. Therefore, the method is accurate. The accuracy data are depicted in Table 6.

Table 6. Accuracy study results

Conc. (%)	Expected amount (µg/mL)	UV			HPLC			HPTLC		
The actual amount found (µg/mL)	% Recovery	% RSD	Actual amount found (µg/mL)	% Recovery	% RSD	Actual amount found (µg/mL)	% Recovery	% RSD	50	15
14.96	99.2	0.51	14.97	99.80	0.27	14.96	99.20	1.46	50	15
14.90	99.33		14.89	99.27		14.92	99.50		50	15
14.92	98.4		14.95	99.65		14.97	99.40		100	20
20.05	100.52	1.57	19.95	99.75	0.18	20	100	0.97	100	20
19.79	98.95		19.98	99.90		19.85	98.50		100	20
20.05	100.5		20.02	101.18		20.03	100.30		150	25
24.80	98.67	0.83	24.89	99.56	0.75	24.79	98.01	0.27	150	25

25.02	100.13		24.95	99.80		24.84	99		150	25

Precision

For this study, six standard solutions of LMU were analyzed by UV, HPLC and HPTLC methods. The % RSD was less than 2, which significantly assures the precision of the proposed methods. Intraday and interday data are reported in Table 7.

Table 7. Precision data

UV method				
Concentration (10 µg/mL)	Intraday study (n=6)		Interday study (n=6)	
0 Hour	6 Hours	2nd Day	3rd Day	*Mean absorbance
0.415	0.400	0.409	0.421	Standard deviation
0.0039	0.0037	0.0038	0.0042	% RSD
0.94	0.91	0.94	0.99	HPLC method
Concentration (10 µg/mL)	Intraday study (n=6)		Interday study (n=6)	
0 Hour	6 Hours	2nd Day	3rd Day	*Mean area
286,645.5	292,654.5	336,242.8	284,331.8	Standard deviation
2106.99	1933.56	3151.59	3535.95	% RSD
0.74	0.66	0.94	1.24	HPTLC method
Concentration (10 µg/mL)	Intraday study (n=6)		Interday study (n=6)	
0 Hour	6 Hours	2nd Day	3rd Day	*Mean area
0.01144	0.01152	0.01156	0.01162	Standard deviation

2.88	2.73	3.16	2.92	% RSD

n = number of measurements, *mean of six observations

Robustness

LMU's six working standard solutions were used for analysis. In the proposed UV method, to validate the robustness parameter, slight variation was employed in wavelength (±2 nm); for HPLC robustness was assessed by introducing little, variation in the percent of methanol (±2%), flow rate (±0.2 mL/min), sonication time (±5 min) and using different Whatman filter no. (40, 42). Similarly, for HPTLC, there was a small change in chamber saturation time (± 5 min) and the mobile phase composition (±0.5). The data are presented in Table 8. The % RSD was not more than 2, hence significantly representing methods to be robust (Table 9).

Table 8. Robustness data

Parameters	*Mean of six observation	Standard deviation	% RSD
UV			
Wavelength at 269 nm	0.687	0.0062	0.90
Wavelength at 273 nm	0.658	0.0059	0.89
HPLC			
Mobile phase Methanol:water (68:32)	283,160	1519.04	0.54
Mobile phase Methanol:water (72:28)	296,229	2833.96	0.96
Flow rate (0.8 mL/min)	363,663	2110.50	0.58
Flow rate (1.2 mL/min)	246,713	2380.96	0.96
Sonication time: 25 min	246,817	1728.86	0.70
Sonication time: 35 min	247,887	2041.86	0.83
Whatman Filter no. 40	252,347	4160.51	1.67
Whatman Filter no. 42	230,568	3367.76	1.43
HPTLC			

Mobile phase Chloroform:methanol(9.5:0.5)	0.01143	2.5166	0.22
Mobile phase Chloroform:methanol(8.5:2.5)	0.01144	3.5119	0.31
Chamber saturation time: 15 min	0.01146	1.5275	0.13
Chamber saturation time: 25 min	0.01148	2.3094	0.20

Table 9. Sensitivity data

Sensitivity	UV method (µg/mL)	HPLC Method (µg/mL)	HPTLC method (µg/mL)
LOD	0.58	0.33	0.44
LOQ	1.75	1.01	1.32

Sensitivity

Sensitivity data

Results of analysis of tablet formulation of LMU by UV, HPLC and HPTLC methods

LMU standard and sample solutions absorbance was measured at 271 nm. In HPLC, both standard and sample solutions were autoinjected into the HPLC system, similarly for HPTLC 2 µl of standard, and sample solutions were employed as bands 4 mm on the HPTLC plate. The amount of LMU present per tablet was determined by UV, HPLC and HPTLC by comparing the absorbance and peak area of the sample with that of the standard, respectively. The obtained results of % labeled claim are reported in Table 10. Percent content obtained by UV HPLC and HPTLC was statistically compared by ANOVA, as depicted in Table 11.

Table 10. Results of analysis of tablet formulations

Method	Brand name	Label claim	Manufacturer name	Batch No	% Label claim estimated (% content)	Mean	% RSD
UV	Lamivir 150	150 mg Lamivudine	Cipla	SA12696	98.76	99.02	0.36

99.34	98.53	99.38	98.82	99.01	HPLC	Lamivir 150	150 mg Lamivudine
Cipla	SA12696	99.97	99.90	0.83	98.79	100.87	99.16
100.77	99.85	HPTLC	Lamivir 150	150 mg Lamivudine	Cipla	SA12696	99.24

Statistical comparison between UV, HPLC and HPTLC methods for % contents

ANOVA test

Table 11. Observations and results of ANOVA test for % contents study

Parameter	Variance			*F* Value	*P* Value	F critical
UV	HPLC	HPTLC	% Content	0.1133	0.6970	0.0504

From the statistical data, it could be inferred that the F value is greater than the F critical value indicating there is a remarkable differentiation between the mean % content determined by UV, HPLC and HPTLC methods, and hence, the null hypothesis is rejected.

Table 12. Comparative study of UV, HPLC and HPTLC methods

Parameters	UV method	HPLC method	HPTLC method
Linearity equation	y=0.0421x – 0.0016	y=33177x – 534	y=0.001x+0.0018
Correlational coefficient(r^2)	0.9980	0.9993	0.9988
% Recovery	98.40–100.52%	99.27–101.18%	98.01–100.3%
Sensitivity LOD	0.58	0.33	0.44
LOQ	1.75	1.01	1.32
% label claim	99.02	99.90	99.54

Forced degradation study

For this study, drug was given treatment with various degradation conditions. 1 mL from stock solutions (1000 µg/mL) was treated separately with 1 mL of 1N hydrochloric acid (heated in a water bath for 2 h at 60 °C), 1 mL of sodium hydroxide (heated in a water bath for 2 h at 60 °C), neutral degradation (refluxing with water for 6 h at 60 °C), dry heat degradation (exposure of drug powder in the oven at 60 °C for 10 days) and photostability

degradation (exposure of drug powder in sunlight for 10 days). Samples were taken at regular intervals to monitor degradations. There is no interaction of the degradation peak with that of the LMU peak. Hence, the proposed HPLC method was stability indicating and specific (Figs. 11, 12, 13, 14 and 15). The peak purity index values of LMU peak and degradation peaks were found to be within acceptable limits, Table 13.

Fig. 11 [Images not available. See PDF.]

Acid degradation chromatogram

Fig. 12 [Images not available. See PDF.]

Alkali degradation chromatogram

Fig. 13 [Images not available. See PDF.]

Neutral degradation chromatogram

Fig. 14 [Images not available. See PDF.]

Thermal degradation chromatogram

Fig. 15 [Images not available. See PDF.]

Photodegradation chromatogram

Table 13. Result of forced degradation study

Sample name	% Degradation	Peak purity index	Single-point threshold
Drug	–	0.284	1.00
Acid degradation	1.38	0.309	1.00
Alkaline degradation	9.19	0.128	1.00
Neutral degradation	14.83	0.883	1.00
Thermal degradation	No degradation	–	–
UV degradation	No degradation	–	–

Discussion

The present research work aims to compare all three developed methods UV, RP-HPLC and HPTLC on grounds of sensitivity, accuracy, percent recovery and percent purity and to evaluate the best method among them, since to our knowledge, no such venture had been made earlier. The inference from the study could be briefed as the UV method could be implemented in laboratories that lack high-tech analytical instruments, in developing countries where affording expensive instruments is a big deal along with the availability of highly skilled person so UV spectroscopy is a method of choice, which is cheapest and does not require so-skilled person in comparing with HPLC and HPTLC methods which are complicated, expensive and time-consuming. The HPTLC method utilized not more than 30 mL of mobile phase thus reducing mobile phase consumption when compared with HPLC method, also less mobile phase consumption indicate the eco-friendly nature of the method. After optimization of the method by TLC to develop that new method on HPTLC it takes an average of 1 h which is much less relative to HPLC but HPTLC shows less sensitivity as per analysis data reported in Table 12. Handling HPTLC requires a skilled person

and is expensive. The RP-HPLC method is more sensitive at 0.33 µg/mL and has a high % recovery of 99.27–101.18% and a % label claim of 99.90% in comparison with the other two methods as depicted in the analysis data of Table 12. Complex samples having many ingredients can be separated easily via HPLC showing high separation capacity, since being autosampler enables batch analysis of multiple components; it is an extremely precise and reliable technique. It is an expensive method that requires a large amount of expensive organic solvents and needs regular maintenance of the system. Also, the current research work provides an alternative method to the Anbazhagan S et al. approach [5] where the simultaneous quantification of three drugs was carried out by all three methods while in the present research work the focus is on LMU alone which was never quantified individually by all three methods before as per literature review. Therefore, in comparison with the Anbazhagan S et al. research work, the current research work requires less consumption of solvents for dilutions, chemicals and glassware thus promising the cost-efficiency of the present method, also total analysis time for HPLC is just 5 min with a retention time of LMU 3.125 min while in Anbazhagan S et al. research work it was 10.81 min with retention time of LMU 4.330 min indicating shorter period of analysis, and hence, rapid analysis of more number of samples could be done. ANOVA was applied to validate the information that there is a remarkable differentiation between mean % content determined by UV, HPLC and HPTLC methods. The *P* value (0.0280) is smaller than the alpha value ($\alpha = 0.05$), i.e., significance level therefore rejecting the null hypothesis, and the proposed null hypothesis was there is no remarkable difference. From the forced degradation studies, it could be inferred that all the degradant peaks and LMU peaks were well separated from each other. Peak purity for each solution was passed at the threshold level. Therefore, the proposed HPLC method is confirmed to be stability indicating (Table 13).

Conclusions

The proposed UV, RP-HPLC and HPTLC methods for the quantification of LMU in tablet formulation were linear having a concentration range (2–12 µg/mL) and had perfect accuracy ranging from 98 to 102%, precision with sensitivity and robust in nature. The % RSD was less than 2% thus compliance with ICH guidelines. The proposed methods are free from intervention due to excipients in tablets and thus could be used for regular determination of LMU in tablets. In conclusion, as per the aim of the study comparison between the methods was carried out, and based on analytical results and statistical tests, ANOVA shows that HPLC is the best method for the quantification of LMU in tablets due to its high reproducibility, sensitivity, good retention time; it has a higher percent recovery and has less analysis time, i.e., 5 min. Hence, forced degradation study by RP-HPLC was carried out by employing many stress conditions to assess the method's stability. The developed RP-HPLC method successfully separates the drug and its degradation products with good resolution so the method is proved to be stability indicating. The present research work is going to be extended to perform the impurity profile of LMU and to detect the pathway of degradation for the same.

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Author contributions

KS analyzed, interpreted the data and performed experimental work and a major contributor in writing the manuscript. VS and PR performed the bench work, methodology and writing the rough draft. The overall work was carried out under the guidance of PS. All authors read and approved the final manuscript.

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Abbreviations

LMU
Lamivudine
ANOVA
Analysis of variance
% RSD
Percent relative standard deviation
LOD
Limit of detection
LOQ
Limit of quantification

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Quantum chemical modelling, molecular docking, synthesis and experimental anti-microbial activity of 1,4-diazepan linked piperidine derivative

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ABSTRACT (ENGLISH)

Background

In this work, we represent synthesis, in silico analysis and biological activity of 1,4 diazepine linked piperidine derivatives (6a–6o). All the derivatives were screened for their anti-microbial activity against gram-positive (*Staphylococcus aureus, Bacillus Subtills, Bacillus megaterium*) and gram-negative (*Escherichia coli, Pseudonymous, Shigella sp.)* bacteria. Compounds were synthesized from reaction of tert-butyl 1,4-diazepane-1-carboxylic, butyryl chloride and varied aromatic aldehyde, further characterized by ^{1}H NMR and LCMS spectral techniques.

Result

Using ampicillin as a positive control, the synthetic compounds 6a–6o were tested for their in-silico study and experimental anti-microbial activity against gram-positive (*Staphylococcus aureus, Bacillus Subtills, Bacillus megaterium*) and gram-negative (*Escherichia coli, Pseudonymous, Shigella sp.)* bacteria. According to in vitro assay compound 6a, compound 6c, compound 6d, compound 6m and compound 6l showed higher activity against all the tested strains. Molecule 6i, compound 6j, compound 6k, compound 6f has good to moderate antibacterial activity. DFT computations were used to optimize the molecular geometry at the B3LYP/6-31G (d, p) theoretical level. The corresponding energy values of molecular orbitals were visualized using optimized geometries. Moreover, Auto Dock Vina 1.2.0 is used to assess molecular docking against two target proteins, Bacillus subtilis (PDB ID: 6UF6) and Protease Vulgaris (PDB ID: 5HXW). The target molecule 6b displayed the best binding energies for both. Additionally, we calculated the ADME for each molecule (6a–6o).

Conclusion

All fifteen synthesized compounds were screened for their in vitro and in silico analysis. In vitro analysis for anti-microbial activity was carried out against gram-positive (*Staphylococcus aureus, Bacillus Subtills, Bacillus megaterium*) and gram-negative (*Escherichia coli, Pseudonymous, Shigella sp.)* bacteria and compound 6a, compound 6c, compound 6d, compound 6m and compound 6l exhibits more potent activity towards all tested strains. Molecular docking is performed against target proteins, L-amino acid deaminase from *Proteus Vulgaris* and LcpA ligase from *Bacillus subtilis*, representing the Gram-negative bacterium and Gram-positive bacterium, respectively. Compound 6b showed the highest no. of interaction with protein according to molecular docking. With the advent of innovative techniques like ADME, we select their hit compounds early on and anticipate future pharmacokinetic and pharmacodynamic benefits and drawbacks of these promising therapeutic candidates.

FULL TEXT

Background

Heterocycles are crucial chemicals their applications in many domains, including medicinal, agrochemical, and veterinary [1]. Several seven membered heterocyclic rings, with 2 hetero atoms, have shown wide range of

medicinal activity. Among these, 1,4-diazepine,1,4-diazepane, and azepinone have been studied in detail. 1,4-diazepanes, also known as homopiperazine, hexahydyro-1,4 diazepine, were first identified as a fundamental heterocyclic molecule in 1899. Later, a simple method was developed to manufacture these at industrial scale [2]. Molecular formula of 1,4 diazepane is $C_5H_{12}N_2$ and M.P. is 42 °C [3]. 1,4-diazepane is seven membered heterocyclic ring with nitrogen atoms at position 1 and position 4 of diazepan ring [4]. These molecules have been extensively studied owing to their versatile nature, and potential application in medicines, agrochemicals, and materials science [5]. Moreover, the 1,4-diazepane (homopiperazine) ring is prevalent among the various substitutions, linkers, as well as scaffolds used in pharmaceutical hit-to-lead optimization, particularly as a crucial component of optimized lead compounds [6]. Further, as 1,4-diazepane is hydrophobic, facilitate the organotin complex to permeate the cell and enhance the compound's biological activity [7]. In medicinal chemistry, 1,4-diazepane moiety has shown extensive pharmaceutical properties (Fig. 1) as anti-HIV [8, 9], anti-dengue [10], anti-Alzheimer [11], anti-tubercular [12], anti-fungal [13], anti-oxidant [14, 15], anti-bacterial [16], anti-cancer [17], anti-microbial [13, 18], Histamine receptor [19], anti-tumour [7, 20], and as Kinase inhibitor [21].

Fig. 1 [Images not available. See PDF.]

Different medicinal activity of 1,4 diazepane

Further, piperidine is a fundamental heterocyclic part of medical chemistry. Piperidine core compounds exhibit pharmaceutical properties like anti-HIV, anti-cancer. A.R. Zala and colleagues developed piperidine-containing molecules as antibacterial drugs in 2023 [22]. Mahmat Yildiz and colleagues developed piperidine derivatives that act as antimicrobial agents [23]. In contrast, Enrico Casalone and colleagues developed 1,4-diazepane derivatives as antimicrobial agents in 2020 [24]. Since, both piperidine and 1,4 diazepane show potent antimicrobial activities, in this study we created 1,4 diazepane linked piperidine derivatives and have characterize their antimicrobial efficacy. The derivative molecules have also been investigated using a series of computational chemistry tools, such as the Density Functional Theory, Molecular docking and ADME analysis. Here, we aim to facilitate the current research in designing innovative strategies for the discovery and development of good antimicrobial agents.

Methods

All the compounds and solvents were purchased from Sigma Aldrich and used without any additional purification. The gradient of MeOH in MDC was created using the elutes and was used for the thin layer chromatography (TLC) method on silica gel plates (60F254, 0.2 mm thick, Merck). In this study, we used a Bruker Advance II 400 NMR spectrometer, used with the internal standard tetra methyl silane (TMS) as a ^{1}H NMR spectrometer, to perform ^{1}H NMR spectra in $CDCl_3$ solution at 400 MHz. Parts per million, or ppm, are used to compute the value in ^{1}H NMR. As the mobile phase of the LCMS equipment, which used WATERS to record data, 0.15% formic acid in acetonitrile was used as a mobile phase for the LCMS equipment.

Chemistry

The five-step synthesis process outlined in Scheme 1 was created to produce the desired anti-microbial active ingredient. Commercially available tert-butyl 1,4-diazepane-1-carboxylic **(1)** intrigues the production route when it is coupled with butyryl chloride in the presence of triethanolamine (TEA) and methylene chloride (DCM) to produce compound **2** [25]. Compound **3** is produced by carbamate hydrolysis using compound **2** and 6 N HCl in dioxane to remove the boc protecting group [26]. In the presence of TEA, $ZnCl_2$, and MeOH, compound **4** was produced by treating compound **3** with commercially available tert-butyl-4-oxopiperidine-1-carboxylate[27]. Once more, carbamate hydrolysis is performed to remove the boc protecting group in preparation for the transition from compound **4** to compound **5** [26]. With the addition of DIPEA, the carboxylic acid in DMF was reacted with compound **5** in the presence of HATU and generated a high quality desired derivative (**6a-o**).[28, 29]. All the used aldehydes and properties of desired derivatives shown in Table 1.

Scheme 1 [Images not available. See PDF.]

Synthetic route of 1,4 diazepane linked piperidine derivative

Table 1. Properties of compounds

In vitro assay

For the investigation of in vitro anti-microbial activity, the cup plate method [30, 31], which is very popular, was used. Gram-positive (*Staphylococcus aureus, Bacillus Subtills, Bacillus megaterium* and gram-negative (*Escherichia coli, Pseudonymous, Shigella* sp.) microorganisms, as well as Staphylococcus aureus (Gram+), were used to test in-vitro anti-microbial activity. To carry out this approach, all bacterial cultures were first kept in nutrient broth and then incubated overnight at a temperature of 37 °C. A full hour was given for the incubated molten agar to set and solidify before it was transferred to the sterilized petri plates. Bacterial culture was evenly swabbed on the sterile plates using a cotton swab. On agar media, 6 mm broad bores were constructed using sterilized cork. Each bore was filled with the test compound solution, which had a measured concentration of 1000 µg/ml, using a sterile tip and micropipette. A plate was similarly prepared for the positive control ampicillin, which is taken as standard. The prepared plates were incubated for 24 h at 37 °C. Every bore had a clear zone encircling it after the incubation process, demonstrating that the 1,4-diazepane linked piperidine derivative under test had anti-microbial efficacy. The average diameter of these formed clear zones of inhibition was determined and used to report activity in millimeters. A value for the MIC (minimum inhibitory concentration) was obtained using the liquid dilution method [32], All derivatives MIC values were established for *Staphylococcus aureus* (Gram+) bacterium. The investigated substances were dissolved in a suitable solvent at a concentration of 50 mg/ml. Similar to this, a conventional ampicillin solution was made at a 50 mg/ml concentration. Bacterial culture inoculum preparation was carried out. In a series of test tubes, 0.2 ml of inoculum is inserted along with a 1 ml solution of the test substance at a specified concentration. In each test tube, 3.8 ml of sterile water was also added. All these test tubes were maintained under observation and incubated for a day in order to detect the existence of turbidity. A similar process was used to screen ampicillin, which is a commonly used medication. MIC values are those where bacterial growth was not seen to occur.

Computational methods

The electronic structures of all 1,4 diazepane linked piperidine derivative molecules (**6a–6o**) were studied using quantum mechanical calculations. Each molecule was constructed using GaussView 5 software [33] and was subjected to geometry optimization using DFT calculations at the B3LYP/6-31G(d,p) level of theory as implemented in Gaussian 09 quantum chemistry package [34]. The calculations were performed in an implicit PCM water solvent model. No symmetrical constraints were imposed during the optimization. Optimized geometries were used to visualize molecular orbitals and their associated energy values using GaussView 5. In particular, the nature and energetics of the lowest unoccupied molecular orbital (LUMO) and highest occupied molecular orbital (HOMO) were characterized. Several other molecular properties were calculated from the HOMO and LUMO energies, namely, the global hardness (η), softness (σ), electronegativity (χ), and electrophilicity index (ω). The following equations are used to compute these properties:

1

$$\chi=12EHOMO+ELUMO$$

2

$$\eta=-12EHOMO-ELUMO$$

3

$$\sigma=1\eta$$

4

$$\omega=\mu22\eta$$

Next, the optimized geometry of each derivative molecule was used to perform molecular docking against two target proteins corresponding to the *Protease Vulgaris* (PDB ID: 5HXW) [35] and *Bacillus subtilis* (PDB ID: 6UF6) [36]. The starting structure for the target proteins were based on their crystal structure deposited in the RCSB protein data bank. Both proteins were prepared for molecular docking by removing any existing ligand and water molecules, followed by charge assignment using MGL Tools 1.5.7. [37] The grid box corresponding to the whole protein was defined for each target. Each derivative molecule was prepared for docking using MGL Tools 1.5.7.

AutoDock Vina 1.2.0 [38] was used for docking the derivate molecules against the two target proteins. Ten conformations were generated for each derivative molecule and the most favorable conformation, based on the binding affinity, was selected for further structural analysis. The non-covalent interactions between the protein and docked ligand were analysed and visualized using LigPlot software [39].

Further, the Absorption, Distribution, Metabolism, and Excretion (ADME) analysis was carried out for each derivatized molecule using the SwissADME server [40] The ADME analysis provides determines various physiochemical properties to assess the pharmacodynamic properties of a potential ligand molecule. Various relevant parameters, such as the Lipinski's rule of five violations, number of hydrogen donors, hydrogen acceptors, rotatable bonds, total polar surface area, skin permeability (Log Kp), molar refractivity, gastro-intestinal absorption (GI), blood brain barrier (BBB), inhibition of cytochromes P450 isoforms (CYP1A2, CYP2C19, CYP2C9, CYP2D6) were estimated.

Next, we carried out Quantitative Structure–activity relationships (QSAR) to determine the quantitative relationship between the biological activity and the molecule properties of the derivatives. We constructed the QSAR models by performing multiple linear regression analysis, which links selected independent variables with the dependent variable using the following equation:

Where X i are the molecular descriptors, Y is the biological activity, n is number of descriptors, a 0 is the constant and a i are the respective coefficients.

Result

By observing structural alterations brought on by the attachment of various substitutions and their placement on the heterocyclic ring in the generated derivative, the link between structure and activity was investigated (Fig. 2). Because of the varied positions of the aryl group and heterocyclic ring, biological activity was changing. In this study, different substituted phenyl rings linked to piperidine and piperidine directly coupled to 1,4-diazepane were found to have different anti-microbial activities. Distinct substitutions have distinct electrical properties, and these differences are shown in their anti-microbial effectiveness. According to research on SAR, anti-microbial activity can vary depending on the heterocyclic or aryl ring. The heterocyclic ring substitutions of manufactured compounds could either donate electrons or draw them out. When substitutions such halogenated groups at various positions on a heterocyclic ring are made, the anti-microbial action increases. According to Table 2, when phenyl rings are substituted in compounds with different halogenated groups, the chloro group exhibits more activity than the bromo group. The enhanced antimicrobial activity was found to be more in both **6c** and **6h**, due to the heterocyclic moiety in the synthetic derivatives with alkoxy phenyl ring in **6h** and the heterocyclic moiety on the heterocyclic ring with –Br and –F in **6c**. Higher antibacterial inhibitory action was demonstrated in **6m** by the phenyl ring with the chloro group in the ortho position. As opposed to the meta-substituted **6e** and the amine-aryl ring with a bromine substitution in **6g**, the phenyl ring with a bromine and methyl group in **6f** shown stronger inhibitory activity. Higher antibacterial inhibitory action was demonstrated by compound **6a**'s phenyl ring with the nitro group in the ortho position as compared to compound **6d**'s phenyl ring with the electron-donating and withdrawing groups replaced. In contrast, **6l** had reduced activity due to the presence of an electron releasing group at the o-position. The methoxy group, a weak electron donor, was shown to be less active than other groups in position 4 of the phenyl ring in compound **6k**. Other substituted derivatives **6i**, **6b**, and **6j** displayed modest activity, while **6o** was found to be more effective than regular ampicillin against *Staphylococcus aureus* and *E. coli*.

Fig. 2 [Images not available. See PDF.]

Structure activity relationship study

Table 2. Antibacterial activity of tested compounds as a zone of inhibition in MIC(μg/mL) of synthesized compound

Compound number	*Staphylococcus aureus* (Gram+	*Bacillus Subtills* (Gram+)	*Bacillus megaterium* (Gram+)	*Escherichia coli* (Gram−)	*Pseudonymous spp.* (Gram−)	*Shigella sp.* (Gram−)
6a	46	42	44	37	39	20
6b	45	32	38	20	15	–
6c	59	54	48	50	51	33
6d	47	41	40	37	34	18
6e	51	46	45	42	29	22
6f	53	47	49	47	34	26
6g	51	45	45	41	35	21
6h	57	53	50	52	40	31
6i	47	36	40	23	19	8
6j	35	28	–	20	16	–
6k	40	34	37	25	21	11
6l	45	40	41	32	29	18
6m	58	50	52	47	39	28
6n	40	38	34	31	28	16
6o	30	–	–	17	–	–
Ampicilin	48	39	29	40	32	21

Table 2 shows that the majority of the substances tested had varying inhibitory effects on the growth of the bacterial strains that were put to the test, according to the results of the study. Because the compound **6a**, the compound **6d**, and the compound **6m**, compound **6l** both possess electron withdrawing and electron releasing groups, these compounds showed a high level of inhibitory activity against the tested microbial strains. Comparatively to the reference drug Ampicillin, compound **6c** substituted with –Br and –F on its heterocyclic ring as well as compound **6h** containing heterocyclic rings attached to methoxybenzene were found to exhibit superior activity against all Gram-positive and Gram-negative microbial strains. Even though compound **6o** showed no effect against *B. subtilis, Bacilius magneterium, Pseudomonas spp., and Shigella spp.*, compound **6j** lost its antibacterial efficacy due to the absence of an aryl ring substitution. Due to the presence of an electron releasing group and a phenyl ring, **6i**, **6k** demonstrated a modest amount of inhibitory action against the investigated strains of *Pseudomonas spp., B. subtilis,*

E. coli, and *Staphylococcus aureus*. Figure 3 depicts the graphical representation of antibacterial activity.

Fig. 3 [Images not available. See PDF.]

Statistical diagram of antimicrobial activity

DFT calculations

All 1,4 diazepane linked piperidine derivative molecules were optimized using the DFT methods at the B3LYP/6-31G(d,p) level of theory. The optimized geometries are shown in Fig. 4 and the various electronic properties are presented in Table 3. First, we compared the energy gap (E_{HOMO}-E_{LUMO}), which is an important parameter to assess the thermal stability and the chemical reactivity of a molecule. The E_{HOMO} and E_{LUMO} energy gap were computed to be negative for all derivative molecules, indicating their stability and in the following order: **6b>6k>6l>6j>6f>6e>6m>6a>6c>6g>6n>6o>6i>6h>6d**. Further, Frontier Molecular Orbital analysis illustrate that for all molecules, the lowest occupied molecular orbital (LUMO) orbital is localized on the π orbitals of the phenyl ring (Fig. 5). Whereas the highest occupied molecular orbital (HOMO) orbital is localized on the diazepane and piperidine binding site. We also computed additional electronic and structural parameters, such as electronegativity (χ), global hardness (η), global softness (σ) and global electrophilicity index (ω), to ascertain the biological activity of the derivative molecules (Table 3). The large χ and ω for all compounds indicate their excellent bioactivity.

Fig. 4 [Images not available. See PDF.]

The geometry of 1,4 diazepane linked piperidine derivative molecules optimized at the B3LYP/6-31G(d,p) level of theory

Table 3. The calculated E_{HOMO}, E_{LUMO} (eV), E_{HOMO}—E_{LUMO} (ΔE, eV), electronegativity (*χ*), chemical potential (*μ*), global hardness (*η*), global softness (*σ*), and global electrophilicity index (*ω*) for derivative molecules as calculated at the B3LYP/6-31G(d,p) level of theory

No	E_{HOMO} (eV)	E_{LUMO} (eV)	E_{HOMO}–E_{LUMO} (eV)	χ (eV)	η (eV)	σ (eV^{-1})	ω (eV)
6a	−0.3340	0.0365	−0.3706	−0.1487	0.1853	5.3971	0.0597
6b	−0.3058	0.1265	−0.4323	−0.0896	0.2162	4.6264	0.0186
6c	−0.3131	0.0574	−0.3706	−0.1278	0.1853	5.3974	0.0441
6d	−0.3134	0.0276	−0.3410	−0.1429	0.1705	5.8649	0.0599
6e	−0.3080	0.0770	−0.3850	−0.1155	0.1925	5.1951	0.0346
6f	−0.3083	0.0779	−0.3862	−0.1152	0.1931	5.1788	0.0343
6g	−0.2912	0.0794	−0.3705	−0.1059	0.1853	5.3981	0.0303
6h	−0.2892	0.0519	−0.3412	−0.1187	0.1706	5.8620	0.0413
6i	−0.2460	0.1148	−0.3608	−0.0656	0.1804	5.5428	0.0119
6j	−0.3052	0.0873	−0.3926	−0.1089	0.1963	5.0946	0.0302

6k	−0.3056	0.0899	−0.3955	−0.1078	0.1977	5.0571	0.0294
6l	−0.3029	0.0902	−0.3931	−0.1064	0.1965	5.0879	0.0288
6m	−0.3076	0.0766	−0.3842	−0.1155	0.1921	5.2051	0.0347
6n	−0.3105	0.0586	−0.3691	−0.1259	0.1846	5.4184	0.0430
6o	−0.3049	0.0601	−0.3650	−0.1224	0.1825	5.4789	0.0410

Fig. 5 [Images not available. See PDF.]

The HOMO and LUMO orbitals for selected derivative molecules (**6b** and **6c**) as calculated at the B3LYP/6-31G(d,p) level of theory

Molecular docking

The derivative molecules were next subjected to molecular docking to assess their antibacterial potential. To this end, we used the geometry optimized derivative molecules to perform molecular docking against two target proteins, L-amino acid deaminase from *Proteus Vulgaris* and LcpA ligase from *Bacillus subtilis*, representing the Gram-negative bacterium and Gram-positive bacterium, respectively. The relative stability of the target protein – ligand complex was determined by their binding affinities Table 4 and their intermolecular interactions with the target proteins (Additional file 1: Figures S21–S22). Molecules **6b** showed the largest binding affinity for both target proteins (Fig. 6). Further analysis of the protein–ligand complex revealed a higher number of interactions in the case of **6b** (Fig. 7). The derivate **6b** formed H-bonds involving residues Tyr286 and Tyr284 of the L-amino acid deaminase from *Proteus Vulgaris*. Similarly, it formed H-bonds with Asn353 and Gln350 residues of LcpA ligase from *Bacillus subtilis*. These H-bonds, together with several hydrophobic interactions, provide higher binding strength to the complex of **6b** with target proteins.

Table 4. Calculated docking affinities (in kcal/mol) of the derivative molecules and ampicillin against the target proteins from *Bacillus subtilis* (PDB ID: 6UF6), and *Proteus Vulgaris* (PDB ID: 5HXW)

No	5HXW	6UF6
6a	−8.7	−7.7
6b	−9.4	−8.2
6c	−8.8	−7.4
6d	−8.3	−7.2
6e	−8.1	−7
6f	−8.1	−7.3
6g	−8.1	−6.9

6h	−8.2	−7.1
6i	−8.0	−7.1
6j	−8.6	−6.8
6k	−8.4	−6.9
6l	−8.3	−7.2
6m	−8.3	−6.9
6n	−8.5	−7
6o	−8.1	−6.9
Ampicillin	−7.8	−6.9

Fig. 6 [Images not available. See PDF.]

Comparison of the calculated docking affinities (in kcal/mol) of all derivative molecules and ampicillin against the target proteins from *Bacillus subtilis* (PDB ID: 6UF6), and *Proteus Vulgaris* (PDB ID: 5HXW)

Fig. 7 [Images not available. See PDF.]

Overlay of the protein-derivative molecule 6b complexes as obtained from their docking with *Bacillus subtilis* (PDB ID: 6UF6) and *Proteus Vulgaris* (PDB ID: 5HXW) proteins. The docked poses were chosen based on their binding affinities and geometric similarities. Intermolecular interactions between the derivation molecules and the target proteins are shown. The hydrogen bond interactions are highlighted

ADME calculations

Next, we performed the ADME calculations for the derivative molecules using the SwissADME server to characterize their drug-likeness and bioavailability. The ADME analysis showed that none of the molecules violated any of the five Lipinski rule. (Table 5) Therefore, these molecules have good potential to be developed as orally active drugs. The TPSA value, closely related to the bioavailability, for the molecules were observed in the range of 56 $Å^2$ to 102 $Å^2$, which is well below the limit of 140 $Å^2$. Similarly, the number of rotatable bonds (NRB) for all molecules is less than the limit of 10, except for **6i**, suggesting that the molecules are conformationally stable.

Table 5. Drug-likeness predictions for the derivative molecules as computed using SwissADME

C	MW	NRB	NHA	NHD	TPSA	LogP	LV	MR
6a	405.45	7	7	0	102.15	0.98	0	123.03
6b	473.57	9	7	0	85.87	1.28	0	144.39
6c	458.33	6	7	0	69.22	1.87	0	119.66

6d	419.47	7	7	0	102.15	1.31	0	127.99
6e	439.35	6	5	0	56.33	2.18	0	121.9
6f	453.37	6	5	0	56.33	2.5	0	126.87
6g	454.36	6	5	1	82.35	1.78	0	126.31
6h	475.58	8	9	2	89.98	1.18	0	145.88
6i	445.6	10	5	0	59.57	2.25	0	142.45
6j	360.45	6	5	0	56.33	1.56	0	114.2
6k	390.48	7	6	0	65.56	1.57	0	120.7
6l	390.48	7	6	0	65.56	1.35	0	120.7
6m	394.9	6	5	0	56.33	2.07	0	119.21
6n	385.46	6	6	0	80.12	1.33	0	118.92
6o	366.5	6	5	0	56.33	1.76	0	116.22

The number of rotatable bonds (NRB), number of hydrogen donors (NHD), number of hydrogen acceptors (NHA), total polar surface area (TPSA) in $Å^2$, LogP value and Lipinski's rule of five violation (LV), and Molar Refractivity (MR) are reported

Moreover, the low skin permeability value (Log K_p) for all molecules indicates low level of skin permeation (Table 6). All derivative molecules showed high level of gastrointestinal (GI) absorption. The molecule's hazardous or adverse effects also depend on its suppression of cytochromes P450 isoforms (CYP1A2, CYP2C19, CYP2C9, and CYP2D6). SwissADME predictions showed that all the derivative molecules show inhibition propensity against one or more of these isoforms.

Table 6. Absorption, Distribution, Metabolism, and Excretion (ADME) analysis for the derivative molecules as computed using SwissADME

	Log K_p	GI Absorption	BBB Absorption	Inhibitor interaction			
CYP1A2	CYP2C19	CYP2C9	CYP2D6	**6a**	−7.6	High	No
No	Yes	No	No	**6b**	−8.27	High	No
No	Yes	No	No	**6c**	−7.53	High	No
No	Yes	No	No	**6d**	−7.42	High	No

No	Yes	Yes	No	**6e**	−7.19	High	Yes
No	Yes	No	Yes	**6f**	−7.02	High	Yes
No	Yes	No	Yes	**6g**	−7.37	High	No
No	Yes	No	Yes	**6h**	−8.19	High	No
No	No	No	Yes	**6i**	−6.99	High	Yes
No	No	No	Yes	**6j**	−7.21	High	No
No	Yes	No	No	**6k**	−7.4	High	No
No	Yes	No	No	**6l**	−8.03	High	No
No	No	No	No	**6m**	−6.97	High	Yes
No	Yes	No	Yes	**6n**	−7.56	High	No
No	Yes	No	No	**6o**	−6.94	High	No

Skin permeability (Log K_p), gastro-intestinal absorption (GI), blood brain barrier (BBB), and inhibition of cytochrome-P isoforms are reported

Considering all the ADME predictions as well as the binding affinities, molecule **6b** appear as the most effective lead as an antibacterial agent. While it is noted that other derivative molecules, such as 6n, showed high binding affinity and similar binding mode as 6b, the latter is more favourable owing to its higher tendency to form H-bonds with the nearby receptor residues.

Among the 15 molecular descriptors, seven were selected for the regression analysis based on their correlation coefficient values. The seven descriptors are the following: Log P, Log S, NHA, η, molecular weight (MW), TPSA, and Log Kp. The resulting QSAR model based on the regression analysis is given by the following equation:

Where N = 15, R = 0.87, determination coefficient (R^2) = 0.77, mean square error (MSE) = 1.77, statistical confidence degree (F) = 5.68.

The high values of R2 and F indicate that the derived QSAR model is acceptable, and that the biological activity is linear correlated with these descriptors (Additional file 1: Figure S23). These descriptors can be used to predict inhibitory activity of new compounds based on the structure of 1,4 diazepane linked piperidine derivatives.

Discussion

Preparation of tert-butyl 4-butyryl-1,4-diazepane-1-carboxylate (2)

Tert-butyl 1,4-diazepane-1-carboxylate **(1)** (1.0 g, 4.99 mmol), TEA (1.04 mL, 7.48 mmol), and DCM (10 mL) were dissolved at 0 °C in a reaction jar. After then, the solution received a dropwise addition of butyryl chloride (798.0 mg, 7.48 mmol). The mixture was then mixed at room temperature for 30 min. The reaction mixture was then split equally between 100 mL of H_2O and 100 mL of EtOAc. EtOAc (2 × 50 mL) was used to extract the aqueous layer further. Na_2SO_4 was used to mix and dry the organic layers. Tert-butyl 4-Butyryl-1,4-diazepane-1-carboxylate **(2)** (1.2 g, 88.88%) was obtained as the crude product after the solvent was removed under vacuum. The next stage didn't require any purification because the crude product was used immediately.

Preparation of 1-(1,4-diazepan-1-yl)butan-1-one hydrochloride salt (3)

The reaction mixture was agitated at room temperature for 16 h with tert-butyl 4-butyryl-1,4-diazepane-1-carboxylate **(2)** (1.0 g, 3.70 mmol) in 6N HCl-dioxane (10.0 mL). To produce the crude product, 1-(1,4-diazepan-1-yl)butan-1-one hydrochloride salt **(3)** (550 mg), the reaction mixture was then concentrated under vacuum. The crude product was utilized right away in the subsequent step of the reaction without going through any purifying procedures.

Preparation of ethyl 4-(1-(tert-butoxycarbonyl)piperidin-4-yl)-1,4-diazepane-1-carboxylate (4)

Tert-butyl 4-oxopiperidine-1-carboxylate (578 mg, 2.90 mmol), 1-(1,4-diazepan-1-yl)butan-1-one hydrochloride salt **(3)** (500 mg, 2.90 mmol), TEA (1.2 mL, 8.70 mmol), $ZnCl_2$ (8.0 mg, 0.1 mmol), and MeOH (7 mL) were mixed in an RBF. The mixture for the reaction was heated to 60 °C and given 4 h to react. The reaction mixture was then cooled to zero degrees Celsius. $NaCNBH_3$ (540 mg, 8.70 mmol) was added to the reaction mixture at 0 °C, and the mixture was stirred for 16 h as it warmed to room temperature. A residue was produced after the reaction mixture was concentrated under a vacuum. The aqueous layer was extracted with EtOAc (250 mL) after the residue was divided between 500 mL of H_2O and 500 mL of EtOAc. Na_2SO_4 was used to mix and dry the organic layers. A crude product was produced after the solvent was subsequently extracted under a vacuum. Column chromatography was used to purify the crude product. Ethyl 4-(1-(tert-butoxy carbonyl)piperidin-4-yl)-1,4-diazepane was the pure product that was produced.400 mg of −1-carboxylate **(4)**, 38.76% yield.

Ethyl 4-(piperidin-4-yl)-1,4-diazepane-1-carboxylate hydrochloride salt (5)

The reaction mixture was agitated at room temperature for 16 h with ethyl 4-(1-(tert-butoxycarbonyl)piperidin-4-yl)-1,4-diazepane-1-carboxylate **(4)** (400 mg, 3.70 mmol) in 6N HCl-dioxane (4 mL). A crude product of ethyl 4-(piperidin-4-yl)-1,4-diazepane-1-carboxylate hydrochloride salt **(5)** (250 mg, 76.13% yield) was produced when the mixture was concentrated under vacuum. The crude product was utilized right away in the subsequent step of the reaction without going through any purifying procedures.

General procedure of compound (6a–o)

The carboxylic acid (1 equivalent) was mixed in DMF (10 volumes) in a reaction vessel. Next, the resulting mixture was cooled to absolute zero. After adding HATU (1.5 equivalents), the reaction mixture was stirred at 0 °C for 10 min. Ethyl 4-(piperidin-4-yl)-1,4-diazepane-1-carboxylate hydrochloride salt **(5)** (1.1 equivalents) and DIPEA (3 equivalents) were then added to the reaction mixture. The end product was stirred at room temperature for six hours. The reaction mixture was then dumped into ice-cold water after it had finished. The resultant residue was divided between 500 mL of H_2O and 500 mL of EtOAc. EtOAc (250 mL) was used to extract the aqueous layer further. Na_2SO_4 was used to mix and dry the organic layers. The crude product was then produced after the solvent was removed under vacuum. To produce the final chemicals **6a-o**, column chromatography was used to purify the crude product. (Supplementary File).

Ethyl 4-(1-(2-nitrobenzoyl)piperidin-4-yl)-1,4-diazepane-1-carboxylate (6a)

^{1}H NMR (400 MHz, DMSO) δ 1.25–1.29 (3H, t), 1.81 (4H, m), 2.77 (6H, m), 3.06 (2H, m), 3.49 (5H, m), 4.12–4.18 (2H, q), 4.77–4.88 (2H, m), 7.3 (1H, m), 7.57–7.61 (1H, m), 7.72 (1H, m), 8.21–8.23 (2H, d, *J*=8 Hz). LCMS m/z Cal. $[M+H]^+$ 404.21 found $[M+H]^+$405.21.

Ethyl 4-(1-(1-((benzyloxy)carbonyl)azetidine-3-carbonyl)piperidin-4-yl)-1,4-diazepane-1-carboxylate (6b)

^{1}H NMR (400 MHz, DMSO) δ 0.85–0.96 (4H, m), 1.27–1.30 (4H, m), 2.00 (2H, m), 2.57–2.63 (1H, t, *J*=24 Hz), 2.78–2.90 (3H, m), 3.00 (2H, s), 3.49–3.55 (6H, m), 4.16–4.17 (6H, d, *J*=4 Hz), 4.77 (1H, s), 5.11 (2H, s), 7.28–7.37 (5H, m). LCMS m/z Cal. $[M+H]^+$ 472.27 found $[M+H]^+$473.2.

Ethyl 4-(1-(6-bromo-5-fluoropicolinoyl)piperidin-4-yl)-1,4-diazepane-1-carboxylate (6c)

^{1}H NMR (400 MHz, DMSO) δ 1.29–1.32 (4H, m), 1.49–1.55 (2H, s), 1.67–1.72 (2H, m), 2.00 (4H, s), 2.81–2.97 (3H, m), 3.11–3.22 (2H, m), 3.53–3.61 (1H, m), 3.67 (3H, s), 4.18–4.20 (2H, d, *J*=8 Hz), 4.79–4.83 (1H, d, *J*=16 Hz), 7.55–7.58 (1H, t, *J*=12 Hz), 7.72–7.75 (1H, m). LCMS m/z Cal. $[M-H]^-$ 458.12 found $[M-H]^-$457.6.

Ethyl 4-(1-(3-methyl-4-nitrobenzoyl)piperidin-4-yl)-1,4-diazepane-1-carboxylate (6d)

^{1}H NMR (400 MHz, DMSO) δ 1.23–1.34 (3H, m), 1.42–1.58 (4H, m), 2.01–2.31 (4H, m), 2.64 (3H, s), 2.82–2.90 (1H, d), 3.15–3.23 (4H, m), 3.43–3.46 (1H, m), 3.51–3.57 (2H, s), 3.72–3.79 (2H, m), 4.13–4.18 (2H, m), 4.83 (1H, s), 7.28 (1H, s), 7.34–7.40 (1H, m), 8.00–8.02 (1H, d, *J*=8 Hz). LCMS m/z Cal. $[M+H]^+$ 418.22 found $[M+H]^+$419.2.

Ethyl 4-(1-(3-bromobenzoyl)piperidin-4-yl)-1,4-diazepane-1-carboxylate (6e)

^{1}H NMR (400 MHz, DMSO) δ 1.26–1.29 (3H, t, *J* = 12 Hz), 1.48–1.53 (4H, m), 1.88 (4H, s), 2.80 (4H, d), 3.03 (1H, s), 3.16–3.22 (1H, m), 3.50–3.59 (2H, m), 3.70–3.77 (2H, m), 4.13–4.18 (2H, m), 4.76 (1H, s), 7.23–7.33 (2H, m), 7.55–7.58 (2H, d, *J* = 12 Hz). LCMS m/z Cal. $[M-2+H]^-$ 438.13 found $[M+H]^-$405.21.

Ethyl 4-(1-(4-bromo-3-methylbenzoyl)piperidin-4-yl)-1,4-diazepane-1-carboxylate (6f)

^{1}H NMR (400 MHz, DMSO) δ 1.26–1.30 (3H, t, *J* = 16 Hz), 1.47–1.52 (4H, m), 1.87–1.99 (2H, m), 2.10 (3H, m), 2.44 (3H, s), 2.93–2.98 (4H, m), 3.51–3.54 (4H, m), 3.65 (1H, m), 4.15–4.17 (2H, m), 4.80–4.90 (1H, s), 7.08 (1H, s), 7.20–7.28 (1H, s), 7.57–7.59 (1H, d, *J* = 8 Hz). LCMS m/z Cal. $[M-H]^-$ 453.15 found $[M-H]^-$452.0.

Ethyl 4-(1-(2-amino-5-bromobenzoyl)piperidin-4-yl)-1,4-diazepane-1-carboxylate (6 g)

^{1}H NMR (400 MHz, DMSO) δ 1.29–1.32 (4H, m), 1.60–1.63 (4H, m), 2.01 (4H, s), 2.88–2.94 (6H, d, *J* = 24 Hz), 3.16–3.17 (1H, d, *J* = 4 Hz), 3.53–3.76 (2H, m), 4.16–4.21 (2H, m), 4.33 (2H, s), 6.64–6.66 (1H, d, *J* = 8 Hz), 7.20 (1H, s), 7.30 (1H, s). LCMS m/z Cal. $[M+H]^+$ 452.14 found $[M+H]^+$453.2.

Ethyl 4-(1-(3-(3-methoxyphenyl)-5-methylisoxazole-4-carbonyl)piperidin-4-yl)-1,4-diazepane-1-carboxylate (6h)

^{1}H NMR (400 MHz, DMSO) δ 0.88–0.92 (4H, m), 1.30 (8H, s), 2.32–2.75 (6H, m), 3.48 (4H, s), 3.69 (1H, s), 3.87–3.90 (2H, d, *J* = 12 Hz), 4.17–4.18 (2H, d, *J* = 4 Hz), 4.82 (1H, s), 7.04 (1H, s), 7.24–7.42 (3H, m). LCMS m/z Cal. $[M+H]^+$ 470.25found $[M+H]^+$471.2.

Ethyl 4-(1-(1-phenylpiperidine-4-carbonyl)piperidin-4-yl)-1,4-diazepane-1-carboxylate (6i)

^{1}H NMR (400 MHz, DMSO) δ 1.28–1.32 (3H, m), 1.53 (4H, m), 1.81–1.84 (3H, m), 1.91–1.98 (7H, m), 2.52–2.60 (1H, m), 2.73–2.79 (1H, m), 2.84–2.98 (3H, m), 3.04–3.11 (1H, t, *J* = 28 Hz), 3.16–3.22 (1H, m,), 3.51–3.52 (2H, m), 3.55 (1H, m), 3.70–3.77 (2H, m), 4.02–4.05 (1H, m), 4.13–4.18 (2H, m), 4.73–4.76 (1H, d, *J* = 12 Hz), 6.87 (1H, s), 6.95–6.97 (2H, m), 7.26–7.28 (2H, m). LCMS m/z Cal. $[M+H]^+$ 442.29 found $[M+H]^+$442.60.

Ethyl 4-(1-benzoylpiperidin-4-yl)-1,4-diazepane-1-carboxylate (6j)

^{1}H NMR (400 MHz, DMSO) δ 1.29–1.33 (3H, t, *J* = 12 Hz), 1.47–1.72 (2H, m), 2.23–2.26 (3H, d, *J* = 12 Hz), 2.43 (3H, s), 3.15–3.25 (3H, m), 3.62 (4H, s), 3.93 (3H, s), 4.19–4.22 (2H, t, *J* = 12 Hz), 7.31–7.45 (6H, m). LCMS m/z Cal. $[M+H]^+$ 359.22 found $[M+H]^+$359.47.

Conclusion

In this research study, synthesis, characterization, in silico and anti-microbial activity been done on a series of 1,4-diazepane linked piperidine derivatives. In vitro assay for anti-microbial activity done with Gram-positive (*Staphylococcus aureus, Bacillus Subtills, Bacillus megaterium*) and gram-negative (*Escherichia coli, Pseudonymous, Shigella* sp.) bacterial strains of synthesized compounds were compared to that of standard drug ampicillin and found to have good activity. The geometry optimization in gas phase at the B3LYP/6-31G (d,p) level of theory as implemented in Gaussian 05 DFT study. The negative values of EHOMO and ELUMO calculations for all derivative indicate their stability which favors for the chemical reactivity. The molecular docking against target proteins, L-amino acid deaminase from *Proteus Vulgaris* and LcpA ligase from *Bacillus subtilis* in identifying the relative stability of the target protein—ligand complex. Molecules **6b** showed the largest binding affinity for both target proteins. Also, the ADME predictions for each derivatized molecule favors for the highest binding affinity. Additional electronic properties, such as electronegativity (χ), global hardness (η), global softness (σ) and global electrophilicity index (ω), was computed to ascertain the biological activity of the derivative.

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Author contributions

KA and TMP: conceptualization, Methodology writing, Data curation, editing. ST and KP: In vitro biological activity, writing, editing. SM: Software, Formal analysis, writing.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

There are no competing interests to declare for all authors.

Abbreviations

M.P
Melting point
MeOH
Methanol
TLC
Thin Layer Chromatography
LCMS
Liquid chromatography-mass spectrometry.
TEA
Triethanolamine
DCM
Methylene Chloride
DIPEA
N,N-Diisopropylethylamine
DMF
N,N-Dimethylformamide
HATU
Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium
MIC
Minimum inhibition concentration
HOMO
Highest occupied molecular orbital
LOMO
Lowest unoccupied molecular orbital
ADME
Absorption, Distribution, Metabolism, and Excretion

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Hypoglycemic and hepatoprotective activity of Phellinus fastuosus on streptozotocin-induced diabetic rats and carbon tetrachloride-intoxicated rats, respectively

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ABSTRACT (ENGLISH)

Background

Phellinus fastuosus is a wood-eating medicinal fungus from Western Ghats of India. Therefore, we investigated hypoglycemic and hepatoprotective effects of *P. fastuosus* aqueous extract on streptozotocin-induced diabetic and carbon tetrachloride (CCl_4) induced hepatotoxicity in rats, respectively.

Result

As compared to the diabetic control group, a 400 mg/kg dose had significant hypoglycemic effects, including a reduction in blood glucose (24.44%) and gain in body weight. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity reduced by 31.81% and 32.84%, respectively, were also noted, along with decreases in triglycerides (24.32%) and cholesterol (25.89%) levels. The albumin, bilirubin and creatinine levels were also significantly reduced after administration of *P. fastuosus* extract in diabetic rats. Administration of *P. fastuosus* extract showed a substantial decrease in the activity of ALT, AST, alkaline phosphatase (ALP), catalase (CAT) and superoxide dismutase (SOD) in addition a decrease in the level of lipid peroxidation (LPO) as compared to CCl_4-intoxicated rats. The cumulative effect of CCl_4 increased the erythrocyte membrane peroxidation, whereas *P. fastuosus* extract reduced the cholesterol and increased phospholipid, thus preventing the alteration of membrane fluidity as compared to CCl_4-intoxicated rats. FTIR and HR-LC-MS-based metabolic profiling revealed the presence of various functional groups and bioactive metabolites.

Conclusion

The extract showed the hypoglycemic and hepatoprotective effects due to the presence of various bioactive

metabolites. Exploration of therapeutic potential of *P. fastuosus* using bioassay-guided fractionation is needed.

FULL TEXT

Background

Medicinal mushrooms are higher fungi with additional qualities including low-fat content and a trans-isomer of unsaturated fatty acids, followed by high fiber, terpenes, phenolics, sterols, etc. [1]. Owing to this, they are widely used in pharmaceutical and nutraceutical industries, particularly for their bioactive potential such as anticancer, immunomodulatory, antibacterial, hypoglycemic and hepatoprotection [2, 3]. Among medicinal mushrooms, the *Phellinus* spp. possess great variability in chemical composition within individual species, hence, are widely used in traditional medication and exploited to evaluate their bioactive potential against cancer, cardiovascular diseases, and diabetes [4–6]. Though some species are explored for their bioactive potential, many of them still required scientific evaluation, for instance, *Phellinus fastuosus*, which also forms a part of folk medicine.

Diabetes mellitus is an endocrine metabolic disorder that disturbs the metabolism of proteins, carbohydrates, and fats. Worldwide more than 425 million individuals are affected by this chronic disorder, which can turn fatal in comorbid conditions [7]. Further, it is a major cause of blindness, kidney failure, cardiac arrest, and lower limb amputation. Numerous traditional remedies have been developed to treat diabetes. Among them, mushrooms are widely used as a natural source of biomolecules for treating ailments, including diabetes [7]. Studies have demonstrated that medicinal mushrooms particularly *Phellinus* spp. has beneficial effects with regards to diabetes. For instance, The *P. badius*, *P. linteus*, and *P. rimosus* showed hypoglycemic activity in experimental diabetic rat/mice models [6, 8, 9].

Liver is a large complex organ in the human body that is vital for detoxification, which is also involved in fat metabolism, protein synthesis and secretion of important enzymes. Drugs, toxic chemicals, or bacterial infections that might affect the liver's normal function are the most common causes of liver damage. Carbon tetrachloride (CCl_4) is a hepatotoxic agent widely used as a toxin to induce liver fibrosis in laboratory investigations [10]. Endoplasmic reticulum structural modifications result from the metabolic activation of CCl_4 by cytochrome P450-dependent mixed oxidases, which further results in the loss of enzymatic machinery, thus impairing normal functioning of liver. Mushroom extracts are reported to prevent CCl_4-induced hepatotoxicity in rats [11].

P. fastuosus is a wood-eating fungus (Fig. 1A), used in traditional formulations to treat ailments such as diabetes, diarrhea, and stomachache as well as to boost the immune system and promote health. It is found across continents, such as Asia and Australia, and is also studied for anti-cariogenic activity [12], plausible use in food preservation [13], and physiochemical characteristics [14]. However, there is no scientific evidence for its bio-prospects, for instance to treat hypoglycemia as well as hepatotoxicity. Therefore, as supported by the traditional knowledge and scientific literature, here we seek to explore the hypoglycemic and hepatoprotective activity of *Phellinus fastuosus* mushroom from Western Ghats of India. The hypoglycemic and hepatoprotective activity of *P. fastuosus* aqueous extract was tested in streptozotocin (STZ) induced diabetic rats and carbon tetrachloride induced erythrocyte-/liver-damaged wistar rats, respectively. Based on the folklore of India's Western Ghats, the aqueous extract was created to resemble the conventional way of administration of medicine (Fig. 1B and C).

Fig. 1 [Images not available. See PDF.]

*Phellinus fastuosus***A** fruiting body, acquired from Devgad **B**, is located in the Western Ghats of India **C**. [Source: https://www.google.com/maps]

Methods

P. fastuosus (Kii 19) basidiocarps were gathered from Western Ghats of Maharashtra, India. The samples were authenticated (SPPU/Bot/42) from Mycology Laboratory, Savitribai Phule Pune University. Further the samples were washed, oven dried at 40 ± 2 °C and grounded using a commercial grinder (Restsch Ultra Centrifugal Mill and Sieving Machine, Germany) and stored in sterile reagent bottles (Borosil®, India).

Extract preparation

Hundred grams powder of *P. fastuosus* fruit body was suspended in distilled water at 90 ºC for four hours and filtered with the help of Whatmann no. 1 paper. The procedure was carried out twice. Combining and filtering the filtrates via Whatmann no. 1 paper. The filtrate was concentrated in vacuum followed by addition of 90% ethanol (4:1 of filtrate) and stirred vigorously. The mixture was allowed to stand overnight at 4 ºC. Next day, centrifuged the mixture at 10,000 rpm for 20 min. Subsequently, equal volume of 10% trichloroacetic acid was added and kept overnight. Next day, centrifuged the sample at 7000 rpm for 5 min and precipitate was wash with chilled 80% ethanol, dissolved in distilled water, and dialyzed for three days against running tap water and one day against distilled water, then centrifuge at 7000 rpm for 15 min. Obtained precipitate was lyophilized and used for bioactivity studies [11, 15].

Hypoglycemic study

Test animals and experimental conditions

Male wistar rats (NTC, Pune, India) of approximately 200 g housed at National Toxicology Center (NTC) were used for animal studies. The rats were housed in a room with stainless steel cages in a controlled environment. Throughout the trial, the rats were fed a commercial pellet meal and given unlimited access to water.

Diabetes was artificially induced by the administration of diabetogenic agent. After a week of acclimatization, the rats had a 16-h fast. Streptozotocin (STZ, or a diabetogenic drug) was injected intraperitoneally at a dosage of 50 mg/kg body weight, diluted in a buffer solution of sodium citrate buffer (pH 4.5) from Sigma, India. The determination of fasting blood glucose was made two days after injecting STZ [16, 17]. When their fasting blood glucose level exceeded 300 mg/dL, the rats were deemed to have diabetes. Rats were divided into six groups, Normal control (NC); Diabetes control (DC); Standard Glibenclamide (STND) (10 mg/kg) [18]; STZ + doses of 100 mg/kg (Kii 19 100); STZ + doses of 200 mg/kg (Kii 19 200); STZ + doses of 400 mg/kg (Kii 19 400) [11, 15, 19]. A single oral dosage of extracts was administered to diabetic rats every day for 14 days. The animal study was conducted at National Toxicology Center (NTC), Pune, India and overall experimental protocol was approved by the institution animal ethical committee (IAEC 805).

Body and organ weight

The change in body weight was measured by calculating the initial and final body weight of the animals. The weight of body organs such as heart, liver, spleen, kidney, pancreas, and lungs were measured after sacrificing the animals.

Biochemical analysis

To estimate glucose level, the tail vein was pricked and followed by blood collection and monitoring using glucometer (Contour®TS, India). In heparinized tubes, blood was drawn, and the plasma was separated by centrifugation at 10,000 rpm for 10 min. An enzymatic colorimetric test kit (Merck Bioline, India) was used to assess the levels of triglycerides and cholesterol. Enzyme kits were used to measure the activity of alanine aminotransferase and aspartate aminotransferase (Merck Bioline, India) based on Reitman-Frankel method [20]. Albumin, bilirubin, creatinine and urea levels were measured using diagnostic kits (Crest Biosystems, India).

Hepatoprotective study

Test animals and experimental conditions

Male wistar rats (150–200 g) were selected and maintained in environmentally controlled conditions. Prior to the experiment, the animals were kept in wire-meshed cages for a week to acclimatize and were given access to regular food and deionized distilled water. Hepatoprotective and erythrocyte protective activity.

Rats were divided into four groups, with ten in each group. Group I (served as control), which received olive oil p.o. three times a week for 14 days. Group II were administered silymarin 200 mg/kg. Group III received CCl_4 1 mL/kg body weight (BW), intraperitoneal (CCl_4 control), three times a week for 14 days. Group IV animals were administered with 400 mg/kg BW along with CCl_4 (Kii19 + CCl_4) for 14 days.

The rats were terminated via cervical dislocation 14 days into the experiment. Blood samples were drawn from the jugular vein, placed in heparinized tubes, and centrifuged for 15 min at 3000 rpm.

The packed cells were rinsed three times with physiological saline (0.9% w/v NaCl), suspended in cold distilled

water (2–5 °C), then centrifuged at 7000 rpm for 30 min to lyse them. The particle that resulted represented the hemolysate, while the supernatant represented the erythrocyte membrane. Aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase were tested in the plasma that was left over after the initial centrifugation in order to measure lipid peroxidation [20]. Using standardized kits, the enzyme levels were determined.

Catalase [21] and superoxide dismutase [22] activity were estimated using the hemolysate. Using a procedure previously described [23], the lipids from the erythrocyte membrane were extracted, and the amounts of cholesterol and phospholipids were measured [24, 25]. The ratio of cholesterol to phospholipids was then determined.

Histological examination

After the animal was sacrificed, the liver was taken out and washed in ice-cold saline. Before sectioning, the tissue samples were embedded in paraffin wax, treated with 10% formaldehyde, and dehydrated in a graduated series of ethanol. After cutting the paraffin slices, they were dewaxed and rehydrated. Light microscopy was used to examine the sections after they had been stained with hematoxylin and eosin [11].

Fourier transformed infrared spectroscopy (FTIR)

The aqueous extract of *P. fastuosus* was analyzed with FTIR. The FTIR spectra was obtained in the range of 4000–450 cm^{-1}using a Bruker, 3000 Hyperion Microscope with Vertex 80 FTIR System (Bruker, Germany).

HR-LC–MS-based metabolic profiling

The aqueous extract of the *P. fastuosus* was characterized using LC–MS at Indian Institute of Technology, Mumbai (IIT-Powai), India. TOF/Q-TOF mass spectrometer (Model—G6550A; Agilent Technologies) with dual AJS ESI as ion source was used. The details of are: Column—Hypersil GOLD C18 100 × 2.1 mm—3 µM, injection volume: 3 µl, flow rate: 0.3 ml/min, mobile phase A (0.1% formic acid) and mobile phase B (90% Acetonitrile + 10% H_2O + 0.1% formic acid), minimum range: 125 m/z and maximum range 1000 m/z, scan rate 1.00spectra/sec. The peaks were identified using inbuilt mass library.

Statistical analysis

Using the statistical package of the Social Science (SPSS) programmed, one-way analysis of variance (ANOVA) test was used to determine the results' statistical significance. Standard Deviation (SD) was used to represent all data as mean ± SD. A one-way analysis of variance and Duncan's multiple range tests were used to compare the group means. At $p<0.05$, statistical differences were considered significant.

Results

Hypoglycemic activity

Phellinus fastuosus sample's hypoglycemic impact was tested for 14 days in diabetic rats induced by STZ.

Body and organ weight

Figure 2 depicts the effect of *P. fastuosus* administration on the body (Fig. 2A) and organ weight (Fig. 2B) in STZ-induced diabetic rats. When compared to diabetic groups, Kii- 19 400 showed a considerable gain in body weight. Further, body organ weight, i.e., heart, liver, kidney, pancreas and lungs significantly increased at a dose of 200 mg/kg, whereas when compared to the diabetic control group, the 400 mg/kg dosage revealed a substantial change in the weight of the liver and heart.

Fig. 2 [Images not available. See PDF.]

Effect of *P. fastuosus* aqueous extract on **A** body, and **B** organ weight of extract fed and control rats after 14 days. Values are mean ± SD (n = 6), and small letters on the bars denote the significant difference between treatments at $p<0.05$

Blood glucose

Figure 3A shows the activity of *P. fastuosus* samples on the blood glucose level in STZ-induced diabetic rats over a period of 14 days. In the diabetic control group (STZ), the blood glucose level showed continuous increase throughout the experiment, finally reaching a level of 454 mg/dL. In contrast, the administration of *P. fastuosus* extract significantly lowered the blood glucose level compared to that of the diabetic control group. Doses of

400 mg/kg of Kii 19 showed reduction in glucose level by 24.44%. Kii 19 shown a decrease in glucose level of 23.05% when compared to the diabetic control group at a dosage of 200 mg/kg.

Fig. 3 [Images not available. See PDF.]

Effect of *P. fastuosus* aqueous extract on the **A** blood glucose, **B** triglycerides and cholesterol, **C** Alanine aminotransferase (ALAT) and Aspartate aminotransferase (AST), **D** Bilirubin and albumin, **E** Creatinine, **F** Urea and, **G** Alkaline phosphatase in streptozotocin-induced diabetic rats for 14 days. Values are mean ± SD (n = 6), and small letters on the bars denote the significant difference between treatments at $p<0.05$

Studies on lipid metabolism

The level of triglyceride was markedly reduced after the administration of *P. fastuosus* extract compared to diabetic control (Fig. 3B). Dose of 400 mg/kg of Kii 19 showed significant reduction of triglycerides level, i.e., 24.32% as compared to diabetes control group. As shown in Fig. 3B, the administration of *P. fastuosus* extract showed significant reduction in total cholesterol level. Kii 19 dose at 400 mg/kg reduced cholesterol by 25.89% as compared to diabetic control. Throughout the investigation, there was no discernible difference in the total cholesterol levels between the Kii 19 100 mg/kg and Kii 19 200 mg/kg groups.

Aspartate aminotransferase (AST) and Alanine aminotransferase level (ALT)

The plasma AST and ALT activity in the STZ group were higher than those in the NC group. Therefore, the remarkable reduction in levels of AST after oral administration of Kii 19 by 31.81% at a dose of 400 mg/kg which revealed a remedial role in liver function (Fig. 3C). The ALT activity was significantly reduced under the influence of *P. fastuosus* samples. The maximum reduction of 32.84% was observed in 400 mg/kg dose.

Bilirubin and Albumin

The level of bilirubin in diabetic rats were increased as compared to normal control rats (Fig. 3D). Administration of the *P. fastuosus* extracts signifciantly lowered the bilirubin level at a dose of 400 mg/kg by 25.13%. Albumin level was also significantly decreased in all groups as compared to diabetic control group. among all groups Kii-19 sample showed significant effect after 14 days.

Creatinine, urea and alkaline phosphatase activity

Figure 3E and F shows that the significant decreased in creatinine level and urea in 400 mg/kg *P. fastuosus* extract fed rats compared to diabetic control.

Hepatoprotective and erythrocyte protective activity

After CCl_4 intoxication, the levels of plasma hepatic enzymes significantly increased. After administration of *P. fastuosus* extract to animals, significantly reduced the elevated levels plasma ALT, AST and ALP (Fig. 4A).

Fig. 4 [Images not available. See PDF.]

Effect of *P. fastuosus* extract on **A** Plasma ALT, AST and ALP and **B** lipid peroxidation products and primary antioxidant enzymes of the erythrocytes of carbon tetrachloride-intoxicated rats in rats after 14 days of treatment. Values are means ± SD for ten rats per group. Means in the same column having different superscript are significantly different ($p<0.05$)

The increased lipid peroxidation products, superoxide dismutase and catalase activity, and decreases in membrane fluidity were evidence of carbon tetrachloride damage to erythrocytes. The accumulation of hydrogen peroxide caused by the elevated superoxide dismutase activity prompted an increase in catalase activity.

In comparison to the control group, the concentration of lipid peroxidation increased in the carbon tetrachloride-treated animals, indicating higher activity of lipid peroxidation. SOD activity increased significantly in carbon tetrachloride-treated animals compared to the normal group (Fig. 4B). CAT activity also increased significantly after administration of carbon tetrachloride (Fig. 4B). The accumulation of lipid peroxidation products in the plasma was dramatically reduced during treatments with the extracts. Superoxide dismutase and catalase activities significantly increased in the rats after carbon tetrachloride intoxication; however, these activities diminished when carbon tetrachloride was administered concurrently with the extracts (Fig. 4B).

Carbon tetrachloride intoxication increased the ratio of cholesterol to phospholipid, decreased membrane phospholipid, and increased membrane cholesterol (Table 1). The findings of this investigation show that the membranes are stiff. The administration of *P. fastuosus* extracts stopped alterations in membrane fluidity and lipid composition.

Table 1. Effect of *P. fastuosus* extract on erythrocyte membrane lipids and cholesterol/phospholipid ratio of carbon tetrachloride-intoxicated rats

Groups	Cholesterol (mg/100 µL)	Phospholipid (mg/100 µL)	Cholesterol /Phospholipid
Group I (Control)	0.58 ± 0.01^{c}	1.12 ± 0.03^{a}	0.58 ± 0.02^{c}
Group II (Kii19)	0.58 ± 0.03^{c}	1.15 ± 0.05^{a}	0.58 ± 0.06^{c}
GroupIII (CCl_4)	0.74 ± 0.05^{a}	0.80 ± 0.02^{c}	0.92 ± 0.05^{a}
GroupIV (CCl_4 + Kii19)	0.62 ± 0.01^{b}	0.92 ± 0.01^{b}	0.70 ± 0.04^{b}

Values are means ± SD for ten rats per group. Means with different superscripts in the same column are substantially different ($p<0.05$)

Histopathology

The histological examination (Fig. 5) reveals cellular damage compared to the CCl_4-intoxicated rats (Fig. 5C), for instance, administration of *P. fastuosus* extract (Fig. 5D) preserved the architecture of hepatocytes and sinusoidal spaces, which is similar to that of the controls (Fig. 5A and B), whereas CCl_4-intoxicated rats showed moderate parenchymal cell hypertrophy, dilatation of sinusoidal spaces (Fig. 5C).

Fig. 5 [Images not available. See PDF.]

The liver histopathological sections **A** control group; **B** animals treated with silymarin 200 mg/kg; **C** CCl_4 control; **D** Kii19 + CCl_4

Fourier transformed infrared spectroscopy

The FTIR spectra (Fig. 6) reported the range of 990–1200 cm^{-1}, indicating the presence of polysaccharide, while the peaks at 1150–1160 cm^{-1} indicate stretching of glycosidic bond. Furthermore, other peaks at 1384 cm^{-1} indicate presence of β-D glucan. The peak at 1628 cm^{-1} represents vibration of proteins. Other peaks at 2851, 2920 cm^{-1} are C–H stretching vibration and hydroxyl stretching vibration, respectively. However, the peaks for N–H stretching vibration were found between 3194 and 3448 cm^{-1}, whereas amide A (N–H stretching) was found at 3282 cm^{-1} and amide N–H stretch at 3441 cm^{-1}, respectively, indicating the presence of polysaccharide bound proteins.

Fig. 6 [Images not available. See PDF.]

FTIR study of *P. fastuosus* aqueous extract

HR-LC–MS-based metabolite profiling

The aqueous extract of the *P. fastuosus* subjected to HR-LC-MS profiling which showed the presence of bioactive compounds like acronidine, flaccidine, pongamoside A, epoxyfumitremorgin C, dihydrodeoxystreptomycin, tetradecylamine, hexadecylamine, docosanedioic acid, 4-keto myristic acid,meta 1-hexadecylamine, N-isovalerylglycine, 2-amino-2-norbornanecarboxylic acid, etc. with potential bioactivities were reported (Table 2). Many unknown compounds were present in the metabolite profiling of *P. fastuosus* extract shown in the chromatogram (Figure S1).

Table 2. List of metabolites identified using HR-LC-MS-based metabolic profiling and their biological activities as mentioned in the literature

Metabolite	RT	Mass	*m/z*	Formula	Biological activity	References
TPPU	1.031	359.14	360.1484	C16H20F3N3O3	Antidepressant effect	[26]
N-isovalerylglycine	1.031	159.0886	160.0959	C7H13NO3	ROS inhibitor	[27]
2-Amino-2-Norbornanecarboxylic acid	1.112	155.0936	156.1008	C8H13NO2	Anti-obesity	[28]
Acronidine	5.6	311.1148	312.122	C18H17NO4	Antimicrobial and anticancer	[29]
2'-Hydroxyfurano[2",3":4',3']chalcone	5.631	264.0777	265.0849	C17H12O3	Antimicrobial	[30]
Pyridine-2-azo-pdimethylaniline	5.697	226.1219	249.1111	C13H14N4	Chromogenic reagent	[31]
Flaccidine	6.421	442.1269	465.1162	C23H22O9	Antimicrobial	[32]
Epoxyfumitremorgin C	7.327	393.1671	465.1163	C22H23N3O4	Anticancer	[33]
Pongamoside A	7.942	440.1117	394.1744	C23H20O9	Antihyperlipidemic *Activity*	[34]
Dubamine	8.081	249.0777	250.085	C16H11NO2	Cytotoxicity	[35]
Dihydrodeoxystreptomycin	9.158	567.2865	568.2937	C21H41N7O11	Antifungal	[36]
Tetradecylamine	10.449	213.2447	214.2521	C14H31N	Hypolipidemic	[37]
1-Hexadecylamine	11.88	241.2759	242.2832	C16H35N	Antimicrobial activity	[38]
Docosanedioic acid	19.671	370.3067	371.314	C22H42O4	Antibacterial activity	[39]

Vitamin E Succinate (tocopherol succinate)	21.325	530.3977	531.4055	C33H54O5	Antitumour activity	[40]
4-keto myristic acid	26.666	242.1866	265.1759	C14H26O3	Anxiolytic-Like Effects	[41]
1-Hexadecylamine	26.713	241.2759	242.2831	C16H35N	Anticancer activity	[42]

Discussion

Phellinus spp. (family: Hymenochaetaceae) gained attention due to their potent medicinal properties [6, 43, 44]. *P. fastuosus*, a member of this family is used as folk medicine, and is reported to be an important source of drosophilin A and drosophilin A methyl ether [45, 46]. Therefore present work demonstrates the hypoglycemic and hepatoprotective potential of *P. fastuosus* aqueous extract on Streptozotocin-induced diabetic and carbon tetrachloride-intoxicated rats, respectively.

Streptozotocin treatment affected the secretion of insulin by the pancreas through selective destruction of β-cells of islets of Langerhans. This induced hyperglycemia in rats leading to type-1 diabetes mellitus [6]. The significant increase in body weight and decrease in blood glucose level observed in *P. fastuosus* extract administered rats compared to diabetic control group demonstrates its glucose-lowering property. It can be hypothesized that the blood glucose-lowering effect of extract is due to increased glucose utilization in diabetic rats as a result of insulin secretion [47]. Consequently, the increase in glucose utilization positively correlated with digestion, which resulted in body weight gain. Similar hypoglycemic activity and increased in body weight was reported in rats administered with different medicinal mushroom extract [6, 15, 17].

Most drugs designed to treat diabetes mellitus focuses on controlling and lowering blood glucose levels [47]. Moreover, the various studied mushroom species prove their hypoglycemic property [15] and emerged as an ideal food for the prevention of hyperglycemia [47].

The increased in cholesterol and triglyceride level and AST and ALT in diabetic control group may be due to streptozotocin-induced liver and metabolic malfunction [47]. However, significant reduction in cholesterol and triglyceride level and AST and ALT level after extracting administration as compared to diabetic control group can be attributed to the reduction in fat emission by liver. Bilirubin and albumin levels serve as possible indicators of liver damage as well as normal liver, gallbladder, and bile duct function [15]. There was not much variation recorded in bilirubin content but albumin level significantly decreased in extract administered rats. Elevated blood levels of urea and creatinine, which are important indicators of renal impairment, are brought on by diabetic hyperglycemia [48]. The result demonstrated that there is no significant variation in animal groups for creatinine level. However, after administration extract, significant decrease in the urea level was observed in comparison with diabetic control group, which it a clear indication of improved liver and kidney function. Further alkaline phosphatase is a membrane bound important enzyme mainly found in liver and bone as well as few other tissues. In diabetes, the level of alkaline phosphatase increases due to ruptured of cell membrane followed by leakage [49]. The *P. fastuosus* extract significantly reduced the alkaline phosphatase activity in a concentration dependent manner. The increase in organ weight such as kidney, heart, liver and lungs indicates that the *P. fastuosus* extract strongly promotes growth of organ tissue irrespective of hyperglycemic condition. This finding suggests that along with hypoglycemic effect there are few components which promote tissue growth, which may act via other protective mechanism such as hepatoprotection, antioxidation and anti-inflammation. However, change in oragn weight is one of the important and indirect diabetes diagnosis markers [47]. The findings presented for hypoglycemic activity of *P. fastuosus* are similar to the recent reports on *Phellinus* spp. including *P. baumii, P. pini* [50] and *P. linteus* [51].

ALT, AST and ALP are important liver enzymes that are responsible for efficient functioning of liver and also act as important markers for hepatocellular damage [52]. CCl_4 is one of the most commonly used hepatotoxins to

experimentally induce liver damage [53]. Lipid peroxidative degradation of bio-membrane is one of the principal causes of hepatotoxicity caused by CCl_4 [54].

A popular herbal remedy for antiaging is *P. fastuosus* extract use [8]. Using CCl_4 to cause lipid peroxidation in rat plasma, we were able to demonstrate that *P. fastuosus* aqueous extract greatly reduced the levels of lipid peroxidation and further substantiated the extract's antilipid peroxidation function. The delivery of CCl_4 to the rats causes the liver and other tissues to produce free radicals such CCl_3, CClO, or C_2H_3O, which induce lipid peroxidation, cell death, and tissue damage, which result in severe liver disorders [55]. The degree of lipid peroxidation was represented in the levels of chain reactions that free radicals caused on the cell membrane to produce lipid peroxidation. This measure could offer an easy way to test for lipid peroxidation and damage to cellular membranes. The findings of this investigation showed that administration of *P. fastuosus* extract had an antilipid peroxidative effect and shielded red blood cells from damage brought on by carbon tetrachloride. According to the findings, *P. fastuosus* extract had an antilipid peroxidation impact on normal rats since it somewhat (not significant) lowered the levels of lipid peroxidation in non-toxication rats.

Low levels of lipid peroxidation products are present in tissues and cells under typical physiological conditions. More products from lipid peroxidation are produced as a result of cell damage when there is oxidative stress [55]. Superoxide dismutase, glutathione peroxidase, and catalase are examples of cellular antioxidant enzymes that typically fight oxidative stress [56]. Increases in lipid peroxidation products, superoxide dismutase and catalase activity, and decreases in membrane fluidity in this study's erythrocytes were evidence of carbon tetrachloride damage. The buildup of hydrogen peroxide caused by the elevated superoxide dismutase activity prompted an increase in catalase activity [55].

Superoxide, i.e., O_2^- is harmful to the body and can become one of the primer reasons to induce inflammation, aging and terminally to cancer [57]. SOD is an important enzyme that catalyzes the elimination of reactive oxygen species (ROS). SOD protects human erythrocyte membrane from ROS, which can elevate the fluidity of membranes by decreasing the cross-linking between the membrane proteins. Similarly, catalase are involved in the conversion of hydrogen peroxide to molecular oxygen and water molecules [55]. Due to CCl_4 cumulative impact, erythrocyte membrane peroxidation is increased, which may potentially result in hemolytic alterations [57]. The elevated level of CAT and SOD after the administration of extract provides reliable evidence to its protective action on the erythrocyte membrane.

The FTIR spectra peak at 1628 cm^{-1} represents vibration of proteins. The peaks at 2851, 2920 cm^{-1} are C–H stretching vibration and hydroxyl stretching vibration, respectively [58–60]. However, the peaks for N–H stretching vibration were found between 3194 and 3448 cm^{-1}, whereas amide A (N–H stretching) was found at 3282 cm^{-1} [61] and amide N–H stretch at 3441 cm^{-1}, respectively, indicating the presence of polysaccharide bound proteins [62].

The HR-LC-MS-based metabolite profiling of *P. fastuosus* aqueous extract reported various compounds like acronidine, pongamoside A, flaccidine, dubamine, dihydrodeoxystreptomycin, docosanedioic acid, etc. (Table 2) have bioactive potential [29, 32, 34–36, 39], which interns support the hypoglycemic and hepatoprotective activity of *P. fastuosus.*

Conclusion

The present study confirmed the hypoglycemic and hepatoprotective activity of *P. fastuosus* aqueous extract on streptozotocin-induced diabetic and carbon tetrachloride-intoxicated rats, respectively. Meanwhile, the metabolites identified through HR-LC-MS-based metabolic profiling of aqueous *P. fastuosus* extract support its bioactivities. The physio-biochemical and metabolic analysis indicate that *P. fastuosus* has the potential to provide hepatoprotective and hypoglycemic compounds; however, further bioassay-guided fractionation followed by molecular docking is warranted to identify the active ingredients for pharmaceutical applicability.

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Author contributions

HS, DS and SA conducted the experiment, wrote original draft of manuscript and review the manuscript. VG, BB and SG design the study, supervised the work and finalized the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this article.

Declarations

Ethics approval and consent to participate

The study protocol was reviewed and approved by the Animal Ethics Committee of National Toxicology Center (NTC), Pune, India. The consent to participate are not applicable for this study.

Consent for publication

Not applicable.

Competing interests

The authors declare no any competing interest.

Abbreviations

AST

Aspartate aminotransferase

ALT

Alanine aminotransferase

ALP

Alkaline phosphatase

LPO

Lipid peroxidation

CAT

Catalase

SOD

Superoxide dismutase

CCl_4

Carbon tetrachloride

STZ

Streptozotocin

Kii 19

P. fastuosus

NC

Normal control

DC

Diabetes control

FTIR

Fourier transformed infrared spectroscopy

HR

High resolution

LC

Liquid chromatography

MS

Mass Spectroscopy

Publisher's Note

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A critical examination of advanced approaches in green chemistry: microbial bioremediation strategies for sustainable mitigation of plastic pollution

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ABSTRACT (ENGLISH)

Background

The escalating concern regarding the environmental impact of plastic waste necessitates the adoption of biodegradable methodologies to curtail its adverse effects. A profound comprehension of the intricate interplay between bacteria and polymers becomes imperative for devising effective solutions to address plastic-induced environmental challenges.

Main body of the abstract

Numerous microorganisms have evolved specialized mechanisms for the degradation of plastics, rendering them amenable to application in green chemistry for the elimination of hazardous plastics from the ecosystem. This article offers a comprehensive survey of contemporary microbial bioremediation approaches geared towards augmenting plastic waste management and ameliorating plastic pollution. Emphasis is placed on elucidating the potential of microorganisms in mitigating the deleterious repercussions of plastics on ecosystems and human health, underscoring the significance of advanced strategies in green chemistry for sustainable plastic pollution mitigation.

Short conclusion

Current research emphasizes the effectiveness of naturally occurring soil microorganisms, particularly fungi like *Aspergillus* and bacteria like *Bacillus*, in breaking down plastics. To harness this potential on a broader scale, optimization of microbial activity conditions and pre-treatment with environmentally beneficial compounds are essential.

FULL TEXT

Background

According to recent statistics, the world's industries produce roughly 140 million tonnes of plastic annually, with a more significant proportion of that amount being released into the environment as garbage [1–4]. The use of chemicals, detergents, cosmetics, medicines, and food packaging accounts for thirty percent of these tonnes [5]. Approximately 64% of synthetic plastics are made of polyethylene, which has a high molecular weight and hydrophobicity. A considerable amount of the 500 billion to 1 trillion polythene materials manufactured annually worldwide end up in the natural environment (land and water) [6–9]. This raises serious environmental concerns. Approximately 10% of municipal garbage produced worldwide is attributed to packaging materials like polythene [10–12]. However, only 5% of the trash is recycled, and the remainder is buried underground, where it takes roughly 100 years for the material to decompose naturally without the aid of bacteria [13–15]. This results from their resistance, perseverance, and inability to degrade [16–18]. Humans may be subjected to severe health and ecological stresses as a result of this pollution. Particularly worrying microplastics have been linked to instantaneous mortality when ingested by aquatic creatures [19–22].

Due to the rise in environmental issues caused by plastics, the usage of physical [23, 24], and chemical [25–27] methods to break down plastic garbage has been condemned. The biological destruction (Biodegradation) of plastic using bacteria and fungus [28–30] has gained popularity recently owing to their efficacy, affordability, environmental friendliness, and sustainability. Multiple factors, including substrate accessibility, polymer surface area, shape, and molecular weight, all play a role in the biodegradation process [31, 32]. One may use a variety of metrics to assess this deterioration, including by the amount of carbon dioxide released into the atmosphere, the percentage change in the mechanical characteristics and/or chemical structure of the polymer, and the amount by which the substrate itself degrades. At the beginning of research into microbial biodegradation, scientists looked into how microbes may affect the physical qualities of plastics, such as water absorption, crystallinity, and tensile strength. Plastic waste may be assimilated into carbon sources or degraded into important alkane compounds using microbial biotechnology. This offers a promising possibility to increase plastic recycling and, by extension, to minimize

environmental plastic pollution [33–35].

Microbes may break down plastics by first producing extracellular enzymes, then attaching those enzymes to the surface of the plastic, then hydrolyzing the plastic into short polymer intermediates, and finally ingesting those intermediates as a carbon source in order to produce carbon dioxide (Fig. 1). In recent years, several bacteria capable of degrading these polymers have been found, despite the synthetic nature of these polymers. Together with the bacterial consortia, abiotic factors facilitate the mineralization, assimilation, depolymerization, and fragmentation of environmental plastic wastes into carbon dioxide, nitrogen, methane, and water molecules, monomers, dimers, and oligomers [36–38]. Since the 1970s, certain strains of bacteria from the genera *Aspergillus* [39–41]*, Penicillium* [42–44], *Streptomyces* [45–47], *Pseudomonas* [48–50], and *Bacillus* [51–53] have been utilized to break down plastic trash. Though the microorganisms responsible for plastic breakdown have been narrowed down, further study is required to confirm the identities of the specific causes.

Fig. 1 [Images not available. See PDF.]

Biodegradation process of plastic waste

Global concern has been raised about the enormous amount of poly(ethylene terephthalate) (PET) [54–56], polyvinyl chloride (PVC) [51, 57], polyamide (PA), polyethylene (PE), polypropylene (PP) that appears to take centuries to break down in the environment. Moreover, the COVID-19 pandemic has amplified the already alarming issue of plastic pollution, driving an unprecedented surge in the demand for single-use plastics such as personal protective equipment (PPE) [58]. This surge has further strained an already overwhelmed waste management system and exacerbated the pollution of our natural environments. In response to this, there is a growing emphasis on exploring innovative strategies to tackle plastic pollution. One such strategy gaining traction is plastic bioremediation. As the imperative for sustainable solutions intensifies, the focus on biodegradable methodologies gains prominence, necessitating a nuanced understanding of the intricate symbiosis between bacteria and polymers. This discourse delves into advanced microbial bioremediation strategies grounded in green chemistry, offering a comprehensive exploration of cutting-edge approaches to enhance plastic waste management and alleviate the escalating spectre of plastics pollution.

The primary contributions of this research article are as follows:

1. From the standpoint of developing plastic waste management, the study provides substantial information regarding microbes capable of effectively digesting polymers.
2. The study describes several ways in which bacteria may be used to break down plastic.
3. The study's principal goal is to understand how bacteria are used in the control of trash plastics.
4. The study also seeks to uncover existing developments in the microbial breakdown biodegradation of plastic trash.

The remainder of the study is structured as follows: Section "Research approach" provides specifics on the methodology used, which was derived from best practises for critical literature reviews. In the section titled "Biodegradation of Plastic Waste by Microorganisms," we briefly address the bioremediation by various microbes. The section under "Limitations" describes the restrictions that this study had to operate within. The last section of the paper, "Conclusions," outlines the entire work.

Research approach

The Web of Science, Scopus, PubMed, and Google Scholar were among the databases searched as criteria for including and excluding the study. The search was conducted using key terms related to the microbial bioremediation of plastic waste. Additionally, the "AND" and "OR" Boolean operators were used to construct relevant words. After data source evaluation, all filtered sources were collected and checked for duplication using Mendeley

Desktop Version 2.61.1. Titles and abstracts served as the primary criteria for screening. Full-text screening was also applied to the remaining articles. Studies evaluating incomplete publications (In press) and papers on the auxiliary subject were disregarded. We further excluded correspondence, discussions, editorials, books, systematic reviews, book chapters, conference abstracts, doctoral dissertations, and brief communications. This study included papers that discussed the role of microbial bioremediation in the removal of plastic waste. Additionally, we manually looked through relevant and referenced papers from the research and reviews that were included.

Main text

The process of plastic waste decomposition by microbes is closely linked to the chemical makeup of the polymers, environmental factors, and microbial behaviour. Microorganisms are essential in the process of decomposing plastic polymers into smaller pieces, which eventually results in the transformation of these pieces into innocuous chemicals such as carbon dioxide and water. The degradation of polymers is an intricate process that is affected by both inherent characteristics of the polymer and external environmental influences. The chemical composition of a polymer, which includes its arrangement, presence of different atoms, and other substances, greatly affects its vulnerability to degradation [59]. Polymers consisting only of carbon chains, particularly those containing double bonds, exhibit greater inertness in comparison to polymers including heteroatoms or additives [60]. Their high level of purity reduces their susceptibility to external influences, hence decelerating the process of deterioration.

The length and content of the carbon backbone are significant factors. Polypropylene, which has longer chains, often demonstrates resistance to degradation [61]. However, the inclusion of heteroatoms might potentially undermine this resistance. Moreover, the degradation rates are influenced by the polarity of the polymer, with nonpolar molecules exhibiting lower susceptibility to degradation. The degree of crystallinity of a polymer also impacts its degradation. Crystalline polymers have a higher resistance to degradation compared to amorphous compounds [62]. They require less water and oxygen to start decomposing. The molecular weight of polymers is a significant factor that affects their degradation rate. Polymers with larger molecular weights have smaller relative surface areas, resulting in slower degradation [63].

The degradability of a polymer is further influenced by the production method and the additives employed. Within landfills, the combination of UV radiation and heat can trigger breakdown by auto-oxidation, causing polymers to break down into microplastics [64, 65]. These microplastics are then further degraded by microbes, resulting in the production of carbon dioxide and water. Polymers such as polyethylene, polypropylene, and polystyrene mostly break down in the presence of oxygen and exposure to UV radiation [66]. This process results in the formation of different end products, which vary depending on the kind of polymer.

The process of anaerobic degradation that occurs in landfills leads to the generation of methane and water, which is facilitated by microbial enzymes that aid in the breakdown of polymers [67]. During the process of degradation, petrochemicals undergo changes such as increased brittleness, discoloration, and the formation of new functional groups. Microorganisms have a tendency to attack the shapeless parts of plastics, whereas the structured sections break down at a slower rate.

Bioremediation by *Achromobacter* sp.

Achromobacter (Alcaligenaceae family) is a bacterial genus belonging to the Burkholderiales order. The cells are straight rods motile by one to twenty percent of flagella. They are aerobic and may be found in fresh and saltwater and soil. Also recognized as a contaminant in laboratory cell cultures [68, 69]. In a study published in 2022, to expedite the biodegradation of thermo-oxidatively pretreated PVC and Low-Density Polyethylene (LDPE), researchers have successfully identified *Achromobacter denitrificans* from compost [70]. In bacterial flasks made of PVC and LDPE, the percentage of dry weight lost was 12.3% and 6.5%, respectively, and the amount of

extracellular protein was 326.4 and 112.32 mg/L, respectively. PVC underwent treatment that caused its pH to rise to 5.12, and its thermal stability was enhanced by 29 °C. Fourier Transform Infrared Spectroscopy (FTIR) results show that chain breakage in the major backbone, synthesis of new groups, and oxidation of antioxidants have all altered the chemical composition of LDPE. The carbonyl groups formed as a byproduct of LDPE breakdown are responsible for the appearance of peaks between 1700 and 1850 cm^{-1}. Scanning Electron Microscopy (SEM) verified surface changes in LDPE and PVC.

Another research found that a novel bacterial *Achromobacter xylosoxidans* influences the structure of High-Density Polyethylene (HDPE) [71]. By studying the coding sequences of the 16S ribosomal subunit, a hitherto undiscovered strain of *A. xylosoxidans* known as PE-1 was extracted from the soil and identified. Degradation of the HDPE chemical structure was seen in analyses of foil samples performed using SEM and Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR). As a consequence, HDPE foil was found to lose around 9% of its weight. On the basis of a comparison between the spectrum of the raw material before the bacterial treatment and the range from a database of spectra, it was anticipated that the microorganisms primarily depended on the HDPE for their carbon and energy needs.

Bioremediation by *Aspergillus* sp.

Fungi of the genus *Aspergillus* are often found living as saprophytes in the soil, where they consume dead plants and other organic matter, including seeds and grains. The individuals that belong to this genus can flourish in environments with high osmotic pressure. Because of the high oxygen tension, species of the genus *Aspergillus* may be found in almost all environments rich in oxygen. In these environments, they often take the form of moulds on the surface of the substrate [72–74]. An investigation was carried out on the biodegradation of black LDPE sheets by a fungus isolated from several Egyptian landfills [75]. For 16 weeks, minimum salt medium and LDPE sheets were heated to 30 °C and rotated at 120 rpm in a rotary shaker. The fungal strains *A. fumigatus* MF 276893 and *A. carbonarius* MH 856457.1 were found to be promising LDPE biodegradation agents. The sheet weight loss percentage was much higher in a mixed culture of the two strains compared to a single isolate. Physical and chemical treatments were also used to increase the degradation capacity. By 5.89% (chemical treatment), 17.76% (HNO_3 treatment), and 39.1% (heat treatment), biodegradation was found to be accelerated. New functional groups associated with hydrocarbon biodegradation were validated by FTIR, demonstrating the essential involvement of manganese peroxidase in the process. In addition to surface changes in biodegraded LDPE (as determined by SEM), differences in FTIR spectra of mixed culture biomass before and after biodegradation proved that LDPE was depolymerized. It has been reported that these strains are capable of complete biodegradation of plasticizers such as tributyl acetylcitrate, 1,2-benzenedicarboxylic acid diisooctyl ester, diisssctyl phthalate, and bis(2-ethylhexyl) phthalate, using Gas Chromatography-Mass Spectrometry (GC–MS). Another research determined five fungal isolates, including Brown rot, White rot, *A. flavus*, and *A. Niger* fungi isolated from various landfills in Peshawar, Pakistan [29]. Weight loss percentage analysis after 30 days of incubation was used to determine the biodegradation potential of these isolates against LDPE polymers. white rot, brown rot, *A. flavus*, and *A. niger* fungus all demonstrated biodegradation percentages of 22.7%, 18.4%, 16.1%, and 22.9%, respectively.

Further research used *Fusarium solani*, *A. versicolor*, and *A. flavus*, all of which were retrieved from a municipal waste yard in Chennai, India, to study the biodegradation of LDPE [76]. The polymers were tested for degradation by exposure to microbial cultures for 60 days in the lab. FTIR spectra verified the biodegradation of LDPE, whereas Field Emission Scanning Electron Microscopy (FESEM) micrographs demonstrated that the fungi had colonized the polythene matrix as a result of their metabolic activities. Sturm test results suggest *A. versicolor* strain is a more promising LDPE-degrading option than the *F. solani* and *A. flavus* strains. Under controlled laboratory conditions,

the biodegradation rate of LDPE sheets was measured after being inoculated with bacteria and fungi collected from various locations around the Dandora dumpsite [77]. Researchers incubated the LDPE sheets for 16 weeks at 37 °C for bacteria and 28 °C for fungus. *A.s oryzae* strain A5 showed the greatest fungal degradation activity, decreasing the average weight by 36.42±5.53%. Findings suggest that *Aspergillus*, *Bacillus*, and *Brevibacillus* are promising candidates for biodegrading LDPE. Moreover, a group of researchers extracted fungal candidates from a nearby dumping site. Mushrooms were grown in a broth made of mineral salts and LDPE powder. In broth medium supplemented with LDPE, only two (RH06 and RH03) of the nine isolates showed the maximum growth response. The findings showed that after 45 days of culture, there was a 5.13% drop in the weight of LDPE film when using isolate RH03, and there was a 6.63% decrease when using isolate RH06. In addition to this, the tensile strength of the treated film was found to be reduced by 58% over the board and 40% in each isolate. The LDPE film's surface developed a groove and a roughness, as shown by an electron microscopy analysis. Moreover, DNA sequencing and Polymerase Chain Reaction data confirmed that strains RH06 is *A. nomius and* RH03 is *Trichoderma viride,* with a 96% and 97% degree of similarity, respectively. The ability of *A. clavatus* to degrade LDPE in an aqueous medium was observed for 90 days [78]. PE mass loss, CO_2 evolution measured by the Strum test, FTIR, and SEM/Atomic Force Microscopy (AFM) morphological alterations all corroborated the deterioration. Researchers used enrichment culture and screening processes to identify two strains of *Lysinibacillus* sp. and *Aspergillus* sp. from waste soils in Tehran, which showed outstanding capacities to break down LDPE [79]. UV-irradiated and non-irradiated pure LDPE films without pro-oxidant additives underwent 126 days of biodegradation in soil with and without mixed cultures of selected microorganisms. As seen by carbon dioxide soil measurements taken after 126 days, biodegradation was moderate in the absence of microorganisms; UV-irradiated and non-UV-irradiated LDPE mineralization was only 8.6% and 7.6%, respectively. Biodegradation was much more effective when the targeted microorganisms were present, with biodegradation percentages for UV-irradiated and non-UV-irradiated films being 29.5% and 15.8%, respectively. When UV-irradiated LDPE was biodegraded in soil containing the designated microorganisms, the percentage decline in the carbonyl index was more pronounced. X-ray diffraction (XRD), FTIR, and SEM confirmed that the chosen microorganisms were able to alter and colonize both kinds of PE. An *A. flavus* fungi PEDX3 was identified from the digestive tract of the wax moth, *Galleria mellonella* [41]. The results of a 28-day incubation period demonstrated that strain PEDX3 was capable of breaking down HDPE MPP (microplastic particles) into the MPP with reduced molecular weight. As measured by FTIR, the breakdown of PE was further confirmed by the presence of carbonyl and ether groups of MPP. Additionally, Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was used to look for possible degradation enzymes. At the end of the degradation process, two genes, AFLA 053930 and AFLA 006190, encoding laccase-like multicopper oxidases, were found to have had their expression levels increase, indicating that they encode probable PE-degrading enzymes. In another investigation, *A. flavus* VRKPT2 and *A. tubingensis* VRKPT1 isolated from the PE trash deposited in marine coastal regions were tested under in vitro conditions to be efficient in HDPE breakdown [80]. The isolated fungus was identified based on internal transcribed space (ITS) homology sequence analysis. Even after 1 month of incubation, the biofilm development detected using an epifluorescent microscope revealed the vitality of fungal strains.

Bioremediation by *Bacillus* sp.

Bacillus species are rod-shaped, aerobic, sporulating bacteria abundant in nature. They may be either obligatory aerobes, which are oxygen-dependent, or facultative anaerobes and may thrive without oxygen [81]. A study was set out to determine how effective bacterial isolates were in degrading microplastics in the Vaigai River in Madurai, India [82]. After being properly processed, the isolates were included in the degradation of UV-treated PE and PP. Four

bacterial isolates, including *Bacillus* sp. (BS-2), *Bacillus paramycoides* (BP), *Bacillus cereus* (BC), and *Bacillus* sp. (BS-1), passed the first screening and were evaluated for the 21-day degrading experiment. Bacterial isolates were stuffed into the microplastics, and a shake flask experiment was conducted using two different methods, each with a control. Degradation of the microplastics was demonstrated by a decrease in their weight, an increase in their fragmentation, and a shift in their surface area compared to control studies (microplastics without isolates). Although PP degradation was most significant with BP (78.99 ± 0.005%) and BC (63.08 ± 0.009%) when used separately, the greatest PP and PE degradation were achieved when BC and BP were used together (78.62 ± 2.16% and 72.50 ± 20.53%).

Activated sludge was studied as a potential biocatalyst for the degradation of microplastics in water [83]. It was initially tested for its ability to hydrolyze PET polymers pretreated at 100 °C for an hour. To assess degradation potential, the consortium undertook a typical CO_2 evolution test at pH 7–7.5, 30 °C, 168 days reactor residence time, and 2.63 g/L PET concentration. After being incubated, the group was able to break down 17% of the PET. Surface erosion was responsible for the unaltered molecular weight. Biodegradation was also noticeably accelerated in the presence of abundant oxygen. *Agromyces mediolanus* PNP3 and *B. cereus* SEHD031MH were discovered to be two of the consortium's isolated bacterial strains. Even though growth was optimal for both strains when grown on PET medium alone, only *B. cereus* showed enzyme activity in a clear-zone assay. The bacterial degradation of polyhydroxybutyrate (PHB) was studied in a solid-media culture setting over a range of temperatures and salinities [53]. After 14 days of cultivation on PHB film, studies show that Bacillus sp. JY14 can destroy around 98% of PHB. This species was shown to be able to biodegrade P(3HB-*co*-3HV) and P(3HB-*co*-4HB).

In a study, sixty marine bacteria were tested for their capacity to digest LDPE [84]. When tested using polythene as the only carbon source for growth, only three were discovered to be effective. Positive isolates were identified by comparing their 16S rRNA gene sequences. The researcher determined that they belonged to the genus *Kocuria*, species M16; genus *Bacillus*, species M27; and genus *Bacillus subtilis*, species H1584. During a 30-day incubation period with H1584, M27, and M16 isolates, PE lost 1.7%, 1.5%, and 1% of its weight. Hydrophobicity on the cell surface was highest (32% in M16), then 15% in H1584, and finally 27% in M27. A triphenyltetrazolium chloride reduction assay was used to verify the vitality of the isolates grown on the PE surface. Calculations of the Keto Carbonyl Bond Index, Ester Carbonyl Bond Index, and Vinyl Bond Index from FTIR spectra showed increases consistent with PE biodegradation.

Bioremediation by *Collectotrichum* sp.

Colletotrichum (sexual stage: Glomerella) is a genus of endophytes or phytopathogens that are symbionts to plants. Some species in this genus may have a symbiotic relationship with their host plants [85]. Thirty fungi were tested for biodegradability of LDPE films in mineral salt medium agar [86]. *Stagonosporopsis citrulli*, *Collectotrichum fructicola*, *Thyrostroma jaczewskii*, and *Diaporthe italiana* grew much faster than *Aspergillus niger* when grown on LDPE film as the sole carbon source. For a further 90 days, they were grown in a broth made of mineral salts and LDPE film instead of any other carbon source. CO_2 emissions ranged from 0.45 to 1.45 g/L for *D. italiana*, 0.36 to 1.22 g/L for *T. jaczewskii*, 0.33 to 1.26 g/L for *C. fructicola*, 0.37 to 1.27 g/L for *S. citrulli*, and 0.33 to 1.27 g/L for *A. niger* when they were cultured on LDPE film. Compared to the levels of lignin peroxidase and manganese peroxidase secreted by the same fungus, the quantity of laccase enzyme produced was reported to be much higher. It was further investigated how these fungi degrade LDPE sheets when cultured. Weight loss was recorded as 28.78, 45.12, 48.78, 46.34, and 43.90%; tensile strength as 3.34, 1.86, 0.43, 1.78, and 1.56 MPa for LDPE films cultured with *A. niger*, *S. citrulli*, *C. fructicola*, *T. jaczewskii*, and *D. italiana*, respectively. After incubation with various fungi, especially *C. fructicola*, FTIR measurement revealed an increased carbonyl index in LDPE films. The biodegradation

of LDPE films was validated by SEM analysis, which revealed morphological changes on the film's surface, including cracks, scions, and holes. The Volatile Organic Compounds, 1,1-dimethoxy-decane, 1,3-dimethoxy-5-(1-methylethyl)-benzene, and 1,3-dimethoxy-benzene were found in these fungi. In terms of biodegradation of LDPE, *C. fructicola* shows promise as a resource and may be incorporated in fungal-based plastic degrading systems.

Bioremediation by *Comamonas sp.*

Gram-negative, rod-shaped spirilla (often called “rods”) are found in bacteria of the genus *Comamonas*. These microorganisms are chemoorganotrophic, meaning they feed off of organic matter rather than sugars, and they are aerobic [87]. The breakdown of dimethyl phthalate (DMP) by a *Comamonas testosterone* bacterial strain, DB-7, was investigated in a study [88]. The results indicate that DMP at varying doses was quickly destroyed, with over 99% degradation occurring within 14 h at 450 mg/L. The breakdown rate of DMP was found to be positively proportional to the inoculum volume of the bacteria, with the ideal degradation temperature being 30–35 °C and pH 9.0, respectively. According to HPLC (High-performance liquid chromatography) and LC/MS (Liquid Chromatography-Mass Spectrometry) studies of metabolic products, phthalic acid (PA) and mono-methyl phthalate (MMP) are the primary degrading intermediates formed by DB-7 during the breakdown of DMP.

Bioremediation by *Enterobacter* sp.

The genus *Enterobacter* includes rod-shaped, non spore-forming, gram-negative, facultatively anaerobic bacteria of the Enterobacteriaceae family. The type genus of the family Enterobacterales [89]. A group of researchers conducted research on the breakdown of LDPE by the recently discovered Enterobacter cloacae AKS7 [90]. A progressive rise in Extracellular Polymeric Substance (EPS) production by the organism (AKS7) was also identified, indicating the establishment of an effective biofilm on the LDPE surface. In addition, two AKS7 mutants with significantly reduced cell-surface hydrophobicity compared to their wild type were screened. The results of which contrasted to wild-type AKS7 cells, the mutants exhibited lower levels of LDPE breakdown. Further analysis showed that, in contrast to wild-type cells, AKS7 mutant cells lacked the ability to adhere to LDPE. The findings showed that AKS7's hydrophobic cell surface promotes the growth of microbial biofilm on LDPE, leading to more efficient breakdown of the plastic by the microbe. Given these results, the organism may be evaluated as a bio-remediating agent for the long-term degradation of polythene-based toxic waste.

Bioremediation by *Halomonas sp*.

Halomonas is a genus of salt-tolerant (halophilic) bacteria. They are rod-shaped gram-negative bacteria and develop in the presence of oxygen. However, it has been reported that some may grow without oxygen [91]. Four bacterial strains with the ability to biodegrade LDPE were identified by a research group [92]. The 16S rRNA gene sequencing technique indicated that bacterial isolates H-265, H-256, H-255, and H-237 were closely related to *Alcanivorax* sp., *Exigobacterium* sp., *Halomonas* sp., and *Cobetia* sp., respectively. Researchers used the Bushnell-Haas medium to incubate these bacterial strains separately for 90 days while providing them with LDPE sheets as a carbon source. Bacterial isolates were able to develop a viable biofilm on the surface of LDPE during the biodegradation experiment, reducing the films' thermal stability. After the incubation research, the bacterial isolate H-255 was shown to have caused a maximum LDPE film weight decrease of 1.72%. FESEM and AFM demonstrated that bacterial adhesion to the film altered its physical structure (surface erosion, roughness, and deterioration). When compared to a control LDPE film, the spectra obtained using ATR-FTIR demonstrated a shift in the peaks associated with C–H stretching and C=C bond stretching and the development of additional peaks associated with C–O stretching and –C=C– bond stretching. Furthermore, carbon remineralization and enzymatic activity validated the biodegradation of LDPE film. This research demonstrated that some marine bacteria actively biodegrade LDPE film, and that these bacteria have the potential to lessen marine plastic pollution.

Bioremediation by *Klebsiella sp.*

The gram-negative, encapsulated, non-motile, facultatively anaerobic, lactose-fermenting, rod-shaped bacterium *Klebsiella pneumoniae* is characterized by its unique characteristics. It occurs naturally in the soil, and around 30% of strains are capable of fixing nitrogen under anaerobic environments [93]. *Klebsiella pneumoniae* CH001, a clinical isolate, was screened for bioremediation of HDPE [94]. After 60 days of growth in nutritional broth at 30 °C and 120 rpm, results indicated that this strain could develop a substantial biofilm on HDPE surfaces. The Universal testing machine (UTM) results indicated a considerable drop in HDPE film's tensile strength (60%) and weight (18.4%). In addition, SEM research revealed surface fractures in the HDPE, while AFM findings demonstrated an increase in surface roughness during bacterial incubation. Taken together, findings suggest that *K. pneumoniae* CH001 is a promising option for the environmentally responsible breakdown of HDPE in natural settings.

Bioremediation by *Penicillium* Sp.

Penicillium is a genus of ascomycetous fungus that is an integral component of the mycobiome of several species. Certain species of the genus generate penicillin, an antibiotic chemical that kills or inhibits the development of certain types of bacteria. Other species are used in cheese production. According to the tenth edition of the Dictionary of the Fungi (2008), the broad genus has more than 300 species [95]. Because of its rapid colony development in the screening medium, the isolate *Penicillium citrinum* was chosen for biodegradation research. In a research, 16 plastic-degrading fungi were isolated from plastic-laden landfill soil in Bhopal, India [44]. Fungi capable of decomposing PE were screened for using a mineral salt agar medium spiked with 3% LDPE powder. Untreated LDPE fragments lost 38.82 ± 1.08% of their weight when exposed to *P. citrunum*; however, after being pretreated with nitric acid, biodegradation increased by 47.22 ± 2.04%. New functional groups ascribed to hydrocarbon biodegradation appeared in FTIR spectra, suggesting enzymatic participation in the process. Depolymerization of LDPE was validated by changes in the FTIR spectra and FE-SEM of LDPE samples (both untreated and pretreated) before and after biodegradation. Variations in the rates of thermal breakdown between biodegraded and control samples provide more evidence of biodegradation. The remarkable competence of *P. citrinum* in LDPE degrading without any pre-treatment has been reported for the first time in this work.

To effectively biodegrade polyvinyl alcohol (PVA) in vitro, researchers set out to discover and broadly screen endophytic fungi (from specified plants) [42]. Seventy-six endophytic fungi were cultured in total on a PVA screening agar medium. Using a combination of phenotypic traits, ITS ribosomal gene sequences, and phylogenetic analysis, 10 isolates were found to have a potential biodegrading effect and were subsequently identified. After 10 days of growth at 150 rpm and 28 °C, four strains showed maximal PVA-degradation in the liquid medium. *Penicillium brevicompactum* OVR-5 removed 81% of PVA, *Talaromyces verrucosus* PRL-2 removed 67%, *Penicillium polonicum* BJL-9 removed 52%, and *Aspergillus tubingensis* BJR-6 removed 41%. OVR-5 was found to be the most promising PVA biodegradation isolate, producing laccase, manganese peroxidase, and lipase enzymes at an ideal pH of 7.0 and an optimal temperature of 30 °C. This work hypothesized a possible PVA breakdown mechanism for OVR-5 in light of investigations of its metabolic intermediates, which GC–MS discovered. Both SEM and FTIR verified the biodegradation findings.

The antarctic filamentous fungus was studied for its ability to degrade polyurethane (PU), polystyrene (PS), and PE samples in a liquid solution [96]. Plastic samples were either inoculated with Antarctic fungus (*Mortierella*, *Geomyces*, *Penicillium* species), treated, left untreated, or artificially aged in a UV chamber for 500 h per ASTM G155. All samples were kept in an incubator for 90 days at 18 °C. The rate of weight loss was examined as a function of time to evaluate the physical–chemical and biological degradation of plastics. In the artificial ageing

chamber, polymers suffered an oxidative breakdown, which sped up their biodegradation (seen as morphological and structural alterations). *Penicillium* sp., of the three fungal strains, showed the most significant breakdown at 28.3% in PU, 8.39% in PS, and 3.5% in LDPE.

In a study, the researcher used garbage bags to isolate fungi and their ability to degrade LDPE. In this case, ethanol-treated LDPE was used alongside untreated LDPE [43]. F1 isolation demonstrated the most degradation out of the three fungal isolates, and this isolate damaged the untreated sheet similarly. Areas of degradation were seen in the surface morphology of F1-treated LDPE as analyzed by SEM. FTIR testing revealed that F1 affected the polymer's production of carbonyl and C=C groups. F1 fungus, when grown in the laboratory, was discovered to release the lipase enzyme. Molecular testing confirmed that isolate F1 was indeed *P. simplicissimum* strain Bar2. In another study, *P. simplicissimum* was discovered in a Shivamogga district landfill by a group of researchers [97]. Findings indicate that treated PE (38%) was more easily degraded by *P. simplicissimum* than autoclaved (16%) or surface-sterilized (7.7%) PE. *P. simplicissimum* was tested for enzymes that degrade PE. Laccase and manganese peroxidase were shown to be active enzymes. Based on these findings, *P. simplicissimum* was reported as a potential answer to the world's PE crisis.

A group of investigators evaluated "Bionolle®" polyester-modified PET films biodegradation in comparison with unmodified PET films in terms of time to decompose [98]. The films' weight was recorded before and after being incubated with the filamentous fungus *P. funiculosum* or their extracellular hydrolytic enzymes released by "Bionolle®" for 84 days. FTIR and X-ray Photoelectron Spectroscopy (XPS) studies revealed significant chemical alterations in polymeric chains. In addition to hydrolytic enzymes, oxidative ones were likely involved in the degradation of films by fungi, as shown by the significant decrease in the number of aromatic rings formed from terephthalic acid. Additionally, "Bionolle®" did not accelerate modified film deterioration.

Bioremediation by *Phanerochaete sp.*

Phanerochaete is a crust fungus genus belonging to the Phanerochaetaceae family. It has historically been classified based on the fruit body's general shape and microscopic features, such as the spores, cystidia, and hyphal structure. According to molecular analyses, the genus is polyphyletic, with members scattered over the phlebioid clade of the Polyporales order [99, 100]. A study examined the biodegradability of starch-blended PVC films using controlled laboratory studies utilizing selected fungus isolates and *in-situ* burial in soil [101]. SEM revealed the surface anomalies such as colour change and mild disintegration in PVC films after 90 days. Isolation of fungal strains characterized by robust growth and adhesion to plastic sheets. *Phanerochaete chrysosporium* PV1 was chosen among the strains exhibiting the highest levels of activity and later confirmed to be this species by rDNA sequencing. FTIR and Nuclear Magnetic Resonance (NMR) studies revealed new peaks, suggesting substantial structural changes and transformation in the films. Gel permeation chromatography (GPC) backed this up by showing a considerable reduction in the molecular weight of polymer film from 80,275 to 78,866 Da (treated). The release of more carbon dioxide (7.85 g/l) than the control (2.32 g/l) in the respirometric technique provided further evidence of the biodegradation of starch-blended PVC films. Hence, suggesting *P. chrysosporium* PV1 is a fungal strain with excellent potential for bioremediation of plastic waste.

Bioremediation by *Pseudomonas* sp.

There are 191 different species of the genus *Pseudomonas*, which are all gram-negative gamma-proteobacteria in the family Pseudomonadaceae. Members of this genus exhibit a high degree of metabolic variability, allowing them to colonize a wide variety of habitats [102, 103]. It is suggested by research that the *Pseudomonas* sp. found in the digestive tracts of superworms might effectively biodegrade Polyphenylene sulphide (PPS) [49]. The biodegradation time of the bead form of plastic was drastically reduced due to its superior degradation efficiency compared to the

standard film type of plastic. Therefore, this work employed plastic beads with a diameter of 300 m to assess the *Pseudomonas* sp. mediated PPS biodegradation over 10 days instead of film-type plastics. This technology not only compares and verifies the biodegradation performance of different polymers in 10 days, but it also quickly identifies the best bacteria for plastic biodegradation.

As reported, another research set out to examine the biodegradation capabilities of five bacterial strains against PVC, PS, PP, and PE films under aerobic conditions [51]. A generalised aerobic breakdown mechanism for plastics is shown in Fig. 2. *B. flexus* and *P. citronellolis* were chosen as suitable PVC film degraders after preliminary screening. Biodegradation of PVC films was tested using the two strains in 2-L flasks. Fragmentation of the film was found after 45 days of incubation, indicating PVC biodegradation. PVC incubated with *P. citronellolis* had a 10% decrease in average molecular weight, as determined by GPC, suggesting that PVC polymer chains were attacked. These findings led to the selection of the *P. citronellolis* strain for biodegradation experiments. As determined by chemical evaluation of the films after 30 days of incubation, the waste PVC polymers had biodegraded, resulting in a gravimetric weight loss of up to 19%. In conclusion, this study documents *B. flexus* and *P. citronellolis* ability to biodegrade PVC sheets. Both strains were shown to have a negligible effect on PVC polymer, suggesting that they work primarily against PVC additives.

Fig. 2 [Images not available. See PDF.]

A generalised aerobic breakdown mechanism for plastics

A soil-dwelling bacteria capable of degrading polyester PU was isolated and characterized as strain MZA-85 [104]. It was determined that the bacterium was *Pseudomonas aeruginosa* by 16S rRNA gene sequencing. The strain MZA-85 altered the surface morphology of PU film, as shown in SEM. The FTIR spectrum exhibited an augmentation of the organic acid functional group and a concomitant diminution of the ester functional group. Results from GPC showed a rise in polydispersity, suggesting that microorganisms break down PU polymer chains. After conducting a p-Nitrophenyl acetate hydrolysis experiment, it was discovered that the bacteria produced cell-associated esterases. GC–MS confirmed the synthesis of adipic acid and 1,4-butanediol monomers. Cell growth in the presence of breakdown products and the Sturm test showed that strain MZA-85 mineralized PU into H_2O and CO_2. These results suggest that strain MZA-85 and its enzymes may recycle pure monomers in biochemical monomerization. On the other hand, *Pseudomonas* sp. AKS2 can degrade 5 ± 1% LDPE in 45 days without pre-oxidation, which is much quicker than the degradation rates reported in previous investigations [105]. This could be attributable to agents modifying the hydrophobic contact between the polythene and the microbe, which may affect the breakdown rate. Accordingly, this research links the capacity for biofilm formation among bacteria to their ability to degrade polymers and shows a connection between hydrophobic contact and polymer breakdown.

Bioremediation *by Rhizopus* sp.

The fungus genus *Rhizopus* is well-known for its extensive plant saprophytic and its role as a specialist animal parasite. They are present in several organic things, including mature fruits and vegetables, tobacco, peanuts, bread, leather, syrups, and jellies [95]. A fungal lab isolate, *Rhizopus oryzae* NS5 was studied for the biodegradation of LDPE [106]. One month of incubation in a potato dextrose broth at 120 rpm and 30 °C resulted in the development of fungi on the surface of PE. Approximately 8.4 ± 3% of the weight and 60% of the tensile strength of PE was shown to decrease gravimetrically. The SEM study of the PE surface revealed hyphal penetration and degradation. After fungal isolation, AFM showed increased surface roughness. Fungal hyphae formed a biofilm on PE fragments. This research demonstrates the potential of *R oryzae* NS5 for eco-friendly and sustainable PE breakdown.

Bioremediation by *Streptomyces* sp.

Streptomyces is the most populous genus of Actinomycetota and the type genus of the Streptomycetaceae family. There are around 500 recognized species of *Streptomyces* bacteria. The genomes of streptomycetes are gram-positive. Most streptomycetes generate spores and have a unique “earthy” odour due to the synthesis of the volatile metabolite geosmin, primarily found in soil and decomposing plant matter [107]. As PET trash, drinking bottles were pulverized and categorized into four particle sizes in research work. In their work, they investigated the biodegradation of PET by *Streptomyces* species [47]. Extracted samples totaling 50 mg were divided into four groups based on particle size, each of which was then incubated with a different set of microorganisms in a culture medium at 28 °C for 18 days. Degradation values were then calculated on particular days. The biodegradation percentages for 500, 420, 300, and 212 m PET particle sizes were reported to be 49.2%, 57.4%, 62.4%, and 68.8%, respectively. To further verify the biodegradation process, the byproducts were analysed by GC–MS. Experimental results may be better predicted using the Michaelis–Menten activation or inhibition model, according to the kinetic modelling of biodegradation.

Researchers isolated microorganisms from Andhra Pradesh and Telangana waste soil to prevent plastic buildup and rid the environment of plastic [108]. The degrading activity of these microorganisms is determined using the clear zone approach and polythene powder. Changes in the granules' physical and structural properties occurred over time after microbes had attached to polymer particles. To determine the effectiveness of biodegradation, the weight technique was used in the laboratory for 2, 4, and 6 months. Experimental results demonstrated that *Streptomyces* sp. had the greatest plastic degradation ability, degrading up to 46.7%; this was followed by *A. flavus* (16.45%), *Pseudomonas* sp. (24.22%), and *A. niger* (26.17%) during a 6-month period. The results of this study demonstrate the critical function that Streptomyces sp. plays in the breakdown of polythene powder and polymer granules.

Bioremediation by *Zalerion sp.*

The marine fungus, *Z. maritimum*, was discovered in the waters off the coast of Portugal [109]. The researcher assessed mass changes in the fungus *Zalerion maritimum* and PE pellets after different exposure times in a minimum growing medium [110]. Results indicated that *Z. maritimum* is able to use PE under test circumstances, resulting in a reduction in both pellet mass and size. These results point to a naturally occurring fungus that, with its low food requirements, might play an active role in the biodegradation of microplastics.

Bioremediation by the synergistic effect

Scientists evaluated the PET-associated lipase activity of bacteria isolated from petroleum-contaminated soils [48]. Bacterial strains and consortiums were cultivated on a liquid carbon-free basal medium (LCFBM) using PET as the only carbon source. Consistent with the ATR-FTIR findings, this work found hydrolysis byproducts of PET using ^{1}H NMR analysis. Together, PET and its cleavage product bis(2-hydroxyethyl) terephthalic acid (BHET) supported the growth of five strains of *Bacillus* and *Pseudomonas* species. The consortium's secreted enzymes could completely convert BHET to the biologically functional monomers ethylene glycol and terephthalic acid (TPA). Strains with different enzymatic abilities for the metabolic breakdown of ethylene glycol and TPA, the building components of PET polymers, were discovered in draught genomes, cooperating and cross-feeding in a nutrient-limited environment utilising PET as the primary carbon source.

Two distinctive cultures of *Arthrobacter* and *Streptomyces* sp. were extracted from farming soils and found to thrive solely on PE film [45]. The suspension phase of culture was very fruitful for the growth of *Arthrobacter* sp. Streptomyces sp. produced huge biofilms on the PE film, showing that the two strains had distinct metabolic types and lived in different microenvironments with differing nutritional availability. CO_2 evolution, increased carbonyl index, reduced hydrophobicity, and the biofilm development on the film surface were all indicators that a 90-day

inoculation experiment might deteriorate PE film. However, a combination of the two strains had a far greater effect on these negative characteristics.

Yet another study utilized a synergistic system consisting of *Thermobifida fusca cutinase* (TfC) and *Microbacterium oleivorans* JWG-G2 to decompose a high crystalline PET film and BHET oligomers [111]. Ethylene glycol terephthalate (EGT) has been discovered as the unique degradation product of *M. oleivorans* JWG-G2 alone. The synergy degrees for the degradation of PET film and BHET oligomers with the addition of TfC as a second biocatalyst were determined to be 2.26 and 2.79, respectively. After treating PET film with *M. oleivorans* JWG-G2 at 5×10^3 μL/cm^2 and TfC at 120 g/cm^2, the highest concentrations of TPA (47 nM) and mono(2-hydroxyethyl) terephthalate (MHET) (330 nM) were found. The degree of surface degradation of PET film was higher than that generated by each treatment on its own. Synergistic microbe-enzyme treatment is based on the occurrence of extracellular PET hydrolases, and a whole genome sequencing research of *M. oleivorans* JWG-G2 showed the presence of extracellular PET hydrolases, including three a lipase, an esterase, and carboxylesterases.

Microplastics composed of LDPE and PS were biodegraded using pure bacterial strains *Lysinibacillus massiliensis* and *Bacillus licheniformis*, as well as a mixed bacterial culture of *Bacillus* sp. and *Delftia acidovorans* [112]. The biodegradation of Microplastic-PS and Microplastic-LDPE with particle sizes between 300 and 500 μm was evaluated for 22 days at 25 ± 2 °C, 7.15 pH, and 160 rpm. Microplastic-LDPE and Microplastic-PS were both more efficiently decomposed by mixed bacterial cultures than by pure bacterial cultures, and the biodegradation efficiency of Microplastic-LDPE was found to be larger than that of Microplastic-PS, as evidenced by a greater decrease in peak intensity and spectrum distortion, as well as higher inorganic carbon values and colony forming unit.

In another research breakdown of Linear Low-Density Polyethylene (LLDPE) plastic using a microbial culture comprising *Brevibacterium* sp. and *Pseudomonas aeruginosa* was studied [50]. Pieces of 1 × 1 cm^2 LLDPE plastic weighing 10 g were placed in containers containing Nutrient Broth growing material. Gravimetric test at pH 7.0, 25 °C for 30 days demonstrated that a mixed bacterial culture could degrade LLDPE plastic by 2–7%. The results of this study show that LLDPE plastic may be degraded by mixed bacterial cultures by being used as a carbon source.

Also, novel thermophilic consortiums of *Aneurinibacillus* sp. and *Brevibacillus* sp. isolated from sewer treatment plants and waste management landfills were evaluated for their ability to degrade PP, HDPE, and LDPE films and pellets [113]. Over the course of 140 days, researchers tested the degradation ability of 36 plastic-degrading isolates. To test the efficacy of degradation, multiple combinations of the eight isolating factors that showed the highest percentage of degradation were tested. For the three types of plastic that were selected for further examination of degradation under varying temperature settings, the combination of IS1, IS3, ISA, and ISC revealed the best % weight loss. At 50 °C, the weight reduction percentages for PP, LDPE, and HDPE strips treated with the consortia of four isolates were 56.3 ± 2, 46.6 ± 3, and 58.21 ± 2% and, for pellets treated with the consortia, they were 44.2 ± 3, 37.2 ± 3, and 45.7 ± 3%, respectively ($p \leq 0.05$). After 140 days, new adsorption bands could be seen by FTIR scanning of the plastic sheet. AFM and SEM showed biofilm formation and structural alterations on treated plastic strips, while Energy Dispersive X-ray Spectroscopy (EDS) showed a considerable drop in carbon content. NMR revealed methyl and aldehyde groups, whereas GC–MS showed fatty acid byproducts. Four strains—ISC, ISA, IS3, and IS1—identified as *Brevibacillus brevis* btDSCE04, *Brevibacillus* sp. btDSCE03, *Brevibacillus agri* btDSCE02, and *Aneurinibacillus aneurinilyticus* btDSCE01, respectively were found (Table 1).

Table 1. An overview of microbial bioremediation of plastic waste

Type of plastic	Microorganism	Place of isolation of microorganisms	Degradation temperature	Degradation time	Microorganism isolation media	Reported degradation	Researchers	References
PE	*Aspergillus flavus*	The gut of wax moth *Galleria mellonella*	24 ± 3 °C	14 d	SCS	Up-regulated expression	Zhang et al. (2020)	[41]
Streptomyces sp. and *Arthrobacter* sp.	Agricultural soils	25 °C	90 d	CDM and LCFBM	The weight losses of plastic films ranged from 0.22 to 0% in CDM and from 0.49 to 0% in LCFBM medium	Han et al. (2020)	[45]	*Penicilliumsimplicissimum*

Local dumpsite of Shivamogga district	–	3 months	NH_4NO_3 + K_2HPO_4 + NaCl + $MgSO_4.7H_2O$ + agar	Rather than autoclaved (16%) or surface (7.3%) polyethene degradation is 38% more effective	Sowmya et al. (2015)	[97]	*Zalerion maritimum*	Marine
25 °C	28 d	Glucose + malt extract + peptone	43 % degradation	Paço et al. (2017)	[110]	LDPE	*Penicillium citrinum*	Municipal landfill soil of Bhopal, India

		Mineral salt agar medium	38.82 ± 1.08% weight loss	Khan et al. (2022)	[44]	*Alcanivorax* sp., *Exigobacterium* sp., *Cobetia* sp., and *Halomonas* sp.	Water column from Diu Island, India	30 °C
60 d	ZMB and ZMA	1.76% weight loss	Khandaret al. (2021)	[92]	*A. fumigatus* MF 276893 and *Aspergillus carbonarius* MH 856457.1	Landfills sites in Sharqiyah Governorate, Egypt	30 °C	16 weeks
Minimal salt agar and CDM (without sucrose)	The biodegradation rates after thermal, HNO_3 and Gamma-irradiation treatment were 39.1%, 17.76%, and 5.79%, respectively	El-Sayed et al. (2021)	[75]	*Collectotrichum fructicola*	Thailand Institute of Scientific and Technological Research, Bangkok, Thailand	27.0±2.0 °C	90 d	MSM

LDPE films cultured with *A. niger, S. citrulli*, *C. fructicola*, *T. jaczewskii*, and *D. italiana* showed weight loss of 28.78%, 45.12%, 48.78%, 46.34%, and 43.90%, respectively	Khruengsai et al. (2021)	[86]	Brown rot, White rot, *Aspergillus flavus*, and *Aspergillus Niger*	Disposal sites at Peshawar, Pakistan	28 °C	30 d	PDA	Weight loss percentage showed that white rot, Brown rot, *Asp*

								e r g il l u s fl a v u s , a n d *A s p e g il l u s N i g e r* s h o w e d 2 2 . 7 % , 1 8 . 4 % , 1 6

								.1%, and 22.9% biodegradation, respectively

Hyder et al. (2021)	[29]	*Penicillium simplicissimum*	Municipality garbage	28 ± 2 °C	150 d	PDA and RBA (Rose Bengal Agar)	F1 and F2 lost 28.72 ± 2.55 and 12.96 ± 2.00 percent of their initial weight compared to untreated LDPE after 80 d	Ghosh and Pal (2021)
[43]	*Enterobacter cloacae* AKS7	Agricultural land of South 24 Parganas, West Bengal, India	30 °C	45 d	Yeast extract + $MgSO_4 \cdot 7H_2O$ + $(NH_4)_2SO_4$ + $CaCl_2 \cdot 2H_2O$ + $FeSO_4 \cdot 7H_2O$ + NaCl + $Na_2WO_4 \cdot 2H_2O$ + $Na_2MoO_4 \cdot 2H_2O$ + K_2HPO_4 + $MnSO_4$ + KH_2PO_4	Cell-surface hydrophobicity	Sarker et al. (2020)	[90]

Bacillus subtilis H1584, *Bacillus pumilus* M27 and *Kocuria palustris* M16	Pelagic waters, Arabian Sea, India	–	30 d	Bushnell–Haas medium	M16 has a hydrophobicity of 32% on its cell surface	Harshvardhan and Jha (2013)	[84]	*Fusarium solani*, *Aspergillus versicolor*, and *Aspergillus fla*

								vus,
Municipal dump yard in Chennai, India	25 °C	60 d	SDA	17% weight loss by *A. flavus*, 19% by *A. versicolor* and and 13% by *F. solani*	Das et al. (2018)	[76]	*Aspergillus*, *Bacillus* and *Brevibacillus*	Dandora dumpsite Nairobi-Kenya

37 °C	16 weeks	Bacteria: 15% glycerol slants; Fungi: PDA	*Aspergillus oryzae* strain A5, 1 (MG779508) was responsible for a 36.4 ± 5.53% decrease in average weight	Muhonja et al. (2018)	[77]	*Trichoderma viride* and *Aspergillus nomius*	Local landfill soil in Medan	26 ± 2 °C
45 d	SDA	LDPE film weight was decreased by 5.13% and 6.63%, respectively when treated with RH03 and RH06	Munir et al. (2018)	[40]	*Rhizopus oryzae* NS 5	Lab isolated	30 ° C	30 d

PDA	A reduction in weight of 8.4 ± 3.0% and a weakening in tensile strength of 60%	Awasthi et al. (2017a)	[106]	*Aspergillus clavatus* JASK1	Landfill soil	25–30 °C	90 d	PDA

35% weight loss	Gajendiran et al. (2016)	[78]	*A. flavus*, *Pseudomonas* sp., *Aspergillus niger*, and *Streptomyces* sp.	Andhra Pradesh and Telangana area's garbage soil	30–35 °C	6 months	MSM	The plastic degradation capacity of *Streptomyces* sp. is the egr

								eatest, at 46.70%; this is followed by the plastic degradation

capacities of *Aspergillus niger* (26.17%), *Pseudomonas sp.* (

								24.22%), and *A. flavus* (16.45%)
Deepika et al. (2015)	[108]	*Pseudomonas* sp. AKS2	Kolkata municipal solid waste dumping ground soil	30 °C	45 d	Yeast + $(NH_4)_2SO_4$ + $MgSO_4 \cdot 7H_2O$ + NaCl + $CaCl_2 \cdot 2H_2O$ + $FeSO_4 \cdot 7H_2O$ + $Na_2MoO_4 \cdot 2H_2O$ + $Na_2WO_4 \cdot 2H_2O$ + $MnSO_4$ + K_2HPO_4 + KH_2PO_4	5 ± 1% weight loss	Tribedi and Sil (2013)

[105]	*Aspergillus niger* and *Lysinibacillus xylanilyticus*	Landfills	30 °C	126 d	Mineral medium-agar	29.5% weight loss for the UV-irradiated films and 15.8% for non-UV-irradiated films	Esmaeili et al. (2013)	[79]
LDPE and PP	*Bacillus cereus* and *Bacillus paramycoides*	Vaigai River, Madurai, India	–	21 d	–	–	Nanthini Devi et al. (2021)	[82]
LDPE and PS	*Lysinibacillus massiliensis* and *Bacillus licheniformis*, and a mixed bacterial culture of *Bacillus* sp. and *Delftia acidovorans*	Municipal wastewater treatment plant Vrgorac—Split—Dalmatia County, and the sediment from the river Kupa, Karlovac County	25 ± 2 °C	22 d	MSM	Microplastic-LDPE and Microplastic-PS were degraded more effectively by mixed bacterial cultures than by pure bacterial cultures	Kučić Grgić et al. (2021)	[112]
LDPE and PVC	*Achromobacter denitrificans* Ebl13	Compost	–	–	–	12.3% (PVC) and 6.5% (LDPE) weight loss	Maleki Rad et al. (2022)	[70]
LLDPE	*Pseudomonas aeruginosa* and *Brevibacterium* sp.	Laboratory isolate	25, 30, and 35 (°C)	30 d	Nutrient Broth	Weight reduction of 5.22% occurred at 25 °C	Dwicania et al. (2019)	[50]
HDPE	*Klebsiella pneumoniae* CH001	Plastic waste dumpsite, Diesel Locomotive Works (DLW), Varanasi, India	30 °C	60 d	Nutrient agar	60% reduction in tensile strength and an 18.4% decrease in weight	Awasthi et al. (2017b)	[94]

Aspergillus sp.	Gulf of Mannar, India	30 °C	30 d	PDA	In fungi isolates, VRKPT1 caused a weight loss of 6.02 ± 0.2%, whereas VRKPT2 caused a loss of 8.51 ± 0.1%	Devi et al. (2015)	[80]	*Achromobacter xylosoxidans*
Landfill along the Mleczna River in Radom, Poland	27 °C	50 d	CDM and the modified Davis Minimal Broth medium	Mass loss percentages varied from 3.64 to 9.38%, with an average loss of 6.10 ± 0.13%	Kowalczyk et al. (2016)	[71]	PVA	*P. brevicompactum*

	30 °C		PVA screening agar medium	–	Mohamed et al. (2022)	[42]	PET	*Aspergillus* sp. NP3 and *Bacillus cereus* SEHD031MH

Activated sludge	30 °C	168 d	Yeast extract + trace elements	17% of PET degradation	Torena et al. (2021)	[83]	*Pseudomonas* and *Bacillus* Species	Petroleum-polluted soils
30 °C	40 d	Rhodamine B agar containing olive oil	3% weight loss	Roberts et al. (2020)	[48]	*Streptomyces* species		28 °C
18 d		Sizes 500, 420, 300, and 212 μm in PET particles showed biodegradation percentages of 49.2%, 57.4%, 62.4%, and 68.8%, respectively	Farzi et al. (2019)	[47]	PET modified with polyester “Bionolle”	*Penicillium funiculosum*	Dump in Sosnowiec	30 °C
84 d	CDM	30-fold higher weight loss	Nowak et al. (2011)	[98]	BHET and PET	*Thermobifida fusca cutinase* and *Oleivorans* JWG-G2	Laboratory isolate	35 °C

16 d	MSM	BHET oligomers had 2.79 synergy and PET film degradation 2.26	Yan et al. (2021)	[111]	PU, PS, and PE	*Penicillium*, *Geomyces*, *Mortierella* species	Antarctic soils	18 °C
90 d	PDA	A breakdown analysis of plastics over time reveals that PU degrades at a rate of 28.3 percent, PS at 8.39 percent, and LDPE at a meagre 3.53 percent as they age	Oviedo-Anchundia et al. (2021)	[96]	Polyhydroxyalkanoates (PHAs)	*Bacillus* sp. JY14	Marine soil of Korea	30 °C
14 d	Sodium dodecyl sulfate	98% PHB degradation	Cho et al. (2021)	[53]	2,6-Dimethylphenol (2,6-DMP)	*Mycobacterium neoaurum* B5-4	Topsoil from a dimethylphenols-contaminated area in Suqian city, Jiangsu province, China	30 °C

12 h	MSM	More than 90% degradation	Ji et al. (2020)	[114]	polyphenylene sulfide	*Pseudomonas* sp.	The gut of superworms (*Zophobas morio*)	–
10 d	LCFBM	15 times smaller size beads	Li et al. (2020)	[49]	PVC	*Pseudomonas citronellolis* and *Bacillus flexus*	Laboratory isolate	–
45 d	MSM	After being exposed to *P. citronellolis*, the average molecular weight of PVC decreased by 10%	Giacomucci et al. (2019)	[51]	Starch blended PVC	*Phanerochaete chrysosporium* PV1	Soil	30 °C
90 d	SDA	Polymer film molecular weight dropped from 80,275 to 78,866 Da	Hameed et al. (2014)	[101]	PE and PP	*Brevibacillus* sp. and *Aneurinibacillus* sp.	Cow dung	55 °C

120 d	Ammonium sulfate, di-potassium phosphate, potassium phosphate, magnesium sulfate	Treatment with a consortium of four isolates was shown to reduce the weight of LDPE, HDPE, and PP strips by 58.21±2, 46.6±3, and 56.3±2%, respectively, while the same treatment reduced the weight of PP pellets by 45.7 ±3, 37.2±3%, and 44.2±3%	Skariyachan et al. (2018)	[113]	Polyester PU	*Pseudomonas aeruginosa* MZA-85	Dumping area of Gujranwala	37 °C
4 weeks	MSM	Degradation of the polyester diol portion	Shah et al. (2013)	[104]	Dimethyl phthalate	*Comamonas testosterone*	Soil from agricultural fields in Chongqing, China	30–35 °C

ZMB Zobell Marine Broth, *ZMA* Zobell Marine Agar, *PDA* Potato Dextrose Agar, *CDM* Czapek-Dox medium, *SDA* Sabouraud Dextrose Agar, *MSM* Mineral Salt Medium, *SCS* Sole Carbon Source, *PE* Polyethylene, *LCFBM* liquid carbon-free basal medium, *LDPE* low-density polyethylene, *PP* polypropylene, *PS* polystyrene, *PVC* polyvinyl chloride, *LLDPE* linear low-density polyethylene, *HDPE* high-density polyethylene, *PVA* polyvinyl alcohol, *PET* poly (ethylene terephthalate), *BHET acid* bis(2-hydroxyethyl) terephthalic acid, *PU* polyurethane, *PHB* polyhydroxybutyrate, *PHAs* polyhydroxyalkanoates, *d* days

Recent advances and challenges

Reengineering of microbes

New possibilities for developing game-changing biorecycling solutions have emerged as a result of recent developments in biology and biotechnology. First, cutting-edge synthetic biology techniques and metabolic engineering methods have created many potential for reengineering and enhancing bacteria that can successfully digest solid plastic wastes and directly utilise the degraded products for biomanufacturing. Enzyme engineering techniques have been used to improve a number of plastic-degrading enzymes. Also, emerging approaches to protein engineering, such as AI-guided protein design and mutation and direct evolution, may increase the likelihood

of creating novel enzymes with resistance to inhibitors or contaminants, temperature tolerance, stability, specificity, and superior activity. Additional methods employ protein alignment data to compare plastic substrate architectures. Modifying cellulosome structure to create a multi-enzyme complex that can effectively degrade PET is an interesting area of research. Large cellulosomes may be created using modern synthetic biology methods, allowing for increased action toward stubborn cellulose [115]. Similarly, high-crystalline PET might be degraded by microbial cell factories if PETsome were developed. Identifying the substance that might serve as a PET-binding domain is crucial, similar to the cellulose-binding domain [116]. An effective bacterial cell surface expression system has recently been established [117].

Mutagenesis

In addition, site-directed mutagenesis has been widely used for enzyme redesign; nonetheless, the success of this approach is fundamentally tied to the accessibility of three-dimensional protein structures. A carboxylesterase from *Archaeoglobus fulgidus* was modified using in silico site-directed mutagenesis to produce a BTA-hydrolase from *Thermobifida fusca* [118]. Molecular docking analysis was then used to compare the interactions of PET with polypropylene following this study. The findings as a whole suggested that the binding affinity of the mutant carboxylesterase for PET was unaffected by the alterations.

Using the *Is*PETase crystal structure and computational modelling, researchers have performed site-directed mutagenesis on 15 amino acid domains in the enzyme's first contact shell [119]. The enzyme was able to depolymerize 90% of the supplied PET in 10 h after disulfide bridges were added to increase its thermostability, and residues crucial for substrate binding were mutated. When the strain was optimized, it could break down 16.7 g of PET per litre per hour. Enzymatic PET degradation, which takes 10 h and is 90% effective, is comparable to chemical PET degradation, which takes 8 h and is 98% effective [120].

Enzyme modelling and experimental results revealed *I. sakaiensis* PETase's binding pockets and a variety of cutinases [121]. Like *Is*PETase, a cutinase isolated from Thermobifida has its binding pocket residues involved in substrate interaction determined [122]. A study comparing the *Is*PETase enzyme to others, such as Thf42 Cut1, determined that the binding pocket structure of *Is*PETase is responsible for its efficacy. When compared to other cutinases, which can only hydrolyze linear PET molecules, this PETase has a shallower and broader surface, making it possible to attach to aggregated PET molecules [121]. The TfCut2 from *Thermobifida fusca* was analyzed in the same fashion. By using computational modelling to identify critical residues and site-directed mutagenesis to modify these residues on the selected substrates, researchers expanded the disintegration rate of PET film by a factor of 12.7 [123].

Adaptive Laboratory Evolution (ALE)

Initiating and promoting evolutionary adaptation processes, such as ALE, is a potent method for enhancing or creating certain phenotypes in microbial strains [124]. ALE is a powerful strain engineering method for introducing mutations to enhance metabolic pathways and enzymes for fast growth on a range of carbon sources and stress tolerances when combined with omics approaches to characterise the induced changes. Numerous ALE instances have arisen for the better usage of plastic monomers; these monomers are crucial for the construction of plastic-upcycling or -degrading cell factories.

After having its genome sequenced, scientists discovered that *Pseudomonas pseudoalcaligenes* CECT 5344 has the capacity to use furoic acid, furfuryl alcohol, and furfurals as carbon sources. Growth on furfurals, however, was discovered to have a significant delay of many days. The ALE-adapted strain grew better on furfurals and had shorter lag periods [125]. This strain improved due to a point mutation in an AraC family activator gene (BN5 2303) in the HTH protein region (L261R). This mutation regulates the upstream hmfABCDE gene cluster.

In addition, terephthalate-independent *P. putida* KT2440 mutants that were successfully isolated from ALE have been shown to use ethylene glycol, a monomeric component of PET [126]. These mutants have missense mutations and a 15 bp deletion in gclR, a transcriptional regulator of the glyoxylate carboligase pathway (PP 4283). PP 2046- and PP 2662-encoded transcriptional regulators and porins improved ethylene glycol growth in ALE-derived *P. putida* KT2440. Secondary mutations may stabilise flux balances during the first phase of ethylene glycol oxidation to glyoxylate.

Current ALE tactics focus mostly on optimising the use of plastic monomers, but there is enormous potential for ALE to be used in the creation and improvement of plastics depolymerization enzymes. A wide variety of enzymes, including esterase, lipase, and cutinase, have been found to depolymerize PET and PLA but with low selectivity and turnover [127]. Novel plastic depolymerizing enzymes might be developed via ALE or directed evolution, two methods that show promise for acquiring enzymatic activity from promiscuous enzyme families.

Obstacles to overcome

The field of microbial bioremediation for plastic waste management encounters several formidable challenges that necessitate concerted efforts to overcome. One prominent obstacle lies in the substrate specificity exhibited by microorganisms. While certain microbes demonstrate efficacy in degrading specific types of plastics, the vast array of plastic polymers presents a challenge in developing microbial solutions that universally address the diversity of plastic materials. Moreover, the rate of plastic degradation through microbial processes is often sluggish. Accelerating this degradation without compromising efficiency remains a significant research challenge, particularly as the volume of plastic waste continues to escalate. Environmental conditions further complicate matters, with factors such as temperature, pH, and the presence of other chemicals influencing the effectiveness of microbial bioremediation. Optimizing these conditions for widespread applicability and scalability across diverse environments poses a considerable hurdle.

The emergence of biodegradable plastics and biopolymers designed to mimic traditional plastics adds complexity to the field. Microorganisms may struggle to differentiate between these bioplastics and conventional plastics, potentially impacting their effectiveness in degrading target materials. The lack of standardized protocols for assessing and categorizing microbial biodegradation of plastics is another critical challenge. Establishing uniform methodologies and metrics is essential for meaningful comparisons and advancements in the field.

Scaling up microbial bioremediation from laboratory experiments to real-world, large-scale applications presents engineering and logistical challenges. Ensuring the viability of microbial processes on an industrial scale while maintaining cost-effectiveness requires innovative solutions and a thorough understanding of the complexities involved. Additionally, the ecological impact of introducing specific microorganisms into ecosystems needs careful assessment. While microbial bioremediation holds promise, unintended consequences on the environment must be thoroughly evaluated through comprehensive risk assessments.

Furthermore, navigating the evolving regulatory landscape surrounding the use of microorganisms for plastic bioremediation poses challenges. Compliance with regulatory standards and ensuring the safety of processes and end-products are critical considerations for the responsible development and deployment of microbial solutions in plastic waste management. Overcoming these multifaceted challenges demands interdisciplinary collaboration, continuous research and development, and a holistic approach that considers the intricacies of both microbial processes and the environmental contexts in which they are applied.

Limitations

This study has a number of caveats, the first of which is that the search was conducted in a specific segment of the most regularly used libraries. Throughout our search, we skipped to check a few libraries. The decision was made to

restrict attention to studies that appeared in reputable peer-reviewed publications. It was determined not to look through grey publications. Second, only relevant results were obtained since the search terms were restricted to just those most closely associated with the initial query. There is a risk that a manuscript could have been disregarded which may discuss microbial remediation of plastic biodegradation but not using the phrases sought for. By developing a methodology, the authors ensured they would have complete command over the search and selection of papers. Finally, the study only considers the most common microorganisms used in the treatment for plastic biodegradation. The authors have made an attempt to provide bibliographic information for all relevant and well regarded publications.

Conclusion

The annual manufacturing of plastic has topped 300 million tonnes, and recycling has almost failed as a sustainable method for the disposal of plastic trash. With the accumulation of these materials in the environment, particularly in rivers and oceans as macro-, meso-, micro-, and nano-plastics, it is of the utmost significance to discover creative methods to reduce this environmental threat. There have been several efforts to identify and isolate microbes with the ability to use synthetic polymers. Using specific microbial strains for plastic biodegradation has recently been shown to be a viable option.

These findings give fresh insights into LDPE and HDPE biodegradation processes by a consortium of microorganisms with putative metabolic complementarities. Another research found novel bacterial strains that may alter the chemical composition of HDPE. Based on these results, it seems that synergistic microbe-enzyme treatment might be a promising future direction for plastic degradation research. The capability of the microbial strain to digest microplastic particles provides a potential application for the remediation of microplastics. The PET-degrading activity shown by the analyzed bacterial strains holds promise for further study and its application to the successful removal of microplastics from water and wastewater using novel and potent technological approaches. This research also uncovered a thermo- and halo-tolerant bacterium that can degrade PHB in both solid and liquid states. In light of these findings, it seems that this strain of bacteria may be useful for degrading a wide range of PHAs. These methods, when combined, may be used to create a streamlined bioprocessing setup, a microbial system that can effectively break down plastics and upcycle them into high-value compounds. Technological and economic obstacles, such as the toxicity of waste products to degrading enzymes and high operational costs, should be addressed for the benefit of the industry as a whole.

Current research suggests that naturally occurring soil microorganisms, such as bacteria and fungi, are quite effective in breaking down plastic. More often than not, fungi are more potent degraders than bacteria. In the lab, however, fungi and bacteria demonstrated the ability to break down plastic. The maximum degradation capability was found for *Aspergillus* fungi and *Bacillus* bacteria among the studied taxa. For this notion to be utilized commercially and on a wider scale, more work is required to increase its degrading capability by evaluating optimal conditions for microbial activity. Another strategy that might be used to improve plastic biodegradation is the pre-treatment with compounds that are beneficial to the environment.

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Tushar Agarwal: investigation, writing-original draft preparation, editing; Neeraj Atray: reviewing, validation; Jai Gopal Sharma: supervision, conceptualization, methodology.

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None.

Abbreviations

ALE

Adaptive laboratory evolution

ATR-FTIR

Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy

BC

Bacillus cereus

BHET

Bis(2-hydroxyethyl) terephthalic acid

BP

Bacillus paramycoides

CDM

Czapek-Dox medium

DMP

Dimethyl phthalate

EDS

Energy Dispersive X-ray Spectroscopy

EGT

Ethylene glycol terephthalate

EPS

Extracellular Polymeric Substance

FESEM

Field Emission Scanning Electron Microscopy

FTIR

Fourier Transform Infrared Spectroscopy

GPC

Gel permeation chromatography

HDPE

High-Density Polyethylene

HPLC

High-performance liquid chromatography

ITS

Internal transcribed space (ITS)

LC/MS

Liquid Chromatography-Mass Spectrometry

LCFBM

Liquid carbon-free basal medium

LDPE

Low-Density Polyethylene

LLDPE

Linear Low-Density Polyethylene

MHET

Mono(2-hydroxyethyl) terephthalate

MMP

Mono-methyl phthalate

MSM

Mineral salt medium

NMR

Nuclear Magnetic Resonance

PA

Phthalic acid

PA

Polyamide

PDA

Potato Dextrose Agar

PE

Polyethylene

PET

Poly(ethylene terephthalate)

PHB

Polyhydroxybutyrate

PP

Polypropylene

PPS

Polyphenylene sulfide

PS

Polystyrene

PU

Polyurethane

PVA

Polyvinyl alcohol

PVC

Polyvinyl chloride

RT-PCR

Reverse Transcription-Polymerase Chain Reaction

SCS

Sole carbon source

SDA

Sabouraud Dextrose Agar

SEM

Scanning Electron Microscopy

TfC

Thermobifida fusca cutinase

TPA

Terephthalic acid

UTM

Universal testing machine

XPS

X-ray Photoelectron Spectroscopy

XRD

X-ray diffraction

ZMA

Zobell Marine Agar

ZMB

Zobell Marine Broth

Publisher's Note

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Diverse pharmacological actions of potential carbazole derivatives by influencing various pathways of molecular signaling

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ABSTRACT (ENGLISH)

Background

Carbazoles are an important class of heterocyclic aromatic compounds that contain nitrogen atom in the ring. They have a large-conjugated system, attractive "electrical and charge-transport properties", and the ability to efficiently incorporate different functional groups into the structurally inflexible carbazolyl ring.

Main text

Carbazole derivative ECCA acts as an anticancer agent by reactivating the P53 molecular signaling pathway; similarly, some other derivatives of carbazole show antifungal activity by acting on the RAS-MAPK pathway. Carbazole derivatives also show their effect on inflammation by inhibiting the p38 mitogen-activated protein kinase signaling pathway by stopping the conversion of DAXX protein into ASK-1. By modifying the AKT molecular

signaling pathway through boosting protein phosphatase activity in the brain, they show anti-Alzheimer's activity and also by translocating the GLUT4 these are effective against diabetes.

Conclusion

After exploring the literature on carbazole, it was found that carbazole has an immeasurably great potential for the treatment of various diseases as the carbazole nucleus leads to various synthesized derivatives which are used for their pharmacological activities. So there is a need to explore carbazole for some newer drugs.

FULL TEXT

Background

Heterocyclic compounds are composed of cyclic rings containing two or more different types of atoms [1, 2]. Throughout nature, heterocyclic compounds are very common and essential. In carbazole, two benzene rings with 6 carbon atoms are fused on either side of a five-membered heterocyclic ring with nitrogen atom showing vast delocalization of electrons [3].

Carbazole and its compounds are useful types of heterocyclic aromatic compounds that show desirable properties of transporting electrons and charges throughout the ring [4]. Also, many polyfunctional groups can be easily added into the carbazolyl ring for the formation of some other novel derivatives [5]. These properties of carbazole-based derivatives result in a wide range of potential applications in the chemistry field (dyes, photoelectrical materials, supramolecular recognition, etc.), and these derivatives also result in various pharmacological activities like antidiabetic [6, 7], antitumor [8–10], anticonvulsant [11], antimicrobial [12–14], antioxidative [15, 16], antifungal [17, 18] antihistaminic [19], anti-inflammatory [20, 21], antitubercular [22], antiviral [23], carbonic anhydrase enzyme activity [24], neuroprotective activity [25] and antidiarrhoel [26], etc.

C-3 and C-6 positions in carbazoles have the highest electron density so in recent years the synthesis of some novel carbazoles has been done by modification in carbazole at C-3 and C-6, and 9th positions of N [27]. If there is no steric hindrance that occurs, the carbazoles along with unblocked 3rd and 6th positions of Carbon in the ring form at least a dimer upon oxidation. Carbazoles undergo electrophilic substitution reaction like sulfonation, nitration, Friedel–Crafts acylation and Friedel–Crafts alkylation forming different derivatives of carbazole. The problem with this substitution is that a mixture of ortho and para positions is obtained but due to high electron density at the 3rd and 6th carbon positions, 75% of the para product formed concerning C-1 and C-8 (ortho position). Formation of mono along with di-, tri-, and tetra-substituted carbazoles is possible due to the reactivity of carbon atoms to the imine group in carbazole. By using appropriate alkylating or acylating agents at room temperature, the N-9 substituted carbazole derivatives are also formed [4].

Main text

Diseases with neurodegeneration

Neurodegenerative diseases (NDs) are a group of disorders (multiple sclerosis, Parkinson's disease, Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, etc.) that cause loss of neuron's structure or function [28–33]. As a result of these changes in the brain anatomy, the logical or functional decline may result in protein misfolding, diminished mitochondrial functions, oxidative stress, impaired neuro-transmission, and loss of neurons which are just a few physiological indicators of NDs [34, 35].

Anticancer potential

Cancer is the major health problem in individuals. Every year approx 18 million peoples are diagnosed with cancer, and it is the prime cause of death worldwide. In developed countries, the age-standardized rate for diagnosis is 296 cases per 1000,000 peoples [36–40]. Cancer is a pathological condition of the body which is due to various genetic mutations which are characterized by uncontrolled proliferation and growth of cells [41–44]. Every cell of the body can become a cancer cell when a genetic mutation occurs [45]. Carcinogenesis process occurs in three stages; in the first stage alteration has already been identified so it is called the initiation stage, in the second promotion stage these altered cells are mutated and form malignant cells, and in the last stage of carcinogenesis which is continuation stage once the tumor has increased in size and cancer cells begin to split in an accelerated irreversible

mode. And in the last stage the cells instead of growing locally start to disseminate to other body parts (metastasis) [46–48].

In human melanoma cells, the p53 pathway is reactivated by the anticancer effect of carbazole derivative ECCA, (9-ethyl-9*H*-carbazole-3-carbaldehyde). In melanoma therapy, about 84% of human melanomas harbor wild-type p53 is thought to be a supreme target for treating melanoma. Carbazole derivatives enhanced the phosphorylation of c-Jun N-terminal kinase (JNK) and p38-MAPK, and either a p38 mitogen-activated protein kinase or c-Jun N-terminal kinase inhibitor reassured cell proliferation inhibition produced by 9-ethyl-9-*H*-carbazole-3-carbaldehyde (ECCA), whose expression was dependent on p53 gene. Carbazole derivatives selectively and significantly depress the expansion of melanoma cells by persuading the programmed cell death of melanoma cells and senescence through p53 activation [49].

Yonghua et al. designated water-soluble carbazole and sulfonamide derivatives which are tested in vivo against human HepG2 (hepatoblastoma liver cancer cell line) xenograft mouse tumor expansion and found that compound 4c (Sodium 6-(*N*-(2,6-dimethoxypyridin-3-yl)sulfamoyl)-9-methyl-9*H*-carbazol-2-yl phosphate) is the most effective tumor inhibitor [50–52].

Huang shows the in vitro cytotoxic activity of different synthesized carbazole derivatives in which compound 7 g (*N*′-(Benzo[d](1,3) dioxol-5-ylmethylene)-1-methyl-9*H*-carbazole-2-carbohydrazide) and compound 7p (*N*′-(4-Chlorobenzylidene)-1,9-dimethyl-9*H*-carbazole-2-carbohydrazide) is the potent inhibitor of cancer cells but do not affect normal cells. 7g Carbazole derivative with acylhydrazone substituted with 1,3-benzodiooxozole displayed significant selective proliferation inhibition activity in vitro with (IC_{50}<12.24 μM). For the in vitro investigation of cytotoxic activity three cell lines, human melanoma (A875), human hepatocellular liver carcinoma (HepG2), and a subclone of the African green monkey cell line (MARC145) were used [53].

Capan, I et al. synthesized a sequence of carbazole derivatives. Result among all the synthesized derivatives showed that compound 9 [(*E*)-2-(9*H*-carbazol-9-yl)-*N*′-(3-(4-chlorophenyl)-4-oxothiazolidin-2-ylidene)acetohydrazide)] and compound 10 [5-((9*H*-carbazol-9-yl)methyl)-1,3,4-oxadiazol-2(3*H*)-one] are found most potent anticancer agent against HepG2, HeLa, and MCF-7 cancer cell lines with IC_{50} values of 7.68, 10.09 μM. Against HeLa, cancer cell lines found that compound 9 is the best antiproliferative agent [54].

Leu et al. synthesized a novel series of carbazole derivatives that are synthesized by imidazole derivatives along with *N*-alkyl bromide substituted carbazole. Compound 61[1-(5-(9*H*-carbazol-9-yl)pentyl)-3-(2-bromobenzyl)-5,6-dimethyl-1*H*-benzo[d]imidazol-3-ium bromide], which was bearing a 2-bromobenzyl substituent at position-3 of the 5,6-dimethyl-benzimidazole, showed powerful inhibitory activities and found to be more selective to human promyelocytic leukemia cell line (HL-60), human hepatocellular carcinoma cell line (SMMC-7721), breast cancer cell line (MCF-7) and human colon adenocarcinoma (SW480) cell lines with IC_{50} values 0.51–2.48 μM. The results of the research indicated that the presence of 5,6-dimethyl-benzimidazole ring and substitution of the imidazolyl-3-position with a 2-bromobenzyl group, as well as for the antitumor activity the length of alkyl chain between carbazole and imidazole ring were important [55] (Fig. 1).

Fig. 1 [Images not available. See PDF.]

Anticancer mechanism of carbazole derivatives. 9-Ethyl-9-*H*-carbazole-3-carbaldehyde (ECCA) re-activates P53 pathway and causes phosphorylation of P38MAPK

Breast and uterine *cancer*

The natural and synthetic carbazole derivatives represent an exciting heterocycle class that has shown several pharmaceutical properties and is also an excellent antitumor tool in preclinical experiments. The antibodies target several cellular key points, such as Topoisomerases I, II and DNA. Several efforts have been made to plan and synthesize some novel carbazole derivatives that have lesser side effects and good potency. The carbazole derivatives without affecting non-tumor cell lines possess antiproliferative activities against uterine and breast cancer cell lines. Carbazole derivatives through the inhibition of Topoisomerase II trigger cancer cells' intrinsic apoptotic pathway.

Antifungal potential

Fungal infections (mycosis) are increasing throughout the world. Although there are several reasons for this higher increase in mycosis, immune modulation of the host is one of the foremost risk factors for intrusive mycosis [56]. Humans suffer from most of the fungal infections due to the setting of iatrogenic immunosuppression. When these factors are absent fungi cause mild, self-limited mucocutaneous surface infections. When this infection develops in a normal host, it causes adaptive immune dysfunction because of genetic defects [56–58].

Tang et al. tested the antifungal activity of all the synthesized compounds against different fungal strains and found that compound 3f (2-(9*H*-carbazol-9-yl)-*N*-(4,5-dihydro-5-(thiophen-2-yl)-1,3,4-thiadiazol-2-yl)acetamide) and 3i (2-(1-chloro-9*H*-carbazol-9-yl)-*N*-(4,5-dihydro-5-*p*-tolyl-1,3,4-thiadiazol-2-yl)acetamide) exhibited the most potent inhibitory activity against C. wilt having inhibition rates 72.40% and 67.65% [59].

Shaikh et al. synthesized two series of carbazole analogues. The first one is 8-methoxy-*N*-substituted-9*H*-carbazole-3-carboxamides, and the second one is carbazolyl-substituted rhodanines. The final compounds were prepared from these two series and were tested for antibacterial activity against *Bacillussubtilis*, *Staphylococcusaureus*, *Pseudomonasaeruginosa*, and *Escherichiacoli* [60–62] and antifungal activity against *Cryptococcus tropicalis*, *Aspergillus niger*, *Cryptococcus neoformans*, and *Candida albicans* [63–65]. Various substituents, nitrogen-containing heterocyclic systems like pyrimidinyl and piperidinyl on carbazole nucleus exerted a significant antifungal activity in the first series of compounds. 2-Methyl piperidinyl carboxamide derivative compound 6f (8-Methoxy-9*H*-carbazol-3-yl)(2-methylpiperidin-1-yl)methanone found as potent antifungal and antibacterial agent. In second series substitution at 3 positions of rhodanines which is conjugated to -methyl carbazole through an acrylidine linkage, the result showed the effect of bioisosteres coumarin present in compound 15i [(5*Z*)-3-(4-methyl-2-oxo-2*H*-chromen-7-yl)-5-((9-methyl-9*H*-carbazol-6-yl)methylene)-2-thioxothiazolidin-4-one]were shown potent inhibitory activity. It was concluded that electron donating groups such as hydroxy, alkoxy, and alkyl groups on the aromatic ring greatly contributed toward the antifungal and antibacterial activity [64].

The morphogenic process came up with the virulency of *Candida albicans*, an opportunistic human fungal pathogen. Ras1-MAPK pathways are crucial to *Candida albicans* virulence through controlling morphogenesis, cell growth, and biofilm formation.

Several carbazole-containing drugs have been prescribed for infections of *Candida albicans*, leading to the expansion of drug-resistant strains. For that reason, it is needed to properly treat fungal infections, and new compounds must be developed. By suppressing Ras/MAPK-related gene protein and RNA levels, carbazole derivatives prevent morphogenesis [66, 67] (Fig. 2).

Fig. 2 [Images not available. See PDF.]

Antifungal mechanism of carbazole derivatives. *N*-(4-methoxybenzyl)-3(9*H*-carbazol-9yl) propan 1-amine inhibits morphogenesis by suppressing protein and RNA levels of Ras/MAPK-related genes

Anti-psoriasis potential

Psoriasis is a chronic autoimmune disease that is associated with systemic manifestation and characterized by keratinocytes and hyperproliferation mediated by T-cells [68–70]. It is an autoimmune disease with a genetic predisposition. In the psoriatic lesions presence of dendritic cells, cytokines and T lymphocytes has prompted the development of biologic therapies [71, 72].

Carbazole present in coal tar is an active antiangiogenic compound along with that antiangiogenic effect carbazole also withdraws the formation of inflammatory IL-15 by human mononuclear cells. It is believed that IL-15, which is increased in psoriasis, contributes to the inflammation of psoriasis [29, 73]. Moreover, carbazole treatment reduced the activity of nitric oxide synthase (iNOS) (a pro-inflammatory enzyme), which is elevated in psoriasis patients. In a study on human psoriasis, carbazole was found to inhibit the activator of stat 3-mediated transcription, which is relevant to psoriasis. iNOS, IL-15, and stat3 activation rely on the small GTPase Rac for optimum activity. As a mechanism for inhibiting downstream inflammatory and angiogenic pathways, carbazole inhibits Rac activation [74]. The mechanism of action of carbazole could involve the inhibition of pro-inflammatory cytokine synthesis, inhibition

of activator protein-1 (AP-1) activity, and inhibition of EGFR activation. Production of IL-15 by mononuclear cells is also inhibited by carbazole, a key cytokine in psoriasis [75–77] (Fig. 3).

Fig. 3 [Images not available. See PDF.]

Anti-psoriasis mechanism of carbazole derivatives. Carbazole present in coal tar inhibits the P38 signaling pathway by inactivation of Rac

Anti-Alzheimer potential

Generally, dementia is caused by Alzheimer's disease, the primary or cellular phase of Alzheimer's disease happens in parallel with accumulating amyloid β, inducing the spread of tau pathology [78]. Pathological characteristics in Alzheimer's disease (AD) are deposition of β-amyloid (Aβ) peptide and hyperphosphorylated tau in the brain, carbazole derivative (9c) *N*(6-(9*H*-carbazol-9-yl)-hexyl)-1benzylpiperidin-4-amine modulated AKT pathway and boosting protein phosphatase 2A activity in the brain. The compound 9c with (IC_{50} = 26.5 μM for AChE and IC_{50} = 0.18 μM for BuChE) results in the highest inhibitory activity against butyrylcholinesterase and acetylcholinesterase. Structure–activity relationship suggested that attachment to the 4-amino-*N*-benzylpiperidine fragment with C5 and C6 alkyl linkers in compound 9c results in the most potent compound among all [79].

Choubdar et al. designated several carbazole derivatives by using some heterocyclic nucleus like quinoline, pyridine, piperidine, benzyl piperidine, and benzyl piperazine, etc., in which compound 3s (9-(5-(quinoline-1 (2*H*)-yl)pentyl)-9*H*-carbazole) linked with quinoline found as the potent drug against BuChE and AChE and also showed the inhibitory activity for AChE induced β-amyloid (Aβ) aggregation. The most potent compound 3s with IC50 = 0.11 μM and 0.02 μM showed the best activity against AChE and BuChE, respectively. The presence of quinoline moiety in compound 3s more actively inhibited Aβ self-aggregation. Docking studies suggested that compound 3s binds effectively to the PAS and anionic binding site of the enzyme with the assistance of π stacking and hydrophobic interactions [80].

Carbazole derivatives regulating Ca2+/CaMKII/CREB signaling pathway. The brain contains CaMKII (Calcium/Calmodulin-dependent Protein Kinase II) which is a specific protein kinase of serine/threonine, which is regulated by Ca2+/calmodulin [81].

Shaikh et al. synthesized a series of α-amino phosphonate-based carbazole derivatives that were in silico and in vitro tested for their cholinesterase activity. All the compounds present better AchE activity [0.475–7.781 μM] than BuChE (3.306–21.32 μM). Against AChE as well as BuChE compound 4j [Diethyl(9-ethyl-9*H*-carbazol-3-yl)amino) (3nhydroxyphenyl)methyl)phosphonate] was the most potent derivative with IC_{50}¼0.475 ± 0.12 mM and IC50¼3.306 ± 0.21 μM, respectively [82] (Fig. 4).

Fig. 4 [Images not available. See PDF.]

Anti-Alzheimer mechanism of carbazole derivatives. AKT pathway regulation by carbazole derivative *N*-(6-(9-*H*-carbazol-9yl) hexyl)-1-benzylpiperidine-4-amine by boosting protein phosphatase activity in brain

Anti-inflammatory potential

Inflammation is a result of a complex biological response that includes inflammatory mediators, sensors, inflammation-inducing factors, and affected target tissues [83, 84]. Physical injuries, toxic chemicals, viruses, fungi, and bacteria are some harmful stimuli under inflammation-inducing factors. Pathogen-associated molecular patterns (PAMs) triggered the inflammatory response [85, 86]. Inflammatory pathways are mediated by some inflammatory mediators like cyclooxygenase (COX), chemokines, vasoactive amines nitric oxide (NO), etc. [87]. The inflammatory responses finally lead to symptoms like heat, pain, redness, and swelling, etc. [88, 89].

Bandgar et al. designated a novel series of carbazole(5a–o) which was tested against the anti-inflammatory activity. Compound 5c via hydrophobic interactions potently binds at the site of COX-II. The oxygen atom of the methoxy group on compound 5c formed two hydrogen bonds with the Asp B:225 and Nag C:671 and the nitrogen atom present in the pyrazoline ring on compound 5c formed two hydrogen bonds with the Leu A:131 [90].

Carbazole derivatives inhibit the lipopolysaccharide-induced inflammatory mediator production in macrophages via

suppression of p38 MAPK (mitogen-activated protein kinase signaling pathway). Carbazole derivatives inhibit the formation of TNF-*α* (tumor necrosis factor *α*), PGE_2 (prostaglandin E_2), and nitric oxide (NO) induced by Lipopolysaccharide (LPS) [91–93] (Fig. 5).

Fig. 5 [Images not available. See PDF.]

Anti-inflammatory mechanism of carbazole derivatives. 3-(3-(2-Methoxy-5-methylphenoxy)-4 5-dihydro-1*H* pyrazole-5yl)-9*H*-Carbazole inhibits the p38 MAPK signaling pathway by stopping the conversion of DAXX protein into ASK-1

Antidiabetic potential

Diabetes is a condition in which due to autoimmune destruction pancreatic β cells slow down the production of insulin results hyperglycemia due to excess availability of glucose [94–98]. α-glucosidase inhibitory activity of compound 7k (1-(5,6-di(furan-2-yl)-1,2,4-triazin-3-ylthio)-3-(3,6-dibromo-9*H*-carbazol-9-yl)propan-2-ol)was found most effective and the result revealed that compound7k is the potent compound with h IC_{50} values of 4.27 ± 0.07 μM among all the synthesized compound by Wang et al. The potent compound with high-density van der Waals contact binds at the bottom of the α-glucosidase pocket, whereas near the entrance of the pocket, furan ring was positioned which made only a few contacts. Detailed analysis showed that the carbazole ring of the potent compound formed arene-cation interactions with Arg-439 and Arg-312 residues, respectively. Furan ring of a potent compound located at the hydrophobic pocket, surrounded by Leu-176, Phe-177, Phe-157, and Pro-240 residues. All these interactions helped 7k to anchor in the binding site of α-glucosidase [99].

Carbazole derivative koenidine translocates the GLUT 4 which is mediated through the AKT-dependent signaling pathway (4) in L6-GLUT-4 myc myotubes [100].

The in vitro testing of separated carbazole alkaloids on uptake of glucose and translocation of GLUT 4 in L6-GLUT-4 myc myotubes was done on streptozotocin-induced diabetic rats for their activities. Therefore, the in vitro study of the koenidine suggested that koenidine, a carbazole derivative, possesses a promising antidiabetic activity through managing diabetes and insulin resistance [6, 101] (Fig. 6).

Fig. 6 [Images not available. See PDF.]

Antidiabetic mechanism of carbazole derivatives. Koenidine 4 translocates GLUT 4 for antidiabetic activity

Conclusion

Carbazole moiety itself is responsible for various types of pharmacological activities; due to high electron delocalization, carbazole shows better physicochemical properties. Derivatives having carbazole pharmacophore are responsible for various therapeutic activities like anti-Alzheimer, antioxidant, antidiabetic, anticancer, anticonvulsant, antimicrobial, and anti-inflammatory, etc., by specifically acting on potential molecular level proteins and factors such as RAS-MAPK, DAXX, ASK-1, AKT, and JNK, either by inhibiting or by activating them by de-phosphorylation or phosphorylation (Fig. 7). Due to all these important activities, the carbazole nucleus has attracted the attention of researchers in the discovery of other novel derivatives of carbazole.

Fig. 7 [Images not available. See PDF.]

Molecular mechanisms of carbazole derivatives for different pharmacological activities

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Author contributions

AT has done various works on synthetic chemistry on carbazole derivatives and collected important information from her research and various literature for this review. BM has conceptualized the idea for this review, prepared the outlines and did the refinement of the paper.

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Competing interests

The authors declare that they have no competing interests.

Abbreviations

GLUT 4
Glucose transporter type 4
ASK-1
Apoptosis signal-regulating kinase 1
ECCA
9-Ethyl-9*H*-carbazole-3-carbaldehyde
RAS-MAPK
Ras/mitogen-activated protein kinase
DAXX
Death-domain-associated protein
AKT
Protein kinase B
JNK
Jun N-terminal kinase
MCF-7
Michigan Cancer Foundation
DNA
Deoxyribonucleic acid
RNA
Ribonucleic acid
iNOS
Inducible nitric oxide synthase
IL-5
Interleukin-5
EGFR
Epidermal growth factor receptor
AChE
Acetylcholinesterase
BuChE
Butyrylcholinesterase
A875
Human melanoma
HepG2
Human hepatocellular liver carcinoma
MARC145
A subclone of the African green monkey cell line

HL-60
Human promyelocytic leukemia cell line
SMMC-7721
Human hepatocellular carcinoma cell line
MCF-7
Breast cancer cell line
SW480
Human colon adenocarcinoma cell lines

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Green analytical chemistry-based spectrophotometric techniques for ternary component analysis of pain relievers

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ABSTRACT (ENGLISH)

Background

The management of pain presents a significant challenge in healthcare, particularly in cases where conventional therapies prove inadequate. In response to this need, this study aims to devise two innovative UV spectrophotometric techniques rooted in the principles of green analytical chemistry for the analysis of Aceclofenac (ACE), Paracetamol (PAR), and Tramadol (TRM) in both bulk and tablet forms.

Results

Utilizing advanced mathematical methodologies such as the double divisor ratio spectra method and area under the curve, the concentrations of these drugs were accurately determined. Validation of the developed methods adhered to the guidelines outlined by the International Council for Harmonisation in the Q2 (R1), revealing linear calibration curves for ACE (8–12 µg/mL), PAR (22.75–35.75 µg/mL), and TRM (2.62–4.12 µg/mL). Furthermore, statistical analyses employing Student's *t* test and *F* test were conducted to ensure the robustness of the proposed method. The evaluation of environmental impact through green metric tools confirmed the eco-friendliness of the proposed methodologies.

Conclusion

The assessment performed utilizing green metric tools has substantiated the environmental sustainability of the

proposed approach. Thus, this methodology offers accurate and reliable outcomes for the determination of three drugs, as indicated by the complete overlap observed in the zero-order spectra.

FULL TEXT

Background

Pain is prevalent in today's society, serving both as a valuable warning to prevent harm and as an unwelcome aspect of our lives. Its impact on our quality of life is influenced by its intensity and duration. The opioid crisis has prompted discussions on pain treatment. While opioids are highly effective in suppressing pain signals, they also pose potential harm [1]. Nowadays treatment using a tri-drug therapy has shown a prominent effect over the treatment for pain management rather than using single and dual regimens due to the tolerance effect of the individuals. Among several combinations, two Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) and one opioid have shown effective treatment in the management of pain.

Paracetamol (PAR), chemically N-(4-hydroxyphenyl)acetamide [2] (Fig. 1a), acts as a centrally and peripherally acting non-opioid analgesic and antipyretic [3]. The mechanism of action involves inhibiting prostaglandin synthesis. This inhibition is achieved by targeting COX-1 and COX-2 in conditions with low levels of arachidonic acid and peroxides. Beyond treating headaches, muscular aches, arthritis, backaches, toothaches, and fevers, PAR is frequently used as an over-the-counter medication to alleviate pain and reduce fever. However, a PAR overdose may lead to severe side effects, such as fulminant liver necrosis [4].

Fig. 1 [Images not available. See PDF.]

Structure of **a** PAR, **b** ACE **c** TRM

Aceclofenac (ACE) is an orally administered NSAID, chemically known as 2-[2-[2-[(2,6-dichlorophenyl) amino] phenyl] acetyl] oxyacetic acid (Fig. 1b). It demonstrates a favorable tolerability profile and produces analgesic effects in various painful situations. ACE is employed in the treatment of rheumatic arthritis and soft-tissue injuries [5]. Its potent inhibitory impact on the cyclo-oxygenase (COX) enzyme disrupts the synthesis of prostaglandins, which are inflammation mediators responsible for heat, pain, edema, and inflammation [5–7].

Tramadol (TRM) is chemically (1R, 2R)-2-[(dimethyl amino) methyl]-1-(3-methoxy phenyl) cyclohexanol (Fig. 1c). It acts as an opioid agonist, effectively reducing pain without suppressing prostaglandins or inducing cardiovascular or respiratory depressive effects. Due to its complementary enantiomers, enhancing effectiveness and tolerance, it is utilized as the racemate [5]. The agonistic activity of (+)-tramadol and its main metabolite, (+)-O-desmethyl-tramadol (M1), on the μ opioid receptor (μOR), inhibiting serotonin and norepinephrine reuptake, synergizes to control pain perception and reaction [8]. TRM is efficacious for a broad spectrum of pain, ranging from moderate to severe, including pain during child birth [5].

Misuse of TRM raises concerns about overdose, opiate addiction, and physical dependence. Its connection to addiction in sports athletes is notable, as it is reportedly used in professional sports to endure discomfort and enhance performance, recognized even as a doping substance in cycling. Despite its hazards, TRM is frequently employed to treat injuries and alleviate perceived exertional pain and fatigue, particularly in cycling, as evidenced by the World Anti-Doping Agency (WADA) Monitoring Program [9]. Athletes, coaches, sports physicians, and pharmacists should familiarize themselves with WADA's 2024 Prohibited List, effective from January 1, 2024 [10].

TRM is commonly prescribed in combination with non-opioid analgesics like PAR for the treatment of moderate-to-severe pains. A fixed-dose combination of PAR, ACE, and TRM provides multimodal analgesic effects with an extended half-life and quick onset. Patients experiencing both chronic and moderate to severe pain are often prescribed and taken orally every 4–6 hr, with a maximum daily intake of 400 mg. The instructions were very clear on the label as the drug cannot be used for more than 5 days [11].

The goal of the current research is to develop an ecologically friendly strategy for analyzing this specific medication. Green chemistry, pioneered by Paul Anastas, aims to replace harmful solvents with less or non-toxic alternatives [12, 13]. Sample preparation and separation science in analytical chemistry often involve solvents, leading to the

development of the “3” approach—Reduction, Replacement, and Recycling. This has led to the redesign of instruments, the use of substitute solvents, and economic benefits in large-volume applications [14]. Additionally, environmentally friendly solvents meeting the US Environmental Protection Agency’s (EPA) criteria for reducing hazardous environmental consequences have fueled the growth of green analytical chemistry (GAC). This focus addresses environmental, social, and economic objectives, emphasizing pollution control, sustainable industrial ecology, pollution prevention, and environmental safety [12].

The combination of these three drugs forms a marketed formulation categorized as a Schedule H1 drug. While the literature search indicates the detection of these three drugs as single components or in combination with other medications using UV and other techniques [15–27], this novel medication lacks a valid pharmacopeia method but is purported to effectively reduce inflammation, swelling, and discomfort in conditions such as ankylosing spondylitis, osteoarthritis, and rheumatoid arthritis. It also aids in managing temporary discomfort, including toothache, ear, and throat pain, as well as muscular and back pain, by suppressing substances in the body that cause edema and pain [17].

This work aims to develop two simple UV spectrophotometric methods for estimating drugs in combination. The double divisor ratio spectra method (DDRSM) and area under the curve (AUC) are easy to understand, accurate, precise, and appropriate for regular QC analysis and identification in bulk and tablet pharmaceutical dosage forms.

Theoretical background

Method 1: double divisor ratio spectra method (DDRSM)

In this novel separation technique, spectra containing varying concentrations of mixture ABC are scanned separately, stored, and then subjected to mathematical operations involving standard spectra with double divisors B′ and C′ in case to separate and analyze A. Double divisor was prepared by the addition of standard spectra B′ and C′ which is divided by the mixture spectra. The resulting ratio spectra of A were further multiplied by using the same double divisor to obtain a zero-order spectrum of A which was further utilized for estimating the concentration of A in the ternary mixture; the overall process is illustrated in Eqs. 1–3.

The stored spectra are divided by the standard spectrum, which consists of double divisors such as A′ and C′, to find the concentration of B in the ternary mixture; it is prepared by the addition of standard spectra A′ and C′ which is divided by the mixture spectra yielding the ratio spectra of B, which were further multiplied by using the same double divisor to obtain a zero-order spectrum of B which were further utilized for estimating the concentration of B in the ternary mixture; the process is illustrated in Eqs. 4 and 5. Similarly, to determine the concentration of C in the ternary mixture, the spectra obtained from Eqs. 3 and 5 were combined and subtracted from the initial mixture spectra, as shown in Eqs. 6 and 7.

This approach, utilizing double divisors and ratio spectra, provides a method for analyzing and quantifying the individual components (A, B, and C) within the ternary mixture [28, 29]. The mathematical procedures involved in these calculations contribute to the accurate determination of each component’s concentration in the complex mixture

1

$$A+B+CB'+C'=AB'C'+BB'C'+CB'C'=AB'+C'+\text{constant}$$

2

$$=AB'+C'+\text{constant}-\text{constant}=AB'+C'\times B'+C'$$

3

$$=A$$

The same steps are used to find the standard spectrum of B, by using the double divisor A′+C′

4

$$A+B+CA'+C'=AA'+C'\times BA'+C'\times CA'+C'$$

5

$$=B$$

6

=A+B+C-A+B

7

=C

Method 2: area under the Curve (AUC)

In this method, the area under the curve (AUC) is calculated by determining the integrated absorbance value between two specified wavelengths, denoted as $\lambda 1$ and $\lambda 2$. These wavelengths serve as the starting and ending points of the curve region in the spectra. The AUC is then computed within the wavelength range of ± 20 nm, representing a specific study region. The concentration of each drug in the ternary mixture is determined solely based on its absorption characteristics within this integrated region. This method provides a straightforward approach for quantifying the amount of each drug in the mixture, relying on the cumulative absorbance values over the specified wavelength range [30]. The combination of these two methods will have an advantage over the HPLC methods because Method 1 helps in the separation of the mixture were as Method 2 helps in determining the concentration of the drug based on the area of the spectra.

Experimental

Apparatus

The JASCO UV–visible double monochromator model number V-730 is utilized, sealed, and quartz-coated with Czerny–Turner monochromator optics, providing a wavelength range of 190–900 nm. Its incredibly low stray light percentage (0.00008%) allows for precise measurements across the largest photometric range (up to 8AU). The apparatus also boasts a narrow spectral bandwidth of 0.1 nm and extended photometric linearity up to 8Abs, enabling the measurement of highly absorbing samples. The photomultiplier tube serves as the detector, and data output is facilitated using the software Spectra Manager™ version 2.5.

Materials and chemicals used

Therapeutic pharmaceutical products, namely PAR, ACE, and TRM, were acquired as gift samples from, Shree Icon laboratory, Vijayawada, India, by re-analyze the content of PAR, ACE, and TRM using an official method; the result was obtained as 99.5 ± 1.25. The same products were employed as working standards without any additional treatment. Hayman premium grade ethanol is used for diluent preparation.

Marketed formulation

The commercially available Zerodol-PT, with the batch number of JTZ013011AS containing 325 mg of PAR, 100 mg of ACE, 37.5 mg of TRM, and additional excipients in sufficient quantities, was obtained from the local pharmacy. This medicine is manufactured and marketed by IPCA Laboratories Limited.

Diluent preparation

For analysis, a diluent is prepared by adding 20 ml ethanol (Hayman premium grade ethanol) in distilled water to reach 100 ml.

Procedure

Standard solution

Standard stock solutions for spectrophotometric analyses were prepared by accurately weighing 10 mg of pure ACE, PAR, and TRM, individually, and dissolving each in a 10-mL standard volumetric flask. To achieve complete dissolution, the solutions were prepared up to the mark using a diluent after being sonicated for three to five minutes (Dilution 1). Further, 1 mL of ACE, 3.25 mL of PAR, and 0.375 mL of TRM were taken from the above solution and diluted with 10 ml in the standard volumetric flask were made accordingly to achieve the concentration of working standards to obtain 10, 32.5, and 3.75 + 6.25 µg/mL (standard addition), respectively. Furthermore, 6.25 µg/mL of known concentration of TRM was added because TRM concentration was very less [31–34] when compared to the other two drugs ACE and PAR which is difficult to interpret in the presence of other. So, to achieve a maximum concentration equal to ACE and PAR, a 6.25 µg/mL concentration was raised by standard addition to achieve a nearer concentration of ACE (10–3.25 µg/mL = 6.25 µg/mL) (Dilution 2). From Dilution 2, 1 ml of each solution was taken and made up to 10 mL of the diluent (Dilution 3); these solutions were then scanned in a UV–visible spectrophotometer within the wavelength range of 200–400 nm. For linearity 0.5, 0.75, 1, 1.25, and 1.5 mL of each

solution was taken from Dilution 2 in standard volumetric flask and was made up to mark with 10 mL diluent and these solutions were then scanned in a UV–visible spectrophotometer.

Preparation of sample solutions from marketed formulations

A total of 20 tablets from the marketed formulation were finely ground, and the powder equivalent to 100 mg, 325 mg, and 37.5 mg of ACE, PAR, and TRM, respectively, was accurately weighed. This drug was transferred into a 100 mL standard volumetric flask, and 6.25 mg of TRM was added as a standard addition technique to avoid the hindrance of the other two drugs in determining TRM. To the flask added a small amount of diluent and sonicated the mixture for approximately twenty minutes. Finally, the volume was made up to 100 mL using the diluent. The resulting solutions were further diluted to obtain final concentrations of 10, 32.5, and 3.75 + 6.25 µg/mL of ACE, PAR, and TRM, respectively, and were designated as sample solutions.

Results

After the preparation of the drug solution, UV scanning was conducted over the wavelength range of 200–400 nm. The spectra of all three drugs exhibited absorbance within the challenging range of 240–280 nm, as depicted in the zero-order spectrum (Fig. 2). To distinguish individual drugs within the ternary mixture, specific methods were applied to each D^0 spectra individually. This step is crucial for accurate identification and quantification of the individual components in the complex mixture. According to the reported method, the UV spectrophotometric methods can be divided into different windows as the methods selected in the determination of three drugs fall in Window 1 (W1), which utilizes zero-order absorption spectra, and Window 3 (W3), which is based on ratio spectra.

Fig. 2 [Images not available. See PDF.]

Overlay of three drugs of UV spectra, ACE (10 µg/mL)—red, PAR (32.5 µg/mL)—blue, and TRM (3.75 µg/mL)—green

Method 1: double divisor ratio spectra method (DDRSM)

Determination of ACE

In this method, the first step involves generation of double divisor by adding the standard spectra of PAR′ and TRM′ to obtain a PAR′ TRM′ followed by a constant generation, where the standard spectra of PAR and TRM are divided by the double divisor PAR′ TRM′. The resulting ternary mixture spectrum is divided by the double divisor to obtain a spectrum, which is then subtracted from the constant and subsequently multiplied by the double divisor PAR′ TRM′ to yield a D^0 spectra of ACE at a wavelength of 276 nm. The overall separation process of ACE from the ternary mixture with the help of spectrums is depicted in Fig. 3

Fig. 3 [Images not available. See PDF.]

The process of how the ACE (10 µg/mL) was separated from the ternary mixture

Determination *of PAR*

To determine the D^0 spectra of PAR, the double devisor was prepared by adding the standard spectra of ACE′ with TRM′ to yield ACE′ TRM′ and then constant is prepared by dividing the standard spectra of ACE and TRM by the double divisor (ACE′ TRM′). The mixture spectrum is then divided by the double divisor, subtracted from the constant, and multiplied by the double divisor to obtain a D^0 spectra of PAR at a wavelength of 247 nm. The overall separation process of ACE from the ternary mixture with the help of spectrums is depicted in Fig. 4.

Fig. 4 [Images not available. See PDF.]

The process of how the PAR (32.5 µg/mL) was separated from the ternary mixture

Determination of TRM

This is achieved using spectra manager software. The above-obtained spectra of ACE and PAR are added together which are subtracted from ternary mixture spectra to obtain D^0 spectra of TRM at a wavelength of 270 nm. The overall separation process of ACE from the ternary mixture with the help of spectrums is depicted in Fig. 5.

Fig. 5 [Images not available. See PDF.]

The process of how the TRM (3.75 µg/mL) was separated from the ternary mixture

Method 2: Area under the curve method (AUC)

Determination of ACE

AUC is calculated for the D^0 spectra obtained from the method 1 to determine the concentration of the separated spectra. This can be performed by selecting a wavelength range of approximately 205–225 nm for the D^0 spectra (Fig. 6a).

Fig. 6 [Images not available. See PDF.]

Area under the curve selection for the three drugs **a** ACE (10 µg/mL), **b** PAR (32.5 µg/mL), and **c** TRM (3.75 µg/mL) at different wavelength ranges

Determination of PAR

AUC is calculated by selecting a wavelength range of approximately 235–255 nm for the AUC spectra (Fig. 6b) which is obtained in the above method. Furthermore, the spectra were utilized to calculate the marketed formulation unknown concentration.

Determination of TRM

AUC is determined for the obtained TRM spectra by selecting a wavelength range of approximately 260–280 nm (Fig. 6c).

Method validation

In accordance with ICH recommendations Q2R1, the developed methods underwent thorough validation, and specific parameters were assessed, including linearity, limit of quantification, detection, accuracy, and precision [30, 35–37].

Precision

The ACE, PAR, and TRM weights were measured precisely. Six repetitions of the identical process were carried out to ensure method repeatability, accounting for both intra- and interday fluctuations. Analyzing the drug solution three times in a single day allowed for the determination of intraday precision. By examining solutions with the same concentrations on three distinct days in a week, interday precision is determined in Table 1.

Table 1. Results for ACE, PAR, and TRM in terms of accuracy and precision

Parameter	Double divisor ratio spectra method		Area under the curve			
ACE	PAR	TRM	ACE	PAR	TRM	*Precision*
Interday precision (mean ± SD)	99.64 ± 1.27	99.30 ± 0.95	99.47 ± 0.87	99.33 ± 0.76	99.10 ± 0.93	99.04 ± 1.09
Intraday precision (mean ± SD)	99.62 ± 1.26	99.45 ± 1.00	99.30 ± 0.85	98.87 ± 0.79	98.60 ± 0.87	98.69 ± 1.05
Accuracy (mean ± SD)	97.76 ± 1.32	98.50 ± 0.59	97.26 ± 1.28	97.71 ± 1.00	98.01 ± 0.53	97.92 ± 1.25

Accuracy

Applying the standard addition method to a medication sample containing known concentrations of ACE, PAR, and TRM standard drugs is equal to 50%, 100%, and 150% of the label claim allowed for the assessment of the method's accuracy. The experiment's results are listed in Table 1.

Stability

Working solutions of ACE, PAR, and TRM were stored in tightly sealed containers at approximately 4 °C for three weeks. Volumetric flasks containing these solutions were covered with aluminum foil to ensure protection.

Linearity

The method's linearity was determined by evaluating five different concentrations ranging from ACE (8–12 µg/mL), PAR (22.75–35.75 µg/mL), and TRM (2.62–4.12 µg/mL), respectively. The linearity spectra for each separated drugs are depicted in Fig. 7, and the results are depicted in Table 2. The detection limit (LOD) and quantification limit (LOQ) for ACE, PAR, and TRM were estimated using the calibration curve approach. LOD represents the smallest detectable amount of an analyte, while LOQ is the smallest quantifiable amount with appropriate precision and accuracy. The results are shown in Table 2.

Fig. 7 [Images not available. See PDF.]

Linearity spectra for the three drugs **a** ACE (8–12 µg/mL), **b** PAR (22.75–35.75 µg/mL), **c** TRM (2.62–4.12 µg/mL) at different concentration ranges

Table 2. Linearity data for the proposed method

Parameter	Double divisor ratio spectra method			Area under the curve		
ACE	PAR	TRM	ACE	PAR	TRM	Wavelength (nm)
276	247	270	205–225	235–255	260–280	*Y*
0.013*x*–0.042	0.040*x*–0.049	0.062*x*–0.132	0.077*x*–0.226	0.042*x*–0.263	0.413*x*–0.883	Slope
0.013	0.04	0.062	0.077	0.042	0.413	R^2
0.999	0.998	0.999	0.999	0.998	0.999	LOD (µg/mL)
0.080	0.859	0.026	0.136	0.745	0.025	LOQ (µg/mL)
0.243	2.603	0.078	0.411	2.259	0.077	Linearity range (µg/mL)
8–12	22.75–35.75	2.62–4.12	8–12	22.75–35.75	2.62–4.12	%RSD

Assay of marketed formulation

The proposed spectrophotometric techniques were applied to analyze a commercially available tablet, Zerodol-PT. The mean drug content ranged from 99.5 ± 1.25. No interference peaks were observed in the spectra, indicating accurate approximation of the medication without considering excipients, and the results are depicted in Table 3.

Table 3. Assay of tablet formulation

Brand	Content	Amount present (µg/mL)	Amount added (µg/mL)	Double divisor ratio spectra method		Area under the curve	
Amt. founded (µg/mL)	Percentage recovered	Amt. founded (µg/mL)	Percentage recovered	Zerodol—PT	ACE		5
5.05	101	5.1	102	10	10	9.95	99.5
10	100		15	15.08	100.53	15.04	100.26
Average:			100.75		99.45		PAR
	16.25	16.1	99.07	16.18	99.56	32.5	32.5
32.43	99.78	32.55	100.15		48.75	48.6	100.3
48.7	99.89	Average:		99.86			99.71
	TRM		1.875	1.88	100.26	1.87	99.73
3.75	3.75	3.72	99.2	3.8	101.33		5.625
5.63	100.08	5.62	100.05	Average:			

Statistical analysis

A statistical comparison between the proposed method and reported method [38] is suggested for ACE, PAR, and TRM analysis in pharmaceutical dosage form with respect to assay, and the results are depicted in Table 4. The calculated Student's *t* and *F* test with a null hypothesis (H_0) stating no difference observed between the values and an alternative hypothesis (H_a) stating the maximum difference between the values is observed. The values obtained were found to be less than the tabulated ones.

Table 4. Determination by Student *t* test and *F* test for the proposed method and reported method [38]

Reported method	Proposed method	*t* test	*F* test	*t* test critical value	*F* test critical value

DDRSM	AUC	DDRSM	AUC	DDRSM	AUC	97.26	99.45	100.75
0.318	0.95	0.128	0.502	4.303	19	100.49	99.71	99.86

Green assessment for the proposed method

The development of the method was rooted in adherence to the twelve principles of analytical green chemistry. Initially, the solvent selection was done based on the G score; it was found that ethanol has a G score of 6.6 which is near to water (7.3). To assert the eco-friendliness of an analytical approach, substantiation through assessment tools is imperative. In this case, two distinct assessment tools were employed for evaluating the developed method. The first, AES, underwent manual computation for its assessment. Conversely, the GAPI assessment utilized software termed the GAPI chart generator version 1.0 which was employed for crafting the GAPI chart, while AGREE metric calculations were conducted utilizing AGREE calculator version 0.5 beta. This comprehensive approach ensured a thorough evaluation of the method's environmental sustainability [39–44]

Green analytical procedure index (GAPI)

GAPI stands as a qualitative gauge utilized to appraise the ecological soundness of an analytical methodology. Operating as a semi-quantitative instrument, it employs pictorial depictions to gauge the environmental sustainability of analytical procedures. By integrating eco-friendly ethanol solvents into the methodology, a method was developed to symbolize a green emblem, highlighting its environmentally mindful characteristics. The GAPI assessment incorporates a color-coded representation of the GAPI value within the final pictogram, offering a visual indicator of its eco-friendliness. Comprising 15 discernible stages, the GAPI evaluation framework is meticulously structured within the GAPI software interface. The proposed method underwent a comprehensive assessment via the GAPI software, with the outcomes elucidated in Table 5 showcasing its environmental viability.

Table 5. Comparison of green metric tools between developed method and proposed methods

Author name	Instrument	Solvent	GAPI	AGREE	ECO SCALE	Refs.
P. Chouhan	RP-HPLC	Methanol: 0.5% Triethanolamine			6+1+1+3+3=14 100–14=86	[38]
Preeti Chandra et al.	HPLC	40: 60 (v/v); phosphate buffer (pH 6.0): methanol			6+1+3+5=15 100–15=85	[5]
V.V. Vaidya et al.	RP-HPLC	0.02M potassium dihydrogen orthophosphate buffer pH 3.0: acetonitrile in the ratio (40:60) v/v			6+1+3+3=13 100–13=87	[46]
Proposed method	UV–visible spectrophotometer	20% ethanol; 80% distilled water			6+0+0+0+0=6 100–6=94	

Analytical eco-scale (AES)

The AES assessment relies on the assignment of penalty points (PP) to chemicals involved. PP is derived through a graphical representation illustrating the chemicals employed in the process. It encompasses four primary evaluation stages, culminating in the AES computation formula: AES = 100—PP.

In the initial stage, ethanol is depicted with three pictograms, resulting in an overall PP of 3. Proceeding to the second stage, as the quantity of solvent or reagent utilized per sample is less than 10 mL, the PP is calculated as 3 × 2 (pictogram score), yielding 6.

Moving to the third stage, with the UV energy consumption per analysis falling below 0.1 kWh, the PP is set at 0. Subsequently, in the fourth stage, the method's waste of solvents, known to have environmental ramifications, is evaluated based on the employed wastage recycling approach, leading to a PP of 0.

Consequently, with the cumulative PP loss for the devised technique totaling 9, the resulting AES score for the developed method stands at 94.

Analytic GREEnness (AGREE)

AGREE metrics is an innovative tool crafted to gauge how environmentally friendly. It provides a detailed look at how these methods impact the environment. The results from AGREE are displayed as a circular graph split into 12 sections, each reflecting one of the twelve green analytical chemistry principles. Every section gets a score between 0 and 1, where 1 signifies the highest level of eco-friendliness. The average score appears at the center of the graph, and the closer it is to 1, the better it is for the environment. The software is designed to fully integrate the established methodology, ensuring that the outcomes reflect the most environmentally friendly approach possible. You can see a summary of the AGREE findings in Table 5. Application of the present method to the AGREE tool yielded a score of 0.91, indicating its strong alignment with GAC principles and highlighting its environmentally friendly analytical attributes.

The developed method underwent a thorough comparison with established HPLC techniques to evaluate its efficiency. The term "greenness" denotes not only the absence of efficiency drawbacks in the developed method but also its exceptional ecological safety profile. When scrutinized for environmental impact alongside other methods, the developed approach exhibited significantly higher greenness scores [45]. These findings, including the comparative analysis results, are detailed in Table 5.

*ACE and PAR dissolve readily in organic solvents and completely insoluble in water. Based on this aspect, any method which utilized water as a solvent to dissolve these drugs was not considered into account because it is practically not possible [47]. Pharmaceutical formulations typically use organic solvents such as ethanol, methanol, chloroform, and acetone to dissolve active ingredients. Both compounds are more soluble in organic solvents than in water; however, this might vary depending on the solvent and other conditions such as temperature [48].

Discussion

UV spectrophotometry, a widely employed and efficient analytical tool in both industry and academia, forms the basis of this study. The primary focus is on the Multicomponent Analytical Technique, showcasing its ease of transferability. To mitigate environmental and safety concerns associated with the typical solvent methanol, a 20% concentration of biodegradable and environmentally friendly ethanol in water is employed. This approach aims to reduce solvent consumption and enhance affordability for responsible consumption of the solvents which is an US SDG goal 12 [49]. Notably, all three medications demonstrated full solubility in the 20% ethanol solution without any issues.

The application of DDRSM facilitated the accurate determination of individual drug components within the mixture. By carefully manipulating the spectra and utilizing specific wavelengths, we isolated the D^0 spectra of each drug, enabling their quantification. Additionally, AUC provided another avenue for determining the concentration of separated spectra. By selecting appropriate wavelength ranges, we could accurately calculate the concentration of each drug component. One significant advantage of these methods is their ability to estimate overlapping spectra, particularly when utilizing environmentally friendly solvents. This ensures accurate analysis of the ternary mixture in tablet dosage form, contributing to both analytical efficiency and environmental responsibility.

In terms of method validation, precision results were found to be accurate for both the proposed methods at various time intervals. Research has demonstrated that the low percentage RSD value and recovered concentration work synergistically, with %RSD below 2%. There was no significant variation in the accuracy results between the drug's various concentrations, despite six determinations being conducted at each level. Stability studies over a period of three weeks revealed no spectrophotometric alterations in the drug solution. The linearity assessment of the proposed method demonstrates its ability to accurately measure concentrations of ACE, PAR, and TRM over their respective linear ranges. The high correlation coefficients (R^2 values close to 1) indicate excellent linearity between concentration and response for each drug. LOD and LOQ values indicate the method's sensitivity, with low values suggesting its capability to detect and quantify trace amounts of the analytes with good precision and accuracy. The %RSD values, representing the relative standard deviation, reflect the method's precision, with low %RSD values obtained indicating good repeatability and reproducibility of the method. Overall, the linearity results validate the suitability of the proposed method for accurate and precise quantification of ACE, PAR, and TRM in pharmaceutical formulations. Statistical comparison between the proposed methods shows no significant difference in terms of the assay, as evidenced by calculated student *t* and *F* tests with H_0 (no difference observed between the values) accepted. Furthermore, there was no discernible difference seen when comparing the dosage form analysis results using the reported methodologies, hence supporting the acceptance of H_0.

The results of the green assessment for the proposed method demonstrate its eco-friendliness and adherence to GAC principles. The utilization of ethanol solvents and the implementation of efficient waste recycling approaches contributed to the high scores obtained from GAPI, AES, and AGREE assessments. The developed method’s AES score of 94 indicates minimal environmental impact, further supported by its AGREE score of 0.91. These scores signify strong alignment with green analytical chemistry principles and highlight the method's environmentally friendly attributes. Comparative analysis with established HPLC techniques reaffirms the superiority of the proposed method in terms of greenness. The method not only offers efficient analytical performance but also ensures exceptional ecological safety, making it a promising choice for environmentally conscious analytical practices.

Conclusion

After dwelling on the above research, it becomes apparent that the proposed techniques are straightforward and uncomplicated to implement, requiring no specialized methods or tools. These analytical methods were very specific, exact, linear, accurate, and consistent in detecting ACE, PAR and TRM in tablet formulation. In contrast to the organic solvents typically utilized in UV spectroscopy, this approach is environmentally benign. UV–visible spectroscopy proves to be superior, cost-effective, and eco-friendly for determining the three medications. Ethanol serves as the solvent, and both methods have been validated according to ICH requirements, even with varying concentrations of the three drugs without pre-separation. The %RSD value, determined from the results and discussions, is less than 2%. Notably, this method poses no negative environmental impact, making it easily adaptable for the determination of ACE, PAR, and TRM medications. The method underwent additional evaluation utilizing environmentally conscious assessment techniques, yielding notably eco-friendly outcomes, particularly evident when employing ethanol and water as solvents. With negligible adverse environmental impacts, the energy demand for UV–visible spectroscopy instrumentation remains low. Its versatility extends to the analysis of ACE, PAR, and TRM, along with other drug compounds, owing to their solubility in ethanol and water. Consequently, this technique is highly adaptable and favored by pharmaceutical industries and quality control departments for routine research and sustainable development endeavors.

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Author contributions

All authors have read and approved the manuscript. Thirumalai Arunagiri contributed to method development, Alagammai Ganesan performed manuscript draft and writing, Vamsi Ravi Kumaran performed manuscript draft writing, Bharathraj Masilamani performed experiments and writing, Kanaka Parvathi Kannaiah* contributed to

supervision, manuscript correction, method development, and software detailing, and Damodharan Narayanasamy was involved in supervision and manuscript corrections.

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Abbreviations

GAC
Green analytical chemistry
ACE
Aceclofenac
PAR
Paracetamol
TRM
Tramadol
COX
Cyclo-oxygenase
μOR
μ Opioid receptor
WADA
World anti-doping agency
EPA
Environmental Protection Agency’s
DDRSM
Double divisor ratio spectra method
AUC
Area under the curve
ICH
International Council for Harmonization
LOD
Limit of detection
LOQ
Limit of quantification
μg/mL
Microgram per mL
RSD
Relative standard deviation
SDGs
Sustainable development goals
AGREE

Analytic GREEnness
GAPI
Green analytical procedure index
AES
Analytical eco-scale

Publisher's Note

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Targeting small druggable compounds against 3RZE histamine H1 receptor as potential of anti-allergic drug applying molecular modeling approach

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ABSTRACT (ENGLISH)

Background

Allergic disorders, prevalent global health concerns, afflict a substantial portion of the world's population. These maladies result from an exaggerated immune system response to ordinarily innocuous substances, such as pollen, dust mites, and specific dietary components. Clinical manifestations of this heightened immune response include itching, swelling, and respiratory impairment, often accompanied by releasing mediators like histamine. The pathophysiological mechanisms of allergy disorders are intricate, arising from a complex interplay between genetic and environmental factors. While clinical presentations may vary, all allergy conditions share a common foundation in the dysregulated immune response to allergens.

Result

The current aim of this study was to identify innovative anti-allergic agents capable of inhibiting histamine and effectively mitigating allergic reactions by utilizing the computer-aided drug design approach by discovery studio (DS) 2022 v 23.1.1 package. The overarching aim was identifying potential drug candidates targeting the active site within the histamine H1 receptor complex; therefore, a collection of 4000 small druggable compounds was curated from ZINC, PubChem, and DRUG BANK databases sources. Four compounds appeared as promising candidates after assessing docking scores and binding energies. Notably, Compound ID 34154, recognized as tymazoline, showed the highest affinity for the H1 receptor of 3RZE, suggesting it may be the most promising choice for more research. Further chemoinformatic and ADMET (absorption, distribution, metabolism, excretion, and toxicity) analyses were conducted to assess the drug-like qualities of this chosen molecule. In addition, bioisosteric substitution techniques were employed to enhance tymazoline's ADMET characteristics.

Conclusion

Tymazoline shows strong binding affinity with 3RZE and verified all the drug-likeness criteria to inhibit the allergic disorders. Furthermore, molecular dynamics (MD) studies corroborated tymazoline's potential as an anti-allergic agent, demonstrating contact between the ligand and the receptor that is well defined and stable.

FULL TEXT

Background

Allergic responses are an abnormal and overactive immune reaction triggered by exposure to allergenic substances can lead to tissue damage or dysfunction [1]. Critical actors in this process include IgE antibodies, mast cells, and cytokines, serving as mediators that coordinate immune cells and chemical signals crucial to the allergic response [2]. Estimates write down that these conditions affect 20–30% of the global population, with variations influenced by geographic location, age groups, and other factors, including genetic predisposition, environmental exposures, and changes in dietary and lifestyle choices [3]. Histamine is a significant mediator in localized allergic hypersensitivity reactions [4]. Besides, histamine receptors are found in four distinct types known as H1, H2, H3, and H4, which are

expressed differently on different cell types and regulate many physiological processes. The activation of H1 receptors causes vasodilation, increased vascular permeability, and bronchoconstriction, all defining characteristics of allergic reactions [5]. Allergic reactions are typically categorized into two primary types: one is IgE-mediate, and the other is non-IgE-mediate. The most frequent allergic reactions, known as IgE-mediate reactions, are brought on by the binding of allergen-specific IgE antibodies to basophils and mast cells. This causes the release of histamine and other inflammatory mediators, which lead to the manifestation of allergy symptoms [6, 7]. Current medications and treatments used to manage persistent allergies often show limited effectiveness [8, 9].

This study proposed the most significant anti-allergic small druggable compounds that have undergone comprehensive investigation for their ability to suppress receptor cells [10]. Small druggable compounds that function as druggable ligands hold significant promise for allergy treatment due to their multifaceted properties, which can neutralize allergens and other beneficial effects [11, 12]. A substantial proportion, approximately 60%, of anti-allergy medications trace their origins to these small druggable compounds because these small druggable compounds have mechanisms of alleviating the inflammatory or allergic responses triggered by allergenic substances [13].

To find the most promising drug molecule, four thousand small druggable compounds were collected in this study, which have characteristics related to suppressing the hypersensitivity from diverse databases (ZINC, PubChem, DRUG BANK) to find potential anti-allergic compounds. Molecular modeling investigations of these compounds were performed using the Computer-Aided Drug Design approach, employing the DS 2022 v 23.1.1 package. The molecular modeling study includes the molecular docking, drug assessment, and dynamics simulations of the histamine H1 receptor of PDB ID is 3RZE. Tymazoline, used as an antihistamine to help reduce swelling and inflammation [14], showed the most excellent affinity for the H1 receptor of 3RZE. The good affinity between tymazoline and the 3RZE H1 receptor implies that tymazoline would be the best inhibitor to suppress the allergic response [15]. Comprehensive analyses were performed to evaluate tymazoline’s drug-likeness, ADMET characteristics, and toxicity. A Molecular dynamics (MD) simulation was performed to confirm tymazoline’s anti-allergic potential against the histamine H1 receptor. Molecular dynamics simulation results were analyzed on the base of root mean square deviation (RMSD) and root mean square fluctuation (RMSF) that was kept at a standard value of 2.25 Å, and tymazoline made stable and satisfactory interaction with 3RZE. Tymazoline complex with 3RZE keeps RMSD lower than the standard value, which reveals a more vital acceptable interaction of complex while slight fluctuation of residues during root mean square fluctuation (RMSF), which confirms that the compound tymazoline gave more promising results than the reference co-crystal inhibitor compound. To our knowledge, tymazoline was first revealed to potentially have the function of an anti-allergic drug by inhibiting the 3RZE H1 receptor cell based on the in silico study.

Material

Bioactive compounds

Since the 3RZE H1 receptor is selected as the target protein and the binding site, 4000 small druggable compounds are collected from various databases (ZINC, PubChem, DRUG BANK) to find potential anti-allergic compounds. These anti-allergic compounds were investigated to check their inhibitory capabilities against the hydrolase enzyme associated with the histamine receptor, explicitly targeting the protein structure represented by PDB ID 3RZE.

Preparation of 3RZE H1 receptor structure

The histamine H1 receptor 3RZE complex’s three-dimensional structure with the ligand doxepin was obtained from Protein Data Bank (www.rcsb.org) and then downloaded file open into DS 2022 v 23.1.1 for further analysis. To enhance the accuracy and relevance of the structural information, computational procedures were applied, including energy minimization, 3D protonation, water molecule removal, and chiral gradient with a resolution of 0.05 Å [16]. By the DS 2022 v 23.1.1 package, the amino acid (THR 112, TYR 108, PHE 432, ILE 454, ASP 107, TRP 158, SER 111) in the 3RZE protein's active site was identified. Computational analyses were conducted using the DS 2022 v 23.1.1 package in an HP laptop 348 G7 with a 1.6 GHz frequency, up to 4.2.1 GHz with intel turbo technology, 8 GB RAM, and 250 GB hard disk.

Molecular docking

After the mentioned protein 3RZE and ligands were prepared by DS 2022 v 23.1.1, molecular docking is ready for processing. The compounds' 3D structures were created, and CHARMM (Chemistry at Harvard Molecular Mechanics) energy minimization was applied within DS 2022 v 23.1.1 using standard smart minimizer protocol [17]. The cleaning water content and energy minimization by CHARMM method within DS 2022 v 23.1.1 are necessary steps taken to prepare the protein molecules by following the standard protocol of smart minimizer in 2000 steps associated with gradient threshold 0.01 kcal/mol [18]. The binding cavity, or active sites, of the energy-minimized protein molecule was surrounded by a receptor grid. This was accomplished by locating important residues of amino acids. These amino acid residues were defined by the choice of the co-crystal ligand, or active inhibitor, linked to the corresponding protein molecule. This process helped to aid in the prediction of binding locations. For the protein structure appointed as 3RZE, the receptor grid boxes that were created were set up with a binding site sphere that had a radius of 2.90 Å and dimensions of 10.858743, 12.558267, and 85.699954 along the *x*, *y*, and *z* axes, respectively [19].

A standard protocol of CDOCKER in DS 2022 v 23.1.1, a molecular docking method based on CHARMM's position, provides highly exact docking results; it is used to find the precise position of drugs inside the active site of a target protein. Some default values, such as simulated annealing and forcefield for all docking and scoring-related parameters, were kept to efficiently generate the docked conformations for the compound of interest [20]. The prediction of binding affinities for the docked compounds was achieved [21]. Furthermore, the interaction types corresponding to the highest docked position were scrutinized in the context of the three-dimensional complex of ligand and receptor. Various non-bonding interplay, including hydrophobic and hydrogen bonding interactions, were assessed using two-dimensional diagrams depicting the receptor–ligand complexes. Each ligand was subjected to generating ten distinct poses within the active site of the receptor molecule, and those ligand molecules showing higher binding affinities were selected as potential drugs for further analysis and consideration.

Drug-likeness properties

Drug-likeness properties and drug-related factors were evaluated using theoretical methods in DS 2022 v 23.1.1. These drug-likeness characteristics included the LogP, molecular polar surface area, molecular weight, number of hydrogen bond donors, and number of hydrogen bond acceptors, all essential elements of Lipinski's rule of five (RO5) [22]. The number of rotatable bonds, aromatic rings, and other physicochemical characteristics were also estimated. The drug-likeness score was used to recognize and confirm the criteria for evaluating pharmaceutical compounds.

ADMET analysis

The ADME-toxicity parameters are figured out by ADMET descriptor tool within DS 2022 v 23.1.1. Distinct types of mathematical modules are used to determine the drug properties, which help to predict drug molecules' pharmacokinetics (pk) and ADMET characteristics. These models encompass plasma protein binding (PPB), cytochrome P450(CYP)2D6 inhibition, aqueous solubility, intestine solubility, blood–brain barrier (BBB), and hepatotoxicity. The mentioned six valuable indices insights into the compound's behavior about safety and pharmacokinetics within biological systems.

Toxicity assessment

Toxicity analysis is virtually assessed by the TOPKAT tool of the DS 2022 v 23.1.1. package. The following toxicity parameters computed to assess carcinogenicity and mutagenicity include rat female or male NTP(National toxicology program potential), mouse female or male FDA (Food and Drug Administration), rat female or male NTP, Ames test prediction, mouse female and male FDA, and rat oral LD50. These toxicity parameters provide valuable information on the compound's safety profile, particularly concerning its potential to cause cancer or mutagenic effects.

Molecular dynamics simulation

Since tymazoline exhibited the most favorable bonding with the H1R target molecule; therefore, it underwent further investigation for MD simulations by DS 2022 v 23.1.1. The target proteins' first crystal structures bound to co-crystal

inhibitors, as well as the complexes of 3RZE and tymazoline, were chosen for inclusion in the MD simulation. The necessary preparations, such as solvent environment using explicit periodic boundary conditions within an orthorhombic box filled with water molecules, were made to ensure the integrity and accuracy of protein and ligand complex by using standard protocol within DS 2022 v 23.1.1 [23]. CHARMM-based smart minimizer, which executes 2000 steps of steepest descent followed by conjugate gradient algorithm with RMSD gradient of 0.01 kcal/, was used to minimize the energy. The distance between the solute molecules and the box boundary was set to 5 Å to create a sufficient buffer zone, as well as 0.15 M sodium chloride was applied to the system during solvation to keep charge neutrality and physiological ion concentration. The stability of complex molecules associated with energy minimization was confirmed by default. MD simulation of ligand and receptor molecule complex was gradually started over a heating period of 10 picoseconds and equilibrated for 10 picoseconds at 300 K temperature using the standard dynamics cascade in DS 2022 v 23.1.1. The main molecular dynamics production run was conducted for 500 picoseconds in the NPT ensemble, and snapshots of the system were saved at regular intervals of 2 picoseconds throughout this process.

RMSD and RMSF

The root mean square deviation (RMSD) calculates the average distance between a molecule's atoms at various simulation time periods in relation to a ligand structure. To calculate RMSD during MD simulation, the initial structure of molecule coordinates is compared with the coordinates of ligand structure, and the distance between the atoms is calculated. RMSD is computed in DS 2022 v 23.1.1 by tool trajectory analysis, which indicates how much the molecule shows deviation from initial conformation during simulation periods.

The root mean square fluctuations (RMSF) calculate the individual atoms' fluctuation or flexibility throughout the course of a simulation in a molecule. The average deviation of each atom's position from its mean position during the simulation is used to calculate it. Which areas of the molecule are more flexible or rigid can be determined using RMSF. High RMSF values denote highly flexible areas, while low RMSF values point to more rigid or lowly flexible areas. After simulation, a trajectory analysis tool was used within DS 2022 v 23.1.1 to calculate the values [24].

Binding free energy calculation

The free binding energy of each protein–ligand complex was determined using the Binding Free Energy Single Trajectory Tool in DS 2022 v 23.1.1 after molecular dynamic simulation. The average binding free energy of each ligand complex is determined by calculating the binding free energy of all the produced conformations during the analysis.

Redocking of selected compound

Once the MD simulation was finished, tymazoline was chosen, and it was rocked with 3RZE to examine the stability of the ligand molecule, which showed signs of new hydrogen bonds and other hydrophobic interactions. In DS 2022 v 23.1.1, the CDOCKER default protocol was used. Additionally, all grid generation and docking analysis settings were left at their default values, as stated in the previously reported technique was used for docking analysis.

Results

Molecular docking analysis

Histamine receptor 3RZE underwent thorough validation procedures to ensure its suitability for later docking studies. Before the commencement of the docking experiments, a receptor grid model was generated, and binding site spheres were optimized to enhance the predictive accuracy of affinities between the ligand and receptor molecule, as the figure indicates the generation of the binding site/active site Sphere around the H1R protein molecule for protein–ligand docking.

All ligand compounds docked in DS 2022 v 23.1.1 by default, CDOCKER protocol, and total binding energies were calculated based on CDOCKER binding energy and ligand and receptor molecule interaction energy. After the docking process, four compounds out of 4000 were selected based on the docking score and interaction between the receptor and ligand molecules. Among four selected compounds, the compound tymazoline, known as PUB CHEM ID 34154, showed the most vital binding energy with the receptor, which is − 66 kcal/mol, and interaction energy of − 12 kcal/mol with the receptor active site, and the last compound is referred as reference compound as

shown in Table 1. In contrast, other ligand molecules show weak binding energies and positive interaction energies, which is incompatible with further analysis. Tymazoline was chosen as a potential candidate against receptor molecule 3RZE (Fig. 1).

Table 1. CDOCKER Binding energy and interaction energy of ligand molecules with H1R

Protein (3RZE) histamine receptor 1	Bioactive compound	CDOCKER binding energy(kcal/mol)	CDOCKER interaction energy(kcal/mol)
H1R	Tymazoline compound CID: 34154	−66.2616	−12.1047
H1R	Cyanidin compound CID:128861	−46.0177	−8.9929
H1R	Theogallin compound CID:442988	−43.2617	+4.26146
H1R	1-Propanamine, 3-dibenz(*b*,*e*)oxepin-11(6H)-ylidene-*N*,*N*-dimethyl-(Reference compound)	−32.8309	−2.04392

Fig. 1 [Images not available. See PDF.]

Receptor grid model of histamine receptor 1 (3RZE); the figure indicates the generation of binding site/active site sphere around the H1R protein molecule for protein–ligand docking

The interaction between the ligand and the residues of the receptor binding site that interacts with the ligand molecule was checked by a 2D interaction diagram. Hydrogen bonding and hydrophobic interactions, known as secondary interactions, displayed a well-defined pattern, displaying the compatibility between the ligand atom and residues of the receptor molecule. Some other interactions, such as hydrophobic bonds and non-bonded interactions, were also checked. Still, these interactions are less critical than hydrogen bonding interactions, as shown in Fig. 2.

Fig. 2 [Images not available. See PDF.]

2D interactions of selected compound **A** (tymazoline), **B** (cyanidin), **C** (theogallin), **D** (1-Propanamine, 3-dibenz(*b*,*e*)oxepin-11(6H)-ylidene-*N*,*N*-dimethyl-)(Reference drug) within the binding site of receptor compound 3RZE, the dotted green line representing conventional hydrogen bond and others representing different types of hydrophobic bond

These visual depictions emphasize the potential binding modes and interactions between the ligand and interacting residues of the binding site in the receptor molecule. Furthermore, a detailed compilation of critical residues, interaction types, RMSD, and the atoms involved in several types of bonding interactions is shown in Table 2.

Table 2. Prediction of RMSD, interaction types, and interacting residues within the binding site of H1R

Compound	RMSD (Å)	Interaction types	Interaction residues

Tymazoline	0.54	Van der Waals Conventional Hydrogen Bond Carbon Hydrogen Bond Pi-Alkyl Pi-Pi T Shaped	THR(A:112) TYR(A:108) ASN(A:198) PHE(A:432) SER(A:111)
Cyanidin	0.97	Van der Waals Conventional Hydrogen Bond Attractive Charges Pi-Alkyl Pi-Cation	TYR(A:458,) TYR(A:431,108) ASP(A:107) ILE(A:454) LYS(A:179)
Theogallin	1.57	Van der Waals Conventional Hydrogen Bond Carbon Hydrogen Bond Pi-Alkyl Pi-Pi T Shaped	THR(A:112) TYR(A:108,431) ASP(A:107) TRP(A:158) SER(A:111) LYS(A:179)
1-Propanamine, 3-dibenz(*b*,*e*)oxepin-11(6H)-ylidene-*N*,*N*-dimethyl-(Reference compound)	0.79	Van der Waals Carbon hydrogen bond Pi-Pi T Shaped Pi-Anion	THR(A:112) TYR(A:108) ASP(A:107) ASN(A:198) TRP(A:428)

Docking analysis of selected compound with protein 3RZE

The specific molecular interaction in the active site of the protein 3RZE with the tymazoline molecule is meticulously documented in the first row of Table 2. To mitigate the tymazoline toxicity, a series of modifications were performed through fragment-based design using a built-in function in DS 2022 v 23.1.1. This approach helps to increase drug molecules' alkaline properties and to decrease tymazoline's toxicity. Interactional changes and the position of interacting ligands within the binding site of receptor molecules were analyzed in 2D and 3D diagrams, as depicted in Fig. 3.

Fig. 3 [Images not available. See PDF.]

A 2D interaction of selected compound tymazoline interaction after fragment base implementation with H1R, **B** 3D structure represents the interaction of residues with H1R active site or hydrophobic cloud around the ligand molecule In particular, the square box colors in Fig. 3 delineate the specific interactions between individual residues and the

drug within the protein's active site.

Drug-likeness

To analyze the drug-like properties of the ligands that showed the highest docking scores for the target molecule, we employed RO5 (Lipinski's rule of five). According to RO5, the molecular weight of the ligands should be equal to or less than 500 Da, have less than ten hydrogen bond acceptors, less than five hydrogen bond donors and miLogP value should not exceed five. Tymazoline shows the same result as having two hydrogen bond acceptors and one hydrogen bond donor, 232.32 g/mol molecular weight, and miLogP value 2.39, as shown in Table 3. Consequently, it received a drug-likeness score of 0.55 according to RO5, which falls within the acceptable range of 0–1.

Table 3. Prediction of RO5 (Lipinski's rule five) of promising drug candidate tymazoline

Compound	MW g/mol	HBA	HBD	Mi Log Value	Lipinski's rule violation
Tymazoline	232.32	2	1	2.39	0

ADMET analysis

ADMET analysis and structural modifications were employed in DS 2022 v 23.1.1 to mitigate the potential toxicity of tymazoline, which exhibited notably poor blood–brain barrier permeability (BBB) and less aqueous solubility (AS), producing no central nervous system (CNS) toxicity. The cytochrome P450 2D6 (CYP 2D6) enzyme is pivotal in drug metabolism, which shows the positive effect of tymazoline and acts as an inhibitor. At the same time, predictive assessments revealed a considerable potential for hepatotoxicity associated with tymazoline, as shown in Table 4.

Table 4. Predicted ADMET properties of tymazoline AS (high), BBB4 (very low), HEPTOX (true, toxic), PPB (true, highly bounded), CYP P450 2D6 (yes, inhibitor)

Pharmacokinetics properties	Effect
AS	High
BBB	4
HEPTOX	True
IA	0
PPB	True
Log Kp	−6.01 cm/s
CYP1A2 inhibitor	No
CYP2C19 inhibitor	No
CYP2C9 inhibitor	No
CYP3A4 inhibitor	No

CYP P450 2D6	Yes

Furthermore, assessments of intestinal absorption (IA) indicated a favorable level, which is 0, ranging from good to better, and plasma protein binding studies unveiled high protein binding for tymazoline.

Toxicity prediction

Toxicity analysis elucidates that the target compound tymazoline demonstrated non-carcinogenic and non-mutagenic properties. The specific toxicity parameters computed for tymazoline include mouse female NTP non-carcinogenic or male NTP carcinogenic, mouse female or male FDA non-carcinogenic, rat female or male NTP non-carcinogenic, Ames prediction (test of mutation in DNA) non-mutagenic, and the Bayesian score (Bs) which represent the dosage activity is less than 1 in all the computed animal except mouse male NTP as shown in Table 5. In-depth analysis using diverse in silico models for non-ruminant animals substantiated their safety and non-toxic nature.

Table 5. TOPKAT toxicity data and Bayesian score of the selected compound tymazoline

Compound (tymazoline)	Effect	BS
Mouse male NTP	Carcinogen	0.68
Mouse female NTP	Non-carcinogen	−1.45
Rat male NTP	Non-carcinogen	−1.36
Rat female NTP	Non-carcinogen	−454
Mouse male FDA	Non-carcinogen	−2.59
Mouse female FDA	Non-carcinogen	−6.55
Rat male FDA	Non-carcinogen	−2.24
Rat female FDA	Non-carcinogen	−3.39
Ames prediction	Non-mutagen	−9.93

Molecular dynamics simulation

MD simulations were meticulously conducted for the compound showing the highest binding affinity, namely tymazoline, with the 3RZE. Simulation of the 3RZE complex was executed under solvated 0.145 NaCl salt concentration for 500 picoseconds by the standard dynamics cascade module integrated within the DS 2022 v 23.1.1. Subsequently, the trajectory of the simulated outcomes was subjected to an exhaustive analysis employing the DS 2022 v 23.1.1 tool, precisely the "Analyze Trajectory" function. Trajectory analysis primarily centered on assessing two critical parameters, focusing on RMSD and RMSF as crucial metrics to identify the stability of ligand molecules within the active site of 3RZE.

Root means square deviation

MD simulations were performed on the protein complexed with tymazoline alongside the reference molecule as co-crystal inhibitor doxepin inside the protein binding site to investigate alterations in protein dynamics and the compliance stability of ligand–protein complexes. During simulations, RMSD was used as a critical parameter to

gauge the conformational changes of ligands within the active site of the 3RZE complex, which showed initial oscillations in the beginning simulation. Also, free protein is supplied to check the comparison between the complex of ligand-bounded protein and reference ligand-bonded protein, while in the free state, it shows more fluctuation throughout time. Still, the stabilization phase in ligand-bonded protein was achieved after approximately 42 picoseconds, as shown in Fig. 4.

Fig. 4 [Images not available. See PDF.]

MD simulation indication of RMSD, the blue line represents the selected ligand bounded within H1R, gray line represents the protein H1R, and the orange line represents the reference ligand bonded within H1R

Subsequently, there was a progressive decrease in RMSD values as the simulation progressed, with only minor fluctuations seen toward the end. The protein complex bound with co-crystal inhibitors displayed a more substantial and persistent fluctuation throughout the simulation trajectory, which manifested higher RMSD values. In contrast, a lower RMSD value in the tymazoline with the 3RZE protein structure implies enhanced stability and satisfaction within the binding site of 3RZE than the co-crystal inhibitor.

Root means square fluctuation

Root means square fluctuation analysis was conducted by considering the backbone atoms of each amino acid residue, and the resulting RMSF plot was employed to visualize residue-level fluctuations. The RMSF plot for the tymazoline complex revealed a consistent pattern of stability within the binding site, with no significant impact on the overall protein flexibility observed throughout the simulation also free protein is supplied to check the stability of the complex molecules. While the in contrast to the reference inhibitor show more fluctuations during the simulation period, as shown in Fig. 5.

Fig. 5 [Images not available. See PDF.]

MD simulation indication of RMSF, the blue line represents the selected ligand bounded within H1R, the gray line represents the protein H1R, and the orange line represents the reference ligand determined within H1R

A notable observation is the heightened residue fluctuation within the loop region spanning from residues ILE148 to LYS191, shown in Fig. 5. Notably, the RMSF values for the protein complex with the ligand remained within the acceptable range (below 2.25 Å), indicating stability in this complex. Conversely, the residues binding the reference drug exhibited fluctuations exceeding the specified threshold.

Molecular dynamics studies reinforced tymazoline's potential as an anti-allergic agent, displaying stable interactions with the 3RZE. The findings suggest a promising avenue for developing novel anti-allergic drugs, with tymazoline warranting further exploration and clinical investigation. Future outcomes may include refining tymazoline's properties, potential synthesis of derivatives, and eventual translation into effective therapeutic interventions for allergic disorders.

Binding free energy calculation

After the completion of the MD simulation, the binding free energy of each ligand complex molecule was calculated for all the generated conformations. The binding free energy is shown in Fig. 6, in which the blue line represents the tymazoline complex molecule's binding energy is − 240059 kcal/mol, the gray line represents the free protein binding free energy is − 264847 kcal/mol while the orange line represents the binding free energy of reference molecule is − 292443 kcal/mol. This shows that the ligand tymazoline complex molecule binding free energy is thermodynamically stable or near to the free protein total binding energy as compared to the reference molecule complex which shows huge gap of energy.

Fig. 6 [Images not available. See PDF.]

Binding free energy calculation of ligand complexes, the blue line represents the ligand-bonded protein, the gray line represents the free protein 3RZE, and the orange line represents the reference-bonded protein

Redocking of selected compound

The detailed intermolecular interactions after redocking the tymazoline with 3RZE active are shown in Fig. 7. We can identify that three hydrogen bonds (LYS179, THR194, ASN198), five hydrophobic bonds(ALA 195, LYS191, SER111, ASP107, TYR108) and some alkyl interactions (PHE435, TRP103, PHE432, TYR431) were found which involve in the stability of tymazoline in active site of protein to make it more stable, which indicate it as promising candidate to consider it as anti-allergic drug.

Fig. 7 [Images not available. See PDF.]

2D interaction diagram of tymazoline with H1R intermolecular interactions, the green dotted line represents the H-bonds, light green dotted lines represent hydrophobic interactions, while pink dotted line represents the alkyl interaction between the tymazoline and H1R(3RZE)

Discussion

Histamine, a pivotal mediator in histamine receptor production (H1R), is crucial in allergic responses. This study leveraged the crystal structure of H1R with the co-crystal inhibitor molecule to identify an inhibitor for allergic reactions. This study aimed to conduct an in silico analysis of small druggable compounds to find an optimal molecule inhibiting histamine for treating allergic symptoms. Docking studies involved 4000 small compounds against the 3RZE histamine receptor 1 protein, with tymazoline selected as the final drug compound based on the least CDOCKER docking and interaction energies. Druggable characteristics of tymazoline were further confirmed through drug analysis, including the Rule of 5 (RO5), ADMET, and MD simulation.

Docking studies revealed that tymazoline binds to TYR 108, ASN 198, and THR 112 amino acid residues with the least binding energies compared to others. MD simulation of 500 ps on the 3RZE-tymazoline complex, alongside the reference co-crystal inhibitor, demonstrated strong and stable confirmations in their interactions. Trajectory analysis based on RMSD and RMSF indexes indicated that the docked complex-maintained stability, with slight fluctuations in RMSD at 42 ps, remaining below 2.4 Å thereafter. In contrast, the co-crystal inhibitor fluctuated from the start, indicating poorer stability than tymazoline. RMSF interpretation suggested normal residue fluctuation for tymazoline compared to the reference molecule. Molecular dynamics studies reinforced tymazoline's potential as an anti-allergic agent, displaying stable interactions with 3RZE. Following MD simulation, the redocking of tymazoline with 3RZE and the computation of binding energy revealed that tymazoline interacted more with the 3RZE active site or formed a more thermodynamically stable complex than the reference molecule.

From our work, we could confirm that Drug assessment of tymazoline, including RO5, showed no violations, and ADMET analysis confirmed its inhibition against H1R without carcinogenicity and mutagenicity effects. The less energy complex indicated greater stability, suggesting improved drug efficacy by binding to the protein for an extended duration. These findings propose a promising avenue for developing novel anti-allergic drugs, with tymazoline warranting further exploration and clinical investigation. Future efforts may involve refining tymazoline's properties, potential synthesis of derivatives, and eventual translation into effective clinical applications.

Conclusion

Allergic mediator 3RZE receptor with a library of 4000 small druggable compounds was investigated by molecular modeling studies, including molecular docking, drug-likeness, ADMET, toxicity assessment, and molecular dynamic simulation studies. Among the library of small druggable compounds, tymazoline was selected, which showed binding solid affinities against the 3RZE regarding CDOCKER interaction energy and binding energy. Tymazoline exhibited satisfactory in silico drug-likeness, ADMET, and toxicity properties. Tymazoline did not violate any RO5 rule, and the drug-likeness score of the compound was within an acceptable range. MD simulation further confirms the anti-allergic potential of the compound tymazoline with the formation of well-defined and stable receptor–ligand interaction. Furthermore, the compound tymazoline exhibited superior scoring values compared to the reference co-crystal inhibitor and maintained standard RMSD values below 2.25 Å throughout the simulation, which confirms that tymazoline is a promising candidate for anti-allergic reactions. These findings underscore the potential of tymazoline as a favorable drug candidate for the targeted medicaments of allergic diseases, demonstrating its good molecular properties, stability, and adherence to established drug development guidelines.

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Author contributions

Preparing, experimental analysis and drafting the manuscript were performed. All authors read and approved the final manuscript.

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All necessary data generated or analyzed during this study are included in this article. Any additional data could be available from the corresponding author upon request.

Declarations

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Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

MD
Molecular dynamics
RMSD
Root means square deviation
RMSF
Root means square fluctuation
CHARMM
Chemistry at Harvard Molecular Mechanics
FDA
Food and Drug Administration
RO5
Lipinski’s rule of five
NTP
National toxicology program potential
TOPKAT
Toxicity prediction by computer-assisted technology

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A simple method for the determination of acyclovir concentrations in human plasma using high-

performance liquid chromatography

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ABSTRACT (ENGLISH)

Background

Acyclovir is an anti-viral medication given to treat herpes simplex and herpes zoster infection. In some severe conditions such as herpes encephalitis, acyclovir is administered intravenously. However, high acyclovir doses may cause acute kidney injury and low acyclovir dose may predispose the patient to inadequate exposure to acyclovir which could be fatal in some conditions. In such cases, the acyclovir plasma concentrations will potentially guide the diagnosis and management of the kidney injury. In this study, we provide a simple and time-efficient method for analyzing acyclovir in human plasma using high-performance liquid chromatography (HPLC).

Results

The process starts with a single protein precipitation step by adding acetonitrile to deproteinize 300 µL of plasma. The chromatographic separation conditions consist of a mobile phase of water: methanol (97:3, *v/v*), a flow rate of 1 mL/min, a run time of 17 min, and a detection wavelength of 254 nm. The calibration curve was linear over the range of (0.70–60 mg/L) ($r^2 > 0.99$). The retention times of acyclovir and the internal standard were around 15 and 12 min, respectively. The intra-day and inter-day analysis of acyclovir in plasma using this method exhibited accuracy and precision of less than 7%, which lies within the acceptable range. Different greenness assessment tools confirmed that the proposed method is eco-friendly.

Conclusion

The proposed method of analysis of acyclovir in the plasma using HPLC is simple, green and accurate method. This method could be applied in clinical settings where monitoring acyclovir concentrations is essential as it has wide range of the concentrations that could be detected.

FULL TEXT

Background

Acyclovir (9-[2-hydroxyethoxymethyl]-9H-guanine) (Fig. 1) is a synthetic nucleoside analog that has an anti-viral activity against herpes simplex and varicella zoster virus [1]. Patients with severe infections such as herpes encephalitis may require careful monitoring of the plasma acyclovir level. High acyclovir concentrations could predispose the patient to acyclovir nephrotoxicity and neurotoxicity. On the other hand, low acyclovir concentration that is below the 50% inhibitory concentration (0.56 mg/L for herpes simplex virus and 1.125 mg/L for varicella zoster virus) could lead to treatment failure and worsen the patient's condition [2–4].

Fig. 1 [Images not available. See PDF.]

Acyclovir chemical structure

Acyclovir follows a two-compartment model with first-order elimination kinetics [5]. The primary elimination pathway for acyclovir is through the renal route, making it significantly influenced by kidney function [6]. Impaired kidney function could result in acyclovir accumulation in the body and the development of either nephrotoxicity, neurotoxicity, or both, while enhanced kidney function could lead to subtherapeutic concentrations of acyclovir and subsequently treatment failure [7, 8].

The most common reported methods for the detection of acyclovir in the plasma were high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC–MS) [9–12]. Most of these methods

used a mobile phase with very low acidic pH [13, 14], which by time could degrade the stationary phase of the column.

Green analytical chemistry (GAC) is an area of activity that ensures that the analytical practices are environmentally friendly [15]. The assessment of greenness is crucial to ensure that analytical methods produce the least threats to the environment [16]. There are different developed tools to assess the greenness of the analytical methods such as analytical method GREEnness score (AMGS) [17], analytical eco-scale [18], and analytical GREEnness (AGREE) [19].

This method aimed to develop a simple, sensitive, and time-efficient method for analyzing acyclovir in plasma. The advantages of our method over the previously developed method are that it utilizes a single protein denaturation step, does not include buffers for the preparation of the mobile phase and it provides higher range of concentrations that could detect acyclovir toxicity. The development of buffer-free HPLC method offers a greener, more efficient, and more versatile approach to chemical analysis which protects the column for a longer time and avoids time-consuming cleaning process and waste generated. Furthermore, the absence of buffers allows for a simplified and more cost-effective analytical process. To our knowledge, this is the first acyclovir analysis method using the HPLC that used the greenness assessment tools to ensure that the method is environmentally friendly.

Methods

Reagents and chemicals

Acyclovir with a purity of (95.8% ± 0.9) and the internal standard (IS) ganciclovir with a purity of (99.3% ± 0.2) were obtained from Sigma-Aldrich (Oakville, ON, Canada). HPLC-grade acetonitrile, water, and methanol were purchased from Fisher Scientific (Edmonton, AB, Canada). Drug-free human plasma was acquired from Cedarlane Laboratories (Burlington, ON, Canada).

Instruments

HPLC–UV system (Shimadzu, Kyoto, Japan) was used to perform the analysis. It consisted of a system controller (SCL-10Avp), an autosampler (SIL-HTC), two pumps (LC-10 AD), and a UV–vis detector (SPD-10AV). The chromatographic separation was achieved by using a C18 reverse phase Supleco Discovery® C18 column (5 µm, 250 × 4.6 mm) (Supleco Inc., Mississauga, ON, Canada) protected by a Discovery® C18 Supelguard™ guard column (5 µm, 20 × 4 mm) (Supleco Inc., Mississauga, ON, Canada). Clarity® software version 8.7 (DataApex, Prague, The Czech Republic) was utilized for data collection and analysis.

Chromatographic conditions

The chromatographic separation was conducted through isocratic elution of a mobile phase mixture consisting of water and methanol (97:3, *v/v*). The flow rate was maintained at 1 mL/min for a total run time of 17 min for elution, and the detection wavelength was set at 254 nm. All the steps were conducted at room temperature.

System suitability

The system suitability was tested through the calculation of the tailing factor (symmetry factor), resolution, capacity factor and the height of theoretical plate (HETP) of five replicates. The results were compared with the guidelines to ensure that the chromatographic condition in optimal conditions, and the method is suitable for its intended purpose.

Preparation of stock and working solutions

Acyclovir and ganciclovir were dissolved in HPLC-grade water to prepare stock solutions of 400 mg/L and 500 mg/L, respectively. Working solutions of 50 mg/L and 100 mg/L of acyclovir as well as 100 mg/L ganciclovir were prepared by further diluting the stock solutions. All solutions were prepared fresh daily.

Preparation of calibration concentrations and quality control samples

Serial dilutions of acyclovir in blank human plasma were prepared to obtain the calibration concentrations of (0.7, 2, 5, 15, 25, 60 mg/L) of acyclovir. Four quality control (QC) concentrations were prepared for the method validation. The quality control samples were the lower limit of quantification (LLOQ, 0.7 mg/L), low-level QC (2 mg/L), middle-level QC (25 mg/L), and high-level QC (45 mg/L).

Sample preparation

Sixty µL of 100 mg/L IS were added to 300 µL of blank plasma spiked with acyclovir and vortex mixed for 30 s.

Then, 2 mL of acetonitrile were added to the prepared plasma spiked with acyclovir and ganciclovir and vortex mixed for 1 min for the purpose of the plasma proteins denaturation. Then, the prepared samples were centrifuged (Eppendorf centrifuge 5804, Eppendorf SE, Barkhausenweg, Hamburg, Germany) at 5000 rpm for 20 min. The obtained supernatant was then transferred to clean tubes and concentrated using SpeedVac® Vacuum Concentrator (Thermo Fisher Scientific, Waltham, MA, USA). Reconstitution was performed by the addition of 200 µL of the mobile phase (water: methanol, 97:3, *v/v*) and vortex mixed for 15 s. A volume of 50 µL of the prepared samples was injected into the HPLC for the chromatographic separation.

Method validation

The validation was done following the Guideline on bioanalytical method validation guidelines developed by the European Medicines Agency (EMA, 2011) [20]. The method validation included linearity, selectivity and sensitivity, precision and accuracy, carry-over, stability, and recovery.

Linearity

The linearity of the method was determined by plotting the calibration curves of the peak height ratios (acyclovir /ganciclovir) vs. the calibration concentrations. Linear regression was performed to obtain the slope, intercept, and coefficient of determination (r^2) of the calibration curve.

Selectivity and sensitivity

The selectivity of the developed method was assured by the absence of any plasma peaks interfering with acyclovir and ganciclovir peaks when comparing chromatograms obtained from blank plasma with those obtained from acyclovir-containing samples. The sensitivity was determined in terms of the LLOQ, in which its response must be at least 5 times higher than the plasma response.

Precision and accuracy

The intra-day and inter-day precision and accuracy of the developed method were tested by injecting five replicates of each of the four QC samples mentioned earlier on three consecutive days. The method's precision was presented as the coefficient of variation (CV, %), and the accuracy was expressed as a percentage error.

Carry-over

Carry-over was assessed by injecting drug-free plasma after the injection of the upper limit of quantification (60 mg/L). Based on the EMA guidelines, the blank plasma response must not exceed ± 20% of acyclovir LLOQ response and 5% of the internal standard response.

Stability

The stability of the method was assessed in either plasma spiked with acyclovir or final prepared samples for HPLC injection (e.g., concentrated and reconstituted) over two weeks of three replicates of two QC samples (2 and 55 mg/L). The stability of the plasma spiked with acyclovir was determined at the preparation time and after three hours and 24 h at room temperature. Furthermore, the stability after 1 and 2 weeks stored at 4–8, − 20, and – 80 °C was also assessed. Moreover, the stability of the final prepared samples was examined over one week and stored at room temperature (autosampler), 4–8, − 20, and − 80 °C. Also, the freeze and thaw stability of acyclovir in plasma was assessed by initially freezing the three replicates of the two QC samples at − 80 °C for 24 h followed by thawing them at room temperature. This cycle was repeated for three days before preparing the samples to be injected into the HPLC. In addition, the stability of stock and working solutions kept in the fridge were tested after 2 months by preparing a working solution from the stock solution stored at 2-8° C and comparing the results of samples prepared from these working solutions.

Recovery

The average extraction recovery of acyclovir was measured by injecting three replicates of three QC samples (5, 15, 25 mg/L) and comparing their chromatographic peaks with those of plasma samples spiked with equivalent concentrations of acyclovir after protein precipitation and sample concentration.

Assessment of greenness

The eco-friendliness of the proposed method was tested using different greenness assessment tools. Three were used to evaluate the greenness which are analytical method GREEnness score (AMGS) [17], analytical eco-scale

[18], and analytical GREEnness (AGREE) [19].

Results

Method development

Preliminary experiments were done to optimize the chromatographic conditions of acyclovir analysis. Various solvents and different compositions were tested to obtain the optimum mobile phase composition to run the analysis. Different compositions of acetonitrile and water as well as different compositions of methanol and water were tested as mobile phases, and it was found that the composition of 97% water and 3% methanol gave the best chromatograms of acyclovir samples. The selected mobile phase composition resulted in increasing the retention time compared to more methanol percentage in the mobile phase. Nevertheless, it had the advantages of better peak separation, decreasing the cost of the analysis and reducing the environmental impact of methanol. Moreover, different wavelengths over the range of 200–800 nm were tested to select the wavelength that provides maximum acyclovir UV absorbance and less plasma absorbance which was found to be 254 nm. The effect of different flow rates was studied, and a flow rate of 1 mL/min was chosen for the method. Using flow rates of more than 1 mL/min resulted in increasing the pressure, while using flow rate of less than 1 mL/min resulted in increasing the retention time and hence the run time. Furthermore, acetonitrile and methanol were tested as protein precipitation solvents, and acetonitrile gave better results. The retention times of acyclovir and ganciclovir using the optimum conditions were around 15 and 12 min, respectively. The ratios of the peak heights of acyclovir to ganciclovir were used in all the calculations as they gave more accurate results than the peak area ratios. Although column temperature affects the resolution of the samples, it was not used to make the study applicable to different systems, and the measurements were all in the room temperature.

System suitability

The results of the system suitability (Table 1) were obtained and compared to the reference ranges. All of the obtained values are within the acceptable ranges.

Table 1. System suitability parameters of the proposed method

Parameter	Obtained value	Reference value [21, 22]
Resolution (R_s)	2.03	≥2
Tailing factor (T)	1.35	0.8–1.8
Height of theoretical plate (HETP)	0.0018	The smaller the value, the better the system suitability
Capacity Factor	3.2	>2

Method validation

Linearity

The calibration curves of the plasma samples of acyclovir were done to test the linearity of the developed method. The peak height ratios of acyclovir to ganciclovir showed linearity over the range of 0.7–60 mg/L with r^2>0.99 (Fig. 2).

Fig. 2 [Images not available. See PDF.]

Calibration curve of peak height ratios of acyclovir to the internal standard versus acyclovir concentrations

Selectivity and sensitivity

There were no interfering plasma peaks with the acyclovir or the ganciclovir as shown in Fig. 3. The lower limit of quantification of acyclovir that gives accurate and precise results was 0.70 mg/L.

Fig. 3 [Images not available. See PDF.]

Different chromatograms of **a** Blank plasma; **b** Plasma containing internal standard (20 mg/L)-retention time 12.1 min; **c** Plasma containing acyclovir (25 mg/L) and internal standard (20 mg/L) retention times 15 and 12.2 min, respectively

Precision and accuracy

To assess the intra-day and inter-day precision and accuracy, we prepared five replicates of four quality control samples and analyzed their concentrations in three different days. The method is precise and accurate as shown in Table 2. The intra-day coefficient of variation was less than 2.2%, and the percentage of error was less than 7%. On the other hand, the inter-day coefficient of variation was less than 4.6%, and the percentage of error was less than 6%.

Table 2. Intra-day and inter-day precision and accuracy

Concentration(mg/L) ($n=5$)	Observed concentration(mg/L) (mean ± S.D)	CV%	Error percentage
Intra-day precision and accuracy			
0.7	0.73 ± 0.01	1.30	− 3.67
2	2.07 ± 0.04	1.96	− 3.57
25	24.67 ± 0.20	0.34	1.36
45	41.87 ± 0.90	2.15	6.96
Inter-day precision and accuracy			
0.7	0.74 ± 0.06	7.38	− 5.99
2	2.07 ± 0.04	1.90	− 3.42
25	25.40 ± 1.16	4.57	− 1.61
45	43.86 ± 1.92	4.37	2.54

S.D standard deviation; *CV*% Coefficient of variation (percentage)

Carry-over

There were no peaks appearing in the blank plasma chromatogram after injecting the upper limit of quantification either in the retention time of acyclovir or ganciclovir. This indicates that there were no carry-over effects of high acyclovir concentrations.

Stability

Plasma samples spiked with acyclovir were stable for 2 weeks at different temperatures. Also, the finally prepared samples for injection showed stability for one week stored at different temperatures. Acyclovir showed stability after three freeze and thaw cycles. All the % remaining of the acyclovir in samples compared with the initial samples (zero time) ranges from 88 to 113% (Table 3) which lies within ± 15% indicating the stability of acyclovir samples. Moreover, the stock and working solutions were stable for 2 months.

Table 3. Stability of acyclovir in plasma and in the finally prepared samples for HPLC injection

Concentration (mg/L) (*n*=3)	Time (storage temperature)	Mean concentration(mg/L)± S.D	% Remaining
Finally prepared samples for HPLC injection			
2	0 h	2.01±0.08	100.00
3 h (Room temperature)	2.11±0.08	105.09	24 h (Room temperature)
2.09±0.06	103.72	1 week (Room temperature)	2.05±0.04
101.84	1 week (2–8)	2.02±0.06	100.37
1 week (− 20)	2.28±0.10	113.60	1 week (− 80)
2.10±0.09	104.30	55	0 h
57.10±7.57	100.00	3 h (Room temperature)	56.94±7.91
99.73	24 h (Room temperature)	50.58±2.28	88.59
1 week (Room temperature)	50.63±1.88	88.67	1 week (2–8)
55.40±2.46	97.03	1 week (− 20)	57.64±7.23
100.94	1 week (− 80)	53.15±0.24	93.08
Plasma spiked with acyclovir stability			
2	0 h	2.01±0.08	100.00
3 h (Room temperature)	2.21±0.21	109.89	24 h (Room temperature)
2.21±0.20	109.81	1 week (2–8)	2.09±0.02
106.23	1 week (− 20)	2.01±0.10	102.56

1 week (− 80)	2.10 ± 0.06	106.75	2 weeks (2–8)
1.91 ± 0.04	94.75	2 weeks (− 20)	2.29 ± 0.24
113.79	2 weeks (− 80)	2.28 ± 0.26	113.21
55	0 h	57.10 ± 7.57	100.00
3 h (Room temperature)	54.76 ± 3.43	95.91	24 h (Room temperature)
48.91 ± 4.79	85.66	1 week (2–8)	53.81 ± 2.10
94.24	1 week (− 20)	52.21 ± 0.56	91.45
1 week (− 80)	53.76 ± 3.55	94.15	2 weeks (2–8)
56.31 ± 1.68	98.63	2 weeks (− 20)	53.39 ± 3.33
93.50	2 weeks (− 80)	58.97 ± 1.22	103.28

S.D standard deviation; % remaining, percentage of drug remaining compared to the initial concentrations

Recovery

The mean percentages recovery of the three QC of 5, 15, 25 mg/L ($n=3$) concentrations is in Table 4. It ranged from 88 to 90%.

Table 4. Mean percent recovery of acyclovir

Concentration (mg/L) ($n=3$)	Recovery %, mean ± S.D	CV%
5	88.40 ± 5.68	6.43
15	90.22 ± 1.07	1.18
25	88.43 ± 4.74	5.36

S.D standard deviation; *CV*%, Coefficient of variation (percentage)

Assessment of greenness

The web application version of the AGREE tool provided the result based on the 12 criteria of green analytical chemistry. The score was 0.64 which indicates that the method is green. The AMGS score, which calculates the instrument energy score, solvent energy score and solvent EHS score were 216.34 which also indicates the greenness of the method. The analytical eco-scale resulted in a score of (100-16) = 84 of which means that the

method is green.

Discussion

Acyclovir is the anti-viral nucleoside analog drug used for the treatment of herpes infections. Acyclovir is given intravenously to patients suffering from herpes encephalitis at a dose of 10 mg/kg/dose every 8 h [23]. High concentrations of acyclovir in plasma above 25 mg/L could highly predispose the patient to nephrotoxicity and neurotoxicity [24]. This paper describes a simple, rapid, accurate, and precise method for the quantifying of acyclovir in human plasma.

This method utilizes a small volume of the plasma 0.3 mL compared to other methods which use larger volumes (0.5–1 mL) [9, 13, 14, 25]. The run time was 17 min which could aid in the analysis of large numbers of samples in a short time. The wavelength that showed the best chromatograms was 254 nm after scanning the UV range (200–800 nm). Although the mobile phase composition was 97% water and 3% methanol which resulted in increasing the retention time compared to more methanol percentage in the mobile phase, it has the advantages of better peak separation, decreasing the cost of the analysis and reducing the environmental impact of methanol. The mobile phase used to elute acyclovir did not contain buffers, which provides the advantages of the simplicity of the method and protection of the column without affecting the results' selectivity and sensitivity.

The greenness of the developed method was assessed using different tools. The AGREE tool was first developed in 2020 by Pena-Pereira, et al. [19]. It involves the 12 concepts of green analytical chemistry in its evaluation [15]. The analytical eco-scale is also an interesting tool to assess if the method is environmentally safe, and it has a score of hundred and good results expected to be >75 [18]. Analytical method GREEnness score (AMGS) is a semi-quantitative user-friendly tool to evaluate the greenness of the analytical methods [17]. The proposed study is determined as green and environmentally safe based on the results obtained from this tool. This was the first acyclovir HPLC analysis study that evaluated the greenness of the proposed method.

It has the advantage of a single protein precipitation step which also reinforces the uncomplicatedness of the developed method compared to other methods [14]. Moreover, the stability of the developed method was assessed, and acyclovir showed stability in stock and working solutions kept for two months in the fridge. In addition, it showed stability in the plasma over two weeks and finally prepared samples over one week in different storage conditions. The validation of the developed method showed precision and accuracy results within the acceptable range (± 15%) as reported by the EMA guidelines [20].

The linearity range of the method (0.70–60 mg/L) covers a wider range than previous study, including high acyclovir concentrations, which are known to be associated with potential adverse effects [14, 26, 27]. Acyclovir levels in herpes encephalitis should be maintained above the 50% inhibitory concentration which is 0.56 mg/L for herpes simplex virus and 1.125 mg/L for varicella zoster virus. On the other hand, high acyclovir concentrations above 25 mg/L could cause acyclovir adverse effects [2, 4, 28]. These concentrations could be readily measured in patients experiencing adverse effects, such as acute kidney failure, neurotoxicity, or worsening symptoms, using this method.

Conclusion

This article represents a quantitative method that is fully validated based on the EMA guidelines for the analysis of acyclovir in the plasma. It has a linearity range of (0.7–60 mg/L) which make it applicable in the clinical practice. The method has undergone evaluation for its eco-friendliness using diverse green assessment tools, positioning it as a novel method of acyclovir detection in the plasma assigned to be green. The developed method is simple and utilizes a single protein precipitation step and excludes the use of the buffered mobile phase. Overall, it is a rapid, selective, accurate, and precise method that could be used widely in clinical settings.

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Author contributions

Conceptualization was done by S.H.M.; methodology was done by S.H.M., A.A and M.K.; validation was done by A.A and M.K.; formal analysis was done by S.H.M. A.A and M.K.; writing—original draft preparation was done by

A.A.; writing—review and editing was done by S.H.M., A.A and M.K.; supervision was done by S.H.M.; funding acquisition was done by S.H.M. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The data used to support the findings of this study are available from the corresponding author upon request.

Declarations

Ethical approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

The authors declare they have no any competing interests.

Studies involving plants

Not applicable.

Abbreviations

HPLC
High-performance liquid chromatography
LC-MS
Liquid chromatography- mass spectrometry
AMGS
Analytical method GREEnness score
AGREE
Analytical GREEnness
IS
Internal standard
QC
Quality control concentration
LLOQ
Lower limit of quantification
EMA
European Medicines Agency
r^2
Coefficient of determination
CV
Coefficient of variation
Rs
Resolution
T
Tailing factor
HETP
Height of theoretical plate
S.D
Standard deviation

Publisher's Note

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Pomegranate extract-loaded surfactant-free zein nanoparticles as a promising green approach for hepatic cancer: optimization and in vitro cytotoxicity

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ABSTRACT (ENGLISH)

Background

Hepatic cancer endures a major health scourge as the consequence of a high incidence of >1 million cases by 2025. Plant-based products are typically effective in ameliorating health conditions. Pomegranate peel extract (PE) with its high polyphenolic content has anticancer effects against different types of cancer. Herein, we aimed to maximize the

PE chemotherapeutic efficacy by loading it in a suitable delivery system to overcome the limitations of PE, to control its release and to achieve liver targeting.

Method

A nanoprecipitation procedure was adopted to incorporate PE into biodegradable and biocompatible natural polymeric zein (ZN)-based nanoparticles (NPs) (PE-ZN NPs). A full factorial design ($2^2 \times 3^1$) was developed to study the effects of the formulation variables, namely pH of dispersion, PE-to-ZN ratio and surfactant concentration.

Results

The optimization revealed a surfactant-free stable PE-ZN NPs formula with a small particle size of 99.5 ± 6.43 nm, high PE encapsulation efficiency % of 99.31% ± 3.64 (w/w) and controlled release of PE over 24 h.

Conclusion

Moreover, the cytotoxicity of the optimum formula against hepatic cancer HepG2 cell lines was assessed and attained about a 2.5-fold reduction in the inhibitory concentration (IC_{50}) values compared to the free PE affording a promising green platform to combat hepatic cancer.

FULL TEXT

Background

Cancer is considered one of the main causes of mortality worldwide as a consequence of millions of new reported cases yearly. Hepatic cancer is the sixth most common cancer globally, ranking the fifth in men and the ninth most commonly occurring cancer in women [16]. The prognosis for hepatic cancer is poor, and there are many challenges for its cure. Liver resection and liver transplantation are the best options of cure for only 5–15% of early-stage patients; however, the high postoperative recurrence and the severe shortage of organ donors limit this option. In more advanced stages patients, chemotherapy is the only available treatment option; however, the traditional cytotoxic drugs affect both cancer and normal cells causing numerous side effects, including gastrointestinal tract ulcers, bone marrow depression, nausea, and hair loss [8]. As a result, neither current ablation therapies nor chemotherapy is appreciably effective in improving outcomes of this devastating disease. Considerable attention is needed for better therapy alternatives for hepatic cancer patients. A European study showed that prevention, development, progression, and treatment of cancers is associated with the diet of patients and a higher dietary intake of fruits and vegetables is associated with a lower risk of cancer development [7, 33]. There is always an increase in researchers' interest and efforts to identify new anticancer treatments with a scope of less toxic, more potent, more selective, and hence a more effective approach than other traditional ones [25].

Nature has been the best source of drugs since ancient ages; natural products are highly considered for their anticancer properties that do not only protect against cellular damage and disease but also have vital roles in the treatment of many diseases such as cancer [4, 17]. As a result of the aforementioned facts, it became necessary to explore natural and plant-based products as a good substitute for chemotherapeutics to overwhelm the major adverse effects of chemotherapeutics, in addition to exhibiting improvements in therapeutic indices.

Punica granatum L. plant (Pomegranate) has been known for its nutritional values, medicinal properties and anticancer activities. Pomegranate parts such as peel, bark, root, and flower encompass various active phytochemicals. Among these parts, the pomegranate peel corresponds to around 50% of the fruit weight which is abundant in high molecular weight polyphenols including flavonoids, ellagic acids, gallotannins and ellagitannins (ETs) [50, 55]. ETs are the most bioactive components in pomegranates. Punicalagin (PU) is considered the most valuable and abundant ET in pomegranates [48]. The anticancer activity of pomegranate can be exerted in a chemo-preventive and/or chemotherapeutic approach. This activity is accountable for its anti-inflammatory, anti-invasive, anti-proliferative and pro-apoptotic activities in different types of cell lines such as skin, breast, prostate, colon and lung cancers [9]. Moreover, Bishayee et al. highlighted the remarkable chemo-preventive effect of PE against liver cancer using a chemical-induced and clinically relevant two-stage rat liver carcinogenesis model and ascribed this activity to the potent antioxidant and anticancer effects of PE extract [14].

Despite these promising characteristics and the intense therapeutic potency of PE extract, its poor solubility, instability and consequently its low bioavailability may hinder its applications. Nanotechnology has the unraveling

ability to shield the PE extract inside the nanoparticles' (NPs) matrix and accordingly prevent additional degradation, and augment the bioavailability [13], in addition to its radical impact in achieving size-dependent passive targeting to specific organs relying on the enhanced permeability and retention (EPR) concept. To achieve this purpose, the particle size of NPs should be controlled to avoid the recognition by the reticuloendothelial system (RES) and to attain an efficient biodistribution pattern [34].

The employment of natural polymers for NPs preparation facilitates the accomplishment of safe dosage forms with low toxicity, high biocompatibility and biodegradability [26]. Many natural polymers such as collagen, zein and chitosan were extensively studied in previous research works [39]. Zein (ZN), a vegetable protein, is auspicious for the preparation of NPs due to its distinctive properties, including biocompatibility, biodegradability and reproducibility [45]. Its structure promotes the encapsulation of different bioactive compounds with hydrophobic, hydrophilic and amphiphilic characteristics, in addition to its desirable slow digestibility that imparts the formed NPs with long stability in the gastrointestinal tract before being degraded. In this regard, NPs that formed from zein have the suitability and ability to control the release of the encapsulated bioactive and to improve its bioavailability [5].

Hence, our aim is to incorporate PE peel extract into the natural biodegradable and biocompatible ZN NPs to provide an amenable green platform with a surfactant-free strategy able to deliver PE extract and ameliorate its activity against hepatic cancer. Utilization of such controlled system not only would result in augmenting the activity of PE but also would aid in the efficient liver targeting potential by the passive delivery in the future applications. The design and optimization were done using a $2^2 \times 3^1$ full factorial design to study the effect of the different variables on PE-ZN NPs characteristics. The physical properties of the developed NPs and the in vitro PE release from the NPs were investigated. Moreover, the ex vivo permeation of the PE-ZN NPs was examined as a prediction of the bioavailability of the investigated formula for the oral administration. Besides, the anti-proliferative activity (MTT assay) of the optimized formulation was studied in HepG-2 cancer cell lines in comparison with free PE.

Materials and methods

Materials

Pomegranate was purchased from Cairo Farms (Cairo, Egypt). Zein protein was generously obtained by FLO CHEMICAL CORPORATION (Wisconsin, USA) as a kind gift. Methanol was purchased from Sigma-Aldrich (St. Louis, USA). All other used chemicals were of analytical grade.

Cells

Human liver (HepG2) cells (VACSERA Company, Egypt) were cultured in Dulbecco's modified Eagle's medium (Invitrogen, CA, USA) including 10% fetal bovine serum (Gibco, NY, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin at 5% CO_2 atmosphere and temperature of 37 °C.

Preparation of pomegranate peel extract

Pomegranate fruits were cleaned up and washed with tap water; then dried and peeled. The peels were separated from the rind and cut into small pieces then dried at 50 °C in a drying oven (Heraeus, Germany) to dryness. The dried peels were ground to coarse powder of approximately 1 mm size. To prepare PE extract, 100 gm of ground pomegranate peel was soaked in 500 mL methanol and subjected to regular mixing for 6 h; followed by maceration for 48 h at 37 °C with intermittent shaking. The extraction procedure was repeated three times to extract the maximum components from the pomegranate peel. Subsequently, the pooled extracts were filtered on Whatman No.1, UK filter paper. The filtrate was concentrated under vacuum using a rotary evaporator (Heidolph, Germany) at 50 °C to obtain 25 ml (mL). The concentrated extract was stored in a refrigerator at 4 °C [38, 40, 43].

Preliminary screening of surfactants

Surfactants of different hydrophilic-lipophilic balance (HLB), as shown in Fig. 1, were examined to investigate their effects on the PE encapsulation and the physical characteristics of the formed PE-ZN NPs. An amount of 50 mg of the surfactant was sonicated in 5 mL ethanolic dispersion of ZN in case of lipophilic surfactants (HLB<7). For hydrophilic surfactants (HLB>7), they were stirred in 10 mL preheated distilled water until complete dissolution with a magnetic stirrer (Stuart US150, USA). The PE-ZN NPs were prepared by the nanoprecipitation technique reported by [34]. Homogeneity of the formed dispersion and PE encapsulation efficiency were the two screening criteria for

selecting the surfactant.

Fig. 1 [Images not available. See PDF.]

Screened surfactants in the preliminary studies arranged according to their HLB

Quality target product profile (QTPP) and critical quality attributes (CQA)

As a prospective strategy to achieve the aim of the study effectively, a quality target product profile has been determined and is summarized in Fig. 2 with the desired quality attributes that will guarantee the achievement of the appropriate formula. The main target is to develop a green and safe dosage form against hepatic target. The employment of natural extract and natural polymer will be examined. The necessity of use of surfactant will be examined. The appropriate method of preparation that will yield the desired particle size for liver targeting and the adequate encapsulation efficiency with a controlled release, augmented ex vivo permeability and optimum activity will be studied. In addition the critical quality attributes that have a substantial role such as method of preparation, residual methanol, particle size, encapsulation efficiency, zeta potential, ex vivo permeability and in vitro cytotoxicity have been determined and justified as demonstrated in Fig. 2.

Fig. 2 [Images not available. See PDF.]

Quality target product profile (QTPP) and critical quality attributes (CQA)

Factorial design

A $2^2 \times 3^1$ full factorial design was implemented to determine the effects of the selected variables using Minitab® 16.1.0 (Minitab Inc., USA). The three variables were: the dispersion pH (X1), PE: ZN ratio (w/w) (X2), and the surfactant concentration (mg/mL) (X3) using the selected surfactant from the preliminary study. The analysis of variance (ANOVA) was used to estimate the significant effects of the selected variables on the following responses; PE encapsulation efficiency (Y1), particle size (Y2), polydispersity index (PDI) (Y3) and zeta potential (Y4) of the PE-ZN NPs.

Preparation and characterization of PE-loaded ZN NPs

Preparation of PE-loaded ZN NPs

The nanoprecipitation procedure was utilized to prepare PE-ZN NPs; the steps are summarized in Fig. 3 with an asterisk on the critical process parameters (CPP). Separately, amounts of ZN and span 40 according to Table 1 were dissolved in 70% v/v ethanolic aqueous solution (5 mL) and PE extract was dissolved in methanol. After that, the two solutions were mixed and added drop wisely into a pH-adjusted aqueous phase (10 mL) (with either 0.1 N HCl or 0.1 N NaOH) under stirring with a magnetic stirrer (AccuPlate™ Analog Hot Plate Stirrer, UK) at room temperature and 2000 rpm for 1 h until the evaporation of alcoholic solvents.

Fig. 3 [Images not available. See PDF.]

Flowchart of preparation and characterization of PE ZN NPs

Table 1. Full factorial design independent and dependent variables and their levels

Independent variables	Levels		
−1	0	1	X1: pH
2	7	9	X2: PE: ZN ratio (% W/W)

0.2		0.6	X3: Conc. of surfactant (mg/mL)

Dependent variable	Constraints
Y1: encapsulation efficiency	Maximize
Y2: particle size	Minimize
Y3: PDI	Minimize
Y4: zeta potential	Maximize

Characterization of PE-loaded ZN NPs

Differential scanning calorimetry (DSC)

A differential scanning calorimeter (Setline DSC+, Setaram, Switzerland) was utilized to scan the prospective physical incompatibilities between PE and the polymer or the surfactant in the dispersion. PE, physical mixtures of (PE and ZN) and (PE and the selected surfactant) were weighed in 30 µL Al-crucibles and measured in a range of 25–300 °C with heating rate of 10 °C/min. under nitrogen atmosphere [37].

Fourier transform infrared (FTIR) spectroscopy

FTIR spectrophotometer (IR Affinity-1, Shimadzu, Japan) was used to evaluate the possible chemical interaction between PE and the polymer or the surfactant in the dispersion. Potassium bromide was physically mixed with the freeze-dried (Lyovapor L-200, Buchi, Switzerland) PE in addition to the physical mixture of ((PE and ZN) and (PE and the selected surfactant). Then a hydraulic pressing machine was used to compress these mixtures into thin discs. After that, the spectra were performed in the wavelength range from 400 to 4000 cm^{-1} [32].

Particle size (PS), polydispersity index (PDI) and Zeta potential (ZP)

The average particle size accompanied by the polydispersity index (PDI) and zeta potential were evaluated by Zetasizer (Malvern Instruments Ltd, Malvern, UK). Samples were appropriately diluted with deionized water and all values were reported as the mean value ± standard deviation (SD) of three different measurements [1].

Encapsulation efficiency

The centrifugation method was used to separate the free drug from PE-ZN NPs; the prepared dispersion of PE-ZN NPs was centrifuged at 10,000 rpm for 1 h at 4 °C using cooling microfuge (Remi CM-12 Plus, Remi laboratory instruments, India). Then, the supernatant was diluted with methanol and measured spectrophotometrically at the wavelength of maximum absorbance (λmax) 265 nm using an ultraviolet–visible spectrophotometer (V-630, Jasco, Tokyo, Japan) in correspondence to a calibration curve of PE in methanol ($n=3$; $R^2=0.999$). PE encapsulation efficiency (EE%) was calculated by the following equation [9, 11]:

1

PEencapsulationefficiency=InitialamountofPE-amountoffreePE×100InitialamountofPE

Determination of residual solvents (methanol)

Testing should be performed for residual solvents when production or purification processes are known to result in the presence of such solvents. As methanol (Class 2 Solvent) was used in the extraction of bioactive compounds from pomegranate peels, therefore the residual methanol was tested in the PE extract and the final optimum formula. The USP<467>protocol for determination of class 1 and class 2 residual solvents was utilized.

Morphological examination by transmission electron microscope (TEM)

The morphology of the optimum NP formula was studied by TEM (JEM-2100, Japan). Each sample was negatively stained with phosphotungstic acid; the excess stain was removed by filter paper and then left to be air dried for

15 min. Consequently, a drop of the stained NPs was placed on a 200-mesh carbon-coated copper grid and the microscope was operated at an acceleration voltage of 200 kV [10].

In vitro drug release

The dialysis membrane method was used to determine the PE release from the selected formulation and was compared to that of the free PE. Initially, the dialysis bags (MW cutoff of 12,000–14,000 Da) (Spectrum Medica, CA, USA) were soaked overnight in phosphate buffer solution (PBS) pH 7.4. The experiment was carried out using the USP dissolution (Hanson Research Corporation, CA, USA), apparatus II (paddle), at 37 °C, 100 rpm, and in 100 mL PBS pH 7.4 as a dissolution medium [9, 34]. A volume of 2 mL of the formula containing an amount of PE equivalent to 20 mg was placed in the dialysis bag and 2 mL aliquots were taken at predetermined time intervals and restored with fresh buffer to retain the sink condition. The same experiment was followed, but in this case 20 mg free PE/2 mL dissolution medium was placed in the dialysis membrane. A preliminary study was done to ensure that the above conditions provide a sink medium for PE at this dose level. PE concentration in the samples was assessed spectrophotometrically at 265 nm in correspondence to a calibration curve of PE in PBS pH 7.4 ($n=3$; $R^2=0.999$).

Determination of release kinetics

To determine the release of PE extract from the optimized NP preparation, (zero order, first order and Higuchi) mathematical kinetic models have been employed for the in vitro drug release results. The coefficients of determination (R^2) results of the three models for the optimum preparation were calculated [28].

Ex vivo permeability study

Ex vivo permeability of the investigated formula was done to investigate the permeability parameters of pure free PE extract and optimized PE-ZN NPs (Z12) formulation. Small intestines of rabbits that were allowed to fast overnight were used. The duodenal parts were isolated and their contents were removed by flushing with normal saline. Subsequently, they were divided into segment sacs of 5 cm and a volume of 2 mL of the optimum formula containing an amount of PE equivalent to 20 mg was placed in each sac which their ends were tied carefully with sutures [36]. The same was done for free PE and blank ZN NPs (free from PE) to act as a blank. A USP dissolution with amber mini-vessels (Hanson Research Corporation, CA, USA), was used and each sac was tied onto apparatus II (paddle), at 37 °C, 75 rpm, and in 100 mL PBS pH 7.4 as a dissolution medium. Samples of 1 mL were withdrawn at time intervals of (0.5, 1, 2, 3, 4, 6, 8, 10 and 24 h) and replaced with an equivalent amount of the fresh buffer solution. The samples were assayed spectrophotometrically at 265 nm [52] with a calibration curve of PE in PBS pH 7.4 ($n=3$; $R^2=0.998$), and the experiment was repeated in triplicate.

In vitro cytotoxicity study

The cytotoxic effect of both the optimized PE-ZN NPs formula and the free PE on HepG2 cancer cell lines (ATCC® HB-8065™) was studied. Samples of free PE and optimized formulae were diluted on pre-cultured cell lines for 48 h treatment at 37 °C post-decanting growth medium. Treated cell lines were microscopically examined for detection of morphological changes and detached cells. Dead cells were washed-out using phosphate buffer saline (PBS), pH 7.2 ± 0.2 (with 0.05% Tween 20). Residual live cells were treated with 0.5% MTT stain as 25 µL/well. Plates were incubated for 3–4 h at 37 °C. Developed intra-cytoplasmic MTT formazan crystals were dissolved using 0.05 mL DMSO for 30 min on a plate shaker. Optical densities (OD) were read using an ELISA plate reader (Anthos-Elisa-reader 2001, Labtec, Heerhugowaard, Netherlands). The 50% inhibitory concentration (IC_{50}) of both the PE extract and the optimized PE-ZN NPs formula were determined using the Master–plex-2010 program (Hitachi Software Engineering America, Ltd). Data were recorded for three independent experiments. The viability percentage was calculated as follows [6, 54]:

2

$$\text{Cellviability percentage} = \text{OD of treated cells} / \text{OD of untreated cells} \times 100.$$

Results

Preliminary screening of surfactants

The screened surfactants were subjected initially to physical inspection. Only formulations prepared with Tween® 40, Cremophor® RH 40, and Span® 40 displayed homogeneous dispersions, while those formulated with other

surfactants showed precipitates. Afterward, the homogenous formulations were exposed to another screening step of estimation of the PE encapsulation efficiency. As shown in Fig. 1 Span® 40 formula revealed a remarkably higher PE encapsulation efficiency of 70.3% ± 2.3 in comparison with 45.7% ± 1.9 for Cremophor® RH 40 formula and 37.5% ± 3.8 in case of Tween® 40. This may be attributed to that zein is a polymer having three-fourths lipophilic and one-fourth hydrophilic amino acids residues, thus acting as a water barrier, and helping in attaining higher encapsulation efficiency values of PE in NPs, especially those including Span® 40. That verifies that there is a reversible relationship between the PE encapsulation and the HLB values of the used surfactant during ZN NPs formation Therefore, Span 40 was selected to be utilized in further studies [12, 23].

Characterization of PE-loaded ZN NPs

Differential scanning calorimetry (DSC)

As shown in Fig. 4 and supplemental Fig. 1A, B and C, the DSC thermogram of the PE showed a sharp endothermic peak at a temperature of 142.791 °C that can be related to the melting point of polyphenols present in PE [56]. The physical mixture of PE: ZN thermogram had the same peak with a slight shift to 146.047 °C with no other peaks for ZN similar to that reported in previous study [45]. It is worth noting that the mixture with polymers could enhance the thermal stability of PE resulting in this slight higher melting temperature [30]. On the other hand, it is depicted from the thermogram of the physical mixture of PE: Span® 40 the appearance of two endothermic peaks at 56.394 °C and 115.666 °C. The first endothermic peak could be attributed to the melting point of Span® 40 as reported in literature [47]. The another peak could be assigned to the melting of PE and this shift to lower temperature can be justified to the result of the partial dissolution of PE in the physical mixture of PE and Span® 40 when the latter is heated [42].

Fig. 4 [Images not available. See PDF.]

DSC thermograms of PE and the physical mixtures of PE with ZN and span 40

Fourier transform infrared (FTIR) spectroscopy

The FTIR spectra of the freeze-dried PE and the two physical mixtures of PE with ZN and Span 40 as shown in Fig. 5 revealed the wide band recorded at 3346 cm^{-1} corresponding to the stretching vibration of N–H and O–H groups and the peaks between 1046 to 1800 cm^{-1} belonged to the aromatic and aliphatic functional groups of the polyphenolic content of PE including CH, C=O, –C=C–C=O, –C=C– and –C–C–. The investigated spectra showed an insignificant shift in the peaks of PE in the physical mixtures relative to the peaks in the spectrum of the pure extract, indicating the absence of any interactions between PE and the other used excipients. The reduction of the peak intensity of PE in the physical mixture may be due to the dilution effect of the mixing process [51]. Therefore, PE and all the used excipients are compatible to be used collectively.

Fig. 5 [Images not available. See PDF.]

Fourier transform infrared spectra of freeze-dried PE and the physical mixtures of PE with ZN and span 40, respectively

Particle size (PS), polydispersity index (PDI) and Zeta potential (ZP)

The average particle size (PS) values of the obtained PE-ZN NPs ranged from 99.5 ± 6.43 nm (Z12) to 1335.0 ± 1.31 nm (Z4) as shown in Table 2 and supplemental Fig. 5. The ANOVA analysis showed a significant effect of the 2 variables (X1; pH and X2; surfactant concentration) (*p*-values of 0.015 and 0.023, respectively) on the PS, while PE: ZN ratio showed a non-significant effect (*p*-value > 0.05). PS was noticed to be high at the pH of the dispersion near the isoelectric point (PI) of ZN which might be attributed to the aggregation of the colloidal dispersion when the pH gets close to the PI of ZN polymer. Accordingly, the PS was shown to be reduced apart from PI at both pH 2 and pH 9 as shown in Fig. 6A. The incorporation of a surfactant led to an increase in PS. This was ascribed to the fact that surfactants with high and low HLB values are not able to be adsorbed on hydrophobic surfaces of drug particles which led to the aggregation of these particles to agglomerate rapidly increasing the particle size [46]. The PDI values of the obtained PE-ZN NPs alternated from 0.096 to 0.869, indicating a narrow size distribution, since all values were less than 1. The concentration of the surfactant (X3) showed a significant effect on PDI (*p*-value < 0.05).

Formulations free of surfactants showed more homogenous distribution than those containing a surfactant. On the contrary, the other two factors (PE: ZN ratio and pH) showed a non-significant effect on PDI. In the present study, the zeta potential of PE-ZN NPs showed a relatively high zeta potential except Z1 which showed the lowest zeta potential (−8.8±0.7) as shown in Table 2. This may be attributed to the PS observed of Z1 (973.1±89.2 nm) where the attraction between particles may exceed the electrostatic repulsive forces, so the zeta potential decreased and the PS increased [31]. Zeta potential at pH lower than ZN PI (pH 6.8) exhibited a positive charge. However, at PI, the dispersion showed a zeta potential of negative value in the presence of nonionic surfactant (Span® 40). It was stated that at a neutral pH, many nonionic surfactants granted a negative charge on the NPs as a result of the differential adsorption of the produced ions (H_3O^+ and OH^-) on their surfaces [2, 31]. On the other side, Podaralla and Peruma indicated that at high alkaline pH, a very high variation in the zeta potential of the ZN NPs is expected. This variation was observed in formulations prepared at pH 9 [44].

Table 2. Preparations and characterization of PE-ZN NPs

#	pH	PM: ZN (% W/W)	Surfactant (mg/mL)	EE (%)	PS (nm)	PDI	ZP (mV)
Z1	7	0.6	50	64.46±1.23	973.1±9.61	0.869±0.001	−8.8±0.7
Z2	7	0.6	0	80.84±3.56	206.7±7.80	0.129±0.003	32.5±1.2
Z3	7	0.2	0	73.4±0.89	185.0±8.90	0.314±0.002	43.3±2.3
Z4	7	0.2	50	68.36±5.62	1335.0±1.31	0.593±0.002	−22.4±0.6
Z5	9	0.2	0	99.12±2.89	175.0±6.77	0.103±0.001	−37.9±1.5
Z6	9	0.6	50	99.57±1.78	116.2±14.60	0.411±0.001	17.2±2.4
Z7	9	0.2	50	99.05±6.59	152.9±10.31	0.497±0.002	17.6±0.8
Z8	9	0.6	0	99.14±0.23	173.8±9.55	0.096±0.001	−41.4±0.2
Z9	2	0.6	50	99.3±4.23	177.2±5.67	0.101±0.003	46.5±1.7
Z10	2	0.2	50	99.32±1.56	278.1±7.37	0.408±0.001	44.9±2.6
Z11	2	0.6	0	99.29±0.56	131.0±2.54	0.149±0.002	24.8±0.1
Z12	2	0.2	0	99.31±3.64	99.5±6.34	0.237±0.001	37.8±1.3

Dependent variables are reported as average values (n=3)±SD

Fig. 6 [Images not available. See PDF.]

Response surface plot of effect of **A** dispersion pH and **B** conc. of surfactant on particle size

Encapsulation efficiency

EE% was determined by measuring the amount of unencapsulated extract in the aqueous phase after centrifugation

of the developed dispersion systems as displayed in Table 2. It was observed that ZN-NPs entrapped a significant amount of PE (64.46–99.57%). pH significantly affected the PE encapsulation efficiency (p value < 0.05). pH apart from the isoelectric point (PI) of ZN protein polymer, such as pH 2 and pH 9 showed a high amount of encapsulated extract in contrast to pH 7 (isoelectric point of ZN) which showed a lower amount of PE entrapped. McTigue and Perry observed an increase in the encapsulation efficiency of the investigated protein (Hen Egg White Lysozyme) while using a pH away from its PI [15]. On the other side, the other two factors (PE-ZN ratio and surfactant concentration) showed a non-significant effect on the EE as displayed in Fig. 6B.

Selection of the optimum ZN NP preparation

Selection of the optimum preparation was based on studying the effects of the different preparation variables on the four essential investigated responses (EE%, PS, PDI and ZP). According to the ANOVA analysis, pH and surfactant concentration have a principal significant effect on EE% and PS, respectively. It is inferred that pH apart from the PI of ZN was favorable to obtain high EE% and small PS. In addition, increasing the surfactant concentration from zero to 50 mg/mL had a negative effect on PS. Therefore, preparations with a surfactant concentration level of 50 mg/mL were excluded. On the other hand, the priority was attained to NP preparations of pH 2. Basically for two reasons: first, to avoid the obtained variations in ZP at pH 9 and second, to obtain NP with a positively charged surface for the favorable interaction with the negatively charged cell membrane. By the examination of the NP preparations of pH 2 level, the preparation of the maximum EE%, minimum PS and highest positive ZP was obtained by Z12. Therefore, Z12 was selected as the optimized NP preparation and subjected to further characterization.

Determination of residual solvents (methanol)

The determination of methanol as a residual solvent in pharmaceuticals is an important quality control measure. Methanol is classified as a Class 2 solvent which should be limited due to potential toxicity. The compendial method for determination of residual solvents USP <467> was capable to determine the residual methanol in both pure PE extract and the optimized formulation of PE-ZN NPs (Z12). The results were 812 ppm and 237 ppm for the pure PE extract and the optimized formulation (Z12), respectively. According to ICH Q3C (R8) option 1, the acceptable amount of methanol as a residual solvent in the final product should be not more than 3000 ppm based on 30 mg/day permitted daily exposure (PDE) and daily dose does not exceed 10 g. The two results were below the limit indicating the safety of the formula and the compliance with global regulatory requirements.

Morphological examination by transmission electron microscope (TEM)

The examination of the morphology of the optimized formulation of PE-ZN NPs (Z12) under transmission electron microscope showed that the ZN-NPs had a spherical uniform shape and a smooth surface. There was no aggregation, and the size of the particles was in the nanosize, matching the results obtained by the Zetasizer as shown in Fig. 7.

Fig. 7 [Images not available. See PDF.]

TEM image of the optimized PE ZN NP

In vitro drug release

The optimized ZN NP preparation (Z12) was selected to study its release profile in comparison with free PE extract. The free extract showed a rapid release pattern of more than 75% of PE within 2 h as inferred from Fig. 8, while the release pattern of PE from the optimum preparation was relatively slow. This slow release pattern was confirmed by the significant difference (p value < 0.0001) in release between free PE extract and Z12 at 2 h. Moreover, it achieves a controlled release over 24 h with higher stability of the encapsulated PE in Z12 comparable to the obvious decline in the release of PE (p value < 0.0011) in the period between 10 and 24 h which most probably might be justified by the degradation of the released free PE in the release medium [9].

Fig. 8 [Images not available. See PDF.]

In vitro release of pomegranate from PE ZN NPs compared to PE

Determination of release kinetics

The results of the estimation of (R^2) of the utilized three mathematical models revealed that the highest (R^2) was for the Higuchi model pointing to a diffusion-controlled release of the encapsulated PE extract from the hydrophobic matrix of the formed ZN NPs to the external aqueous phase.

Ex vivo permeability study

As illustrated in Fig. 9, the ex vivo cumulative permeation percentages of PE through the rabbit intestine from both optimized PE-ZN NPs (Z12) and free PE extract over 24 h were plotted. It was noticeable that the permeation percentages from both optimized PE-ZN NPs (Z12) and free PE were comparable till the first hour. Thereafter, the permeation rate of free PE was relatively slower than the optimized PE-ZN NPs (Z12) and reached the maximum at 6 h with cumulative percentage of (56.96% ± 1.72) and significant difference (p value < 0.0133) from the optimized Z12 formula that had permeation percentage of (63.62 ± 3.54). Conversely, the permeation of PE from the optimized PE-ZN NPs (Z12) was continuous after 6 h and reached the maximum at 24 h with cumulative permeation percentage of (89.02% ± 4.58). This relative increment in PE permeation in case of PE-ZN NPs could be attributed to several factors. For instance, the small nanosized of the particles and the high positive charge of the NPs surface that aid to increase the interaction with the cell membrane and consequently augment the flux and permeation of the extract. In addition, the hydrophobic properties of the zein molecules due to the γ-zein N-terminal repetitive part promote the extract permeation through interaction with the cell membranes components. Similar finding were reported by [21, 24].

Fig. 9 [Images not available. See PDF.]

Ex vivo permeation of pomegranate from PE ZN NPs compared to PE

In vitro cytotoxicity study

To examine the effect of nanoencapsulation on the biological activity of PE, the cytotoxic effect of the optimized preparation of PE-ZN NPs (Z12) and the free PE on HepG2 cancer cells were compared. It was found that the optimized formula (Z12) had a superior anti-proliferative effect on the HepG2 liver cell line with a 50% inhibitory concentration (IC50) of 266.68 µg/mL ± 23.11 relative to 657.47 µg/mL ± 13.84 in case of free PE extract. As demonstrated in Fig. 10, the optimized PE-ZN NP preparation (Z12) increased the potency of the cytotoxic activity on HepG2 hepatic cancer cells by reducing the IC50 values by about 2.5-fold than the free PE (p value < 0.0001). This finding may be attributed to the more interaction between the negatively charged cell membranes and the positive charge of the optimized preparation [3]. Furthermore, the augmented cytotoxic activity might be due to the high ability of PE NP in internalization of PE into the cells relative to the free extract [36].

Fig. 10 [Images not available. See PDF.]

IC50 values of the PE extract and PE ZN NPs (Z12)

Discussion

To sum up, hepatic cancer with its high morbidity and lethality increases the emergence of developing promising platforms to combat it. Nature is a precious source of natural products that have proven their medicinal properties. Considerable anticancer therapeutics have originated from natural sources. Phytochemicals and their valuable constituents always pave the way to handle chronic health threats. Pomegranate is an ancient fruit with auspicious antioxidant and anticancer properties and is considered an effective remedy for different cancer diseases. Punicalagin is one of the most valuable polyphenols in pomegranates which is not absorbed in its intact form but hydrolyzed into moieties of ellagic acid and rapidly metabolized into short-lived metabolites of ellagic acid [48].These compounds possess many biological activities in the prevention and treatment of cancer [29, 53] because of their free radical scavenging activities [22] as well as their anticancer effects [48].

Accordingly, our goal in this study is to make a formula that stands out for the therapeutic significance of pomegranate and overcome any challenges that may affect the physicochemical properties, bioavailability and stability of pomegranate. Many attempts have been made to augment its biological activities and studies have been performed to assess the safety and efficacy. For example, Shirode et al. [48] have developed poly (d,l-lactic-*co*-

glycolic acid)–poly(ethylene glycol) NPs encapsulated with PE extract with efficient cellular uptake and anti-proliferative activity against different breast cancer cell lines. Another study obtained by Badawi et al. [9] who optimized solid lipid nanoparticles loaded with PE extract and improved its cytotoxic activity against different cancer cell lines relative to the free PE extract. Finally, Soltanzadeh et al. [49] encapsulated the PE extract in chitosan NPs aiming to protect its sensitive constituents but a limitation to encapsulation has been observed when increasing the PE concentration.

In our study, we focused on increasing the encapsulation efficiency, controlling the release of PE extract and achieving a qualified platform suitable for targeted delivery to hepatic cancers. Zein natural polymer was utilized to load PE extract by carrying on a $2^2 \times 3^1$ full factorial design to study the effect of different variables on the properties of the formed NPs.

The screening of the potential physical or chemical incompatibilities as a prerequisite for selection of the most suitable excipients in the dosage form was done by implementing two concomitantly and complementary analytical techniques, namely DSC and FTIR [18]. The FTIR analysis showed the chemical compatibility between the PE extract and the selected polymer and surfactant and the DSC analysis indicated the presence of the sharp melting peak of PE in the DSC of the physical mixtures of both zein polymer and span® 40 with a forward shift in case of zein due to the thermal stability and a backward shift in case of span® 40 on account of the partial dissolution of PE in the melted surfactant.

The pH of dispersion at three levels, namely 2, 7 and 9, parallel with two levels of Span 40 as surfactant concentrations, namely zero and 50 mg/mL were selected to demonstrate the feasibility to rely on tuning the pH of the dispersion and passing up the use of surfactants. The ANOVA analysis revealed the significant effect of pH on PS (being high at pH 7 near the isoelectric point (PI) of ZN and low at pH apart from PI at both pH 2&9), EE% (pH 2&9 showed a high amount of encapsulated extract in contrast to pH 7) and ZP. While the surfactant concentration had a significant effect on PS and PDI, the change of PE: ZN ratio had no significance on any of the investigated responses. The observed effects on each response were in harmony with literature. In detail, the observed effect of pH on PS has been reported by many authors such as [44] that reported that the decrease in PS of ZN NPs is due to the existence of zein polymer in in the monomeric form at pH>PI. On the other hand, [19] attributed the low PS and high porosity of ZN NPs at low pH to the more solubility of zein in acidic pH. For the encapsulation efficiency, the attained EE% at pH apart from the PI was remarkably high around 99%, on the other hand, a noticeable decrease in the EE% at pH 7 (near the PI) from 64.46 ± 1.23 to 80.84 ± 3.56%. This could be appropriately attributed to the refolding behavior of proteins at the PI which could result in the escape of the encapsulated extract leading to less encapsulation efficiency than that obtained at pH far from the PI as reported by [35]. In case of the ZP, the effect of pH on the ZP of the prepared ZN NPs was in agreement with [27] who attributed the obtained change in the ZP of the uncoated ZN NPs from highly positive at low pH to highly negative at high pH to the electrical characteristics dominated by the zein molecules at particles' surfaces, which are cationic at low pH due to protonation of amino and carboxyl groups ($–NH_3^+$ and –COOH) and anionic at high pH due to de-protonation of these groups ($–NH_2$ and $–COO^-$). Finally, the surfactant concentration effect was negative on both PS and PDI with observable increase in PS and less homogeneity with relatively higher PDI values than the surfactant-free formulations. Based on these findings, it is inferable that the integral positive effect of changing the pH of the dispersion medium in comparison of the incorporation of surfactant and the adequacy of the hypothesis of the reliance on tuning the dispersion pH is deduced.

As per the predetermined QTPP and the above ANOVA analysis, the optimization resulted in an optimized ZN NP preparation having a small PS of 99.5 ± 6.43 nm (<100 nm) that a prerequisite for size-dependent passive targeting to liver as reported by [34] who studied the distribution of 5-fluorouracil zein nanoparticles and discussed the optimum average size required for accumulation in liver after IV administration taking into consideration the fenestrae size and the necessity of the unrecognition by RES and high EE% of 99.31 ± 3.64. In addition, a PDI value of 0.237 ± 0.001 indicates narrow size distribution and a high positive ZP charge of 37.8 ± 1.3 that imports good physical stability and ability to interact efficiently with negatively charged cell membranes. Then the performed in-

vitro study on the optimized ZN NP preparation showed a distinguishable and stable in vitro release pattern comparable to the free PE extract in a controlled manner for 24 h achieving sustained release profile. Additionally, as the methanol which is a class 2 solvent was used in the PE extract preparation and it is known that residual solvents have no therapeutic value and may affect physicochemical properties of the active constituents and have potential toxicity [20]. Taking into consideration exceeded quantities of such solvents can consequently result in risks for patients. Methanol (Permissible Daily Exposure 30 mg/day) can be used in the pharmaceutical products with permissible limit of 3000 ppm as a residual solvent in the final products according to ICH guideline for Residual Solvents Q3C (R9). GC analysis was performed to detect the quantity of the residual solvent in both the PE extract and the optimized NPs. The obtained results showed that both the extract and the formula were below the permissible limit indicating the safety of the formula and the compliance with global regulatory requirements. Nanosized formulations exhibit a large loading capacity together with a larger surface on which the loaded drug can conjugate with targeting organs with a controlled permeation pattern [41].This was exhibited through the ex vivo permeability study that has revealed an enhancement of 1.5 folds of the cumulative PE permeation from the optimized ZN NP over 24 h in comparison with PE extract that delivered its content through only 6 h.
Oxidative stress is one of the main causes of the development of human hepatic cancer. Bishayee et al. [14] demonstrated the potential effect of using pomegranate products in the chemoprevention and treatment of hepatic cancer through overcoming the oxidative damage trough modulation of Nrf2 signaling. In our study, the anti-proliferative activity (MTT assay) of the optimized formulation was studied in HepG-2 cancer cell lines in comparison with free PE. The potential of the optimized preparation to enhance the cytotoxic activity of PE extract was evidenced by an in vitro cytotoxicity study that revealed the superiority of PE-loaded ZN NP over Free PE extract on HepG2 liver cell line by significantly decreasing the IC50 by 2.5 fold than the free PE which in turn proves our endeavor and presents a promising platform for future utilization in preclinical and clinical studies against hepatic cancer.

Conclusion

Successful treatment for hepatic cancer was aimed in this study by loading pomegranate peel extract in nanosized particles with the aid of zein polymer which achieves high drug encapsulation efficiency with small particle size. This developed system attained more effective and more retardation of pomegranate extract release than the free extract. The in vitro drug release study demonstrated that the free extract showed a high percentage of release of about 80% within 2 h, while the optimized preparation exhibited more retardation till 24 h. The in vitro cytotoxicity study showed that PE-ZN NPs attained reduced IC_{50} values by 2.5-fold than the free PE; thus, formulated pomegranate extract in zein nanoparticles has a more effective and cytotoxic effect than free extract. The study demonstrated that the pomegranate zein nanoparticles could be a promising green drug delivery system, where both the active ingredient and the carrier polymer are derived from plants with a surfactant-free strategy, thus protecting the liver against toxins and can be safely and effectively used to treat hepatic cancer. Finally, future stability studies, more in vitro tests to understand the underlying mechanisms of the hepatic anticancer properties of prepared NPs of the optimum formula and in vivo experiments are required to validate PE-ZN NPs efficacy and bioavailability. Consequently, the approach may be worth considering for scale-up to expand its applications in clinical studies against hepatic cancer.

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Author contributions

S.M. contributed to the conceptualization. D.M.N.A. and M.M.B. was involved in the methodology and experimental design; S.M. assisted in screening, formulation, and characterization. M.A.E. was involved in the plant extraction. S.M. and M.M.B. prepared the original draft literature. D.M.N.A. contributed to the discussion. A.N.E. and D.M.N.A. contributed to the review and editing. A.N.E., D.M.N.A. and A.R.F. were involved in the supervision.

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Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

The authors declare that they have no competing interests.

Abbreviations

ANOVA
Analysis of variance
CPP
Critical process parameters
CQA
Critical quality attributes
DMSO
Dimethyl sulfoxide
DSC
Differential scanning calorimetry
EE
Encapsulation efficiency
EPR
Enhanced permeability and retention
ETs
Ellagitannins
FTIR
Fourier transform infrared (FTIR) spectroscopy
HLB
Hydrophilic lipophilic balance
ICH
International Council on Harmonization
IC50
The 50% inhibitory concentration
NPs
Nanoparticles
Nrf2
Nuclear factor erythroid 2-related factor 2
OD
Optical density
PBS
Phosphate buffer solution
PDI
Polydispersity index
PE
Pomegranate extract

PI
The isoelectric point
PS
Particle size
PU
Punicalagin
QTPP
Quality target product profile
RES
Reticuloendothelial system
RPM
Rotate per minute
SD
Standard deviation
TEM
Transmission electron microscope
ZP
Zeta potential
ZN
Zein

Publisher's Note

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Exploring the antimicrobial activity of Origanum majorana L . against the highly virulent multidrug-resistant Acinetobacter baumannii AB5075 : UPLC-HRMS profiling with in vitro and in silico studies

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ABSTRACT (ENGLISH)

Background

The infamous multidrug-resistant (MDR) bacterium *Acinetobacter baumannii* is becoming a nightmare in intensive care units across the globe. Since there are now very few effective antimicrobial agents, it is necessary to explore unconventional resources for novel antimicrobials. This study investigated the potential antimicrobial activity of *Origanum majorana* L. against *A. baumannii* employing multiple approaches including antimicrobial susceptibility, fractionation, ultra-performance liquid chromatography–high-resolution mass spectrometry (UPLC-HRMS) dereplication, and in silico analysis for target/ligand identification.

Results

On the extremely pathogenic MDR strain *A. baumannii* AB5075, *O. majorana* L. has shown a significant growth inhibitory effect (MIC = 0.675 mg/mL). The polar 50% methanol fraction was the most active (MIC = 0.5 mg/mL). The UPLC-HRMS dereplication of the bioactive fraction detected 29 metabolites belonging to different chemical classes. Justicidin B, one of the identified metabolites, was projected by preliminary in silico analysis to be the most highly

scoring metabolite for binding with molecular targets in *A. baumannii* with a Fit score = 8.56 for enoyl-ACP reductase (FabI) (PDB ID: 6AHE), suggesting it to be its potential target. Additionally, docking, molecular dynamics simulation, and bioinformatics analysis suggested that this interaction is similar to a well-known FabI inhibitor. The amino acids involved in the interaction are conserved among different MDR *A. baumannii* strains and the effectiveness could extend to Gram-negative pathogens within the ESKAPE group.

Conclusions

Origanum majorana L. extract exhibits antimicrobial activity against *A. baumannii* using one or more metabolites in its 50% methanol fraction. The characterized active metabolite is hypothesized to be justicidin B which inhibits the growth of *A. baumannii* AB5075 via targeting its fatty acid synthesis.

FULL TEXT

Background

Healthcare practitioners everywhere give careful consideration to *Acinetobacter baumannii* as it is a major cause of nosocomial infections. *A. baumannii* is frequently responsible for outbreaks in intensive care units that show up as urinary tract infections, surgical site infections, pneumonia linked to ventilator use, and sepsis [1]. Based on pooled data from various regions globally, *A. baumannii* was found to produce approximately 25 hospital-acquired infection cases per 1000 patients; in intensive care units, this ratio rose to 54 cases per 1000 patients [2]. Furthermore, the problem has gotten worse due to its resistance to several antimicrobial agents that are currently available, as well as, its acquired resistance to their alternatives.

Multidrug-resistant (MDR) *A. baumannii* are increasingly reported worldwide reaching 45% of *A. baumannii* infections, exceeding the MDR rates reported for all other common nosocomial pathogens [3]. This has placed *A. baumannii* among the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus*, *Klebsiella pneumoniae, A. baumannii, Pseudomonas aeruginosa,* and *Enterobacter* spp.) [4–6]. For multidrug-resistant Gram-negative bacteria, including *A. baumannii*, colistin and carbapenems like doripenem, imipenem, and meropenem, are typically regarded as the last resort treatments [7–9]. Nevertheless, the administration of colistin has been restricted due to its toxicity, including nephrotoxicity and neurotoxicity [10, 11]. The frequency of isolation of carbapenem-resistant *A. baumannii* (CRAB) is increasing with currently few alternatives for treatment. The Centres for Disease Control and Prevention (CDC) has listed CRAB as an “urgent threat” to human health [12, 13]. In a developing country like Egypt, the rate of isolating CRAB from patients reached 80% and higher [14]. After the COVID-19 pandemic, the reported infections by CRAB increased by 78%, which is highly alarming [13].

The development of novel antibiotics is essential due to the high mortality rates associated with CRAB infections [15, 16]. This has warranted WHO to include *A. baumannii,* along with other carbapenem-resistant pathogens, *P. aeruginosa* and Enterobacteriaceae*,* to be critically prioritized in the research and development of new antibiotics [17].

Plant-produced secondary metabolites, sometimes referred to as phytochemicals, may offer chemical resistance against infectious agents. The abundance of these metabolites, some with efficacies comparable to that of synthetic antibiotics, provides a repertoire of chemicals that could be used to successfully create and deploy new antimicrobial agents against MDR-ESKAPE pathogens [18–20].

Origanum majorana L. commonly referred to as Sahtar, Zaatar or sweet marjoram is a photoautotrophic medicinal perennial herb of the *Origanum* genus, which belongs to the Lamiaceae family [21]. This plant is found broadly all around the Mediterranean region, but especially in Morocco, Algeria, Egypt, Spain, and Portugal [22]. Traditional medicine makes extensive use of it, as a possible treatment for a wide range of illnesses including allergies, respiratory infections, hypertension, diabetes, and stomach pain, as well as, an intestinal antispasmodic, [23]. Pharmacological research revealed that this plant’s essential oils and extracts possessed a variety of biological qualities, including hepatoprotective, antimutagenic, anticancer, antiparasitic, antibacterial, antifungal, antidiabetic, antioxidant, anti-inflammatory, and analgesic effects [24]. The plant extracts’ safety and therapeutic advantages have been verified by a toxicological evaluation [25]. In rats, *O. majorana* L. extracts did not result in any fatalities,

which further confirms their safety [26, 27]. *O. majorana* L. has previously been found to contain a wide range of bioactive compounds from various chemical classes, including hydroquinone, sterols, terpenoids, tannins, phenolic acids (arbutin and methyl arbutin), fatty alcohols, and flavonoids (diosmetin, orientin, luteolin, apigenin, vitexin, and thymonin) [28]. Thymol, carvacrol, cis-sabinene hydrate, limonene, terpinene, and camphene are among the plant's abundant volatile oil constituents [28]. It is important to bear in mind that different plant organs, growth stages, and even harvesting techniques can have different effects on the amount and composition of the aforementioned metabolites and volatile oil components within the same species [29]. Antibacterial activity has been demonstrated by the plant against a variety of pathogenic bacteria, including *E. faecalis*, *Bacillus subtilis*, *Escherichia coli*, *K. pneumoniae*, *Serratia* sp. and *Salmonella choleraesuis* [30–32].

In search of prospective treatments for MDR *A. baumannii*, this study aimed to investigate the antibacterial activity of *O. majorana* L. extract and its fractions. UPLC-HRMS was used to analyse the chemical profile of the active 50% methanol fraction (50% MF) in order to establish a relationship between the antibacterial activity and the bioactive secondary plant metabolites. Justicidin B, a secondary metabolite identified in *O. majorana* L. bioactive fraction, has been identified as a potential novel antimicrobial agent against *A. baumannii* and other ESKAPE pathogens, which targets the essential FAS-II fatty acid biosynthesis pathway component FabI.

Methods

Bacterial strains and culture conditions

The multidrug-resistant highly virulent *A. baumannii* strain AB5075 [33] was obtained from Prof. Dr. Lory, Harvard University, USA as a gift, and was used as the test microorganism in all the experiments. The bacterial strain was typically grown in tryptic soy broth (TSB) with shaking at 180 rpm at 37 °C or plated on tryptic soy agar (TSA) and incubated at 37 °C.

Preparation, extraction and fractionation of *O. majorana* L. aerial parts

Origanum majorana L. aerial parts were collected from the Experimental Station of the Faculty of Pharmacy, Cairo University, Giza, Egypt. Mrs Therese Labib, Consultant at Orman Botanic Garden, Dokki, Giza, Egypt, kindly authenticated the plant material. Air-dried powder of *O. majorana* L. aerial parts (0.5 kg) was exhaustively extracted with methanol (5 × 7 L) and evaporated on a rotary evaporator to yield the total methanolic extract (ME, 75 g). Part of the residue (45 g) was suspended in 350 mL of distilled water and then fractionated using dichloromethane (3 × 750 mL), which was then evaporated to get DCM-F (25 g). The mother liquor (ML) was applied on the Diaion-HP20 column and eluted firstly with distilled water (1L, discarded), followed by 50% methanol (1 L), and then 100% methanol (1 L). The fractions were evaporated to yield 50% MF (4 g) and 100% MF (6 g), respectively.

Preliminary screening of *O. majorana* L. extract for antimicrobial activity

An overnight culture of *A. baumannii* AB5075 was grown in TSB at 37 °C and 180 rpm. The culture was diluted in TSB to OD600 = 0.1 arbitrary units, then diluted 1:1000 in TSA which was prewarmed to 50 °C. The inoculated TSA (50 mL) was distributed in a 15-cm-wide petri dish. After agar solidification, 10 µL of the extract, or the fraction, were spotted on the agar surface, the spots were dried in a laminar flow cabinet, and the plates were incubated at 37 °C. After 24 h, the plates were inspected visually, and the diameters of the growth inhibition zones were measured using a ruler.

Determination of the minimum inhibitory concentration (MIC)

Following the criteria of the Clinical and Laboratory Standards Institute (CLSI), the MIC was ascertained using the broth microdilution method [34]. The dried extract or fraction was dissolved in DMSO to a final concentration of 100 mg/mL. Then, it was diluted in Muëller-Hinton broth to yield a series of twofold dilutions ranging from 0.001 to 2 mg/mL. One hundred µL aliquots from each concentration were distributed in a 96-well plate. Each well was inoculated with 10 µL (~10^5 CFU as determined spectrophotometrically by measuring absorbance at 600 nm and verified by viable count) of a freshly prepared bacterial suspension. The plates were incubated for 24 h at 37 °C. The MIC was the minimum concentration of the tested crude extract or its fractions that inhibited visible bacterial growth. Muëller-Hinton broth containing the equivalent of DMSO served as a negative control and the experiment was performed in triplicate.

UPLC-HRMS analysis of the active fraction

The *O. majorana* L. active subfraction (50% MF) was subjected to UPLC-HRMS analysis followed by dereplication (identification of the secondary metabolites present in the extract) using the Dictionary of Natural Products and Reaxys online databases. The dereplication depended on the HRMS isotope profiles, MS/MS fragmentation, and the databases were filtered to include only the plant genus or family.

A Bruker MAXIS II Q-ToF mass spectrometer connected to an Agilent 1290 UHPLC system was used to analyse the samples. The column used was a Phenomenex Kinetex XB-C18 (2.6 mM, 100 × 2.1 mm). The following LC gradient profile was used to achieve separation: 5% MeCN + 0.1% formic acid to 100% MeCN + 0.1% formic acid in 15 min at a flow rate of 0.1 mL/min. MS parameters were: mass range *m/z* 100–2000, capillary voltage 4.5 kV, nebulizer gas 4.0 bar, dry gas 9.0 L/min, and dry temperature of 250 °C. MS/MS experiments with a step collision energy of 80–200% were carried out using the Auto MS/MS scan mode.

Virtual target identification

PharmMapper was used to characterize potential target(s) of justicidin B [35]. This platform uses an extracted pharmacophore model and stores it as a library ligand dataset in mol2 format. Then, it rates each molecule in the PDB according to how well it fits the model. This approach yields a pure Fit score that is far more reliable and significant than chance pharmacophore matching. The query structure was submitted to the platform in the pdb format, and the results were sorted based on the Fit scores.

Docking study

Using an AutoDock Vina docking machine, the crystal structures of FabI (PDB ID: 6AHE) were utilized for the docking study [36]. The co-crystallized ligand AFN-1252 was used to determine the binding site and the docking grid-box. The coordinates of the grid-box were set to be x = −36.404, y = −8.512, z = −3.293.

A criterion of 2.0 Å was established for the ligand to binding site shape matching root-mean-square deviation (RMSD). The Charmm force field (v.1.02) with a distance-dependent dielectric and a non-bonded cut-off distance of 10.0 was used to obtain the interaction energies. Then, an energy grid was set at 5.0 Å from the binding site [36]. Inside the chosen binding pocket, the studied compound, justicidin B, was energy-minimized. Pymol software was used to edit and visualize the generated binding poses [37].

Molecular dynamics simulation (MDS)

NAMD 3.0.0. software was used to perform MDS [38, 39]. Protein systems were constructed with the VMD software's QwikMD toolkit [39, 40], and the protonation states of the amino acid residues were set (pH 7.4). The protein structure was examined for any missing hydrogens, and the co-crystalized water molecules were then removed. Following that, the entire structure was immersed in an orthorhombic box of TIP3P water together with 0.15 M Na^+ and Cl^- ions in a 20 Å solvent buffer. The systems were then energy-minimized and allowed to equilibrate for five nanoseconds. With the help of the VMD plugin Force Field Toolkit (ffTK), the ligand topologies and properties were determined. Afterwards, the produced parameters and topology files were loaded into VMD so that the simulation steps could be carried out and the protein–ligand complexes could be interpreted without errors.

Binding free energy calculations

The molecular mechanics Poisson–Boltzmann surface area (MM-PBSA) method from the AMBER18's MMPBSA.py module was used to calculate the docked complex's binding free energy by applying the following equation complex $\Delta G_{Binding} = \Delta G_{Complex} - \Delta G_{Receptor} - \Delta G_{Inhibitor}$ [41].

Conservation of the FabI sequences

The amino acid sequence of the FabI (PDB ID: 6AHE) enzyme, which showed the best-Fit score with justicidin B, was retrieved from the NCBI database. To confirm the conservation of FabI in different strains of *A. baumannii* and different strains of ESKAPE pathogens, the protein sequence of FabI was analysed by the BlastP tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) [42], using a nonredundant protein sequences database against each organism (*A. baumannii*, *E. faecium*, *S. aureus*, *K. pneumoniae*, *P. aeruginosa* and *Enterobacter* spp.) with an expect threshold of 0.001. Proteins were considered conserved if they had a max alignment score of more than 200.

The conservation of amino acids in the predicted binding site for justicidin B was further confirmed by the alignment of the sequences of representative proteins from MDR *A. baumannii* strains (including those from *A. baumannii* ATCC 19606 (PDB ID:6AHE) and *A. baumannii* AB5075) and from representative strains of different ESKAPE pathogens. The sequences of the target proteins were retrieved from the NCBI database. The protein sequences were aligned using the multiple sequence alignment Clustal Omega tool with default settings (https://www.ebi.ac.uk/Tools/msa/clustalo/) [43].

Results

O. majorana L. total methanolic extract and its fractions have potent growth inhibitory effects against *A. baumannii*

The *O. majorana* L. total methanolic extract was spotted on the trypticase soy (TS) agar seed inoculated with *A. baumannii* AB5075. It showed a 14-mm zone of growth inhibition after 24-h incubation (Fig. 1A). Using the broth microdilution method, the minimum inhibitory concentration (MIC) of the total methanolic extract of *O. majorana* L. was determined at 0.675 mg/mL. Chromatographic techniques were used to prepare subfractions by partitioning with solvents of different polarities and applying them on a Diaion column. The 50% methanol fraction showed considerable antimicrobial activity (Fig. 1B). Upon measuring the MIC for this fraction, it showed a value of 0.5 mg/mL. On the other hand, the non-polar dichloromethane fraction had a higher MIC value of 0.875 mg/mL.

Fig. 1 [Images not available. See PDF.]

Growth-inhibitory effects of *O. majorana* L and its fractions against *A. baumannii*. Ten µl of the total methanolic (TM) extract (**A**) of *O. majorana* L or the 50% methanol fraction from Diaion-HP20 (50% MF) (**B**) were spotted on TSA plates inoculated with *A. baumannii* AB5075 and the plates were incubated overnight at 37 °C. In both cases, equivalent amounts of the solvent (DMSO) were spotted on the same plates. The experiment was repeated three times, and representative images were presented

UPLC-HRMS analysis of *O. majorana* L. active fraction reveals multiple metabolites.

To unlock the chemical diversity of the active fraction of the *O. majorana* L., UPLC-HRMS profiling revealed the presence of 29 metabolites belonging to different structural classes, comprising two terpenes, two alkaloids, one lignan, nine flavonoid aglycones, four flavonoid glycosides, seven phenolic acid derivatives, and two alicyclic derivatives. Moreover, three hits were not identified, indicating new compounds or compounds that were not reported before in the family Lamiaceae (Table 1).

Table 1. LC-HRMS analysis of the 50% methanol fraction (50% MF) of *Origanum majorana* L

HRMS m/z	Mol. formula	Tentative identification	Compound structure
165.0911	$C_{10}H_{12}O_2$	Eugenol	
181.04954	$C_9H_8O_4$	Caffeic acid	
227.1279	$C_{12}H_{18}O_4$	12-hydroxyjasmonic acid	
271.0603	$C_{15}H_{10}O_5$	Apigenin	
273.0758	$C_{15}H_{12}O_5$	Naringenin	
283.0812	$C_{13}H_{14}O_7$	No hit from the family	
285.0757	$C_{16}H_{12}O_5$	Genkwanin	

289.0706	$C_{15}H_{12}O_6$	Aromadendrin; dihydrokaempferol	
299.2005	$C_{20}H_{26}O_2$	Majoradiol	
303.0499	$C_{15}H_{10}O_7$	Quercitrin; sophoretin; flavin	
312.0502	$C_{16}H_9NO_6$	Aristolochic acid II	
315.0865	$C_{17}H_{14}O_6$	Salvigenin; cirsimaritin	
329.1019	$C_{18}H_{16}O_6$	5-Hydroxy-4',6,7-trimethoxyflavone	
331.0812	$C_{17}H_{14}O_7$	5,6,4'-Trihydroxy-7,3'-dimethoxyflavone	
342.0608	$C_{17}H_{11}NO_7$	Aristolochic acid I; aristolochic acid	
355.1023	$C_{16}H_{18}O_9$	Chlorogenic acid; 5-*O*-caffeoylquinic acid	
359.1125	$C_{19}H_{18}O_7$	Gardenin B	
361.0917	$C_{18}H_{16}O_8$	(R)-(+)-rosmarinic acid	
365.1032	$C_{21}H_{16}O_6$	Justicidin B	
375.1074	$C_{19}H_{18}O_8$	Rosmarinic acid methyl ester	
379.1023	$C_{18}H_{18}O_9$	No hits from the family	
389.1806	$C_{18}H_{28}O_9$	12-Hydroxyjasmonic acid 12-*O*-hexoside	
407.0972	$C_{19}H_{18}O_{10}$	No hits from the family	
423.1285	$C_{20}H_{22}O_{10}$	Amburoside A	
439.1235	$C_{20}H_{22}O_{11}$	Oreganol-A; protocatechuoyl calleryanin	
447.0921	$C_{21}H_{18}O_{11}$	Apigenin-7-*O*- glucuronide	
453.1391	$C_{21}H_{24}O_{11}$	Oreganol-B	
463.0873	$C_{21}H_{18}O_{12}$	Luteolin-7-*O*- glucuronide	
593.1864	$C_{28}H_{32}O_{14}$	Acacetin-7-*O*-rutinoside	
595.1656	$C_{27}H_{30}O_{15}$	Vicenin-2; apigenin 6,8-di-C-hexoside	

Virtual screening-based target characterization

PharmMapper was employed to suggest a suitable protein target for the identified metabolites. The retrieved results were arranged according to their Fit scores. Only bacterial targets related to the *Acinetobacter* genus were considered. A threshold Fit score of 7 was set to select the best-scoring hits. As a result, *A. baumannii*-derived enoyl-ACP reductase (FabI) (PDB ID: 6AHE) was found to be among the top-scoring hits for justicidin B (Fit score = 8.56), where all the remaining bacterial hits were below the cut-off score of 7. Hence, this target (i.e. FabI) was proposed as its potential target, and justicidin B was the only compound among the 29 LC-HRMS-dereplicated metabolites that scored above the threshold Fit score.

Re-docking justicidin B modelled structure into the FabI active site achieved binding mode and docking score comparable to those of the co-crystalized inhibitor AFN-1252 (docking scores = −10.67 and −11.75 kcal/mol, respectively). Justicidin B was able to establish two H-bonds with TYR-149 and TYR-159. In addition, it was involved in multiple hydrophobic interactions with PHE-96, LEU-102, TYR-149, and ILE-203. Similarly, the co-crystallized inhibitor was involved in the same hydrophobic interactions and formed H-bonds with both TYR-159, as seen in justicidin B, and with ALA-97 (Fig. 2A, B).

Fig. 2 [Images not available. See PDF.]

Binding modes of justicidin B (brick red-coloured structure) (**A**) and the co-crystalized inhibitor AFN-1252 (Cyan-coloured structure) (**B**) inside the binding site of FabI (PDB ID: 6AHE). **C** RMSDs of both structures (i.e. Justicidin B and AFN-1252) inside the binding site of FabI throughout 100 ns-long MD simulation. The yellow-coloured structure is the co-factor Nicotinamide Adenine Dinucleotide (NAD)

According to subsequent molecular dynamic simulation (MSD) experiments (100 ns-long), the justicidin B structure was able to achieve stable binding mode throughout the simulation, with an average Root Mean Square Deviation (RMSD) of 1.96 Å, which was similar to that of the co-crystallized inhibitor AFN-1252 (average RMSD = 1.65 Å; Fig. 2C). Such stable bindings of both justicidin B and AFN-1252 were translated into stable and significant interaction energies (electrostatic and van der Waals) inside the enzyme's binding site, where both compounds showed total interaction energies averaged around − 26.16 and − 44.27 kcal/mol, respectively (Fig. 3A, B). Hence, their calculated absolute binding free energies were comparable as well ($\Delta G_{Binding}$ = -8.11 and -9.52 kcal/mol, respectively).

Fig. 3 [Images not available. See PDF.]

Interaction energies (i.e. electrostatic and van der Waals interaction energies) of justicidin B (**A**) and AFN-1252 (**B**), respectively inside the binding site of FabI over the course of 100 ns-long MD simulation

According to the previous modelling and MD simulation findings, it could be hypothesized that *O. majorana* L. extract was able to inhibit the growth of *A. baumannii* via targeting its fatty acid synthesis by one of its metabolites (i.e. justicidin B).

The conservation of the predicted interaction sites of justicidin B and FabI in *A. baumannii* and among members of the ESKAPE group

To check if the predicted interaction of justicidin B and FabI could be extended to other strains of *A. baumannii*, we performed a blast analysis for the enoyl-ACP reductase (FabI) amino acid sequence against *A. baumannii* strains in the National Centre for Biotechnology Information (NCBI) database. The alignment score was >200, indicating a high degree of conservation of the overall amino acid sequence of this protein. Upon performing a multiple sequence alignment of the amino acid sequences of the FabI protein in fifteen MDR and CRAB strains, all five amino acids involved in the interaction were conserved (TYR-149, TYR-159, PHE-96, LEU-102, and ILE-203) (Fig. 4A). Moreover, to determine if this interaction could be extended to other members of the ESKAPE pathogens, the FabI amino acid sequence of representative MDR strains from the members of this group was retrieved when another multiple sequence alignment was performed (Fig. 4B). The results of this alignment indicated that all the five amino acids involved in the interaction between justicidin B and FabI are conserved (TYR-149, TYR-159, PHE-96, LEU-

102, and ILE-203) in the Gram-negative members: *K. pneumoniae, A. baumannii, P. aeruginosa* and *Enterobacter* spp. On the other hand, for the Gram-positive members *S. aureus* and *E. faecium*, all the interaction sites were conserved except the non-polar amino acid ILE-203 which was replaced with another non-polar amino acid, valine, and TYR replaced PHE-96 in *E. faecium*. This change in one amino acid in these two strains could have a minimal impact on justicidin B binding.

Fig. 4 [Images not available. See PDF.]

Alignment of the protein sequences of the enoyl-ACP reductase (FabI) in (**A**) MDR and CRAB *A. baumannii* strains and (**B**) among ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp.) pathogens. The amino acid sequence of each protein was retrieved from the NCBI database and aligned using Clustal Omega software. The alignment visualization was done using Jalview 2.11.3.1. The amino acids involved in justicidin B binding are highlighted

Discussion

The rise of antibiotic-resistant pathogens and the difficulties in developing antimicrobials have aroused concerns among medical professionals for the well-being of humanity. Plants, a rich source of undiscovered metabolites, offer a solution as a natural source of antimicrobial compounds because they are attainable and generally safe to use. *O. majorana* L. or sweet marjoram is an aromatic, herbaceous, perennial plant in the family Lamiaceae. The plant has been used as a flavouring and herbal spice and is widely reported for its antibacterial activity against multiple pathogens, including *E. coli*, *B. subtilis*, *B. megaterium*, *S. aureus, P. aeruginosa*, and *Proteus vulgaris* [44, 45].

In the current study, the methanolic extract of *O. majorana* L. demonstrated remarkable antimicrobial activity against *A. baumannii* with a MIC of 0.675 mg/mL. The antimicrobial activity of the methanolic and ethanolic extracts and the decoction of *O. majorana* L. was reported previously against many Gram-positive and Gram-negative pathogens and *Candida* spp. [46, 47]. Only one study evaluated and proved its antimicrobial activity against *A. baumannii,* using the disc diffusion method [48]. The MIC of the methanolic extract was reported previously to be >100 µg/mL against *S. aureus* and *E. coli* [49]. A higher MIC (1.56 mg/mL) was determined for the decoction of *O. majorana* L. against *S. aureus* and *K. pneumoniae* [47]. The ethanolic fraction has MIC between 40 and 80 µg/mL, against *E. coli* and *K. pneumoniae* [46]. Additionally, the essential oil fraction of the plant was found to be highly active against different bacteria, fungi and protozoa [31, 49, 50]. However, it was not quantitatively tested against *A. baumannii*.

To pinpoint the active fraction responsible for the antimicrobial activity against *A. baumannii* in this study, the plant was fractionated, where the polar 50% methanol fraction was found to be the most active subfraction (MIC = 0.5 mg/mL). Chemical profiling of the bioactive fraction by UPLC-HRMS analysis revealed 29 metabolites belonging to diverse chemical classes. The dominant metabolites were phenolic acids, flavonoids, and lignans [51]. Phenolic acids such as rosmarinic acid, caffeic acid, and chlorogenic acid were previously reported in *O. majorana* L. [29]. They are beneficial against several chronic diseases such as degenerative diseases, cancer, diabetes, and ageing [52]. However, to the best of our knowledge, no reports are available about the antimicrobial activity of these metabolites from *O. majorana* L. against *A. baumannii*. The antimicrobial activity of many of these metabolites against Gram-positive and Gram-negative pathogens, such as naringenin [53], quercetin [54], and caffeic acid [55], was previously reported.

Two alicyclic derivatives, 12-hydroxy jasmonic acid and its 12-*O*-glucoside, were previously isolated for the first time from *Origanum* species, i.e. polar extracts of *O. vulgare* L [56]. Later, they were isolated from *O. dictamnus* L. Only 12-hydroxy jasmonic acid and not its glucoside exhibited a strong antibacterial effect against *A. hemoliticus* [57]. When screened for its phytotoxic potential against different phytopathogens, bioguided fractionation of the most active *O. majorana* L. fraction led to the isolation of different molecules, including a new compound named majoradiol, a carvacrol dimer, which was characterized but was not tested for its bioactivity due to its scarcity [58]. Aristolochic acid I and aristolochic acid II were isolated from *O. vulgare* and showed potent activity against leukaemia. Additionally, this is the first report to find that aristolochic acid I and aristolochic acid II demonstrated potent antithrombin activity [59]. Justicidin B, an arylnaphthalene lactone, is a plant-derived subclass of lignans

which is distributed in many plants and different families. It is the main active component of *Phyllanthus piscatorum*, exhibiting strong antifungal, antiprotozoal, and anti-proliferative properties [60].
Caffeic acid was isolated from *Origanum dictamnus* L. and demonstrated a good antibacterial effect against *A. hemoliticus* and *P. aeruginosa* [57]. Chlorogenic acid, rosmarinic acid, and rosmarinic acid methyl ester were isolated from the methanol extract of *O. dictamnus* L. and exhibited weak antibacterial activity against *S. aureus* [61]. Amburoside A was previously isolated from *O. micranthum* and exhibited a weak carbonic anhydrase inhibitory effect [62]. Oreganol A and B were isolated from the extract of dried leaves *O. vulgare* and exhibited strong 1,1-dipehnyl-2-picrylhydrazyl (DPPH) radical scavenging activity [63].
Naringenin was previously reported in *O. dictamnus* L. and showed a strong antibacterial effect against *Acinetobacter hemoliticus* [57]. Despite the structural similarity with naringenin, apigenin was also isolated from the same species in the same study by Chatzopoulou and co-workers but did not demonstrate any antibacterial activity [57]. Genkwanin, salvigenin, and 5,6,4'-trihydroxy-7,3'-dimethoxyflavone were isolated from the chloroform extract of glandular hairs of *O. x intercedens* [64]. Genkwanin is known for its potent antiviral activity against African swine fever viral infection in Vero cells in a dose-dependent manner [65]. Gardenin B, quercitrin, 5-hydroxy-4',6,7-trimethoxyflavone, and aromadendrin were isolated from *O. majorana* L. with other flavonoid aglycones [58]. Aromadendrin demonstrated moderate anti-inflammatory activity by the inhibition of COX-1 [66]. Apigenin-7-*O*-glucuronide, luteolin-7-*O*-glucuronide, acacetin-7-*O*-rutinoside, and vicenin-2 were isolated from the methanol extract of *O. dictamnus*. None of them demonstrated antibacterial effects against the Gram-positive or Gram-negative strains tested [61].
It can be challenging to characterize a given molecule's biological target. The success rate of identifying accurate molecular targets has greatly increased due to the ongoing development of in silico methods like virtual screening and molecular modelling. Nowadays, a plethora of ligand-based or structural search methods are employed in various online target identification platforms. PharmMapper is a dependable platform that makes use of its pharmacophore model to screen and propose the most probable protein targets for a query chemical [35]. Pharmacophore-based screening works on the basic premise that pharmacophore maps, which delineate the spatial arrangement of structural features, are the primary determinants of the binding of certain compounds to their protein targets. Therefore, molecules whose shapes match these pharmacophore maps have the best chance of binding to the same protein target.
PharmMapper software was employed to screen the 29 metabolites detected in the 50% methanol fraction for binding with putative protein targets in *A. baumannii*. The metabolite justicidin B recorded the highest Fit score (8.56) with the *A. baumannii*-derived enoyl-ACP reductase (FabI). Justicidin B is an aryl naphthalene lignan previously identified in the Lamiaceae family [67], but it is identified here for the first time in *O. majorana* L. extract. The antimicrobial activity of justicidin B was previously reported against *S. aureus*, *E. coli,* and *P. aeruginosa.* In addition, justicidin B has been proven to have antiviral, antifungal, and antiparasitic properties. It also exhibits anti-inflammatory, antioxidant and anticancer properties [68]. It was originally isolated through the bioactivity-guided fractionation of the dichloromethane extract of *P. piscatorum* and demonstrated non-specific cytotoxicity, in addition to potent in vitro fungicidal and antiprotozoal effects [60]. It recently demonstrated strong antiviral potential against Zika virus [69], an effective inhibitor of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) [70], and strong cytotoxic activity against HeLa cervical cancer cells through targeting key proteins involved in apoptosis regulation [71]. It was even isolated from a marine-derived actinomycete, *Nocardia* sp. ALAA 2000 and exhibited a broad-spectrum antimicrobial effect against a panel of Gram-positive and Gram-negative strains as well as different fungi [72].
FabI was predicted as a possible binding target for justicidin B. FabI is a bacterial catalytic enzyme that reduces a carbon–carbon double bond in an enoyl moiety covalently linked to an acyl carrier protein. This is the rate-determining step in the elongation cycle of fatty acids used in lipid metabolism and biotin biosynthesis in bacteria [73, 74]. Being a unique bacterial enzyme, FabI has been considered a potential target for developing new antibacterial therapeutics [75, 76]. It has been discovered that FabI is necessary for bacterial viability. Recently, a

wide range of compounds has been recognized as FabI inhibitors, such as triclosan, imidazoles, indole naphthyridinones, thiopyridine, and 4-pyridone [77].

Lately, there has been a growing recognition of promising antimicrobial targets involved in fatty acids synthesis pathways [78]. Novel FabI inhibitors are considered promising antimicrobial agents against MDR bacteria which could be identified by using molecular docking and virtual screening with the FabI X-ray crystal structures that are currently accessible [79]. Fabimycin is a previously discovered FabI inhibitor and an antibiotic candidate with in vivo activity against Gram-negative pathogens [80]. Our docking studies and MDS suggested justicidin B as a novel inhibitor against *A. baumannii* FabI; it binds the enzyme in a pattern similar to its co-crystalized ligand.

The sequence of FabI enzyme that bound to justicidin B was found to be conserved in *A. baumannii* and ESKAPE pathogens strains available in the NCBI database with alignment scores >200. The conservation of FabI enzyme among different bacterial species was confirmed previously [81]. The conservation of the amino acids involved in justicidin B binding was confirmed. Only in *S. aureus* and *E. faecium*, the amino acid ILE-203 was replaced with valine, and PHE-96 by tyrosine, respectively, which should have minimal impact on justicidin B binding. This predicts justicidin B as a promising antimicrobial agent through FabI inhibition with potential broad-spectrum activity. However, the impact of changes in the amino acid sequence in these two Gram-positive pathogens is yet to be determined.

Finally, the predicted activity of justicidin B (an aryl naphthalene lignan) as a broad-spectrum antimicrobial against *A. baumannii* and other ESKAPE pathogens through FabI inhibition still needs to be confirmed by different phenotypic and genotypic studies in a step towards the introduction of new antimicrobial compounds. By tracing the aryl naphthalene lignans containing metabolites, we could take a substantial step towards discovering plant-based chemicals as prospective inhibitors of conserved protein targets in MDR-ESKAPE pathogens.

Conclusions

O. majorana L. extract offers a promising source of natural antimicrobial extracts that could be successfully used against the troublesome *A. baumannii* infections. With further experimental confirmations, justicidin B can be employed as a lead molecule for the development of novel inhibitors against MDR *A. baumannii* and other ESKAPE pathogens through interaction with its potential target, the FabI, and the subsequent inhibition of lipid metabolism and biotin biosynthesis in bacteria.

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Statement regarding plants

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Author contributions

Conceptualization, A.S.A., A.M.E. and M.E.R.; methodology, N.H.M., A.E.M.S., A.M.S., R.A.E., N.M.E., M.Y, W.M., M.T.K.; software, A.E.M.S., A.M.S., R.A.E., M.E.R.; validation, A.S.A., A.M.E., A.S.A. and M.E.R.; formal analysis, N.H.M., A.E.M.S., R.A.E., M.Y., W.M., N.M.E., A.M.S.; investigation, N.H.M., A.E.M.S., R.A.E.; resources, A.S.A. and M.E.R.; data curation, A.S.A., A.M.E. and M.E.R.; writing—original draft preparation, N.H.M., A.E.M.S., R.A.E., A.M.S., N.M.E., M.T.K., W.M., M.Y.; writing—review and editing, A.S.A., A.M.E. and M.E.R.; supervision, A.S.A., A.M.E. and M.E.R.; project administration, A.S.A., A.M.E. and M.E.R.; funding acquisition, A.S.A. and M.E.R.. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

DPPH
1,1-Dipehnyl-2-picrylhydrazyl
100% MF
100% Methanol fraction
50% MF
50% Methanol fraction
ACP
Acyl carrier protein
CRAB
Carbapenem-resistant *A. baumannii*
FabI
Enoyl-ACP reductase
ESKAPE pathogens
Enterococcus faecium, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.
ffTK
Force field tool kit
MF
Methanol fraction
MIC
Minimum inhibitory concentration
MMPBSA
Molecular mechanics Poisson–Boltzmann surface area
ML
Mother liquor
MDR
Multidrug-resistant
NCBI
National centre for biotechnology information
NAD
Nicotinamide adenine dinucleotide
RMSD
Root-mean-square deviation
SARS-CoV-2
Severe acute respiratory syndrome coronavirus 2
MSD
Subsequent molecular dynamic simulation
CDC

The centres for disease control and prevention
CLSI
The Clinical and Laboratory Standards Institute
DCM-F
Dichloromethane fraction
ME
Total methanolic extract
TSA
Tryptic soy agar
TSB
Tryptic soy broth
UPLC-HRMS
Ultra-performance liquid chromatography–high-resolution mass spectrometry

Publisher's Note

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DETAILS

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Exploration of different strategies of nanoencapsulation of bioactive compounds and their ensuing approaches

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ABSTRACT (ENGLISH)

Background

Nanotechnology has gained rapid popularity in many fields, such as food science. The labile bioactive is enclosed in a shield that protects it from harmful environmental factors. It also allows for targeted delivery to specific areas. Bioactive compounds in foods are slowly degraded or can change due to external or internal factors such as oxidation. Innovative technologies and novel edible packaging materials can be used to reduce bioavailability. One promising technology for overcoming the problems above is encapsulation.

The main body of the abstract

Nanostructure systems enhances a number of properties, including resistance to degradation and improvements of physicochemical functions like solubility, stability, and bioavailability, among others as the nanosize increases surface area and, consequently, activity. A recently emerged nanoencapsulation technologies, including electro spraying, nano-fluidics, complex coacervation, electrospinning, polymerization, etc. have been briefly discussed. Different bioactive molecules can be nano encapsulated by absorbing, incorporating, chemically interacting, or dispersing substances into nanocarriers. There have also been other characterization techniques and different physico chemical parameters investigated to evaluate the characteristics of encapsulated bioactives. The current article highlights numerous bioactive substances utilized for nanoencapsulation using cutting-edge methods.

Short conclusion

This review examines how different encapsulating bioactive materials can improve encapsulating films or coatings. The advent of nanotechnology has opened up a wide range of possibilities for the development, design, and formulation of innovative pharmaceuticals. The food and pharmaceutical industry can focus its attention on products that have added value through the various enhancements offered by nanoencapsulation.

FULL TEXT

Background

Widespread bioactive substances, also referred to as secondary metabolites, are found in plant matrix, and over the

past few decades, numerous in vitro as well as in vivo researches comprises of epidemiological and cohort designing, have provided the indication that eating plant-based food protects against a number of ailments.
The nanoencapsulation process provides a protective barrier around bioactive substances [1]. It is a system where a suitable nano-carrier, resistant to enzymatic degradation [2], especially in the gastrointestinal tract, including chitosan, zein, and alginate, is widely used to encapsulate bioactive compounds employing several delivery methods [3], including association colloids, nano-particles, nano-emulsions, nano-fibres/nano-tubes, and nano-laminates. Research on innovative formulation methods, particularly the creation of biological capsules, is encouraged by the rising demand for biological products in agriculture [4].This has happened because these bioproducts are more stable and their active components are more reactive, which reduces volatility losses [5].
According to these circumstances, bioactive constituents (volatile oils, metabolites from fungi, different extracts from plants etc.) can be shielded from outside influences and deterioration by being enclosed in food and agricultural zones. The encapsulation enables the products' biological integrity and maintains the environment during storage, maintaining the active ingredients' long-term vitality [6].
Upon UV exposure and high temperatures, for instance, microbial compounds are vulnerable to abiotic and biotic variables that decrease the efficiency of these living things and metabolites of those, results in the loss of toxin integrity and spore viability [7].
Encapsulation functions as a substitute for these difficulties in this way. It enables, among other things, the reduction of volatility-related losses, the improvement of biological integrity, enhancement of efficacy, enhancement ofcommercial viability, and enhancement of stability of different formulations in the agricultural sector [8–10].
Numerous polymers are employed as wall materials for encapsulation in order to safeguard the core, which is typically created by bioactive chemicals. For this, a variety of substances are employed, including chitosan, maltodextrin, gums (such as shellac, gum arabic, gum acacia, and), whey protein, starch, sodium alginate, pectin, cellulose and,sodium caseinate, zein, pullulan, galactomannan [11–15].
Before a new encapsulated product is developed, it is essential to conduct preformulation studies on the materials to be used for encapsulation in order to accommodate the physicochemical behaviour of the active ingredient and produce the desired encapsulation efficiency, as well as the size of the shell or capsule, the surface morphology and functionalities of the capsule, and the behaviour of the encapsulated active ingredient. Encapsulating pharmaceuticals with the appropriate encapsulating material(s) can greatly increase the bioavailability of both currently available and upcoming poorly soluble drugs [16].
For encapsulation, natural polymers are ideal materials. Natural polymers, which are macromolecules with high molecular weights that are derived from nature, are favoured because of their adaptability to change, biodegradability, biocompatibility, renewability, and low toxicity [17].

Main text

Bioactive composites derived from plant

Plants have always been a benediction for maintaining a healthy lifestyle from the dawn of time since them not only offer a safe place to live but also, and perhaps most significantly, food and bioactive components for therapeutic use [18]. Plants and foods derived from them were first employed as a source of food and for its nutritional value; subsequently, their therapeutic properties, which might treat ailments, were discovered and Fig. 1 has shown various types of bioactive compounds from plant for nanoencapsulation [19]. Furthermore, these substances are categorised as follows depending on their clinical and toxicological characteristics:

Fig. 1 [Images not available. See PDF.]

Bioactive compounds from plant

Alkaloids

Alkaloids are heterocyclic chemicals with a small distribution in the plant kingdom that have a bitter taste and can retain nitrogen. Tropane alkaloids having anticholinergic characteristics are found in the Solanaceae family of plants, which also includes *Atropa belladonna*, *Datura spp.*, and *Hyoscyamus niger.* Alkaloids are the focus of research on

plant-based drugs. The investigation of alkaloids from herbal and medicinal plants showed antiproliferative effects and antineoplastic properties in vitro and in *vivo* for a wide range of cancers. The databases that were accessible electronically have been screened for antiproliferative properties in lung cancer treatment [20]. It is frequently employed to lessen muscle pain. Additionally, pyrrolizidine alkaloids are found in Senecio species and other Asteraceae and Boraginaceae plants. With huge application including the treatment of cancer cells, boosting bone marrow leucocyte production, and increasing cardiac contraction. Additionally, *Theobroma cacao* and *Coffee arabica* both contain methylxanthine alkaloids [21].

Glycosides

They are usually attached with a monosaccharides or oligosaccharides or sometimes with uronic acids. Glycone is the portion that is attached to saccharide, while aglycone is the portion that is made up of pentacyclic triterpenoids and tetracyclic steroids. The main subcategories of glycosides are saponins, anthraquinones,cyanogenics, glucosinolates, and cardiac glycosides.

These substances are glycosylated, and the aglycone moieties, which are connected to uronic acid or monosaccharide or oligosaccharide, are constituted of vitamins, terpenoids, alkaloids, polyphenols, steroids, antibiotics, and other substances [22]. The glycosides' glycosidic residue is thought to be the cause of their biological effects [23]. The cardiac glycosides, diterpenoid glycosides, cyanogenic glycosides, and anthraquinone glycosides are among the classes of glycosides that are frequently harmful. Cardiac glycosides are known to produce hyperkalaemia, a disease that results in blood potassium ions that are greater than normal [24].

The Na+/K+-ATPase activity of bio-membranes is inhibited by the cardiac glycoside derivatives digitalis and strophanthus, such as ouabain [25, 26]. Diterpenoid glycoside consumption has been linked to renal proximal tubule necrosis in both humans and animals, as well as centrilobular hepatic necrosis. Adenine nucleoside carriers are hampered by diterpenoid glycosides, which have been connected to the inhibition of oxidative phosphorylation in mitochondria [27].

More than 2,650 plant species produce the cyanogenic glycosides, which are -linked glycosides of -hydroxy nitriles.Plants containing this class of glycosides produce hydrogen cyanide into the bloodstream, which impairs the efficient utilisation of oxygen in the peripheral tissues by reducing the activity of cytochrome oxidase in the mitochondrial electron transport chain. Aside from goitre and congenital hypothyroidism in children, irreversible paralysis, angular stomatitis, damage of the optic nerves, tropical ataxic neuropathy, sensorineural hearing loss, and sensory gait ataxia are further harmful outcomes of cyanide use. Examples of cyanogenic glycosides that can be found in edible plant parts are linamarin and lotaustralin, both of which are present in *M. esculenta* [28].

Tannins

Tannins are bioactive bitter polyphenolic chemicals that bind to and precipitate proteins, alkaloids, and other substances. They are soluble in water. Numerous plant species contain tannins, particularly those in the Polygonaceae and Fagaceae families. Condensed tannins and hydrolysable tannins are the two main categories.Condensed tannin groups are composed of bigger polymers of flavonoids, whereas hydrolysable tannin groups are composed of glucose clusters coupled to various catechin derivatives. Tannin molecules and protein molecules commonly engage in random interactions. Larger groups of tannins are used as drugs to treat skin bleeding, diarrhoea, and transudates [29].

Terpenoids and phenylpropanoids

The isoprenoids, often referred to as terpene derivatives, are made up of numerous combinations of terpene derivatives that are derived from five carbon isoprene units, which are combined together to form terpenoids. Instead of existing as low polar or none terpene aglycones, the majority of terpenoids exist as glycosidic forms [30].

An isoprene with penta carbons is used in the synthesis of terpenoids. Mono-terpenoids contain two isoprene units, whereas sesqui-terpenoids include three isoprene units. They are well-known for having a lower molecular weight and having a large number of groups more than 25,000. Though, phenylpropanoids are a class of compounds having a basic carbon structure that begins at nine and above, strong flavours, and a volatile tendency. These substances are frequently found in the *Lamiacea*e family and are known as volatile oils [31].

It has antibacterial, antiviral, and antitumor properties and is used as a natural medicine. Additionally, it supports gastric stimulation. Diterpenoids are also a cluster of 4 isoprene units with a strong flavour that is a lipophilic non-volatile (odourless) molecule. Since it has antioxidant properties, it is abundantly available in many plants, like coffee arabica [32].

Resins

Resins are the mixtures that include both volatile as well as non-volatile property chemicals, as well as a collection of compounds that are lipid soluble. While volatile resins have mono- and sesquiterpenoids, non-volatile resins are made up of diterpenoid and triterpenoid molecules. The antibacterial and wound-healing abilities of these resins, which are widely distributed in herbaceous plants, are well-known [33].

Proteins

Since proteins are a substantial source of nutrients for both animals and human, plant proteins have grown significantly in favour in the food and pharmaceutical industries. It is commonly known that the Fabaceae family, lentils, and the Euphorbiaceae family all have significant protein content [34].

Categorization of nanoencapsulation schemes

In the area of food science, nanoencapsulation has grown in favour recently. A bioactive substance is used as the core matrix and is encapsulated a wall matrix in inner part that can survive enzymatic and other degradation. Because bioactive substances are very susceptible to heat and the digestive enzymes found in the stomach and GI system of a person, using a substantial wall matrix/nano-carrier protects against them. Encapsulating bioactive compounds has been the subject of increasing research for various reasons. Encapsulations have been achieved using a variety of techniques, including electrospinning and coacervation. These encapsulations are known to enhance the physicochemical characteristics of bioactive compounds, increase bioavailability and stability, control the release, improve bioactivity and disguise flavour. The materials used include lipids and synthetic and natural polymers. They also influence performance and functionality. Additionally, these wall materials aid in sustaining the compound's nutritional activity and aid in disguising some compounds' unpleasant tastes [35]. Depending on how much energy is used to encapsulate these molecules, several methods are used, such as top-down and bottom-down procedures as well as their combination [36].

The top-down method of encapsulation uses equipment like spray-drying, ultrasonication, homogenizers, and many others, which results in high power consumption, as opposed to the bottom-up method, which uses techniques like precipitation, micro-emulsification, conjugation, atom exchange, etc., which use much less energy. Delivery release rate, solubility and stability of the nano-carrier, and manufacturing cost, are significant variables that are crucial in choosing a specific method for nanoencapsulation [37].

Bioactive agents and its encapsulating carriers

Hybrid nano-carriers

Internal (metal and polymer) and exterior (single/multi-lipid layer) networks make up the two main networks that make up hybrid nano-carriers [38]. This nanoparticle's outside coating serves as defence against degradation and water diffusion. These lipid-polymer and organic–inorganic carriers were primarily created for the regulated release of bioactive substances for the treatment of cancer cells.

Seyedabadi et al. reported that when compared to nano-liposomes without chitosan covering, slow-release encapsulated caffeine using chitosan wrapped in nano-liposomes performed better for the encapsulation of caffeine. [39]

Lipid-based nano-carriers

Niosomes, nano-liposomes, particulate carriers are lipid-based nano-carriers, also referred to as vesicular carriers. As a result of the interaction between the surfactant molecule and the aqueous solution, a spherical bilayer is formed. It is employed to encapsulate peptide among other bioactive substances. While liquid and solid lipid are mixed together to create nano-lipid particles, in internal phase solid lipid nanoparticles are created by combining solid lipid [37].

Chaudhari et al. studiedcompritol, a solid lipid, squalene, a liquid lipid, span 80, and tween 80 were used as

emulsifiers and co-emulsifiers to encapsulate the compounds piperine and quercetin. For slow lipid wall matrix erosiveness (12 h), these bioactive chemicals were encapsulated and showed slower release [40].
Another research reported by Abd-Elhakeem et al. demonstrated about employing lipid-based nanoencapsulation to elevate the bioavailability and target delivery of eplerenone orally. After 24 h, rabbit intestinal permeability to eplerenone-loaded nano-lipid capsules were up to two folds higher than that of traditional aqueous medication [41].

Polymeric nano-carriers

They are thought to be an incredibly ideal part for encasing and delivering bioactive chemicals. Currently, natural-based nano-carriers such starch, chitosan, casein, albumin and whey protein are most frequently employed. Ravi et al. in 2018 utilised chitosan as a wall substance to enclose the marine carotenoid named fucoxanthin [42]. The bioactive compound's anticancer activity was improved, and caspase-3 activity was 25.8 times higher as a result. Gagliardi et al. conducted a comparison study for the encapsulation of rutin using natural and synthetic nanoparticles, namely poly (lactic-co-glycolic acid), zein etc. According to the findings, poly release represented slower release (25%) after 60 h in comparison to zein along with 0.8% rutting [43].
Apart from this, in 2021, a research group from Portugal, Costa et al. bioactive extract from grape pomace was encapsulated with nanoparticles of chitosan and alginate, which increased the bioactivity and prevented the bioactive components from being hydrolysed in the gastrointestinal tract [44].

Systems of nanoencapsulation of biologically active composites

Capsulation of Bioactive substances is considereda more difficult technique than microencapsulation. It is broken down into three primary categories: low-energy, high-energy, and a combination of low and high. Low-energy processesinclude precipitation, micro-emulsification, conjugation, etc.[45]. Spray-drying, ultrasonication, homogenization, etc. will fall under the category of high-energyprocesses.
Delivery energy, release rate, solvability, solidity of the nano-carrier, and manufacturingprice are some of the variables that affect the decision of which method to adopt for designing nano-capsules. Several encapsulation methodshave been covered in the current review study [46].

Electro spraying

An alternative to the drying-encapsulation method is the electro-spraying process. At room temperature, it uses high-voltage electric current to operate. The fundamental idea behind electrospinning and electro-spraying is the same, but what sets these two techniques apart is the molecular adhesion of the polymers, which is less for electro-spraying causing the jet to break up into tiny droplets [47]. Due to the surface tension in the air, the jet particles acquire a spherical shape when exposed to it. It has been previously demonstrated that using zein as a wall material during electro spraying increases the permeability and bioactive release characteristics in green tea catechins. Later in 2019, it came to light that resveratrol achieves a 70% encapsulation efficiency when nano-encapsulated with the same wall material [48].

Micro-/nano-fluidics

The fundamental idea behind micro- and nano-fluidics is the interfacial contact between the fluids. It promotes droplet development delaying the emancipation of biologically active substances. Additionally, it aids in the creation of precise nano-droplets of a similar size. It comprises a polydimethylsiloxane glass foundation. This channel helps in transporting the fluid because it is related to all other channels [49]. A syringe is used to inject gas and liquid. This processutilizes nano or microfluidic appliances as emulsion devices. Micro fluidization was used to create a nano-emulsion system under ideal conditions, which included pressures between 40 and 65 MPa and 2–5 cycles. The research suggested that the nano-emulsion included smaller fish oil droplets. After a few years, the system was altered by utilizing micro fluidization and citrus pectin. The results exhibited that the qualities of the nano-emulsion were improved, thus safeguarding cholecalciferol against UV [50] degradation in comparison to the original pectin. Additionally, the modified pectin's molecular weight and hydrodynamic diameter were both decreased to 235 kDa and 420 nm, respectively.

Complex coacervation

In the encapsulation process known as complex coacervation, two polyelectrolytes with opposing charges engage

with one another in an aqueous media. The bioactive substance is encapsulated around a protein or carbohydrate compound [51]. This interaction only occurs at particular parameters like strength, polymeric concentration, biopolymer content, and biopolymer weight. This method enables the creation of particles that have a coating of a capsulating agent thus protecting the biologically active chemicals [52, 53].

Electrospinning

By applying high-voltage electricity to polymeric fluids to process fluids with electric charges, electrospinning creates dry micro- and nanostructures. The instrument essentially consists of three main components: syringe and pump, an electrified needle, and a collection plate [54]. When high-voltage electricity is applied to the nozzle or needle, the liquid is forced inside. The studies were conducted at room temperature, and the nanofibers were stored in a desiccator before being transported after being collected on an electric collector plate [48]. Nano-fibers can be anything between one and many nanometers in diameter [55].

Blend, coaxial, emulsion, high-throughput, and polymer-free are among the five main types of electrospinning techniques. The biologically active component and the solutions of the polymerare assortedtogether using either a blend or emulsion approach, which is effective at managing the release of bioactive substances. Using this method, both hydrophilic and hydrophobic compounds can be easily enclosed [56]. On the other hand, a coaxial electrospinning arrangement utilizesa syringe connected to a single output pump producing enclosed fibers [57]. High-throughput electrospinning, on the other hand, is a pointless process used to create ultra-thin fibers from emulsions. In 2018, a high-throughput process was utilized to create glycoconjugates. In addition to all of these methods, the polymer-free methodutilizes a solution of a polymer having high masswhich is incorporated by a pump to produce a greater yield than the traditional methods [58].

According to Xiao et al. [59], this method can create nanofibers varying from 80 to 50 nm, while Moreira et al. [60] claim that the polymer-free method can widely help the food industry. Additionally, Poornima et al. [61] successfully delivered controlled drug release using this method to capsulate resveratrol with caprolactone and lactic acid. While Zein was used to enclose resveratrol with the best encapsulation effectiveness (96.9%), they are the ideal one according to researchers [62].

Spray chilling

Ascorbic acid, a form of vitamin C, has been shown to have positive benefits in agriculture, including lowering the impact of nickel and cadmium on barley, reducing the stress of drought on sweet pepperand cucumber, and enhancing apple quality [63]. It makes it possible to lessen the effects of salt stress and promote better barley growth. In this regard, spray cooling has been reported as an efficient method for encapsulating ascorbic acid, displaying retentions above 80% [64]. The method entails melting the completely hydrogenated palm oil to 85 °C, mixing in the ascorbic acid, and then letting it instantly flow through the spray drier before being cooled to 4 °C.

Polymerization

In this method of nano-encapsulation, the bioactive ingredient is added after the aqueous fluid's monomers are polymerized to create nano-particles. Additionally, the nano-particles are cleansed during encapsulation by getting rid of excess stabilizer and surfactant that had accumulated on their surface. Typically, this method is used to create poly butyl cyanoacrylate nanoparticles [65].

Supercritical microencapsulation

Modern terminology for supercritical microencapsulation includes "micronization." Mild temperatures are used during supercritical micronization to prevent lowering the quality of bioactive substances. Water and carbon dioxide are the two most common solvents utilized in this method. To microencapsulate bioactive substances of relevance to the food and agricultural industries, carbon dioxide under supercritical circumstances has grown in popularity [53].

It is a different environmentally friendly technique for creating nanoparticles. Since CO_2 is non-toxic, inexpensive, and inflammable, it is frequently utilized as a supercritical fluid. In this procedure, a liquid solvent is used. This liquid solvent isassorted with supercritical liquid (CO_2). Instant precipitation now occurs as a result of the solute's insolubility non the supercritical fluid, which enhances the effect of the produced nanoparticles [66].

According to some studies, depending on the characteristics of the wall matrices and the active components, this

method can be beneficial in a variety of contexts, including the creation of encapsulated products. The processing method employing supercritical CO_2 is chosen according to the main component and polymer to be capsulated. For instance, the way CO_2 reacts with the solvent, the wall material, and the active substance. The contact between CO_2 and the solution of polymer utilized in the biopolymer drug delivery system is crucial to the encapsulation process. While polymers, such as polylactide, are effective for SAS treatment, RESS is challenging to solubilize in supercritical CO_2 [67].

Ultrasonication

The term ultrasound refers to a group of sound waves that are louder than 16 kHz. There are two types of sound waves: low-intensity waves and high-sound waves. Low-intensity waves are utilized for the purpose of detection, butmolecules are modified utilizing high sound waves, including reducing their size and emulsification. It is employed in nanotechnology to create several types of nanostructures [68].

A generator, a transducer, and a titanium horn-shaped sound expeller make up the ultrasound system's components [69]. To create nano-emulsions, nano-liposomes, niosomes, and other types of nano-delivery systems utilizing ultrasound, a variety of nano-delivery strategies have been devised [70].

Homogenization

Homogenization is the process of creating homogeneous-sized nano-fragments at a particularly escalated pressure. It has a pressure range of 100 to 400 MPa, which is 10 to 15 times higher than a typical homogenizer. The study found that the milk and milk product industries frequently use this technique to improve the basic characteristics of the products while also enhancing the anti-microbial quality of certain microbes. At 250 & 350 MPa homogenization pressure and 40 and 50 °C inlet temperatures, a decrease of about 2 and 3 log cycles was found [71].

It also serves as an alternative to thermal processing since it effectively inactivates enzymatic and microbial characteristics [72]. The homogenization technique was also used to create a soy protein emulsion [73]. Figure 2 has shown various types of nanoencapsulation systems for bioactive compounds.

Fig. 2 [Images not available. See PDF.]

Types of nanoencapsulation systems for encapsulation of bioactive composites

Nano encapsulated bioactive agents and its physicochemical features

Encapsulation efficiency and loading capacity

The amount of bioactive compounds encapsulated within the matrix wall is what we call it. Quantifying the amount of mixture in a nanoparticle can be done using UV–Vis spectrum analysis [74]. The perfect nanoparticle has the maximum loading capacity of the compound with minimal wall materials. The incorporation or absorption of bioactive compounds can be done in two ways. Entrapment capacity mainly depends on the solubility and molecular mass of the bioactive compounds encapsulated within the material of the wall [75]. The maximum entrapment capacity is reported for macromolecules and proteins at an isoelectric level.

Particle size

These properties are conscientious for the quality of the nanoparticles, as well as their delivery capability, stability and viscosity [76]. The intracellular capacity of nanoparticles is higher than that of micro-particles due to their relative size and mobility [77].

The size and shape of nanoparticles can be detected using a variety of microscopes. Optical properties of nanoparticles and nano-capsules or micro are determined by laser diffraction &scanning electron microscope techniques. The surface study, which requires extremely powerful analyzers like transmission electron microscopes to determine the quantity of pores, is needed. Mixing fluorescent dyes with the bioactive compounds makes it possible to detect multiple locations using fluorescence or confocal microscopy. The photon correlation spectrum, or dynamic light scattering, is widely used to determine the size of nanoparticles in the 1000 nm range. This helps to determine the particle range and its concentration within a matrix [78]. The charge characteristics of nanoparticles are identified. The electrical properties of nanoparticles can be altered by altering the compounds in the fluid. A nanoparticle with a Zeta Potential greater than (±) 30 mV is stable. It is interesting to note that the Zeta Potential

process can identify whether partition materials are encapsulated within the nano-capsule or if they cover its outer arrangement [64, 79].

Stability

The nanoparticles' stability is the ability to stay intact within the matrix wall until they are released at the time and location desired. The nano-emulsions are more stable caused by the morphological structures of small droplets. The stability of bioactive compounds can also be tested by placing them into various modified environments, such as high/low temperatures, fluids with different ionic charges, and pH levels [80].

Control release

The release of bioactive agents depends on several factors, like as solubility of the mix, the surface binding/adsorption, and the diffusion of the matrix. Other aspects include matrix degradation or a combination of matrix degradation, diffusion, and degradation. The release of nano-spheres containing bioactive compounds in an even distribution is mainly due to erosion of the wall material. Diffusion is the only way to control release if wall degradation occurs slowly. Quick release can lead to poor wall materials or small binding ability [65]. The mixing technique has been reported to play a crucial role in the release profile for nano-capsules, as it slows the release [81]. If the coating is polymer, the release will be by diffusion from the inside of the matrix to the outside. Several other techniques, such as reverse dialysis bags, dialysis bags, and synthetic or artificial membrane diffusion, can release the compound.

Applications of nano-encapsulated compound

The most common encapsulation material is natural or synthetic polymers. Food encapsulation is a technique that has been used for many years in the food industry [82]. Encapsulation is a technique that aims to deliver bioactive compounds directly to target organism tissues. The bioactive compounds are more stable and bioavailable, and their benefits to the body increase [83]. Table 1 shows the various applications of nano-encapsulated compounds in food. Other compounds useful for food encapsulation include dyes and flavors, vitamins, antioxidants, enzymes and bioactive peptides.

Table 1. Applications of nano-encapsulated compound in the food industry

Sl. No	Nano-encapsulated compound	Nanomaterials	Functions	Food materials	References
1	Vitamin D3	Potato proteins	encouragement of human health	beverages solutions	[96]
2	Vitamin E	Edible mustard oil	Health supplement antioxidant	Mango juice	[97]
3	α-Tocopherol	Canola oil and tween 80	Antioxidant agent	Fish sausages	[98]
4	Quinoa peptide	Soyphosphatidylcholine and cholesterol	Natural food preservative	Fresh beef burgers	[99]
5	Pears peel fruit	Sodium alginate-chitosan	Preservative	Guava juice	[100]

6	Olive leaf phenolicsoleuropein	Lecithin cholesterol	Functional food product	Yogurt	[101]
7	Nisin and garlic extract	Phosphatidylcholine and oleic acid	Antimicrobial agent	Whole UHT milk	[102]
8	Nisin and lysozyme	Phosphatidylcholine pectin	Natural antimicrobials	Whole UHT milk	[103]
9	Fish skin peptide	Lecithin	Preservative and vehicle for Entrapping fishy smell	Pork patties	[104]
10	Fish oils	Lecithin sunflower oil	Improvement of the nutritional value	Bread	[105]
11	Chlorogenic acids	β-cyclodextrin	Phenolic compounds Supplementation on aroma volatile profile	Bread, cookies, caramel cottage cheese, nutty filling, and mushroom or meat stuffing	[106, 107]

Other substances, such as those found in essential oils and herbal extracts with insecticidal and antimicrobial properties, are being encapsulated [84]. Biotechnology faces a difficult task in controlling volatile oils and extracts. The essential oil of savoury leaves was combined with natural polymers like apple pectin and gum arabic. All polymers showed a higher efficiency of encapsulation. The herbicidal effect of amaranth, tomato and other plants was subsequently increased [85]. Pepper oil was encapsulated, and its antibacterial activity was tested long-term. Encapsulating this oil with gum arabic/malt dextrin had an inhibitory action against *Pseudomonas aureus, Enterococcus Faecali* and *Staphylococcus aureus.*

The Supercritical Anti-solvent method encapsulated curcumin from turmeric using polymer matrices. This dye contained turmeric extracts. These polymers were Eudragit L100 and Pluronic F127 or mixtures thereof. In this field, economic evaluations were also developed to help boost the application of the technology on a larger scale and to transfer it to an industrial scale.

Gas anti-solvent was used to create poly (caprolactone), containing resveratrol, with heterogeneous properties. The encapsulation process was successful because the resveratrol's chemical structure or antioxidant activity did not change. The microparticles also maintained their release over 48 h [86].

The use of non-hydrolyzed polymers in agriculture has increased due to their accessibility and price. Hydrolyzed starches are the most effective agents for encapsulating pesticides (metabolites made by *Bacillusthuringiensis*) because they protect environmental factors while improving the formulation. Encapsulation is a viable method for formulating biopesticides and/or biofertilizers for agricultural fields. Encapsulation has several uses in agriculture [87] and pharmaceutical, which are shown in Tables 2 and 3

Table 2. Applications of nano-encapsulated compound in the agricultural industry

Sl. No	Nano-encapsulated bioproducts in agriculture	Functions of nano-encapsulated compound

1	Biofertilizer	1. Enhanced formulation stability 2. Better biological properties 3. Slow release of encapsulated bioproducts 4. Reduction of volatility 5. Enhanced in efficiency 6. Increase in commercial viability 7. Protection of encapsulated materials
2	Bioherbicide	3
Biofungicide	4	Bioinsecticide
5	Plant extracts	6

Table 3. Application of nano-encapsulated compound in pharmaceutical industry

Sl. No	Nano-encapsulated bioactive compound	Applications
1	Phenolic compounds	Protection, improvement of their antioxidant and other functional activities, target delivery
2	Carotenoids	Stabilization, efficient controlled release, expansion of their industrial applications
3	Essential fatty acids	Stabilization, better solubility, decrease volatility, use of lower doses, favorable impact on the sensory quality of the final product
4	Vitamins	Protection to oxidation
5	Peptides and enzymes	Improved antimicrobial or antioxidant activity, better absorption
6	Probiotics and prebiotics	Increment of viability, promotion of gastrointestinal health

Trichoderma Harzianum is a very effective biological control agent for agricultural purposes. Its live spores make it highly sensitive to both biotic and non-biotic factors. Encapsulation improves the activity of phytopathog*ens, such as Sclerotinia sclerotiorum. Another study encapsulated spores from the* Trichoderma genus in biologically-based lignin to treat diseases in the vine trunk. In vitro tests showed that the spores remained at rest until the fungus triggered germination at the right time.

The Pickering emulsion microencapsulated *Metarhizium conidia* were found to have a better distribution of cells in the leaves and greater control over the *Spodoptera Littoralis* pest [88]. In a separate study, *Bacillusthuringiensis*

aizawai was encapsulated in Pickering emulsion water-in-oil. It was found that *Spodoptera thuringiensis aizawa* larvae of the first instar were killed with 92% efficiency [89].

Similarly, inoculating potatoes with two strain of *Pseudomonas fluorescens* by an alginate/gelatin capsule led to a larger level of safeguard from harmful soil conditions and a greater establishment in the Rhizosphere. The antifungal effect of salicylic acids containing zinc oxide was shown when the bacteria were encapsulated with sodium alginate. Azadirachtin, a natural insecticide found in neem trees (*Azadirachta indica*), was nano-emulsified with whey isolate. The strategy significantly affected the death of *Spodoptera frugiperda*, a pest caterpillar that attacks soybean crops. The encapsulated orange essential oil also positively affects the mortality of *Spodoptera frugiperda*, affecting soybean crops [90].

Peel inhibits the growth of *Escherichia colitis* and *Staphylococcus aureus* [91]. A study on the encapsulation of biofertilizers showed formulations with *Burkholderia spp*. and Pseudomonas alginate encapsulated in phosphate-alginate provided superior environment for the growth of wheat plants under semi-arid or salt-stressed environments [92]. A study showed that biofertilizers containing *Pseudomonas cepacia* and *Azosprillum brasilense*, encapsulated in polymers made of clay mineral and sodium alginate, led to a higher level of formulation control [93]. The study also showed that the slow release of active compounds positively affected wheat plants' growth, with increased biomass.

Future directions

Nanoencapsulation-based bioactive compounds are unique and current; their operation in both the food and pharmaceutical areas results in new opportunities for commercialization. Their application is primarily the creation of nanostructured ingredients for food and pharmaceutical product, which allows the enhancement of solubility and stability, flavour, texture, and colour of foods. Encapsulation can be used to mask unpleasant aromas or flavours. It also increases the bioavailability of nutrients and allows for controlled release in both food and pharmaceutical products. At the same time, the volatility of compounds encapsulated is reduced. In the near future, food and pharmaceutical industries can gain from incorporating NPs into their new products [94, 95].

Conclusions

It is necessary to strengthen our immune systems to combat the rapid spread of deadly diseases. This can only be achieved by consuming bioactive substances extracted from plants. Nanoencapsulation technologies are unique and recent; their action in both the food and pharmaceutical areas effects in new opportunities for commercialization. The nanoencapsulation with carriers of bioactive compounds is an approach that can be used to improve their bioavailability, stability, and use within the pharmaceutical industry. Nanotechnology used to encapsulate microbial compounds with bioactive properties has many obvious advantages, from packaging to food processing. These include improved bioavailability and stability. The controlled release and defence of bioactive substances also offer safety benefits. To facilitate the safe marketing of health-beneficial new nanotechnology products, global legislation is needed to recognize the safety and toxicology of these nanomaterials. In other words, it is necessary to develop and implement novel extraction and encapsulation technologies for highly effective microbial bioactives that can be used in different industries, such as the food, pharmaceutical and cosmetic industries. Further, it is necessary to understand the safety and toxicology of nanoencapsulation, as well as to implement global legislation to ensure safe marketing and use.

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Author contributions

Dr. SC conceived the idea and outlined the content; Dr. KK collected information, reviewed the literature and developed the manuscript; Dr. SC and Dr. RM proof read and edited the manuscript. All authors read and approved the final manuscript for submission.

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Availability of data and materials

All necessary data generated or analyzed during this study are included in this published article. Any additional data could be available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Phytochemical analysis and evaluation of antibacterial activity of various extracts from leaves, stems and roots of Thalictrum foliolosum

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ABSTRACT (ENGLISH)

Background

Thalictrum foliolosum, a member of the Ranunculus family, is recognized for its therapeutic potential in addressing gastric issues, dyspepsia, tooth pain, abdominal colic pain, and piles. The diverse array of secondary metabolites present in the plant contributes to these therapeutic applications. This study aims to uncover and quantify the bioactive secondary metabolites found in the unexplored leaves, stems, and roots of *T. foliolosum*. Additionally, we also aimed to evaluate the antibacterial activity and MIC values of these extracts against a panel of pathogenic bacteria, such as pathogenic strains, including *Escherichia coli, Pseudomonas aeruginosa, Streptococcus mutant* and *Staphylococcus aureus*.

Result

HPLC analysis suggested all examined compounds were found significantly more in root parts of plant. To

determine the potential antimicrobial activity of different plant parts result suggested chloroform fraction of root most effective with variable potency against each examined pathogen at 25–100 µg/ml extracts which indicated rich content of berberine in this fraction. Minimum MIC (121.26 µg/mL) of the chloroform fraction of the root was also supported the results. Fatty acid methyl ester analysis by gas chromatography revealed that the stem contained high levels of fatty acids, such as palmitic acid, stearic acid, and linolenic acid, all of which have antibacterial properties.
Conclusion
The potential antimicrobial activity of extracts of various plant parts strongly supports the *T. foliolosum* plant's widespread use in folk medicine for the treatment of various chronic diseases and adulterants with various associated medicinal plant species.

FULL TEXT

Background

In nature, plants are the richest resource of bioactive natural products, such as alkaloids, phenolics, flavonoids, carotenoids, triterpenoids and other secondary metabolites for thousands of years [1]. Extensive studies in ancient pharmaceutical practices and contemporary clinical findings consistently affirm that plant-based medicines exhibit a higher safety profile compared to their synthetic counterparts, maintaining a crucial role in healthcare. Derived from botanical sources, these medicinal compounds serve as chemical entities in traditional medicine systems, modern pharmaceuticals, dietary supplements, pharmaceutical intermediates, and synthetic drug formulations [1, 2]. Thalictrum, a genus encompassing approximately 220 species, exhibits a diverse array of secondary metabolites including flavonoids, alkaloids, steroids, triterpenoids, triterpenoid glycosides, carotenoids, lignins, tannins, cardiac glycosides with high molecular weight, fatty acids, phenolic acids and sterol across its various species, highlighting the pharmacological significance of this genus [3–5]. While there isn't extensive research specifically on *Thalictrum foliolosum's*, although this species has been used in traditional medicine for various purposes. Given the relevance of phytopharmaceuticals in today's society, the current study concentrated on *Thalictrum foliolosum* DC, an endemic herb that has received little attention.

T. foliolosum is a potential medicinal herb belonging to the Ranunculaceae family that is found widely in the Northern Hemisphere region in India and the China Pacific [6–8]. Previous studies have shown that *T. foliolosum* roots are used locally as a tonic, antipyretic, diuretic, laxative, and collyrium for the improvement of eyesight as well as in the treatment of gastric problem, dyspepsia, tooth pain, abdominal colic pain and piles [8, 9]. The combination of the dried root of *T. foliolosum* with *Thymus linearis* was very effective in treating colic and gastric problems [10]. Whole-plant extracts of *T. foliolosum* also showed inhibitory effects against cancer cells as well as the progression of malignant malarial fever [11, 12]. *T. foliolosum* plants may be regarded as belonging to an elite group of medicinal plants due to the presence of benzylisoquinoline alkaloids (BIQ), which include berberine, protoberberine, magnoflorine palmatine, jatrorrhizine, and other considered isoquinoline alkaloids along with several groups of polyphenolic compounds that demonstrated strong bioactivity [13–15]. Previously published reports showed the roots and rhizome parts of *T. foliolosum* have alleviative properties. Therefore, unrestricted collection of roots and rhizome parts resulted in the decline of their population in their habitats [6, 7]. Generally, it is observed that complete knowledge about the chemical composition and potential medicinal uses of various parts of a plant will enable systematic harvesting and conservation of declining plant populations. Considering the therapeutic importance of *T. foliolosum*, there were no more reports of bioactive compounds in aerial parts of this plant. The current study used reverse phase HPLC to identify and quantify four therapeutically important BIQ alkaloids, namely magnoflorine, berberine, palmatine, and thalicarpine, in different parts of the plant (leaves, stem, and roots). Additionally, fatty acids were identified using gas–liquid chromatography. Moreover, a comparative study of antimicrobial activity against human pathogenic bacteria, such as *E. coli*, *Pseudomonas aeruginosa*, *Streptococcus mutans*, and *Staphylococcus aureus*, was conducted using extracts or fractions of various parts of the plant. Thus, the present study was undertaken to analyze the alkaloids content and major fatty acids in *T. foliolosum* leaves, stem and roots and to understand the relation between with antimicrobial activities.

Methods

Sample collection of *T. foliolosum* plants and processing

T. foliolosum, a perennial herb, was obtained in September month from the forest areas of the Nanital district (2290-m asl), Kumaon region of Uttarakhand state in India (Fig. 1). Nanital is located at the GPS coordinates of 29° 22′ 49.0944" N and 79° 27′ 48.8520" E. This region has a humid subtropical climate with an average annual temperature of 17.1 °C (62.7 °F) and 1903 mm (74.9 in) of rainfall. The plants were identified by Dr. Harsh Singh (Taxonomist scholar) and a voucher specimen number 257717(LWG) was deposited at CSIR-National Botanical Research Institute (India). The collected plant materials (leaves, stem and roots) were initially carefully washed with saline water, then the samples were placed under running tap water for further washing. After some time, plant materials are rinsed again with autoclave water for half an hour and then put on blotting paper for 48 h to dry. The plant materials were blotted dry before being chopped into small pieces and dried in the shade for 15–20 days. Finally, the dried plant material was ground into a fine powder using an electric grinder (Mill CT 293 Cyclotec TM) and stored in sterilized glass bottles [5, 6].

Fig. 1 [Images not available. See PDF.]

Thalictrum foliolosum plant parts **a** aerial parts (Leaf and stem), **b** Roots of *T. folilosum*

Chemicals

All the standards of BIQ alkaloids (Magnoflorine chloride, berberine chloride, palmatine chloride and thalicarpine) were procured from Sigma-Aldrich (USA). Solvent such as methanol, hexane, ethyl acetate, chloroform and acetonitrile and other chemicals were purchased from Merck (India).

Extraction of plant materials for HPLC analysis

For quantitative analysis, fine powders of different plant materials (1 g) were extracted with 25 ml of acidic (1% HCl) methanol using ultrasonication method (Aczet, Ultrasonic cleaner CUB) at room temperature for 40 min. After ultrasonication, plant materials in extracted solvents were kept at room temperature for overnight. The next day, all sonicated extracts were filtered through a Whatman No 1 filter paper in a round bottle flask, and fresh extracted solvent was added to the same plant materials for additional ultra-sonification. Three times, the same procedure was followed. Later all extracted materials were pooled in round bottom flask and evaporated at 50 °C using a rotary evaporator (Buchi, USA) under low pressure. Finally, the dried extracted plant materials were dissolved in methanol and filtered through a 0.45 µm filter and stored at 4 °C for subsequent HPLC analysis [6, 7]. The acidic-methanol extract of *T. foliolosum* had a yield of 27.42%, 21.10% and 16.24% of the extracted root, leaves and stem respectively. Reference standard solutions (i.e. berberine, palmatine, magnoflorine and thalicarpine) (Sigma-Aldrich) were prepared in methanol. All of the extracted solutions as well as the standard solutions were maintained at 4 °C in the refrigerator.

HPLC method

Plant samples were extracted and subjected to further analysis using High-Performance Liquid Chromatography (HPLC) with a gradient method for identifying bioactive compounds, following the protocol established by Mishra et al. [6, 7]. The analysis was performed using a Shimadzu (Japan) HPLC Prominence system comprising a 20 µL sample loop, a PDA SPD M 20 A photodiode array system, an LC-20AD dual pump system, and a SIL-20 AC Autoinjector with a cooler. Compounds were separated on a Shimadzu RP-C18 column (250 × 4.6, 5 µm pore size) with a guard column of the same packing material. A gradient mobile phase was employed consisting of component A (0.3% formic acid + 0.3% triethylamine) and component B (acetonitrile). The mobile phase gradient program was as follows: 0–25 min, 5–25% B; 25–35 min, 25%-35% B; 35–45 min, 35–45% B; 45–60 min, 45–100% B, at a flow rate of 1 mL/min. Data integration and compound identification were performed using Shimadzu Lab Solution software at a wavelength of 265 nm [6]. Results were compared against standards obtained from Sigma-Aldrich, USA. Quantitative analysis was conducted by averaging the results of three independent analyses of the same sample.

Fatty acid analysis using gas liquid chromatography

500 mg dry weight of plant samples (leaf, stem and roots) were used for FAME preparation. Fatty acid methyl esters (FAMES) were prepared as per method followed by Bureau of Indian Standards IS: 548 (Part-III), 1976 reaffirmed 1994. Prepared FAME dissolved in hexane were analyzed using GC system (7890B GC System, Agilent Technologies) equipped with a flame ionization detector (FID). FAMEs were separated on DB-225 column (30 m × 0.25 mm ID × 0.25 µm film thickness). Nitrogen was used as carrier gas and hydrogen and air as ignition gases. The conditions used for GC analysis were: injector temperature of 230 °C, detector (FID) temperature of 260 °C with split mode injection (1:20). The oven temperature program was started from 90 (2 min hold) to 130 °C at ramping rate of 3 °C min^{-1}. Temperature from 130° to 230° (5 min hold) was achieved with ramp of 2 °C min^{-1}. The peaks were identified by comparing with standards and the results are presented as mean of triplicates. Data were integrated by Open LAB CDS Chem Station Edition software. Reference standards of methyl palmitate, methyl stearate, methyl oleate, methyl linoleate, methyl arachidate and methyl behenate, methyl linoleate were procured by M/s Sigma-Aldrich.

Extracts preparation for antimicrobial assay

For antimicrobial activity various plant parts materials (20 g) were extracted in aqueous methanol (50%) by sonication method. Then extracted solvents were further fractionated by various organic solvents, such as ethyl acetate, chloroform and hexane [5]. All extracted samples were filtered using Whatman filter paper No. 1. The filtrate was then dried using a low-pressure rotary evaporator (Buchi, USA) and lyophilized. All samples were stored at 4 °C in an airtight tube until further analysis was completed.

Microorganism used in study

In vitro antimicrobial activity was determined against four different human pathogenic bacteria i.e. *E. coli* (ATCC 25922), *S. mutans* (ATCC 25175), *S. aureus* (ATCC 4944) *and P. aeruginosa* (MTCC 424). *E. coli* is a gram-negative, rod-shaped bacterium that is commonly found in the lower intestine and most of these strains are harmless. However, pathogenic *E. coli* varieties cause serious food poisoning, septic shock, meningitis, or urinary tract infections in humans [16]. *S. mutans* is a gram-positive coccus bacterium, primarily found in mouth, pharynx, and intestine and significantly involved in tooth decay [17]. *Staphylococcus aureus* is a gram positive, round shape, facultative anaerobe bacterium. *S. aureus* are frequently found in the upper respiratory tract and on the skin and being a common cause of respiratory infections including skin infections [18]. *P. aeruginosa* is encapsulated rod-shaped, gram-negative bacterium. The important characteristic of this bacteria is multidrug resistance. Usually, *P. aeruginosa* found in medical equipment's hence these bacteria associated hospital acquired infections, i.e. ventilator associated pneumonia and other sepsis syndrome the general symptoms of these bacteria are inflammation and sepsis but if their colonization occurs in lungs, kidneys and urinary tract it may be fatal [19].

Screening of antimicrobial activity

In vitro antimicrobial activity was determined against four different human pathogenic bacteria using disk-diffusion method with slight modification [20]. In short, 18–24 h old bacteria culture (0.5 OD_{600} nm) was spreading over the entire surface of nutrient agar plates (90 mm size) using autoclaved steel spreader and sterile paper disks (approx. 6 mm diameter) were placed on them. Then different concentrations of test samples (25, 50 and 100 µg) were loaded on disks. Streptomycin (25 µg) was utilized as a positive control and respective solvents to which extract dissolved was used as a negative control. Then, plates were allowed to incubate at 37 ± 1 °C for 18–24 h. The diameter of the inhibition zone (ZOI) was measured after 24 h of incubation to assess antibacterial activity (measured in mm including disk size). All experiments were carried out in triplicate, and the observed ZOI values are expressed as a mean with standard error of the mean (SEM).

Minimum inhibitory concentration (MIC)

MIC was determined of various extract of leaves, stems and roots parts of *T. foliolosum* against the different pathogenic bacteria by using the previously reported serial dilution method using 96-well microtiter plates. In brief, all pathogenic bacteria were grown in nutrient broth for 6 h before being inoculated, followed by 10^6 cells/mL bacterial culture was inoculated in 200 µL nutrient broth containing tube. Now each extract (leaf, stem, and root) has been added separately in bacterial broth with concentrations ranging from 100 to 800 µg/ml. All tubes were incubated at

37 °C for 24 h and further examined for visible turbidity. The minimum inhibitory concentration (MIC) was determined to be the lowest concentration that inhibited visible growth of the tested bacteria. MIC of berberine, magnoflorine and streptomycin were determined with similar procedure.

Results

Identification and quantification of BIQ alkaloids contents in leaves, stems and roots

Chromatographic analysis aimed to quantify biologically active BIQ alkaloids, namely magnoflorine, berberine, palmatine, and thalicarpine, in extracts obtained from leaves, stems, and roots. Identification of all significant peaks in the chromatogram was accomplished using authentic reference standards of the respective alkaloids. (Fig. 2A) (Supplementary Fig. 1). In terms of quantitative analysis, the root extracts of *T. foliolosum* exhibited the highest total alkaloid content (15.03 mg g-1), followed by the leaves (2.75 mg g-1), while the stem extracts contained a comparatively lower amount (Fig. 2B). Examining individual alkaloids in the root part revealed the following order of content: magnoflorine (10.02 mg g-1)>berberine (3.09 mg g-1)>thalicarpine (1.60 mg g-1)>palmatine (0.31 mg g-1). Similar pattern in alkaloid content were observed in the leaves, with stem-derived extracts displaying lower alkaloid levels compared to roots and leaves.

Fig. 2 [Images not available. See PDF.]

HPLC analysis of antimicrobial responsive alkaloids. **a** Identified BIQ alkaloids i.e. magnoflorine, thalicarpine, palmataine and berberine peaks in HPLC chromatogram of different parts (leaf, stem and root) of *T. foliolosum*. **b** Bar graph represents the comparative observation of identified benzylisoquinoline alkaloids in different plant parts of *Thalictrum foliolosum* plants. The results are expressed as means ± SD of three replicates

Antimicrobial activity of extracted plant materials (leaf root and stem) and standard compounds berberine and magnoflorine

Various extract fractions obtained from different plant parts exhibited a range of antimicrobial activities against the microbial strains employed in the experiment, as detailed in Table 1. The antimicrobial assay indicated that the chloroform fractions of roots and leaves were particularly effective, demonstrating broad-spectrum growth inhibition across all concentration ranges (25–100 µg/ml) against all pathogenic bacteria. The ethyl acetate fractions of leaves and roots displayed remarkable antimicrobial activity at a concentration of 50 µg/ml against all pathogenic bacterial cultures. However, the ethyl acetate fractions from the stem part did not inhibit the growth of gram-negative *P. aeruginosa* (Table 1). Additionally, aqueous methanolic extracts of leaves and roots exhibited impressive growth inhibition against *E. coli*, *P. aeruginosa*, and S. *mutans* at concentrations ≥ 100 µg/ml. Hexane extracts of leaves and stems demonstrated antimicrobial activity against all tested microorganisms except *P. aeruginosa*. However, in our report, hexane root extracts did not exhibit a zone of inhibition against both gram-negative and gram-positive bacteria at concentrations ranging from 25 to 100 µg/ml. Comparatively, when standard compounds berberine chloride and magnoflorine chloride were employed at similar concentrations against the same pathogenic bacterial cultures, berberine emerged as the most effective compound at each concentration. While magnoflorine did not exhibit effective growth inhibition against various pathogens at various concentrations, a slight zone of inhibition was observed against *E. coli* at 100 µg/ml (Fig. 3).

Table 1. Antimicrobial activity of various plant part extracts of *T. folliolosum* against different pathogenic gram-positive and gram-negative microbes

	Plant Parts	Extracts	Inhibition Zone diameter at 50 µg/ml and 100 µg/ml plant extracts in various organic solvents					
Staphylococcus aureus			*Streptococcus mutans*	*Pseudomonas aeruginosa*	*Escherichia coli*	50	100	

50	100		50	100		50	100		(a)	Leaf	Aqueous methanol	–	–	
9.2±0.14[b]	14.4±0.16[f]	++	–	10.1±0.11[c]	+	–	13.5±0.12[e]	++	Ethyl acetate	9.6±0.56[d]	13.7±0.16[b]	++	9.4±0.34[c]	12.3±0.13[e]
++	–	11.5±0.15[d]	++	13.1±0.41[f]	13.8±0.43[ef]	++	Hexane	9.4±0.21[d]	10.3±0.78[d]	++	9.1±0.45[b]	11.9±0.67[cde]	++	–
–	–	12.2±0.18[ed]	14.1±0.46[g]	++	Chloroform	11.8±0.35[c]	13.1±0.43[b]	++	8.3±0.45[a]	15.4±0.56[g]	+++	9.6±0.12	12.3±0.10[e]	++

13.3±0.15^{fg}	14.3±0.43^{h}	++	(b)	Stem	Aqueous methanol	–	–		–	9.2±0.19^{a}	+	–	–	
–	11.4±0.15^{b}	++	Ethyl acetate	9.4±0.14^{d}	12±0.34^{c}	++	9.8±0.14^{e}	11.9±0.79^{cde}	++	–	–	–	0	10.2±0.45^{a}
+	Hexane	13.5±0.31^{a}	14.3±0.89^{a}	++	–	9.8±0.42^{b}	+	–	–	–	12±0.21^{d}	12±0.76^{d}	++	Chloroform
9.2±0.23^{d}	10.1±0.11^{d}	++	–	–	–	–	–	–	0	0		(c)	Root	Aqueous methanol

–	–		–	11.5 ± 0.56^{cd}	++	–	–	–	10.3 ± 0.11^{b}	14.9 ± 0.23^{i}	++	Ethyl acetate	–	–
	9.3 ± 0.21^{bc}	11.2 ± 0.31^{c}	++	–	9.5 ± 0.13^{a}	+	9.6 ± 0.24^{a}	11.8 ± 0.12^{c}	++	Hexane	–	–		–
–	–	–	–	–	–	–	–	Chloroform	12.7 ± 0.18^{b}	13.6 ± 0.35^{b}	++	9.7 ± 0.34^{d}	14.1 ± 0.17^{f}	++

Positive control streptomycin (25 µg/ml) showed inhibition diameter (16.4 ± 0.12) *(S. aureus),* (18.12 ± 0.23) (*S. mutans)*, (15.6 ± 0.18) (*P. aeruginosa)* and (16.2 ± 0.16) *(E. coli)* respectively. Zone of Inhibition (mm) is expressed as mean of triplicates with standard error of means (SEM). Inhibition zones include the circular paper disk diameter (6 mm), (−), negative control (respected solvent)

"–" = <8 mm; "+" = 9 mm, "++" >9 mm <14 mm and "+++" = 15 mm to <18 mm

The data are presented as the mean ± the standard deviation. The values are presented as the mean of three replicates ± the standard deviation. The data marked with different letters share significance at $p < 0.05$ (based on the Duncan test)

Fig. 3 [Images not available. See PDF.]

Growth inhibition assay. Antibacterial activity of magnoflorine (M) and berberine (B) reference compounds against human pathogenic bacteria (*E coli, P. aeruginosa, S. mutans and S. aureus*) using 25–100 µg concentration (B). Streptomycin used as standard antibiotic drug 25 µg

Minimum inhibitory concentrations (MIC's) analysis

In our investigation, we determined the Minimum Inhibitory Concentration (MIC) of potent plant extracts using a 96-well microtiter plate. The MIC values for aqueous methanol, ethyl acetate, and chloroform fractions are detailed in Table 2. Notably, the chloroform fraction of the root exhibited the lowest MIC against all tested strains, with a lesser

value of 121.26 µg/ml observed against *S. aureus* (refer to Table 3). The ethyl acetate fraction from various plant parts demonstrated effective MIC values ranging from 158.15 to 448.78 µg/ml against all tested microbial pathogens. Particularly, the ethyl acetate root fraction displayed the minimum MIC against *S. mutans*. Additionally, the aqueous methanolic extracts showed MIC values ranging from 224.78 to 482.67 µg/ml (refer to Table 3). Furthermore, we analyzed the MIC values of reference alkaloid compounds, as presented in Table 4. The findings suggested that berberine chloride showed less MIC values (123.95 µg/ml) compared to magnoflorine chloride against all pathogenic bacterial strains (refer to Table 4).

Table 2. Minimum inhibitory concentration (MIC) analysis of various plant part extracts against pathogenic bacteria

	Plant parts	Extracts	MIC (µg/ml)			
Staphylococcus aureus	*Streptococcus mutans*	*Pseudomonas aeruginosa*	*Escherichia coli*	(a)	Leaf	Aqueous methanol
474.96 ± 3.25 ^{h}	264.91 ± 2.14 ^{f}	324.56 ± 2.42 ^{e}	298.76 ± 3.12 ^{f}	Ethyl acetate	158.15 ± 1.56 ^{d}	250.54 ± 2.43 ^{e}
245.67 ± 2.65 ^{b}	216.25 ± 2.78 ^{c}	Chloroform	121.26 ± 1.01 ^{a}	235.19 ± 2.13 ^{d}	194.54 ± 1.54 ^{a}	209.84 ± 2.87 ^{b}
(b)	Stem	Aqueous methanol	482.67 ± 3.76 ^{i}	350.73 ± 3.65 ^{h}	450.34 ± 3.56 ^{h}	324.54 ± 3.04 ^{g}
Ethyl acetate	146.28 ± 1.23 ^{c}	225 ± 2.24 ^{c}	448.78 ± 3.46 ^{i}	345.12 ± 2.96 ^{h}	Chloroform	168.67 ± 1.76 ^{e}
402.65 ± 3.42 ^{i}	389.45 ± 3.65 ^{g}	399.98 ± 3.78 ^{i}	(c)	Root	Aqueous methanol	400 ± 2.65 ^{g}
279.98 ± 2.45 ^{g}	364.98 ± 3.32 ^{f}	224.78 ± 2.12 ^{d}	Ethyl acetate	345.56 ± 2.34 ^{f}	189.91 ± 1.76 ^{b}	278.14 ± 2.34 ^{d}

The data are presented as the mean ± the standard deviation. The values are presented as the mean of three replicates ± the standard deviation

The data marked with different letters share significance at $p<0.05$ (based on the Duncan test)

Table 3. MIC analysis of Berberine, Magnoflorine and Streptomycin against studied pathogens

		MIC (µg/ml)			
Staphylococcus aureus	*Streptococcus mutans*	*Pseudomonas aeruginosa*	*Escherichia coli*	(a)	Berberine chloride
113.95 ± 1.45	115.45 ± 1.87	140.78 ± 1.21	150.36 ± 1.45	(b)	Magnoflorine
350 ± 2.41	310.25 ± 3.56	325.67 ± 3.87	254.65 ± 2.76	(c)	Streptomycin

The data are presented as the mean ± the standard deviation. The values are presented as the mean of three replicates ± the standard deviation

Table 4. Identification and quantification of fatty acids in *T. foliolosum* by gas liquid chromatography

		Identified fatty acid in *T. foliolosum*	Quantity of FAME in leaf (%)	Quantity of FAME in stem (%)	Structural characteristics
1	Palmitic acid		5.72 ± 0.83	7.81 ± 1.65	Hexadecenoic acid. It is a fatty acid with a 16-carbon chain. It is the most common saturated fatty acid found in animals, plants and microorganisms
2	Stearic acid		0.99 ± 0.06	2.98 ± 0.26	octadecanoic acid, saturated fatty acid with an 18-carbon chain
3	Olic acid		1.81 ± 0.13	3.42 ± 0.19	Long-chain carboxylic acid; its molecule contains one double bond between C9 and C10 with the cis configuration
4	Behenic acid		1.28 ± 0.26	0.76 ± 0.04	Behenic acid (also docosanoic acid) is a carboxylic acid, saturated fatty acid
5	Arachidic acid		0.98 ± 0.025	1.29 ± 0.11	Naturally occur in *Staphisagria macrosperma*, *Dipteryx lacunifera.* saturated long-chain fatty acid with a 20-carbon backbone
6	Linoleic acid		1.30 ± 0.09	2.72 ± 0.12	An octadecadienoic acid in which the two double bonds are at positions 9 and 12 and have cis stereochemistry
7	Linolenic acid		1.42 ± 0.151	3.27 ± 0.09	carboxylic acid with an 18-carbon chain and three cis double bonds

The results are expressed as mean ± SD of two replicates

Fatty acid analysis

Analysis of fatty acids in various parts of the *T. foliolosum* plant (leaves, stems, and roots) revealed higher levels of FAME in the stems compared to other plant parts. Trace amounts of FAME were recorded in root, in our observation. Table 4 provides detailed information on the identified fatty acids and their respective quantities in different plant parts. Notably, fatty acid esters such as palmitic acid (16:0) (7.81%), stearic acid (2.98%), oleic acid (C18:1) (3.42%), linoleic acid (C18:2) (2.72%), and linolenic acid (C18:3) (3.27%) were found in significant amounts in the stem parts.

Discussion

Plant extracts have been used for centuries as natural medicines due to the diverse array of bioactive compounds they contain. These compounds can have therapeutic effects on the human body. Herbal remedies are often passed down through generations and used to treat various ailments. Extracts of various plant, i.e. *Andrographis paniculata* , *Withania somnifera*, *Panax ginseng*, *Panax quinquefolium, Coptis japonica* and *Carica papaya* have been widely

used in natural medicine to treatment of various chronic disorders [5, 21]. Similarly, a lesser explored medicinal herb *T. foliolosum* widely used as traditional medicine in gastric problem, bloating, toothache, abdominal colic pain and piles (hemorrhoids) [7, 22]. Plants contain a variety of bioactive compounds, such as alkaloids, flavonoids, terpenoids, sterols and polyphenols. These compounds possess medicinal properties and can have antioxidant, anti-inflammatory, antiviral, antibacterial, and antifungal effects. Previous reports and contemporary studies have shown that the therapeutic potential of *T. foliolosum* plants may be due to the presence of bioactive BIQ alkaloids and other non-alkaloidal compounds such as flavonoids, saponins, polyphenolic compounds and steroids, etc. [23, 24].

Quantitative and qualitative analysis of BIQ alkaloids—specifically, berberine, magnoflorine, palmatine, and thalicarpine—was conducted using High-Performance Liquid Chromatography (HPLC) across different plant parts of *T. foliolosum*. The order of alkaloid abundance was consistently observed as magnoflorine>berberine>thalicarpine >palmatine in both the underground and leaf components of *T. foliolosum* plants. Magnoflorine is a quaternary aporphine alkaloid with numerous pharmacological properties, including anti-diabetic, anti-inflammatory, neuropsychopharmacology, immunomodulatory, hypotensive, antioxidant, and antifungal properties [25]. Berberine has long history of medicinal use as folk medicine in Chinese, Indian and Native American. Berberine has potential therapeutic use in metabolic syndrome, type 2 diabetes and dyslipidemia as well as antimicrobial activity [26]. The palmatine alkaloids are also used in traditional Asian medicine for the treatment of jaundice, liver infection, high blood pressure, inflammation and dysentery. Palmatine has recently been shown to be beneficial in the treatment of central nervous system problems [27]. Thalicarpine is a hypotensive alkaloid that has been recognized as being involved in the binding of novel antitumor agents [23, 24, 28].

In addition to biochemical analysis, antimicrobial activity showed that the chloroform fractions of root and leaves have significant activity against different pathogenic micro-organism. It was reported that berberine is the most active compound in the chloroform fractions and has been identified as the most important antibacterial component in various Thalictrum species of all the BIQ compounds identified [7, 14, 22, 24]. Similar reports were also found in other species of Thalictrum (*T. delavayi*, *T. minus*, *T. orientale*, *T. fortune* and *T. avanicum)* further supporting the antimicrobial potential of berberine [29]. Furthermore, our antimicrobial assay using berberine and magnoflorine standard compounds against pathogenic bacteria provided valuable insights into the potential bioactivity of these compounds. The observed superior effectiveness of berberine compared to other reference compounds highlights its promising antimicrobial properties (Table 4). The potential mechanisms of berberine antimicrobial activity may be mediated by the suppressing cell adhesion and migration as well as inhibiting the microbial enzymes [22]. Besides, magnoflorine an aporphine BIQ (usually soluble in water, methanol, and ethanol, and insoluble in low polar organic solvents such as petroleum ether and chloroform) was occurred significant amount in root and leaves but this alkaloid has been showed poor antibacterial activity against various pathogenic bacteria (Fig. 3). However, it was reported that magnoflorine had potent antifungal activity against *Penicillium avellaneum* and *Candida* strain, which may account for the potential antifungal activity in *T. foliolosum* [25].

Additionally, ethyl-acetate extracts of leaves were found to have impressive antimicrobial activity than other parts (roots and stem) of ethyl acetate extract against *E. coli*, *S. aureus*, and *S. mutans.* It has been reported the ethyl-acetate fraction contained significant amount of polyphenolic or flavonoids compounds, which have strong antimicrobial activity and antioxidant activity [5, 30]. Moreover, aqueous methanolic extracts (50%) of leaves and roots also shows the effective antibacterial activity against the *E. coli*, *P. aeruginosa* and *S. mutans*. Inhibition activity in aqueous methanolic extract fraction may be due to soluble fraction of alkaloids, phenol and flavonoids compounds [14, 22]. Furthermore, the non-polar hexane fraction, particularly from stem parts, demonstrated effective antimicrobial activity against both gram-negative and gram-positive bacteria. Fatty Acid Methyl Ester (FAME) analysis revealed significant amounts of fatty acid esters including palmitic acid, stearic acid, and linoleic acid in *T. foliolosum* stem parts, contributing to their potential antibacterial activity [31, 32]. The comprehensive analysis of *Thalictrum foliolosum* extracts highlights the presence of various bioactive compounds with significant antimicrobial potential.

Conclusion

In conclusion, our study provides valuable insights into the medicinal properties of *T. foliolosum*, a lesser-explored herb with a rich traditional history of use in treating various ailments. Through quantitative and qualitative analysis, we identified a diverse array of bioactive compounds in different plant parts, particularly BIQ alkaloids such as magnoflorine, berberine, palmatine, and thalicarpine, alongside substantial presence of both unsaturated and saturated fatty acids. Our findings underscore the potential therapeutic significance of *T. foliolosum* extracts, with particular emphasis on their antimicrobial activity against a range of pathogenic microorganisms. Berberine emerged as a key antimicrobial compound, exhibiting potent activity against various bacteria. Additionally, ethyl acetate and aqueous methanolic extracts displayed significant antibacterial effects, likely attributed to their polyphenolic or flavonoid content. Furthermore, our study highlights the importance of understanding the composition and pharmacological properties of medicinal plants to harness their therapeutic potential effectively. *T. foliolosum* shows promise as a natural remedy for combating microbial infections, and further research into its mechanisms of action and clinical applications is needed.

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Author contributions

Manoj Kumar Mishra; Conception and design the study, performed the experiments, analyzed the data and interpretation of data and wrote the manuscript.

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Availability of data and materials

Yes.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

Abbreviations

BIQ
Benzylisoquinoline
HPLC
High Performance Liquid Chromatography
FAME
Fatty acid methyl esters
GC
Gas chromatography
ZOI
Zone of inhibition
MIC
Minimum inhibitory concentration

Publisher's Note

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Evaluation of antioxidant activity in different Egyptian barley cultivars: an in vitro and in silico study

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ABSTRACT (ENGLISH)

Background

Cereals have historically played a crucial role in the human diet, serving as a significant natural source of energy and offering various health benefits. Barley (*Hordeum vulgare* L.) has been given significant attention in recent years due to its exceptional nutritional value, surpassing that of other cereals. The objective of this research is to evaluate the antioxidant activity of various solvent extracts obtained from three different barley cultivars.

Results

The G.136 variety's acetone extract exhibited the highest level of antioxidant activity in both the DPPH assay, with an IC_{50} of 55.62 µg/ml, and the FRAP assay, with 447 µM trolox/mg extract. The dominant compounds identified before in the acetone fraction were subjected to an evaluation of their docking scores, along with an assessment of ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) and TOPKAT (Toxicity Prediction by Komputer Assisted Technology) studies. Notably, hordatine A1, prodelphinidin B3, hordatine B1, procyanidin B2, and isovitexin 7-*O*-glucoside were the major compounds with the highest LipDock scores compared to trolox the reference drug with polyphenol oxidase.

Conclusions

The findings indicate that the acetone extract from all three cultivars demonstrates noteworthy results, surpassing the efficacy of other solvent extracts against the antioxidant activity.

FULL TEXT

Background

Cereals have long been closely linked to food and drink, acting as a major natural energy source and providing numerous benefits for human health [1]. Barley is one of the oldest cereal crops that are grown. 10,500 years ago, the ancient Egyptians utilized it for the first time near the Nile River [2]. Barley can be classified according to its grain content into several categories, including normal, waxy (characterized by high-amylose starch content), high-glucan, and proanthocyanidin-free varieties [1]. Among the grains, barley has the highest quantities of β-glucan, followed by rye, wheat, and oats in decreasing order [3]. Oxidative stress, which is associated with the development of several diseases such as cancer, anemia, ischemia, diabetes, and cardiovascular diseases, is known to have a significant impact on the body's cellular processes [4]. Studies have demonstrated that barley grains contain phytochemical substances that have significant antioxidant qualities when evaluated in vitro [5]. The type of solvent employed during the extraction process has been discovered to affect the nature and quantity of secondary metabolites recovered from medicinal plants [6]. The structural dissimilarities of phenolic compounds influence their solubility in liquids of variable polarity. As a result, the solvent used for extraction and separation processes can have a substantial impact on the yield of phytochemicals derived from plant sources [7]. The primary objective of this study was to examine the principal phytochemical constituents within the most potent barley plant extract. The extraction process encompassed the use of various solvents, including 70% ethanol, methanol, water, 80% methanol, and acetone. Additionally, the research evaluated the antioxidant capabilities of the barley extracts, and in silico investigations were conducted to assess the potential binding modes of various phytochemicals as ligands with polyphenol oxidases (PPOs) receptor proteins.

Methods

Plant materials collection and extraction

In August 2019, whole barley grains (*Hordeum vulgare* L.) from three distinct cultivars were gathered from the Agriculture Research Center in Egypt, sourced from different geographic locations. The three commonly cultivated *H. vulgare* varieties in Egypt, namely Giza 136 (G.136), Giza 127 (G.127), and Giza 131 (G.131), were utilized. Fifty grams of powdered plant material from each of the three cultivars underwent extraction using five distinct solvents: 100% methanol, 80% methanol in water, 70% ethanol in water, 80% acetone in water, and distilled water. The yield from cultivar G.136 was 5.9% with methanol, 6.12% with ethanol in water, 11.43% with 70% ethanol in water, 8.23% with 80% acetone in water, and 3.8% with distilled water. Meanwhile, for cultivar G.127, the yields were 4.42% with methanol, 7.23% with 80% methanol in water, 8.39%, 8.38% with 70% ethanol in water, 8.45% with 80% acetone in water, and 12.44% with distilled water. As for cultivar G.131, the yields were 3.19% with methanol, 8.52% with 80% methanol in water, 7.42% with 70% ethanol in water, 6.5% with 80% acetone in water, and 5.5% with distilled water. This process resulted in a total of 15 samples (three cultivars in five different solvents). To extract the compounds, all of the samples underwent sonication for 30 min, three times using 500 mL each time [8].

Ultra high-performance liquid chromatography-mass spectrometry analysis (UHLPC-MS)

The combined extracts were concentrated under reduced pressure. Ten milligrams of each extract was accurately

weighed and then subjected to UHPLC-MS/MS analysis and measured their antioxidant activity.

Antioxidant activity measurements

DPPH radical scavenging activity

The three varieties of barley with different solvents were evaluated for their antioxidant capacity using the DPPH as mentioned by Boly et al. [9]. Briefly, in a 96-well plate ($n=6$), 100 µL of freshly made DPPH reagent (0.1% in methanol) was together with 100 µL of the sample. The reaction was allowed to proceed for 30 min in the dark at room temperature. The subsequent decrease in DPPH color intensity was measured at 540 nm after the incubation period. FluoStar Omega, a microplate reader, was used to record the results. The following equation describes how data are expressed as means ± standard deviation, compared to torolox the standard drug.Percentage\,inhibition= Average\,absorbance\,of\,blank-Average\,absorbance\,of\,the\,testAverage\,absorbance\,of\,blank×100Microsoft Excel® was used to analyze the data, and Graph Pad Prism 5® was used to get the IC_{50} value by converting the concentrations to their logarithmic value and choosing a nonlinear inhibitor regression equation. (log (inhibitor) vs. normalized response−variable slope equation) [10].

Ferric reducing antioxidant power assay (FRAP)

With a few minor adjustments to be performed in microplates, the Benzi et al. [11] method for the ferric reducing ability assay was used. In summary, a freshly made TPTZ reagent (300 mM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM$FeCl_3$, respectively) was used. In a 96-well plate ($n=3$), 190 uL of freshly made TPTZ reagent was combined with 10 uL of the sample. The reaction was then allowed to sit at room temperature for 30 min while kept in the dark. The final measurement of the blue color after incubation was made at 593 nm. Data are displayed as means ± SD. FluoStar Omega, a microplate reader, was used to record the results. The ferric reducing ability of the samples is presented as µM TE/mg sample using the linear regression equation extracted from the calibration curve, compared to torolox the standard drug.

Molecular docking studies

A molecular docking study was conducted using the Discovery Studio 4.1 program and the LIPDOCKER methodology. The isolated component was docked against the active site of antioxidant, PDB (ID: 2Y9X). Heavy atoms were created, superfluous chains were eliminated, hydrogens were added, and the protein was purified. The CHARMm forcefield and MMFF94 as a partial charge were used in the simulation. Fixed constraints and protein minimization were applied. The receptor binding site was located using the complicated ligand interaction site.

ADMET/TOPKAT prediction

The in silico ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) investigations were conducted using the Discovery Studio 4.1 program. These analyses aimed to predict the pharmacokinetic properties of the drug under examination, offering insights into its potential behavior within the body. The outcomes also yielded crucial structural information guiding the assessment of potential antioxidant activity. Graphical representations and numerical data were generated and presented. Additionally, the toxicity protocol TOPKAT was applied to the same set of compounds, evaluating various criteria including Ames Prediction, Carcinogenicity, and Rat Oral LD50 g/kg body weight.

Results

DPPH radical scavenging activity

In this experiment, the presence of hydrogen or electrons supplied by the antioxidant constituents in the samples resulted in the initial purple color of the DPPH radical changing to yellow. Figure 1 demonstrates that the antioxidant capacity of various sample extracts varies according to the polarity of the utilized solvents. As IC_{50} values decrease, the degree of antioxidative activity increases [12].

Fig. 1 [Images not available. See PDF.]

DPPH activity of the three barley varieties in different solvents. Significant differences among means of different treatments were determined using Bonferroni posttests at $P<0.001$ ($n=3$) with all solvent extracts compared to each other. a, b, c, d significant difference compared to methanol, water, acetone, and 80% methanol of G.136, a′, b′, c′,

d′ significant difference compared to methanol, water, acetone, and 80% methanol of G.127, a″, b″, c″, d″ significant difference compared to methanol, water, acetone, and 80% methanol of G.131, *corresponding to $P<0.05$, **corresponding to $P<0.01$, and the significance difference with $P<0.001$

All tested varieties exhibited a significant difference with a P value less than 0.0001. However, variety G.131 methanol extract besides varieties G.127 and G.136 acetone extracts did not exhibit a significant difference with $P>0.05$ in comparison with the standard drug, trolox, in terms of their antioxidant activity. This implies that they possess antioxidant properties. Besides, among all the varieties, variety G.136 acetone extract had the highest antioxidant activity (IC_{50}: 55.62 µg/ml), whereas variety G.127 acetone extract had the second-highest antioxidant activity (IC_{50}: 58.77 µg/ml). However, the methanolic extract of variety G.136 did not show any measurable DPPH activity.

Ferric reducing antioxidant power assay (FRAP)

A significant difference was found between the tested cultivars at a significance level of $P<0.0001$, as illustrated by the antioxidant capacity of various sample extracts in Fig. 2. After comparing the acetone extracts of different varieties, the G.136 variety showed the highest antioxidant activity (447 µM trolox/mg extract), while the G.127 variety showed the subsequent highest antioxidant activity (426 µM trolox/mg extract). As expected from the results of DPPH, the ethanolic extracts of cultivars G.136 and G.127 showed the lowest levels of antioxidant activity (132.1 and 91.2 µM trolox/mg extract, respectively).

Fig. 2 [Images not available. See PDF.]

FRAP activity of the three barley varieties in different solvents. Significant differences among means of different treatments were determined using Bonferroni posttests at $P<0.001$ ($n=3$) with all solvent extracts compared to each other. a, b, c, d, e significant difference compared to methanol, ethanol, water, acetone, and 80% methanol of G.136, a′, b′, c′, d′, e′ significant difference compared to methanol, ethanol, water, acetone, and 80% methanol of G.127, a″, b″, c″, d″, e″ significant difference compared to methanol, ethanol, water, acetone and 80% methanol of G.131, *corresponding to $P<0.05$, **corresponding to $P<0.01$, and the significance difference with $P<0.001$

Ultra high-performance liquid chromatography-mass spectrometry analysis (UHLPC-MS)

Previously, sixty-four compounds using various solvents were discovered from all extracts (under publication). Because the acetone fraction has the highest activity in the previously mentioned antioxidant activity, we shed light on its prominent components. In the acetone fraction of the three cultivars, the major identified 18 compounds are shown in Table 1 and Fig. 3. Proanthocyanidin was the most prevalent chemical class among the identified phytochemicals in the three cultivars, followed by flavonoids and hordatines in the acetone fraction. Proanthocyanidin and flavonoid abundance were highest in G.131 of all the cultivars. However, cultivars, G.127, displayed the greatest quantity of hortatines (Fig. 4).

Table 1. Metabolites using ultra high-performance liquid chromatography (UHPLC)-MS/MS of the acetone fraction in the three cultivars and their height

	RT	Compounds	Formula	Chemical class	M–H	Area of the compounds	Ref

G.131	G.127	G.136	1	0.238	Caffeic acid	$C_9H_8O_4$	Organic acids, phenolic compounds, and their derivatives	179.0334	0
0	4417	[13–15]	2	0.271	Guanine	$C_5H_5N_5O$	Amino acids	150.04236	12,033
23,042	17,730	[16]	3	0.486	Gluconic acid	$C_6H_{12}O_7$	Organic acids, phenolic compounds, and their derivatives	195.0506	195,682
133,080	170,648	[17]	4	0.865	Coumaroyle–OH–eagmatine	$C_{14}H_{20}N_4O_3$	Hordatines and hydroxycinnamic acid agmatines	291.1446	0

53,560	0	[18, 19]	5	1.406	Procyanadin B2	$C_{30}H_{26}O_{12}$	Proanthocyanidin, flavonoids, and their conjugates	577.1349	82,440
521,543	3,103,547	[20]	6	2.417	*p*-coumaric acid	$C_9H_8O_3$	Organic acids, phenolic compounds, and their derivatives	163.0391	0
7449	0	[13, 15, 21]	7	2.638	Adenin	$C_5H_5N_5$	Amino acids	134.04855	16,016
9048	0	[22]	8	3.327	Hordatine A1	$C_{28}H_{38}N_8O_5$	Hordatines and hydroxycinnamic acid agmatines	565.28967	17,631

0	0	[18]	9	3.736	Hordatine A glucoside	$C_{34}H_{48}O_9N_8$	Hordatines and hydroxycinnamic acid agmatines	711.34607	0
0	28,424	[18]	10	4.103	Tricin	$C_{17}H_{14}O_7$	Proanthocyanidin, flavonoids, and their conjugates	329.0659	510,883
556,177	7,078,250	[13, 15, 21]	11	4.124	Isovitexin 7-*O*-rhamnosyl glucoside	$C_{33}H_{40}O_{19}$	Proanthocyanidin, Flavonoids and their conjugates	739.2091	47,112
41,444	0	[19]	12	4.768	Prodelphinidin B3	$C_{30}H_{26}O_{13}$	Proanthocyanidin, flavonoids, and their conjugates	593.13025	125,598

1,879,499	74,140	[21]	13	4.971	Hordatine B1	$C_{29}H_{40}N_8O_6$	Hordatines and hydroxycinnamic acid agmatines	595.2935	1,199,896
1,811,832	125,598	[18, 19]	14	5.731	Iso-orientin	$C_{21}H_{20}O_{11}$	Proanthocyanidin, flavonoids, and their conjugates	447.092375	6,005,607
5,952,463	386,493	[18, 23, 24] [19]	15	5.741	Quercetin 3-*O*-glucoside	$C_{21}H_{20}O_{11}$	Proanthocyanidin, flavonoids, and their conjugates	447.092375	40,335,493
13,767,985	4,707,879	[25]	16	5.756	Naringenin	$C_{15}H_{12}O_5$	Proanthocyanidin, flavonoids, and their conjugates	271.0604	268,109
0	0	[26, 27]	17	5.956	Canrenone	$C_{22}H_{28}O_3$	Steroids	339.20102	224,607

0	0	[28, 29]	18	6.937	Ferulic acid glucoside	$C_{16}H_{20}O_9$	Organic acids, phenolic compounds, and their derivatives	355.1031	107,234

Fig. 3 [Images not available. See PDF.]

Acetone fraction chromatogram of the three barley cultivars

Fig. 4 [Images not available. See PDF.]

Chemical classes among the three varieties

Based on a comparison of the detected component amounts in the three cultivars, quercetin 3-*O*-glucoside had the highest abundance among the three cultivars, with the G.131 cultivar exhibiting the highest concentration of the other varieties in addition to iso-orientin and hordatine B1; iso-orientin was also prominent in G.127 cultivar as well as prodelphinidin B3. Moreover, the main compounds in variety G.136 were tricin and procyanadin B2 as shown in Fig. 5.

Fig. 5 [Images not available. See PDF.]

Dominant compounds in the three barley cultivars (G.131, G.127 and G.136)

Molecular docking studies

Molecular docking

The polyphenol oxidase enzyme PPO, which was obtained from the Protein Data Bank (PDB ID: 2Y9X), was docked to the identified phytochemicals and trolox, as reference antioxidant standard, to determine their potential binding mechanisms and virtual binding affinities. Docking of the 18 major detected compounds using the LIPDOCKER protocol after ligand preparation showed a LipDock score ranging from (−61.3546 to−143.402) (Table 2). Trolox showed hydrogen bond interaction with the essential amino acids (His85, Glu322, and Asn81) and hydrophobic interaction with (His 244).

Table 2. LipDock score of the dominant compounds beside their amino acid interactions along with the reference trolox and the ligand drug

	Compounds	LipDock score	Key amino acid interaction	
Hydrogen bond	Pi-bond	1	Caffeic acid	86.5240
	Thr 84	2	Guanine	74.4313

	Thr 84	3	Gluconic acid	93.0249
Trp136, Ile217, Ala221, Ile148, Trp138, Gly149	–	4	Coumaroyle–OH–eagmatine	117.4070
Ser282, Met280, Asn260, His259, Asn81	–	5	Procyanadin B2	132.7140
Glu322, Asn81, Ala246, Asn320, Tyr65	Val283, Ala246	6	*p*-coumaric acid	75.5750
–	Val283	7	Adenin	70.6453
His85		8	Hordatine A1	143.4020
–, Arg321, His85, Thr84, Asn81, Asn320, Thr324, His244	Val283	9	Hordatine A glucoside	110.0050
His259, Asn260, Thr84, Thr324, Glu322	Ala246, Val247, Cys83	10	Tricin	103.7580
His85, Cys83, Ala323 Val283, His85, Ala80	–	11	Isovitexin 7-*O*-rhamnosylglucoside	124.8250

Tyr65, Tyr78	Ala323, Ala80, Pro284, Val283	12	Prodelphinidin B3	141.8140
Cys83, Asn81, Glu322 His244	Val283, Ala246,	13	Hordatine B1	138.9150
Asn81, His85, Arg321, Glu322, Ala323, Thr84	Val283	14	Iso-orientin	105.0620
Cys83, Asn81, Thr324, Ala323, Cys83, His85	Val283, His244,	15	Quercetin 3-*O*-glucoside	122.8930
Ala323, Tyr65, Tyr78	Ala80, Pro284, Val283, Ala323	16	Naringenin	108.7270
Arg321, Gly86,	His251, Ala246, Ala250, Val247, Arg321	17	Canrenone	98.0593
–	His85, His244, Cys83, Val283	18	Ferulic acid glucoside	115.5120
His85, Glu322	Val283, Ala323, Glu322	19	Trolox	80.2885
His85, Asn81, Glu322	His 244	20	2Y9X	61.4536

Hordatine A1, prodelphinidin B3, hordatine B1, and procyanadin B2 showed the highest LipDock interaction energy score relative to trolox (Fig. 6), in addition to their highest abundance in the LCMS/MS results. Moreover, hordatine A1 and prodelphinidin B3 shared the same binding interaction with the essential amino acids as trolox (His85, Glu322, Asn81, and Val283) that showed a better stability along with the LipDock score.

Fig. 6 [Images not available. See PDF.]

2D binding mode of **A**: hordatine A1, **B**: prodelphinidin B3, **C**: hordatine B1, **D**: procyanadin B2, **E**: isovitexin 7-*O*-glucoside along with reference compound, **F**: trolox and the ligand drug, **G**: 2Y9X

In vitro predictive Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) study

The ADMET investigation, carried out using Discovery Studio 4.1 Software, focused on the molecular composition of the compound and included the computation of various parameters [30]. These parameters included: ADMET solubility level, Blood Brain Barrier Level (BBB LEV), and CYP2D6. Most of the compounds in the ADMET plot exhibited BBB levels ranging between 3 and 4. In the HIA plot, a significant portion of the compounds were located outside the 99% ellipse. Furthermore, many of these compounds had an ADME aqueous solubility rating falling between 3 and 4. The CYP2D6 score serves as an indicator of whether a specific chemical structure is inhibitory or non-inhibitory to the cytochrome P450 2D6 enzyme.

The key property, PSA (polar surface area), is a factor associated with drug bioavailability. Generally, molecules with a PSA greater than 240 are assumed to have limited bioavailability when passively absorbed (Fig. 7; Table 3).

Fig. 7 [Images not available. See PDF.]

ADMET Plot of the 2D polar surface area (PSA_2D) against calculated ALogP98 for examined compounds

Table 3. Computer-aided ADMET screening and TOPKAT Ames Toxicity study results of the identified compounds

	Compounds	ADMET solubility level	BBB level	CYP 2D6[f]	Hepatotoxic prediction	TOPKAT WOE prediction	TOPKAT Ames prediction	TOPKAT Rat Oral LD50 g/kg body weight
1	Caffeic acid	4	3	FALSE	FALSE	Non-carcinogen	Non-mutagen	1.63246
2	Guanine	4	3	FALSE	TRUE	Non-carcinogen	Non-mutagen	3.78993
3	Gluconic acid	5	4	FALSE	FALSE	Non-carcinogen	Non-mutagen	2.72903
4	Coumaroyle-OH-eagmatine	4	4	FALSE	TRUE	Non-carcinogen	Non-mutagen	0.864996
5	Procyanadin B2	1	4	TRUE	TRUE	Non-carcinogen	Non-mutagen	3.02939
6	*p*-coumaric acid	4	3	FALSE	FALSE	Carcinogen	Non-mutagen	1.35061
7	Adenin	4	3	FALSE	TRUE	Carcinogen	Mutagen	0.521014
8	Hordatine A1	3	4	FALSE	FALSE	Non-carcinogen	Non-mutagen	5.01923
9	Hordatine A glucoside	1	4	FALSE	FALSE	Non-carcinogen	Non-mutagen	19.2151

10	Tricin	3	3	FALSE	TRUE	Non-carcinogen	Non-mutagen	0.534205
11	Isovitexin 7-*O*-rhamnosylglucoside	0	4	FALSE	TRUE	Non-carcinogen	Non-mutagen	3.85073
12	Prodelphinidin B3	0	4	TRUE	TRUE	Non-carcinogen	Non-mutagen	2.53554
13	Hordatine B1	3	4	FALSE	FALSE	Non-carcinogen	Non-mutagen	7.97171
14	Iso-orientin	3	4	FALSE	TRUE	Non-carcinogen	Non-mutagen	1.32758
15	Quercetin 3-*O*-glucoside	3	4	FALSE	FALSE	Non-carcinogen	Non-mutagen	0.335631
16	Naringenin	3	3	TRUE	TRUE	Non-carcinogen	Non-mutagen	1.57835
17	Canrenone	2	1	FALSE	FALSE	Carcinogen	Non-mutagen	2.70998
18	Ferulic acid glucoside	4	4	FALSE	FALSE	Non-carcinogen	Non-mutagen	4.73868

TOPKAT toxicity studies

The compounds that were previously prepared underwent (TOPKAT) toxicity protocol [31], which involved evaluating them based on specific criteria, including Ames Prediction, Hepatotoxic Prediction, Rat Oral LD_{50}, and Carcinogenic Potency. This methodology was designed to gauge the potential toxicity of newly developed substances (Table 3).

Discussion

Natural antioxidants are significantly more beneficial and efficient in combating oxidative stress when compared to their synthetic counterparts. Medications derived from plant products are considered safer for consumption [32].

The DPPH radical dot assay is commonly employed to evaluate the free radical scavenging capabilities of an antioxidant molecule. It is recognized as a standard and straightforward colorimetric method for assessing antioxidant properties [33].

It is noteworthy that the acetone extract from variety G.136 exhibited the most substantial antioxidant activity. Following closely, the acetone extract derived from variety G.127 demonstrated the second-highest antioxidant potency. Conversely, it is essential to highlight that the methanolic extract obtained from variety G.131 did not manifest any detectable DPPH activity.

The FRAP assay stands out as a straightforward, rapid, and cost-effective direct technique for gauging the total antioxidant activity of reductive antioxidants present in a test sample [34].

In line with the findings from the DPPH assay, it was observed that the G.136 variety exhibited the highest antioxidant activity, followed by the G.127 variety with a slightly lower but still significant antioxidant activity. Interestingly, in contrast with the acetone extracts discussed earlier, the ethanolic extracts of cultivars G.136 and

G.127 displayed the lowest levels of antioxidant activity.

Numerous studies have suggested that pure water is not an efficient solvent for extracting polyphenols due to their higher solubility in solvents that are less polar than water [35]. Comparable findings were published by Zhu et al. [36] concerning the significant antioxidant activity of the Chinese-grown barley acetone extract.

Among the identified phytochemical classes in the three cultivars, proanthocyanidins were the most prevalent, followed by flavonoids and hordatines in the acetone fraction. Specifically, proanthocyanidins and flavonoids were most abundant in G.131, while G.127 exhibited the highest quantity of hordatines. Quercetin 3-*O*-glucoside was the most abundant component across all cultivars, with G.131 having the highest concentration of this compound compared to the other varieties.

A docking study was applied on the most prominent compounds in the acetone fractions, hordatine A1, prodelphinidin B3, hordatine B1, and procyanidin B2 exhibited the highest LipDock interaction energy scores compared to trolox.

Furthermore, these compounds also demonstrated the highest abundance in the LCMS/MS results. Likewise, these compounds previously revealed antioxidant activity [37, 38].

After making ADMET and TOPKAT studies of the dominant compounds in the acetone fractions, the compounds' Absorption, Distribution, Metabolism, and Excretion Toxicity could be determined.

This suggests that they are unlikely to permeate the blood–brain barrier and, consequently, are unlikely to cause adverse effects in the central nervous system (CNS) adverse effects. They are likely to have limited absorption in the intestines suggesting that they possess good aqueous solubility; the expected values for these compounds indicate good passive oral absorption for most of them, which is a positive attribute for their pharmacological effectiveness.

In this context, since these compounds are classified as non-inhibitors of CYP2D6, it suggests that their usage is not likely to lead to adverse effects such as liver impairment.

The results of all TOPKAT Ames probabilities, applications, and scores indicated that these compounds are non-mutagenic and non-carcinogenic, and they fell within the anticipated ranges.

Conclusion

The present study offers valuable insights into the antioxidant applications of different solvents extracts of three different cultivars. The acetone extracts of the three cultivars showed the best results compared to other solvents. G.136 variety showed the highest level of antioxidant activity in both the DPPH and FRAP assays. A docking study was conducted for the 18 major compounds in the acetone fractions of the three varieties followed by ADMET and TOPKAT studies. Hordatine A1, prodelphinidin B3, hordatine B1, procyanidin B2, and isovitexin 7-*O*-glucoside demonstrated the highest LipDock scores when compared to the reference standard drug. Additionally, these compounds exhibited the highest areas among other constituents in the three cultivars. The results of the current study could offer valuable insights for defining new research avenues regarding the utilization and applications of the examined extracts with specific solvents as pharmaceutical and nutraceutical agents.

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Author contributions

Conceptualization was performed by O.E.; S.E.; and W.E.; data curation by O.E.; investigation by O.E.; methodology by O.E.; supervision by S.E.; W.E.; A.E.; and E.A.; visualization by O.E.; S.E.; and W.E.; writing—original draft by O.E. and W.E.; writing—review &editing by O.E.; S.E.; W.E.; and E.A. All authors read and approved the final manuscript.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Ethics approval and consent to participate

Ethical approval was obtained from the research ethics committee, Faculty of Pharmacy, Cairo University, serial number MP (2413).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Plant authentication

The Whole barley grains (*Hordeum vulgare* L.) from three distinct cultivars were gathered from the Agriculture Research Center in Egypt and sourced from different geographic locations in Egypt. The three commonly cultivated *H. vulgare* varieties in Egypt, namely Giza 136 (G.136), Giza 127 (G.127), and Giza 131 (G.131) were utilized.

Abbreviations

DPPH
2,2-Diphenyl-1-picrylhydrazyl
FRAP
Ferric reducing antioxidant power assay
ADMET
Absorption, Distribution, Metabolism, Excretion, and Toxicity
TOPKAT
Toxicity Prediction by Komputer Assisted Technology
PPOs
Polyphenol oxidases
PDB
Protein Data Bank
(UHPLC)-MS/MS
Ultra high-performance liquid chromatography
PSA
Polar surface area

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DETAILS

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Antibacterial activity of medicinal plants and their role in wound healing

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ABSTRACT (ENGLISH)

Background

The study of plant-based medications, or phytomedicine, involves a wide spectrum of biological activities. Due to the existence of secondary metabolites, herbal medicine has been used and practiced throughout history for the

treatment of both acute and chronic conditions. Over the past century or so, numerous novel compounds with medicinal potential have been derived from plants. In the age of growing super infections and the emergence of resistant strains, natural medicines are inspiring optimism.

Main body of the abstract

The review discusses the role of herbal medicine as antibacterial agents and their use in wound care and management of wounds and the critical role of secondary metabolites of herbal plants in fighting bacterial infections. Some medicinal plants such as St. John's wort (SJW) (*Hypericum perforatum*), Rosemary (*Rosmarinus officinalis*), Ginger (*Zingiber officinale*), and nopal cactus (*Opuntia ficusindica* (*L.*)) also possess wide range of biological activities and can give a synergistic effect if combined with antibiotics. In addition, natural biopolymers play an important role in the management of wounds as well as the physiological processes of the skin (hemostasis, inflammation, proliferation, and remodelling).

Method

A narrative review of papers relevant to the use of phytomedicine in treating infections was conducted by using electronic databases PubMed, CrossREF, and Google Scholar.

Short conclusion

Phytomedicine is one of the top options for the treatment of chronic illnesses for millions of people around the world. To learn about the bioactive components of medicinal plants, their medical benefits, and their synergistic or additive effects to enhance the action of medications, substantial new studies are still needed.

Highlights

Phytomedicine involves a wide spectrum of biological activities.
Bioactive compounds extracted from plants are used for the treatment of both acute and chronic conditions.
Natural plant &secondary metabolites play a significant role in the treatment of bacterial infections.
Natural biopolymers are used in wound care and restoring physiological processes of the skin.
Substantial new studies are needed to learn about bioactive components medical benefits, and their synergistic or additive effects.

FULL TEXT

Background

An herbal remedy known as phytomedicine is utilized all over the world to treat or prevent physical and mental illnesses [1]. Herbal medicine or a phytopharmaceutical preparation is a type of medication that is made in a crude form solely from whole plants or specific plant parts [2]. Herbal medicine, which has a history spanning more than 3000 years and was enumerated in Sheng Nong's herbal book "*The Devine Farmer's Classic of Herbalism*" [3], is the foundation of traditional Chinese medicine (TCM). Herbal medicine is one of the most sought-after treatments by 3.5–4 billion people worldwide, mainly in Africa, India, and China, according to the World Health Organization (WHO) [4]. Products made from medicinal plants have greatly increased over the past 10 years, which has revolutionized and improved phytomedicine. There are about 35,000 plant species that are utilized as medicines, but only 20% of them go through the phytochemical analysis stage and only 10% make it to the biological screening stage, leaving the rest in need of further study [5]. As herbal medicine becomes more and more popular, it is important to maintain quality, safety, and to prevent potential toxicity [6–8]. In addition to long-term boiled extract and cold infusion of plants, plants can be extracted using alcoholic, vinegar, and hot water as well as other solvents, times, and temperatures [1]. Due to the existence of secondary metabolites, which are abundant in bioactive substances, herbs are used to treat both acute and chronic illnesses, including depression, cardiovascular disease, inflammation, and others [9, 10]. Plant constitutes are used directly as therapeutic agents or as models for pharmacologically active compounds or as starting materials for the synthesis of drugs (Table 1) such as morphine which was produced from opium extracted from *Papaver somniferum,* digoxin from *Digitalis purpurea*, antimalarials such as quinine from *Cinchona* bark, and over 60% of cancer therapeutics are based on natural products such as paclitaxel from the Pacific yew tree [11–15]. Due to inadequate research methodologies, time-consuming, and expensive isolation techniques, there is little information available regarding the composition of the majority of herbal medications. As a

result, this page discusses the crucial role of medicinal plant extract in the battle against bacterial infections and the management of skin wounds and disorders such as atopic dermatitis (AD) and diabetic foot ulcers (DFUs). Additionally, extracellular matrix (ECM) and biopolymers derived from microorganisms, animals, and plants (cellulose, hyaluronic acid, collagen, alginate, and chitosan) all have bioactive qualities that make them useful in the treatment of wounds and the healing process.

Table 1. List of drugs derived from plant origin and their clinical use

Drug	Plant origin	Clinical uses	References
Aescin (Fig. 1)	*Aesculus hippocastanum*	Aescin has potent anti-inflammatory, antioxidant, antiedematous, and vaso-protective effects. It is used in the management of haemorrhoids and hematoma	[16]
Aesculetin (Fig. 1)	*Fraxinus rhynchophylla*	Aesculetin is a phenolic coumarin derivative compound that has anti-inflammatory, antinociceptive, antioxidative, and anticancer effects, in addition to its effectiveness against allergic asthma	[17]
Agrimophol (Fig. 1)	*Agrimonia Pilosa*	Agrimophol is a phloroglucinol compound identified by high-throughput screening (HTS) method. It acts by disturbing pH_{IB} homeostasis of *Mycobacterium tuberculosis*	[18, 19]
Allyl isothiocyanate (Fig. 1)	*Brassica nigra*	A black mustard volatile oil responsible for the bitter taste and pungent odour. It has the potential to be used as antibacterial, anticancer, antifungal, and antihelminthic, in addition to its antifermentative and antibrowning in food industry	[20, 21]
Anisodamine (Fig. 1)	*Anisodus tanguticus*	An atropine derivative with nonspecific cholinergic antagonist activity. It has a cardiovascular properties that include depression of cardiac conduction and protection against arrhythmia	[22, 23]
Artemisinin (Fig. 1)	*Artemisia annua* L	A sesquiterpene lactone with potent antimalarial activity in addition to its ability to treat some viral infections and various neoplasms	[24]

Aspirin (Fig. 1)	Willow tree bark	Anti-inflammatory and antiplatelet agent	[25, 26]
Atropine (Fig. 1)	*Atropa belladonna*	An Anticholinergic and Cholinergic Muscarinic Antagonist	[27]
Berberine (Fig. 1)	*Berberis vulgaris*	A nonbasic and quaternary benzylisoquinoline alkaloid used for the treatment of skin diseases, inflammatory disorders, respiratory diseases affections of eyes, tumours, microbial pathologies and for wound healing	[28]
Bromelain (Fig. 1)	*Ananas comosus*	Anti-inflammatory, antiedematous, antithrombotic, fibrinolytic, anticancerous, and facilitate the death of apoptotic cells	[29]
Camphor (Fig. 1)	*Cinnamomum camphora*	Camphor used for the treatment of various symptoms such as infection, inflammation, muscle pain, congestion, and irritation in various regions	[30]
Camptothecin (Fig. 1)	*Camptotheca acuminata*	A natural alkaloid acts as a DNA topoisomerase 1 poison with antitumour activity	[31]
Catechin (Fig. 1)	*Camellia sinensis*	Catechins are polyphenol compounds from tea leaves and have a strong antioxidants activity. They can prevent or reduce skin damage	[32]
Cocaine (Fig. 1)	*Erythroxylum coca*	Topical anaesthesia of the mucous membranes of the nasal, oral, and laryngeal cavities in addition to off-label use as vasoconstrictive in the treatment of epistaxis before cauterization or packing	[33, 34]
Codeine (Fig. 1)	*Papaver somniferum*	Codeine is an alkaloid from opium or morphine used as a sedative, hypnotic, central analgesic, antinociceptive and is also used in insomnia and tuberculosis due to incessant coughing	[35]
Colchicine (Fig. 1)	*Colchicum autumnale*	Treatment of gout	[36]

Convallatoxin (Fig. 1)	*Convallaria majalis*	A cardiac glycosides used to treat atrial fibrillation and cardiac failure through inhibition of Na^+/K^+-ATPase	[37, 38]
Curcumin (Fig. 1)	*Curcuma longa*	Curcumin prevents carcinogenesis by affecting angiogenesis, cancer cell growth, in addition to the suppression of cancer cell metastasis and induction of cancer cell apoptosis	[39]
Deslanoside (Fig. 1)	*Digitalis lanata*	A cardiac glycoside used to treat supraventricular arrhythmias congestive heart failure and chronic atrial fibrillation. Moreover, studies showed its ability to inhibit the tumour growth of human prostate cancer cells	[40, 41]
Digoxin (Fig. 1)	*Digitalis purpurea*	A cardiac glycoside that has inotropic effects and is used to manage systolic dysfunction in congestive heart failure (CHF) patients and also work as atrioventricular nodal blocking agent to manage atrial tachydysrhythmias	[42]
Emetine (Fig. 1)	*Cephaelis ipecacuanha*	An alkaloid used to treat amoebiasis	[43]
Ephedrine (Fig. 1)	*Ephedra sinica*	A sympathomimetic drug prescribed as a nasal decongestant. Furthermore, it used as antipyretic and diaphoretic effects	[44, 45]
Etoposide (Fig. 1)	*Podophyllum peltatum*	Podophyllotoxin used as chemotherapeutic drug against various cancers due to its anticancer activity	[46, 47]
Galantamine (Fig. 1)	Amaryllidaceae family (*Galanthus nivalis* and *Galanthus woronowii*)	An oral acetylcholinesterase inhibitor used for therapy of Alzheimer disease	[48, 49]
Glaucarubin (Fig. 2)	*Simarouba glauca*	An antimalarial and anticancer drug	[50]
Glaucine (Fig. 2)	*Glaucium flavum*	Isoquinoline alkaloid used as a cough suppressant	[51]

Glycyrrhizin (Fig. 2)	*Glycyrrhiza glabra*	It is used as a remedy for gastrointestinal problems, cough, bronchitis, arthritis and widely used to treat gastritis and peptic ulcers	[52]
Gossypol (Fig. 2)	*Gossypium species*	A lipid-soluble polyphenol that exhibits significant antineoplastic effects against various cancer types	[53]
Hesperidin (Fig. 2)	*Citrus species*	A bioflavonoid compound with antioxidant, antibacterial, antimicrobial, anti-inflammatory, and anticarcinogenic properties	[54]
Hyoscyamine (Fig. 2)	*Hyoscyamus niger*	An alkaloid used as mild antispasmodic, analgesic, sedative, and mydriatic	[55]
Irinotecan (Fig. 2)	*Camptotheca acuminata*	A topoisomerase I inhibitors used to treat various types of cancer	[56]
L-Dopa (Levodopa) (Fig. 2)	*Mucuna pruriens*	A drug used in the management of Parkinson's disease	[57]
Morphine (Fig. 2)	*Papaver somniferum*	A natural alkaloid with potent and analgesic effects used for severe pain, control of pain from angina pectoris, or acute myocardial infarction and other medical uses	[58, 59]
Ouabain (Fig. 2)	*Strophanthus gratus*	Cardenolide compound used for the treatment of congestive heart failure by inhibiting Na^+/K^+-ATPase. It also has a potential use in the treatment of cancer	[60, 61]
Paclitaxel (Fig. 2)	*Taxus brevifolia* Nutt	A broad-spectrum anticancer compound	[62]
Papain (Fig. 2)	*Carica papaya*	A cysteine protease known for its antibacterial activity, wound healing properties, inhibition of platelet, and inhibition of atherosclerosis	[63, 64]
Papaverine (Fig. 2)	*Papaver somniferum*	An alkaloid used as a vasodilator and direct-acting smooth muscle relaxant	[65]

Physostigmine (Fig. 2)	*Physostigma venenosum*	A reversible acetylcholine esterase inhibitor in both the periphery and central nervous system. It is used to treat glaucoma and anticholinergic toxicity	[66, 67]
Pilocarpine (Fig. 2)	*Pilocarpus jaborandi*	An alkaloid used to treat glaucoma and xerostomia	[68]
Pseudoephedrine (Fig. 2)	*Ephedra sinica*	A sympathomimetic used to treat the symptoms of paranasal sinuses and obstruction in the nasal cavity, in addition to vasomotor rhinitis, allergic rhinitis, and otitis media	[69]
Quinine (Fig. 2)	*Cinchona ledgeriana*	Prevention and therapy of malaria	[70]
Reserpine (Fig. 2)	*Rauvolfia serpentina*	An alkaloid extract used to treat hypertension	[71]
Scopolamine (Fig. 2)	*Hyoscyamus niger*	A natural alkaloid with potent anticholinergic effects that used for the treatment of nausea, vomiting, and motion sickness	[72]
Sennosides A, B (Fig. 3)	*Cassia species*	Anthraquinone glycosides used as Laxative by relaxing and loosening the bowels	[73]
Tetrahydrocannabinol (THC) (Fig. 3)	cannabis	A potent psychoactive compound used as an antiemetic anti-inflammatory and has the ability to reduce neuropathic and chronic pain	[74]
Theophylline (Fig. 3)	*Theobroma cacao*	Used as second-line treatment of asthma and chronic obstructive pulmonary disease (COPD)	[75, 76]
Thymol (Fig. 3)	*Thymus vulgaris*	A phenolic monoterpene used mainly for the treatment of the upper respiratory system. It is used as expectorant, anti-inflammatory, antibacterial, antiseptic, and antiviral	[77]
Tubocurarine (Fig. 3)	*Chondodendron tomentosum*	A competitive blocker of nicotinic acetylcholine receptors used for the relaxation of skeletal muscles	[78, 79]

Yohimbine (Fig. 3)	*Pausinystalia yohimbe*	A monoterpenoid, indole alkaloid act by selective inhibition of presynaptic α2-adrenergic receptors (ARs) and used as a stimulant and aphrodisiac to improve erectile function	[80]

Fig. 1 [Images not available. See PDF.]

Chemical structure of drugs derived from plant origin; Aescin, aesculetin, agrimophol, allyl isothiocyanate, anisodamine, artemisinin, aspirin, atropine, berberine, bromelain, camphor, camptothecin, catechin, cocaine, codeine, colchicine. Convallatoxin, curcumin, deslanoside, digoxin, emetine, ephedrine, etoposide, and Galantamine
Fig. 2 [Images not available. See PDF.]

Chemical structure of drugs derived from plant origin cont., glaucarubin, glaucine, glycyrrhizin, gossypol, hesperidin, hyoscyamine, irinotecan, L-Dopa (Levodopa), morphine, ouabain, paclitaxel, papain, papaverine, physostigmine, pilocarpine, pseudoephedrine, quinine, reserpine, and scopolamine
Fig. 3 [Images not available. See PDF.]

Chemical structure of drugs derived from plants cont. sennosides A, B, tetrahydrocannabinol (THC), theophylline, thymol, tubocurarine, and yohimbine

Method

We evaluated the scientific literature collected from PubMed, CrossREF, and Google Scholar databases, considering all the articles published between 1975 and 2023. The keywords used were “herbal plants”, “phytomedicine”, “secondary metabolites”, “synergistic”, “antibacterial”, and “wound healing”. Article titles and abstracts were manually screened, and studies not related to the topic were excluded.

Main text

Antibacterial activities of medicinal plants

Globally high rates of morbidity and mortality are mostly caused by infectious diseases. Millions of people per year die as a result of the advent of bacterial strains that are resistant [81]. Antibiotic resistance in bacteria evolves through intrinsic or acquired resistance, by chromosomal mutation, or by horizontal gene transfer (HGT) [82, 83]. Several mechanism can lead to the development of antibiotic resistance such as alteration in cell membrane permeability either by reducing antibiotic penetration or increasing its elimination by efflux pumps; moreover, bacteria can deactivate the antibiotic itself or modify the antibiotic targets, in addition to other alternative pathways which were described in literature and are illustrated in Fig. 4 [83–88]. The most harmful microorganisms for human health have been recognized by the World Health Organization (WHO) and are categorized into three priority groups: critical pathogens (*Acinetobacter*, *Enterobacteriaceae*, *and Pseudomonas*), high-priority pathogens (*Campylobacter*, *Enterococcus faecium*, *Helicobacter pylori*, *Nisseria gonorrhoeae, Staphylococcus aureus*, *Salmonella* spp.), and medium-priority pathogens (*Streptococcus pneumoniae*, *Shigella* spp.) [83, 89–91]. Pathogenic plant bacteria can cause diseases on susceptible plant hosts which starts usually with low numbers of pathogen cells and then colonize and multiply to large amounts in living plant tissue. This results in the alteration of plant’s developmental system which eventually leads to reduction of plant growth and yield. Disease severity depends on the host genetic constitute, environmental conditions, and the pathogen [92]. Herbal plants produce unlimited wide variety of secondary metabolites which are mostly aromatic and phenol derivatives that gives them the ability to safeguard plants against pathogens [9, 93, 94]. Plants use oxygen for their growth and development but in stress like pathogen attack; the usage of oxygen causes the production of reactive oxygen species (ROS) in the plant and results in photo-oxidative damage [95–97]. In stress conditions plants induce excessive biochemical changes to activate defence pathways such as changing cell wall composition, detoxification of several ROS

species, induction of enzymatic and nonenzymatic components, and alteration of pathogen activates [98–101]. Numerous studies have demonstrated that various chemicals components of herbal plants possess antibacterial properties that can protect the human body against diseases without being damaging to cells [102]. Herbal or synthetic antimicrobial agents are both possible. The main negative effect of synthetic substances including antibiotics, metals, and metal oxide nanoparticles is the production of ROS, which is extremely hazardous and can lead to cancer [103]. On the other hand, herbal antimicrobial compounds including cinnamon, thyme, chamomile, eucalyptus, lemon balm, garlic, ginger, and others are free scavengers that can prevent ROS generation [102]. Antimicrobial phytochemicals can be divided into several categories such as phenolics and polyphenol (e.g. catechol and caffeic acids (Fig. 5)) [104]; terpenoids and essential oils (e.g. camphor, farnesol and artemisinin (Fig. 5)) [104, 105]; alkaloids (e.g. morphine (Fig. 5)) [104]; lectins and polypeptides (e.g. Thionins (Fig. 5)) [106–108]; polyacetylenes (e.g. 8*S*-heptadeca-2(*Z*),9(*Z*)-diene-4,6-diyne-1,8-diol (Fig. 5)) [109], and many others.

Fig. 4 [Images not available. See PDF.]

Structure of **a** gram-negative bacteria and its mechanism of resistance and **b** gram-positive bacteria and its resistance mechanism

Fig. 5 [Images not available. See PDF.]

Chemical structure of antimicrobial phytochemicals including catechol, caffeic acids, camphor, farnesol, artemisinin, morphine, thionins, 8*S*-heptadeca-2(*Z*),9(*Z*)-diene-4,6-diyne-1,8-diol, luteolin, quercetin, epicatechin, benzoic, cinnamic acids, tyrosol, hydroxytyrosol, sulphoraphane, proanthocyanidin (TPA), quercetin 3-*O*-glucoside, quercetin pentosides, gallic acid, naringenin, salicylic acid, kaempferol, astragalin, tellimagrandin I, II, rugosin D, and spiraeoside

Antibacterial phytochemicals

Phenolics and polyphenol

Polyphenols are a wide class of chemical substances found in plants that have a variety of biological functions [110]. Flavonoids (such as luteolin (Fig. 5), quercetin (Fig. 5), catechin (Fig. 2), and epicatechin (Fig. 5)) and nonflavonoids (such as benzoic, cinnamic acids, tyrosol, and hydroxytyrosol (Fig. 5)) are two categories of polyphenols that can be simple, large, or complex compounds[111]. Phenolic compounds have been used in traditional medicine, and although their mode of action as antibacterial agents is still not completely understood, it does appear to be distinct from that of antibiotics, reducing the risk of cross-resistance and making them a promising agent against resistant pathogens [112–114]. Inhibiting the growth of *Helicobacter pylori* is one of the potential benefits of polyphenols, which are powerful antioxidant and anti-inflammatory compounds found in foods like broccoli, garlic, liquorice, cranberries, and curcumin [115, 116]. Due to antibiotic resistance and issues with patient compliance, *H. pylori* eradication rates with triple therapy, which includes clarithromycin, metronidazole, or amoxicillin in combination with a proton pump inhibitor, are lower than 50–70%. The bismuth-containing quadruple therapy, as well as sequential and hybrid regimens, is now advised for treating *H. pylori* infection. There is not currently a therapy with >90% eradication rates, though [117–121]. To determine the impact of polyphenol components (garlic, liquorice, cranberry, curcumin, and broccoli) on the elimination of *H. pylori* infection, Wang et al. [122] conducted a meta-analysis study. The study discovered that polyphenol compounds may have a positive impact on the elimination of *H. pylori*, since the total elimination rate of the bacteria was higher in the polyphenol compounds group than in the control group. The research backs the adjuvant use of polyphenolic substances in the treatment of *H. pylori*. The polyphenol content of garlic makes it a potent alternative to conventional *H. pylori* treatment, according to in vitro studies [123–125]. Additionally, liquorice has anti-*H. pylori* properties [126]. Studies conducted in vitro revealed that *Glycyrrhiza glabra* and its aqueous extract have anti-*H. pylori* activity. Possible mechanisms of action include blocking dihydrofolate reductase, DNA gyrase, protein synthesis, and *H. pylori* adherence to human stomach tissue. Additionally, the components of cranberry juice prevent the adherence of a number of pathogenic infections, including the influenza virus, *E. coli*, and *H. pylori* [127–129]. According to in vitro research, *H. pylori* was prevented

from proliferating and was caused to produce a coccoid form by the polyphenols in cranberry extract [130–132]. Turmeric root contains the poly-phenolic compound curcumin (diferuloylmethane) (Fig. 5) [133]. Curcumin administration significantly reduced inflammation in gastric mucosa infected with *H. pylori*, according to animal studies [134]. Additionally, in vivo administration of sulphoraphane-rich fresh broccoli (Fig. 5) sprouts to mouse models reduced *H. pylori* colonization and prevented lipid peroxidation in the stomach mucosa [135, 136]. Żurek et al. [137] investigated the biological potential of polyphenolic substances isolated from walnut (*Juglans regia* L.). The edible kernels of walnuts, which also include polyunsaturated fatty acids (PUFA), tocopherols, phytosterols, and vitamins in addition to a number of other bioactive chemicals, are a rich source of polyphenols [138–141]. In addition, other parts of walnuts such as leaves are valuable products as well and their content was assessed spectrophotometrically by UPLC-PDA-MS/MS method. Several phenolic compounds were identified and expressed as total phenolic (TPC), proanthocyanidin (TPA) (Fig. 5), and total flavonoid contents (TFC) with quercetin 3-*O*-glucoside (Fig. 5) and quercetin pentosides (Fig. 5) dominating. Aqueous walnut leaf extracts antibacterial and antifungal activities were tested against Gram-negative bacteria (*E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*), Gram-positive bacteria (*Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus pyogenes*), and fungus. (*C. albicans*). Result showed highest susceptibility (at 10 mg/ml concentration) against *K. pneumoniae*, and *S. pyogenes,* less bactericidal activity against gram-positive and without any antifungal effect. These findings may be explained by the fact that gram-negative bacteria's cell walls contain lipopolysaccharide (LPS), which creates a hydrophilic environment and shields it from hydrophobic molecules. The aqueous and ethanol extracts of walnut leaves may contain more hydrophilic compounds as a result, leading to higher inhibitory activity against gram-negative bacteria [137]. In similar context, Bouslamti et al. [142] tested antioxidant and antibacterial activity of *Solanum elaeagnifolium* leaf and fruit extracts against Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*), Gram-negative bacteria (*Escherichia coli* and *Proteus mirabilis*), and *Candida albicans*. HPLC and colorimetric methods were used to determine the chemical composition of *S. elaeagnifolium* fruits and leaf extracts which are quercetin (Fig. 5), luteolin (Fig. 5), gallic acid (Fig. 5), and naringenin (Fig. 5). Result showed that the extracts generated good antioxidant activity and potent antifungal activity. Nonetheless, further studies are needed to assess potential adverse effects. Meadowsweet plant (*Filipendula ulmaria* (L.) Maxim.) has been also used in traditional medicine due to its wide range of pharmacological effects [143, 144]. For the biological activity of meadowsweet, phenolic secondary metabolites are thought to be responsible, including phenolic acids and their derivatives (such as gallic acid and salicylic acid in Fig. 5), flavonoids and flavonoid glycosides (such as kaempferol and astragalin in Fig. 5), and tannins (such as tellimagrandin I, II, and rugosin D) [145, 146]. Meadowsweet extracts from different organs and parts (leaves, flowers, fruits, and roots) have been studied for their antioxidant and antibacterial properties by Savina et al. [147] Gram-negative bacteria *P. aeruginosa* and Gram-positive bacteria *B. subtilis* were used to investigate the antibacterial activity of flower and fruit extracts. All portions of the meadowsweet plant had a high overall phenol level, whereas the flowers had a high flavonoid concentration. The primary flavonoids in meadowsweet include luteolin, kaempferol derivatives, and spiraeoside (Fig. 5), which all have potent antibacterial properties. Meadowsweet plants were also found to have significant amounts of salicylic acid and its derivatives, which are thought to have anti-inflammatory effects. Furthermore, it was shown that meadowsweet roots had greater total catechin and proanthocyanidin contents than other sections, while the fruits had higher total tannin contents, particularly tellimagrandins I and II and rugosin D. All of the components had anti-inflammatory and antibacterial effects.

Terpenoids and essential oils (EOs)

More than 17,500 plant species can produce essential oils (EOs), which are volatile secondary metabolites with a particular flavour or scent [148]. The cytoplasm and plastid of plant cells produce EOs compounds, which are then stored in intricate secretory structures including glands and secretory cavities before being present as liquid drops in the flowers, leaves, stems, fruits, bark, and roots of plants. In addition to many other substances like fatty acids, oxides, and derivatives of sulphur, the primary constituents of EOs include terpenes, terpenoids, and phenylpropanoids [149, 150]. EOs are produced through mechanical cold pressing of plants, steam distillation, dry

distillation, hydrodistillation, and more recent techniques including microwaves and supercritical fluid extraction. Depending on the method used, different chemical compositions of EO were obtained [150–152]. Due to their biological characteristics, including their antibacterial qualities, EOs have been utilized as perfumes, food spices, and in folk medicine [153]. Terpenes, which make up the majority of essential oils (EOs), are generated from the isoprenoid pathway and are made up of isoprene units (C5). On the basis of this, terpenes are classified as monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30), and carotenoids (C40). Terpenes have a variety of chemical properties, including alcohol (terpineol, menthol, carveol, linalool, and citronellol; Fig. 6), aldehyde (citral and citronellal; Fig. 6), ketone (carvone; Fig. 6 and camphor; Fig. 5), phenol (thymol; Fig. 3; and carvacrol; Fig. 6); ether (eucalyptol; Fig. 6), and hydrocarbon (pinene, and limonene; Fig. 6) groups [154, 155]. An antibacterial, antifungal, antioxidant, anti-inflammatory, analgesic, antimutagenic, and wound-healing compound, thymol is a phenolic monoterpene. Due to its strong antibacterial characteristics, it also enhances digestion, lessens respiratory issues, and is used in dentistry to treat infections of the oral cavity [156]. *Majorana syriaca*, sometimes known as thyme, is a widespread east Mediterranean aromatic species with a high content of essential oils that give the plants their distinct flavour and perfume as well as antibacterial and antifungal properties. Due to its high volatile oil content, *M. syriaca* is used in traditional medicine to cure the flu, colds, and cough. The primary essential oil in thyme is thymol, which is used in mouthwashes as an antibacterial. Additionally, thyme extracts are added to cough syrups to treat coughs and other respiratory issues like bronchial issues. Thyme essential oils' high phenol concentration is what gives them their potent antibacterial properties, allowing for usage as a potent disinfectant, in oral medicinal preparations, and as a flavouring ingredient in numerous food products [157–161]. Abu-Lafi et al. [162] used static headspace-gas chromatography/mass spectrometry (SD-GCMS) analysis to determine essential oils in thyme leaves and identified 29 monoterpenes (oxygenated and hydrocarbons) such as thymol, carvacrol, *a*-phellandrene, *a*-pinene, *c*-terpinene, *o*-cymene, *p*-cymene, and *b*-myrcene (Fig. 6). The primary components of oxygenated monoterpenes in thyme leaves were the phenolic substances thymol and its geometric isomer carvacrol. The investigation also revealed the presence of thymoquinone, which is known to play a protective function against oxidative damage brought on by chemicals that produce free radicals, such as carbon tetrachloride. Free terpenes present in EOs were investigated for their antibacterial activity by Guimares AC et al. [154]. Results showed that hydrocarbons such terpinene, camphene, R-(-)-limonene, and (+)-α-pinene have inferior antibacterial action to oxygenated terpenes like phenolics (Fig. 6), which is consistent with the findings of earlier studies [163–167]. Eugenol and terpineol showed rapid and excellent bactericidal action against *Salmonella enterica* and *S. aureus* strains, respectively. Moreover, carveol, citronellol, and geraniol exhibited rapid bactericidal effect against *E. coli*. Therefore, hydroxyl groups in phenolic and alcohol compounds resulted in higher antimicrobial activity than hydrocarbons [154]. The antibacterial properties of EOs from *Cinnamomum cassia* bark and *Eucalyptus globulus* leaves are well established. These EOs' main secondary metabolites, 1,8-cineole (Fig. 6) and *trans*-cinnamaldehyde (Fig. 6), are what provide these compounds their therapeutic properties. However, ethnobotanical physicians prefer the use of entire EOs over purified components to treat bacterial infections [168–170]. Therefore, a set of 6 g-positive and -negative bacteria were used by Nguyen HTT et al. [171] to evaluate the antibacterial activity of plant EOs to their separated main components. According to the findings, entire oils of eucalyptus and cinnamon with low concentrations of 1,8-cineole (61.2%) and *trans*-cinnamaldehyde (89.1%) have more favorable effects than the active components that have been refined to less than 99%. Additionally, CC crude extract had greater and stronger effects on both gram-positive and gram-negative bacteria compared to EG. The study's findings support the use of complete essential oils for bacterial infections in traditional medicine since they offer advantages over isolated constituents that might not have the same effects when used as medications. To combat foodborne infections, EOs can also stop bacterial growth [172]. Another study by Trinh et al. [173] looked at the antibacterial properties of *trans*-cinnamaldehyde, the major component of Cinnamomum cassia essential oil, in relation to the *Listeria innocua* strain. The accumulation of *trans*-cinnamaldehyde in the hydrophobic core of the cytoplasmic membrane of *L. innocua*, which results in membrane disruption, was found to be the cause of the antibacterial action of *trans*-cinnamaldehyde as well as other minor *C. cassia* essential oil. Although there was no evidence of large hole

creation or cell lysis, viable but nonculturable (VBNC) cells did start to appear. To use *C. cassia* EO and *trans*-cinnamaldehyde rationally in food preservation, a better understanding of their modes of action is required. Kolozsváriné Nagy J et al. [174] tested EOs of cinnamon, eucalyptus, thyme, clove, tea tree, rosemary, lemon grass, lemon balm, and citronella grass against *Xanthomonas arboricola* pv. *pruni* (*Xap*) which is responsible for water-soaked spots on the surface of leaves, and fruits that may lead to severe infections and complete destruction of the plant. High-performance thin-layer chromatography (HPTLC)-*Xap* combined with solid-phase microextraction-gas chromatography/mass spectrometry (SPME-GC/MS) was used to identify active EOs' components. All EOs inhibited bacterium isolates with superiority to cinnamon with MIC values of 31.25 µg/mL and 62.5 µg/mL, while the tea tree EO was the least effective with the highest MIC values. Compounds that are identified to have antibacterial activity by HPTLC zones are *trans*-cinnamaldehyde in cinnamon, thymol in thyme, eugenol in clove, terpinen-4-ol (Fig. 6) in tea tree, borneol in rosemary (Fig. 6), citral in lemon grass and lemon balm, and citronellal and nerol in citronella grass (Fig. 6). Biofilms produced by bacteria are one of the reasons for the resistance of bacteria and can act as reservoirs for spoilage bacteria in food such as *Shewanella putrefaciens* which is the chief spoilage bacteria in fish [175, 176]. Therefore, Xie et al. [177] studied antibacterial effectiveness of *Ocimum gratissimum* L. essential oil (OGEO) in vitro against *Shewanella putrefaciens* to be used as natural preservative. The main active ingredients of OGEO are eugenol and caryophyllene (Fig. 6) which have antibacterial activity according to previous studies. The study demonstrated that OGEO had a positive inhibitory effect on the growth of *S. putrefaciens* and act by disrupting the formation of biofilms and cell membranes of the bacteria with minimum inhibitory concentration and minimum bactericidal concentration of 0.1%. inhibiting *S. putrefaciens* by EOs provide a new method of inhibiting the spoilage of food. *Ocimum basilicum* (Great basil or Saint-Joseph's wort) from *Lamiaceae* family contains many bioactive secondary metabolites such as polyphenols, flavonoids, and terpenes. Main EOs included in the plan are linalool, methyl estragole, methyl cinnamate, and methyl chavicol (Fig. 6) [178–181]. *Ocimum basilicum* has many pharmacological activities and has been used in traditional medicine as antioxidant, anticancer, antiviral, antiaging, and antimicrobial properties [182–184]. Studies showed that the biological activity of O. *basilicum* is related to cinnamic acid (Fig. 5) derivative of the polyphenoid rosmarinic acid (Fig. 6) [185]. Eid et al. [186] studied *O. basilicum* seeds essential oil biological activity and showed that it had a broad-spectrum antibacterial activity. The oil suppressed the development of all tested microbial strains (minimum inhibitory concentrations (MICs) between 1 and 2.3 µg/mL) and fungus strain *C. albicans* (MICs of 1.3 µg/ml for fungus). Antibacterial activity may refer to the presence of phenolic components in the essential oil which trigger intracellular ATP and potassium ion leakage and lead to cell death. Yaldiz et al. [187] also investigated antibacterial, antiquorum sensing, and antibiofilm capabilities of the ethanol extract and essential oil derived from *O. basilicum*. Results revealed that in addition to having antifungal effects on *C. albicans*, basil essential oil also demonstrated antibacterial activity and antiquorum sensing activity against some Gram-positive and -negative bacterial species.

Fig. 6 [Images not available. See PDF.]

Chemical structure of essential oils (EOs) and terpenoids including terpineol, menthol, carveol, linalool, citronellol, citral, carvone, carvacrol, eucalyptol, a-pinene,limonene, a-phellandrene, c-terpinene, o-cymene, p-cymene, b-myrcene , camphene, eugenol, 1,8-cineole, terpinen-4-ol, nerol, caryophyllene, methyl estragole, methyl cinnamate, methyl chavicol, and rosmarinic acid

Alkaloids

Alkaloids are a wide and diverse group of secondary metabolites found in 300 plant families and can be found as well in bacteria, fungi, and animals. Alkaloid name came from their basic nature and is characterized by the presence of a basic nitrogen atom in the form of a primary (RNH_2), secondary (R_2NH), or tertiary amine (R_3N). Alkaloids can be monomers or oligomers and can be classified into three major categories: true-alkaloids with N-atom in the heterocycle, proto-alkaloids without N-atom, and pseudo-alkaloids with a basic carbon skeleton [112, 188–192]. Heterocyclic alkaloids can be divided into 14 subgroups that include, pyrrolizidines, pyrrolidines, indoles, isoquinolines, quinolizidines, purines, piperidines, tropanes, and imidazoles [193]. A rich source of bioactive

compounds is Marine invertebrates which developed a defence chemical system to protect themselves from predation. Marine sponges are a rich source of secondary metabolites which are responsible for their numerous biological activities such as anti-inflammatory,anticancer, antiviral, antibacterial, and anticoagulant activities [194–196]. The sponge *Luffariella variabilis* exhibit antibacterial activity against gram-positive bacteria due to the presence of sesterterpenes manoalide, secomanoalide,and *trans*- and *cis*-neomanoalide (Fig. 7) [197]. Moreover, the sponges *Poecillastra* sp. and *Jaspis* sp., contain psammaplin A (Fig. 7), a bromotyrosine-derived natural product, while the sponge *Siliquaria* sp. contain the motualevic acids (A–F) (Fig. 7) a halogenated glycyl-lipid conjugates which all inhibit gram positive-bacteria only and are inactive against gram-negative bacteria [198, 199]. Antibacterial and antibiofilm activity of another group called the pyrrole-imidazole alkaloids was studied and tested against gram-positive and -negative bacteria. Bromoageliferin (Fig. 7) is a member from pyrrole-imidazole alkaloids family isolated from marine sponges with other members such as oroidin and sceptrin (Fig. 7). Reports documented the inhibition of *Rhodospirillum salexigens* SCRC 113 biofilms formation a marine bacterium by oroidin and bromoageliferin which resulted in using them as templates to develop numerous analogues and study their antibiofilm activity [200–209]. Melander et al. [210] studied pyrrole-imidazole alkaloids (monomeric and dimeric alkaloids) ability to inhibit biofilm formation and suppress antibiotic resistance against gram-negative *Acinetobacter baumannii* and gram-positive methicillin-resistant *S. aureus* (MRSA). Result showed that monomeric alkaloid oroidin exhibited modest activity against both strains, while stevensine (monomeric oroidin analogue) (Fig. 7) was inactive. On the other hand, the dimeric alkaloids Sceptrin was slightly more active against *A. baumannii*, while dibromosceptrin (Fig. 7) and bromoageliferein were both more active against MRSA with bromoageliferin as the most potent compound. Result indicated that both monomeric and dimeric alkaloids can inhibit phenotypic and genotypic bacterial resistance mechanisms; therefore, a high-throughput screening is needed to identify marine natural compounds to be used as adjuvants with antibiotics to restore FDA-approved antibiotics efficacy and to find alternative approaches to combating MDR bacteria. Tuberculosis (TB) is a widespread infectious disease, and due to the increase of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains new antiTB agents are needed. Arai et al. [211] isolated several compounds from marine sponge of *Haliclona* sp. such as tetracyclic alkylpiperidine alkaloid, 22-hydroxyhaliclonacyclamine B, and haliclonacyclamine A and B alkaloids (Fig. 7) to be used as antidormant mycobacterial compounds. Result showed strong antimycobacterial activity under both aerobic and hypoxic condition against *Mycobacterium smegmatis* and *M. bovis Bacille de Calmette et Guérin* (BCG) from both haliclonacyclamine. Haliclonacyclamine B exhibited bactericidal activity against *M. bovis*(BCG), while hydroxyhaliclonacyclamine B showed weaker antimicrobial activities against *Mycobacterium bacilli* and reduced antimycobacterial activity which may be due to the presence of the 22-hydroxy group. Wijaya et al. [212] studied *Dicranostigma franchetianum* plant from Papaveraceae family and isolated a wide spectrum of isoquinoline alkaloids (IAs) such as berberine, chelerythrine, protopine, and sanguinarine (Fig. 7). Alkaloids were tested against *Mycobacterium tuberculosis* H37Ra and four other mycobacterial strains. Most isolated alkaloids exhibited weak or no antimycobacterial activity; however, benzophenanthridine (6-ethoxydihydrochelerythrine) and bisbenzophenanthridine (*bis*-(6-(5,6-dihydrochelerythrinyl))ether) alkaloid derivatives (Fig. 7) showed moderate antimycobacterial activity against all tested strains. Moreover, semisynthetic berberine derivatives resulted in a significant increase in antimycobacterial activity against all tested strains. Further studies are needed to develop more potent berberine derivatives with low cytotoxic profile. On the other hand, Dong et al. [213] conducted a bioassay-guided phytochemical study on the semi-mangrove plant *Myoporum bontioides* A. Gray, which belong to Myoporaceae family and tested its activity against methicillin-resistant *S. aureus* (MRSA). New sesquiterpene alkaloids and furanosesquiterpenes were isolated from the plant, and result showed that sesquiterpene alkaloids displayed potent anti-MRSA activity. Alkaloids also can be used in food system to prevent or treat foodborne diseases such as oliveridine and pachypodanthine (Fig. 7) an aporphinoid alkaloids. Marco Di et al. [214] tested these alkaloids against *Yersinia enterocolitica* an important foodborne pathogen that cause a gastrointestinal disease in humans called yersiniosis. Result showed that both oliveridine and pachypodanthine inhibited the growth of *Y. enterocolitica* with superiority for oliveridine with lower MIC values which open an opportunity to develop

potential antimicrobial agents to prevent or treat foodborne diseases.

Fig. 7 [Images not available. See PDF.]

Chemical structure of alkaloids compounds including secomanoalide, neomanoalide, psammaplin A, motualevic acids (**A**–**F**), bromoageliferin, oroidin, sceptrin, stevensine, dibromosceptrin, 22-hydroxyhaliclonacyclamine B, haliclonacyclamine A and B, berberine, chelerythrine, protopine, sanguinarine, 6-ethoxydihydrochelerythrine, *bis*-(6-(5,6-dihydrochelerythrinyl))ether, oliveridine, and pachypodanthine

Lectins and polypeptides

Naturally antimicrobial proteins and peptides can be found in humans, animals, plants, and microorganisms. Plants include proteins called lectins that attach to certain carbohydrates on the surfaces of microbes, aiding in the defensive mechanisms against pathogens. Plant lectins are classified according to their affinity for monosaccharides and complex glycans, but they do not interact with endogenous cellular glycans. Instead, they have a strong affinity for the sugars on bacteria, fungi, and other organisms, which suggests that they serve as a plant defence molecule [215–218]. Lectins have different types including C-type lectins (e.g. endocytic lectins), S-type lectins (e.g. galectins), L-type lectins, P-type lectins, M-type lectins, Jacalin-related lectin (JRL),siglecs, *Oscillatoria agardhii* agglutinin homolog (OAAH), Cyanovirin-N homologs (CVNHs), *Galanthus nivalis* agglutinin-like (GNA-like) lectins, and others [219]. Lectins have been used in medicine as immunomodulators against tumour cells and microbial infections. Moreover, lectins showed to be able to disrupt quorum sensing (QS) signal transduction and interfere with nonessential functions for cell viability [220, 221]. Lectins purified from *Moringa oleifera* leaf (SLL-1, SLL-2, and SLL-3) showed variable antibacterial potency against *E. coli*, *Shigella dysenteriae,* and *S. aureus*, while water-soluble lectin purified from *Moringa oleifera* seeds significantly reduced *S. aureus* but not *E. coli* [222, 223]. On the other hand, mannose-glucose-binding lectin isolated from *Calliandra surinamensis* leaf did not kill *S. aureus* nor *Staphylococcus saprophyticus* but reduced their growth and their biofilm formation with no activity against *E. coli* [224]. In addition, a rich source of lectins is Marine species that include green algae (22%), red algae (61%), and cyanobacteria (17%) [225]. Few literature studies discussed the potential usefulness of algal or cyanobacterial lectins as antibacterial agents [226–228]. Purified lectins isolated from two red algal species, *Eucheuma serra* (ESA) and *Galaxaura marginate* (GMA), showed strong inhibitory action against marine gram-negative *Vibrio vulnificus*, while no action recorded for other two Vibrio species, *V. peagius* and *V. neresis* according to Liao et al. [229] study. Selectivity in the inhibition is referred to the differences in bacterial surface carbohydrates. Furthermore, Hung et al. [230] isolated lectins from *Eucheuma denitculatum* (EDA) red algae which also exhibited activity selectivity against *V. alginolyticus*, but not against *V. parahaemolyticus* or *V. harveyi* due to the binding of lectins to high-mannose N-glycans. Moreover, Holanda et al. [231] isolated lectins from *Solieria filiformis* red alga and tested its activity against gram-negative and -positive bacteria. Result showed that lectins inhibited the growth of the gram-negative species; *Salmonella typhi, Serratia marcescens, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter aerogenes*, and *Proteus sp.*, while it stimulated the growth of the gram-positive species *Bacillus cereus*. No activity was noticed against *S. aureus, B. subtilis, E. coli*, and *Salmonella typhimurium*. The interaction between the *S. filiformis* lectin and the gram-negative bacteria's cell surface receptors, which encouraged changes in the flow of nutrients, may be the cause of the activity against gram-negative bacteria. These findings raised the possibility of using marine lectins as organic substitutes for antibiotics in the treatment of gram-negative pathogens [232]. As mediators of innate immunity in all living things, antimicrobial peptides (AMPs) or host defence peptides are also recognized to play a significant role in biological processes. Because AMPs are cationic and amphipathic, they can more easily pass through microbial cytoplasmic membranes and cause cell lysis. The usage of AMPs is constrained for a variety of reasons, including their undesirable cell toxicity, restricted availability, high cost during synthesis, sensitivity to protease degradation, and others [233, 234]. Compared to other species, plants have more AMPs, which typically have 20–50 amino acid residues and are abundant in glycine, cysteine, and positively charged residues [234, 235]. Plant AMPs are divided into thionins, defensins, α-hairpinins, nonspecific lipid transfer proteins (nsLTPs), hevein- and knottin-like peptides based on their 3D structure and cysteine signature [236]. A study of

cysteine-rich peptides (CRPs) revealed that they all had a structural component in common called the γ-core that is responsible for their antibacterial activity. This structural element has an antiparallel β-hairpin conformation [237]. Thionins are poisonous to yeast, fungus, and bacteria. Antifungal activity was defined as the development of pores or a specific contact with a particular lipid domain as a result of direct protein-membrane interactions between positively charged thionin and the negatively charged phospholipids in fungal membranes [238, 239]. The antibacterial, antifungal, proteinase, and insect amylase inhibitory properties of plant defensins also classify them as γ-thionins [240, 241]. Although the exact mechanism of action of defensins is yet unknown, and not all plant defensins work in the same way, they almost certainly use glucosylceramides as receptors for fungal cell membrane entry. Ion outflow and membrane rupture are caused by defensins' positive charges repelling one another into the cell membrane [242]. Similar behaviour has been seen with nsLTPs, which interfere with the biological membrane permeability and integrity of pathogens [243]. Gram-positive bacteria (*Bacillus megaterium* and *Sarcina lutea*) and a number of fungi were able to halt the formation of powerful nsLTPs that were isolated from onion seeds [244, 245]. Sunflower (*Helianthus annuus*) seed-derived nsLTP also demonstrated antifungal activity [246]. The α-hairpinins are a different small family of AMPs that includes peptides with a wide range of biological activities, including antifungal and antibacterial activity [247]. The rubber tree (*Hevea brasiliensis*) (which produces latex that is similar to hevein) gave its name to the family of AMPs that resembles hevein. Hevein-like peptides are extremely stable, cysteine-rich substances that withstand heat and protease activity. They are active against a variety of phytopathogens, including bacteria and fungus, due to their capacity to interact with pathogens' cell wall chitin, which is missing in plants [248]. Tk-AMP-K2$_{10\text{-}23}$, a knottin-like peptide isolated from *T. kiharae* seeds, was also discovered to have antibacterial action. According to the results of flow cytometry, there were less *Cr. neoformans* cells, which suggest that the peptide caused cell lysis by interfering with the integrity of the membrane [249–251]. More research and focus are required on peptides that are particularly effective against specific infections in order to create new antimicrobial agents.

Polyacetylenes

A wide variety of biomasses, including plants (particularly species of the Apiaceae family and carrot), marine organisms, fungi, insects, and people, include polyacetylenes, which are naturally occurring molecules characterized by the presence of two or more carbon–carbon triple bonds. There are just a few papers on the antibacterial potential of polyacetylenes, which are renowned for their anti-inflammatory and anticancer properties [252, 253]. A large number of natural polyacetylenes have been isolated such as 8*S*-heptadeca-2(*Z*),9(*Z*)-diene-4,6-diyne-1,8-diol (Fig. 5) from *Bupleurum salicifolium Soland* (Umbelliferae). This polyacetylene showed antibiotic activity against gram-positive bacteria *S. aureus* and *Bacillus subtilis* with no activity against gram-negative bacteria (*E. coli, Salmonella sp., Pseuknas aeuriginosa*) and the yeast *Candida albicans* [109]. Falcarinol-type polyynes (Fig. 8) showed to have antifungal activity that protects carrots from fungi as *Botrytis cinerea Pers*, while panaxydol and panaxytriol (Fig. 8) from *Panax ginseng* found to have cytotoxic effect against numerous cancer cell lines [254–256]. *Exocarpos latifolius Kuntze* stems were used to extract exocarpic acid derivatives (Fig. 8); some derivatives exhibited antimycobacterial activity against *M. tuberculosis*, while others exhibited no activity [253, 257]. Amadaldehyde, a C63 polyacetylenic aldehyde that was isolated from mango ginger (the rhizomes of *Curcuma amada* Roxb.), was shown to have antibacterial and antioxidant activity against microorganisms in addition to cytotoxicity and platelet aggregation inhibitory effect [258]. Several polyynes such as epoxide-ketone are found in one of the most important oriental medicinal plants *P. ginseng C.A. Meyer*. These compounds exhibited antimicrobial activity against several bacteria such as *Bacillus subtilis, S. aureus, Cryptococcus neoformans*, and *Aspergillus fumigatus* which conclude that *P. ginseng* releases antimicrobial polyacetylenes into the surrounding soil from roots to defend the plant[259]. Moreover, triyne carboxylic acid compound (octadeca-9,11,13-triynoic acid) (Fig. 8) isolated from the roots of *Polyalthia cerasoides* (Roxb.), showed antimalarial and antimycobacterial activity against *Plasmodium falciparum* and *M. tuberculosis*, respectively. Furthermore, 13,14-dihydrooropheic acid (Fig. 8) from the extract of *Mitrephora celebica* Scheff, and debilisones A–F (Fig. 8) from the Thai herbal plan *Polyalthia debilis* (Pierre) exhibited antibacterial activity against *Mycobacterium smegmatis* and *M. tuberculosis*, respectively

[260–262].

Fig. 8 [Images not available. See PDF.]

Chemical structure of polyacetylenes compounds including falcarinol, panaxydol, panaxytriol, amadaldehyde, octadeca-9,11,13-triynoic acid, 13,14- dihydrooropheic acid, debilisones (**A–F**)

Medicinal plants with antimicrobial activities

St. John's wort (SJW) (*Hypericum perforatum*)

There are 500 species in the *Hypericum* (Hypericaceae) family, and they are all found worldwide. The most prevalent species is *Hypericum perforatum*, also known as St. John's wort (SJW), which is one of the most well-known and widely used herbs in the world. The herb has been widely used for therapeutic purposes across the globe [263, 264] and has been included into traditional medicine. The aerial parts or flowering tops make up the crude form of SJW medicine, which is employed in multi-ingredient formulations or as a monopreparation (either as is or as an extract) [265]. The upper stem leaves and flowering tops of *H. perforatum* are used in dietary supplements to treat mild to severe depression [266]. The flowering tops are typically prepared and used for its hypnotic and tonic properties or to speed wound healing. Due to varying extraction techniques, individual plant extracts do not include the entire group of phytoconstituents but only some of them, which causes a number of issues that affect the usage of *H. perforatum* in pharmaceutical formulations. Additionally, some of the active ingredients may experience problems with stability with exposure to time or light[267]. Due to the plant's synthesis of bioactive secondary metabolites like naphthodianthrones, flavonoids, bioflavonoids, phloroglucinols, xanthones, proanthocyanidins, acid phenols, and essential oils [263, 268, 269], it has been the subject of phytochemical studies.

Antibacterial activity of SJW

Sherif et al. [270] investigated the antibacterial activity of the *Hypericum perforatum* plant. The plant extract and its fractions were evaluated by Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometric (LC–ESI–MS/MS) and tested against MRSA, *Enterococcus faecalis, E. coli,* and *K. pneumonia* MDR isolates. The results demonstrated that different extract from *H. perforatum* had a promising antibacterial activity against tested pathogens, particularly MDR-*K. pneumoniae*, with inhibition zones ranging from 17.9 to 27.9 mm and strong antioxidant effects, as opposed to sub-fractions extract, which demonstrated lesser inhibition zones and higher MIC values. Bacterial cell membranes are affected by extract of *H. perforatum*, which causes cell shrinkage and deformation that results in cell lysing. Additionally, the extract promoted cell elongation and thickness, which is similar to the actions of penicillin and the antibiotic cefotaxime, according to published research [271–274]. These findings indicate that these herbal extracts can be used to treat resistant bacteria, but further research is required to clarify why the total extract showed the most potent antibacterial activity over the subfraction? Is it an additive effect of specific compounds that act together with different mechanism to inhibit resistance bacteria or something else?. Using *H. perforatum* flower extracts, Okmen et al. [275] investigated the antibacterial activity of the bacteria that cause mastitis, a complicated condition characterized by inflammation of the parenchyma of the mammary glands and bacterial alterations in milk. Along with coagulase-negative *staphylococci* (CNS), *Staphylococcus, Streptococcus*, and *coliform* bacteria are the pathogens that cause the disease [276]. Although this condition is frequently treated with antibiotics, new antibiotics are still needed to combat germs because of the emergence of antibiotic resistance. The study's findings indicated that *H. perforatum* flower methanol extracts exhibit bactericidal action against gram-positive and gram-negative bacteria (*S. aureus, Proteus vulgaris, P. aeruginosa,* and *E. coli*), with the highest inhibition zone against Coagulase-negative *Staphylococci*. The plant extract also demonstrated antimutagenic qualities. However, additional tests are needed to know the extract constitutes and the bioactive compounds responsible for the biological activities. Polyphenolic substances with pharmacological characteristics are called xanthones. Strong free radical scavengers, xanthones have been shown to have activity against a variety of bacteria, including vancomycin-resistant *enterococci*, methicillin- and multidrug-resistant *S. aureus*, and M. tuberculosis [277–283]. When *H. perforatum* was exposed to *Colletotrichum gloeosporioides* cell wall extracts, xanthone (Fig. 9) buildup was seen as a defence mechanism [284]. After elicitation with *Agrobacteriumtumefaciens*,

Franklin et al. [285] demonstrated that the antioxidant and antibacterial properties in *H. perforatum* cells had dramatically increased. The up-regulation of xanthone metabolism, particularly paxanthone (Fig. 9), which increased 12-fold within 24 h, was the cause of the enhanced activity. After 12 h of co-cultivation, the viability of *A. tumefaciens* was reported to have dramatically decreased. As a result of the buildup of xanthones, *H. perforatum* cell antibacterial activity rose ten times. Numerous infectious illnesses are brought on by *S. aureus*. Due to its development of resistance against practically all standard of care (SOC) antibiotics, Methicillin-resistant S. aureus (MRSA) has, regrettably, become a significant problem. Therefore, it is urgently necessary to create new tactics to combat MRSA [286–288]. Wang et al. [289] investigated the effect of hypericin (HYP) from *H. perforatum* on the susceptibility of *β*-lactam antibiotics (cefazolin, oxacillin, and nafcillin) and their synergistic effect with oxacillin in a murine bacteremia model. Result showed that HYP significantly reduced the minimum inhibitory concentrations (MICs) of *β*-lactam antibiotics and SarA (RNA-binding protein) which is a key regulator that bind on target promoters to control *S. aureus* virulence factors. Moreover, HYP enhanced the efficacy of oxacillin in MRSA bacteremia model; therefore, these results might be due to the synergistic effect of HYP with oxacillin.

Fig. 9 [Images not available. See PDF.]

Chemical structure of bioactive compounds found in St. John’s wort (SJW), rosemary, ginger which include, xanthone, paxanthone, hypericin, carnosol carnosic acid, p-cymene-7-ol, suberoylanilide hydroxamic acid, rosmanol, epirosmanol, isorosmanol, rosmaridiphenol, pyrogallol, ellagic acid, benzoic acid, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), α-curcumene, α-farnesene, β-bisabolene, β-sesquiphellandrene, zingiberene, gingerol, paradol, shogaol, gingerenone-A, zingerone, 6-dehydrogingerdione, 10-gingerol, 12-gingerol, *trans*-anethole, and m-phenylphenol

Rosemary (*Rosmarinus officinalis, L.*)

In addition to its use in food preservation to stop oxidation and microbial contamination, *Rosmarinus officinalis*, L., a member of the *Lamiaceae* family that originated in the Mediterranean, is renowned for its antioxidant, hepatoprotective, antiangiogenic, and potential treatment for Alzheimer's disease properties [290–292]. Numerous polyphenolic substances, including rosmarinic acid (Fig. 6), hesperidin (Fig. 2), carnosol, and carnosic acid, are found in rosemary (Fig. 9). According to various studies [293–295], rosemary essential oil contains 1,8-cineole, camphor, and -pinene p-cymene-7-ol in addition to borneol (Fig. 9). Fresh or dried leaves, flowers, fruits, roots, stems, seeds, and bark can all be used to make plant extracts, while dried samples were shown to contain higher quantities of flavonoids. Air, microwave, oven, and freeze drying are all acceptable drying techniques [296–298].

Antibacterial activity of rosemary

Rosmarinus officinalis was found to have antibacterial properties in many research. In their research on the antibacterial properties of several natural extracts, including rosemary, Fernández-López et al. [299] examined how long the shelf life of veal meatballs could be stored for. The results showed that all of the studied microorganisms were susceptible to rosemary extracts (oil extract, water miscible extract, oil, and water-miscible extract), with oil extract having the strongest inhibitory impact. The most vulnerable bacteria were *Brochothrix* spp., which might be a reference to the antibacterial action of nonpolar phenolic compounds against gram-positive bacteria. Govaris et al. [300], Gomez-Estaca et al. [301], and both Camo et al. [302] and Quattara et al. [303] reported that rosemary EOs prevented food-spoileding bacteria from growing. *Rosmarinus officinalis* and *Ocimum basilicum* essential oils have been shown to have antibacterial action against multidrug-resistant clinical isolates of *E. coli* by Sienkiewicz et al. [293]. Since both EOs were effective against every clinical strain of *E. coli*, it can be inferred that they can be used to treat and prevent the emergence of resistance strains. Probuseenivasan et al. [304] and Mihajilov-Kristev et al. [305] found similar results, confirming rosemary essential oil's potent antibacterial action against *E. coli*. Carnosol, carnosic acid, rosmarinic acid, rosmanol, epirosmanol, isorosmanol, and rosmaridiphenol (Fig. 9) interact with the cell membrane and alter the production of nutrients, genetic material, and fatty acids to produce rosemary’s inhibitory effects. Additionally, they affect electron transport, result in cellular component leakage, interact with proteins in the membrane, and cause a loss of membrane functionality [306, 307]. To increase the effectiveness of

antibiotics against multi-drug-resistant bacteria like MRSA, Ekambaram SP et al. [308] looked into the antibacterial activity and synergistic effect of rosmarinic acid (dimer of caffeic acid) with conventional antibiotics. The agar well diffusion method was used to assess the antibacterial activity of rosmarinic acid against microorganisms. In comparison to using an antibiotic alone, the results demonstrated that rosmarinic acid had a synergistic impact with the medications ofloxacin, amoxicillin, and vancomycin against *S. aureus*. However, only the vancomycin and rosmarinic acid combination was effective against MRSA. The activity of rosmarinic acid on surface proteins known as microbial surface components recognizing adhesive matrix molecules (MSCRAMM's) present in *S. aureus* and MRSA was suggested to be the mechanism of action. Pomegranate, rosemary, and antibiotic were combined in a study by Abu El-Wafa et al. [309]. *P. aeruginosa* isolates with significant biofilm producers and antibiotic resistance were examined for the synergistic effects of the combination. Pomegranate and rosemary plant extracts were the most successful at inhibiting biofilm by lowering swimming and twitching motility, which in turn decreased bacterial cells adhering to surfaces and quorum-sensing (QS) signals. The presence of polyphenol molecules such catechol, pyrogallol (Fig. 9), gallic, ellagic, rosmarinic acid, and benzoic acid may be referred to as these activities. There are, however, limited reports of plant extracts' antibacterial and antibiofilm activity [114, 310–312]. As a result, combining plant extracts with antibiotics may be able to prevent and get rid of microbial biofilms. Pomegranate and rosemary plant extracts combined with piperacillin, ceftazidime, imipenem, gentamycin, or levofloxacin had synergistic effects against a *P. aeruginosa* isolate and dramatically reduced biofilm mass after 24 h compared to the use of the plant extracts separately or together. To investigate the effects of medicinal plants and commercial antibiotics against bacterial pathogens, additional in vitro and in vivo investigations are required. In vitro tests using butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), two antioxidant food additives, and rosemary methanol extract were also conducted by Romano et al. [313] to examine the antioxidant and antibacterial properties of the extract (Fig. 9). The outcomes demonstrated that rosemary extracts (rosmarinic acid, carnosic acid, and carnosol) increased the antioxidant activity of BHT and BHA as well as the antibacterial activity of BHA.

Ginger (*Zingiber officinale*)

Zingiber officinale, a member of the Zingiberaceae family, has been used as a spice and a herbal remedy since ancient times [314]. Due to the presence of numerous bioactive components like terpene (e.g. α-curcumene, α-farnesene, β-bisabolene, β-sesquiphellandrene, and zingiberene) (Fig. 9), and phenolic compounds (e.g. gingerols, paradols, shogaols, gingerenone-A (Fig. 9), quercetin (Fig. 5), zingerone, and 6-dehydrogingerdione (Fig. 9)) [315–319], the ginger root is the most significant component that is used to cure a variety of illnesses including nausea, emesis, headaches, and colds. The majority of biological actions, including those that are antioxidant, anticancer, anti-inflammatory, and antibacterial, are caused by phenolic compounds [319–322]. Additionally, a number of studies [323–328] demonstrated that ginger can prevent and treat a wide range of illnesses, including neurodegenerative diseases, diabetes mellitus, obesity, cardiovascular diseases, respiratory problems, and nausea and emesis brought on by chemotherapy.

Antibacterial activity of ginger

Ginger essential oil is derived from the roots and has potential uses in a variety of industries, including pharmaceuticals, cosmetics, and food. *Shewanella putrefaciens* is a Gram-negative spoilage bacterium that can be found in aquatic products. *Zhang* et al. [329] studied the antibacterial activity of ginger essential oil against this bacterium. Result showed that ginger EO (Zingiberene, zingerone and α-curcumene) displayed significant antibacterial activity with MIC and MBC values of 2.0 and 4.0 μL/mL, respectively, against *S. putrefaciens* by disturbing cell membrane integrity. Additionally, ginger essential oil (EO) has the power to alter biofilm metabolism and kill it, supporting its usage as a natural food preservative. By Wang et al. [330], who extracted ginger essential oil using supercritical CO2 and steam distillation techniques, ginger was also demonstrated to be excellent for food preservation. The primary components were identified by GC–MS as zingiberene and α-curcumene, and their antibacterial efficacy was evaluated mostly against the pathogens that cause food deterioration, *E. coli* and *S. aureus*. Through bacterial cell membrane damage that resulting in protein and nucleic acid leaks, ginger EOs demonstrated remarkable bactericidal efficacy. This decreases bacterial metabolic activity, which ultimately leads to

bacterial cell death. The expression of several genes encoding important enzymes, the tricarboxylic acid cycle, DNA metabolism, and proteins involved in cell membranes is all inhibited by ginger EOs. Similar mechanisms of action were also observed by Zhang et al. [331] who found that ginger extract damaged the *Ralstonia solanacearum* cell membrane's permeability and integrity, leading to the leakage of several cell components, including nucleic acids, proteins, and others, as well as changes in the bacteria's shape. Additionally, Atai et al. [332] showed that ethanol ginger extract can be utilized to treat oral candidiasis and suppress the growth of *Candida albicans*. In the same context, Park et al. [333] assessed the ginger's ability to suppress mouth microorganisms linked to periodontitis. Result revealed that ginger extracts including 10-gingerol and 12-gingerol (Fig. 9) exhibited antibacterial activities against anaerobic Gram-negative bacteria, *Porphyromonas endodontalis, Porphyromonas gingivalis*, and *Prevotella intermedia* which causes periodontal diseases. Chairgulprasert et al. [334] extracted ginger EOs by steam distillation of fresh rhizomes and identified its chemical constituents by GC–MS which found the presence of *trans*-anethole, *m*-phenylphenol, (Fig. 9) estragol, and camphor in the extract. Results showed that petroleum ether and dichloromethane extracts in addition to the EOS were able to inhibit different bacterial pathogens such as *E. coli, S. aureus, Bacillus substilis,* and *Sarcina* sp.; on the other hand, no activity was recorded against *P. aeruginosa.* Research investigations are focusing on the effectiveness of combining medicinal herbs with antibiotics for enhanced antibacterial action. Using antibiotics (Ceftazidime), Sagar PK et al. [335] assessed the antibacterial activity of crude methanol extracts of medicinal plants like eucalyptus, clove, and ginger against *P. aeruginosa*. Isolates. With ceftazidime, all plant extracts had a synergistic impact on *P. aeruginosa*. When ginger and eucalyptus extracts were mixed, their separate MICs were not reduced; however, when ginger and clove extracts were combined, a maximum twofold reduction in MIC was discovered. When ginger extract was used with aminoglycosides to treat vancomycin-resistant *enterococci* (VRE), MIC values were also lowered. By increasing membrane permeability and improving the influx of aminoglycosides into enterococcal cells, 10-gingerol has been shown by Nagoshi et al. [336] to be able to lower MICs of many aminoglycosides, including arbekacin, bacitracin, and polymyxin B. The effectiveness of 6-gingerol alone and in combination with amphotericin B against Leishmania in vivo murine models was evaluated by Alireza Keyhani et al. [337]. In addition to its capacity to create an apoptotic index, raise the expression of Th1-related cytokines, and decrease transcription factor levels, the combination demonstrated a strong antioxidant and extreme leishmanicidal activity. When combined with fluconazole, methanol ginger extract demonstrated antifungal activity against drug-resistant *vulvovaginal candidiasis* (VVC) in a mouse model, according to Khan et al. [338]. As compared to fluconazole or ginger extract used alone, which did not entirely cure VVC, in vitro results revealed better activity for the combination of fluconazole and ginger extract against *C. albicans*. Azole-resistant candidiasis may therefore be treated by administering this combination.

Cactus (*Opuntia ficus-indica*)

Opuntia ficus-indica (*L.*), sometimes known as the nopal cactus, is a tropical and subtropical plant found in South Africa, Mexico, Latin America, and Mediterranean nations [339]. All cactus portions contain high levels of polyphenols, which have anti-inflammatory and antioxidant qualities. The most significant source of polyphenols and flavonoids, including gallic acid, is found in flower parts. These compounds have cytotoxic and antioxidant properties that lessen DNA damage [340–342]. Cactus fruit, also known as cactus pear, also contains fibres, ascorbic acid, vitamin E, amino acids, and carotenoids, all of which have hypoglycemic and hypolipidemic effects [342–344]. Due to the abundance of fatty acids, it contains, including oleic acid, palmitic acid, linolenic acid, and linoleic acid (Fig. 10); cactus cladodes can have a hypocholesterolemic impact [345, 346]. Therefore, *Opuntia ficus indica* have been used in traditional medicine due to the abundance natural compounds and derivatives for treating burns, oedema, wounds obesity, and hyperlipidemia, in addition to its antiviral, anti-inflammatory, and hypoglycemic properties[347].

Fig. 10 [Images not available. See PDF.]

Chemical structure of bioactive compounds from nopal cactus including oleic acid, palmitic acid, linolenic acid, linoleic acid, octadecadienoic acid, dihydroauroglaucin, isotetrahydroauroglaucin, cristatumin B, quercetin5,4'-

dimethyl ether, isorhamentin3-O-glucoside, isorhamnetin

Antibacterial activity of Cactus

The extensive use of cactus pears flowers in traditional medicine is a result of their abundance in natural bioactive substances. Protein, fibre, and minerals make up the chemical makeup of cactus hexane extracts, as demonstrated by Ennouri et al. [348]. In the plant extract, octadecadienoic acid and palmitic acid (Fig. 10) were the primary components and potassium was the leading mineral. Antibacterial activity against *E. coli* and *S. aureus* was highly effective which suggest using cactus as a food preservative. There are few studies available on the antibacterial activities of *O. ficus-indica*. The plant has proven to be effective in wound healing and skin conditions such as healing laser-induced skin burns. Khémiri et al. [349] demonstrated that cactus was able to inhibit cutaneous infections by showing antimicrobial effect against *Enterobacter cloacae,* antifungal activity against *Penicillium, Aspergillus, and Fusarium* (opportunistic cutaneous moulds), and antiyeast effect against *Candida sake* and *Candida parapsilosis*. Therefore, cactus-extracted oil is a good healing agent due to its antibacterial effect and the ability to reduce reepithelialization phase. Future investigations are needed to identify the active compounds in the oil extract and its mechanism of action involved in the healing process. Ammar et al. [350] also evaluated cactus flowers extracts (mucilaginous and methanol) antioxidant and antibacterial activities for enhancing wound healing in excision wound model in rats. A beneficial effect was noticed on cutaneous repair which assessed by acceleration in wound contraction and remodelling phases. Both extract showed antibacterial activities against tested gram-negative and gram-positive bacterial strain, *E. coli, S. aureus, Bacillus subtilis,* and *Listeria monocytogenes*, while no antibacterial activity was noticed against *P.aeruginosa.* The result supports the use of *O. ficus-indica* as therapeutic agent for dermal wound healing, but more research is needed to know the exact mechanism of action. Alqurashi et al. [351] explored biological activities of oil from *Opuntia ficus-indica* seed and showed that it possesses an inhibitory action against *Saccharomyces cervisiae* while no activity against *Aspergillus niger.* On the other hand, Elkady et al. [352] isolated endophytic *Aspergillus niger* fungus from cactus fruit peels and tested the effect of endophytic ethyl acetate extract and its isolated compounds (dihydroauroglaucin, isotetrahydroauroglaucin, and cristatumin B) (Fig. 10) on resistant bacterial strains. Result showed excellent activity against Gram-negative and gram-positive resistant bacteria. Another study done by Elkady et al. [353] tested methanol extract of cactus against pneumonial pathogens (*Streptococcus pneumoniae, Klebsiella pneumoniae, P. aeruginosa, Legionella pneumophila, Moraxella catarrhalis, and Stenotrophomonas maltophilia*). The isorhamentin3-O-glucoside and isorhamnetin (Fig. 10) compounds show moderate antibacterial activity against all tested microorganisms, while quercetin5,4'-dimethyl ether, which was isolated from the cactus ethyl acetate fraction, demonstrated the most antibacterial activity. According to these findings, *O. ficus-indica* and the components that were extracted from it can work as new all-natural antibacterial agents for the treatment of infectious disorders. Further studies in vivo are needed to confirm the antibacterial activity of the extracts. Sánchez et al. [353] studied antibacterial and antibiofilm activity of several methanolic plant extracts against nosocomial microorganisms. Methanolic plant extracts were tested against specific clinical bacterial isolates by using well diffusion method, and results showed that Prosopis *laevigata* extract was active against all the clinical isolates with highest inhibition diameter against *S. aureus* strain compared to *Gutierrezia microcephala* and *O. ficus-indica* that showed lesser inhibition diameter and no activity for Nothoscordum *bivalve* extract. *E. coli* was less susceptible, while *K. pneumoniae* and E*. faecalis* were more resistant to the extracts except for P*. laevigata* and *O. ficus-indica.* The major reduction on the specific biofilm formation index (SBF) in dose-dependent manner and on cytotoxic activity (using brine shrimp lethality test) was caused by *O. ficus-indica.* Unfortunately, there are no available studies and data on the synergistic effect of *O. ficus-indica* with antibiotics which highlights the need for researcher to focus on studying synergistic or additive effect of herbal plants, especially *O. ficus-indica* with antibiotic to discover new treatments.

Medicinal plants and wound healing

The epidermis, dermis, and hypodermis are the three layers of the skin, one of the biggest organs in the body. Additionally, it serves as the initial line of defence against aggressors like infections, chemicals, and physical contact. Due to the function of the epidermis, which blocks the entry or exit of water- or water-soluble substances,

and the hypodermis, which blocks heat loss due to the poor thermal conductivity of fat, it also has the capacity to prevent water loss and preserve temperature [354–357]. In order to maintain skin hemostasis during inflammation, immune cells and nonimmune cells form a structure known as skin-associated lymphoid tissue (SALT). Additionally, the skin's microbiome, which includes bacteria, fungi, and viruses, is crucial for immune response, concluding that the skin serves as more than just a physical barrier [354, 358, 359]. Over the years, natural product ingredients such polyphenols, fatty acids, probiotics, polysaccharides, and others have demonstrated their efficacy as immune system modulators. The secret to controlling or curing skin inflammatory problems may lie in using natural products [360–362]. Skin can react to infectious agents through innate and adaptive immune processes, just as other tissues like mucosal surfaces. To isolate the damaged area, stop bleeding, and initiate the coagulation cascade, a clot must first form in order for the wound healing process to begin. Then comes the inflammatory phase, where immune cells begin to infiltrate and high levels of pro-inflammatory mediators are discovered to stop pathogen entry and more serious problems. The next stage is the proliferative phase, which is characterized by a significant growth of skin-resident cells including fibroblasts and high levels of angiogenesis. The remodelling phase, which may last for more than a year after the injury, is the longest and involves the skin regaining its natural structure. Any issue throughout these stages may hamper wound healing, which may then result in infections, excruciating pain, and occasionally neurological damage [363–366]. Several factors can cause impaired wound healing such as local factors that influence the characteristics of the wound like oxygenation and infections and systemic factors in which overall health or disease state affects the ability to heal like hormones and diabetes [367–370]. Impaired wound healing can result from a variety of factors, including local ones that alter the characteristics of the wound, such oxygenation and infections, as well as systemic ones, like hormones and diabetes, that have an impact on overall health or disease states and the capacity to heal. Numerous abnormalities, including fibrosis, scarring, and nonhealing wounds like persistent ulcers, can result from aberrant wound repair [371, 372]. New compounds with antioxidant, anti-inflammatory, and anticarcinogenic properties are being researched to prevent skin damage. Natural substances have been employed as antitumoural, analgesic, anti-inflammatory, and antioxidant agents [373, 374]. Table 2 lists the top plants for healing wounds.

Table 2. Most used medicinal plants in treating skin disorders

Plant name	Active compounds	Therapeutic uses	References
Achillea millefolium L	Flavonoids, monoterpenes, and sesquiterpenes	Skin inflammatory and wound healing	[375–379]
Aloe vera	Acemannan (Fig. 11)	Wound healing	[380, 381]
Bletilla striata	Triterpenoids and polysaccharides	Drug delivery, wound dressing, and wound healing	[382, 383]
Blumea balsamifera	L-Borneol (Fig. 11)	Dermatitis, eczema, skin bruises, and skin injury	[384, 385]
Boswellia sacra	Boswellic acids (Fig. 11)	Improvement of blood circulation, pain treatment, and rheumatoid arthritis	[386–389]

Caesalpinia sappan	Brazilin and Sappanchalcone (Fig. 11)	Improvement of blood circulation, pain treatment, and oedema	[390, 391]
Calendula officinalis	Esculetin, and Quercetin-3-O-glucoside (Fig. 11)	Burns, dermatitis, and wound healing	[392–394]
Celosia argentea	Celosin I and Celosin II (Fig. 11)	Skin sores and ulcers	[395, 396]
Centella asiatica	Asiaticoside and Madecassoside (Fig. 11)	Wounds healing	[397–399]
Cinnamomum cassia	Cinnamaldehyde (Fig. 5)	Analgesia and improvement of blood circulation	[399, 400]
Commiphora myrrha	Furanoeudesma-1,3-diene and Terpene (Fig. 12)	Gastrointestinal diseases, wounds, and pain	[401–404]
Curcuma longa	Curcuminoids (Fig. 12)	Digestive diseases, liver disorders, menstrual difficulties, pain disorders, sprains, and wounds	[405, 406]
Entada phaseoloides	Tannin (Fig. 12)	Aging, atherosclerosis, cancer, diabetes, and neurodegenerative disorders	[407, 408]
Ganoderma lucidum	Ganoderma lucidum polysaccharide	Cancer, diabetes, hepatitis, leukaemia, and ulcer	[409–413]
Ligusticum striatum	Phthalide lactones, and alkaloids	Antiatherosclerotic, antioxidant, neuroprotective, and vasorelaxant	[414–418]
Panax ginseng	Ginsenosides (Fig. 12)	Laser burn, excision wounds models in mice, cell migration, and wound healing assays	[419–424]
Polygonum cuspidatum	Emodin, polydatin, and resveratrol (Fig. 12)	Hepatitis, hyperlipidemia. Jaundice, scald, skin burns, and suppurative dermatitis	[425–428]
Rheum officinale	Emodin (Fig. 12)	Chronic kidney disease, hepatitis, and wounds healing	[429–431]

Sanguisorba officinalis	Polysaccharides, tannins, triterpenoid glycosides, and triterpenoids	Burns, chronic intestinal infections, haemorrhoids, menorrhagia, and scalds	[432–434]
Sophora flavescens	Kushenol, and sophoraflavanone B (Fig. 12)	Asthma, burns, dysentery, eczema, fever, hematochezia, inflammatory Jaundice, oliguria, and vulvar swelling	[435]
Wedelia trilobata	Kaurenoic acid (Fig. 12) and Luteolin (Fig. 5)	Arthritic painful joints, rheumatism, and stubborn wounds	[436, 437]
Zanthoxylum bungeanum	Afzelin, hyperoside quercitrin, and rutin (Fig. 12)	Skin wrinkles	[438, 439]

Fig. 11 [Images not available. See PDF.]

Chemical structure of active compounds found in medicinal plants used to treat skin disorders including, acemannan, L-Borneol, boswellic acids, brazilin, sappanchalcone, esculetin, quercetin-3-O-glucoside, celosin I, celosin II, asiaticoside, and madecassoside

Fig. 12 [Images not available. See PDF.]

Chemical structure of active compounds found in medicinal plants used to treat skin diseases cont. including cinnamaldehyde, furanoeudesma-1,3-diene, tannin, ginsenoside, emodin, polydatin, resveratrol, kushenol, sophoraflavanone B, kaurenoic acid, afzelin, hyperoside, and rutin

Biopolymers

Pathogenic microorganisms (bacteria, viruses, and unicellular and multicellular eukaryotes) can cause several series diseases which are a public health concern [440]. Different pathogens accumulate in acute and chronic wounds such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, and others which can affect wound healing process and increase life-threatening problems [441, 442]. Because they are naturally occurring biomolecules derived from bacteria, animals, and plants, biopolymers play a significant role in wound care. These bioactive features include antimicrobial, cell proliferative, angiogenic, and immune-modulatory polymers. These qualities, along with their biodegradability, renewability, and decreased antigenicity, rendered them more advantageous for the healing process than synthetic materials, which have problems with biocompatibility because of their harmful effects [443–445]. Natural biomaterials like cellulose, hyaluronic acid, collagen, alginate, and chitosan (Table 3) are frequently used in the wound care industry. With the advancement of technology, the characteristics of biopolymers can be improved to meet a variety of wound care needs, including tissue repair, scar-less healing, and integrity restoration of lost tissue [446, 447]. By preventing microbes from directly interacting with the bacterial cell wall, producing ROS to increase oxidative stress, and inducing the leaking of macromolecules like DNA and proteins from microorganisms, antimicrobial biomaterials can aid in the healing of wounds [442, 448].

Table 3. Natural biopolymers used in wound healing process

Biopolymer	Source	Biological role	References
Collagen	Cattle and porcine slaughterhouse wastes	Skin tissue engineering, nerve regeneration, vitreous replacement, coating of bioprostheses, and others	[449–452]

Cellulose	Plant's cell wall (e.g. *Hibiscus Cannabinus*) and in several bacteria (e.g. *Gluconacetobacter,* *Agrobacterium*, and *Sarcina*)	Artificial skin substitute and as regeneration of tissue for wound healing purposes	[453, 454]
Alginate	Brown algae	Biomedical, cosmetics, pharmaceutical, food industries	[455–459]
Hyaluronic acid	Synovial fluid, articular cartilage, and mammalian bone marrow	Bone regeneration, tissue engineering, drug delivery, tumour cell targeting, skin aging treatment, reduced wrinkles, skin firmness, skin hydration, wound healing, and others	[459–462]
Chitosan	Partially deacetylation of chitin	Wound healing, tissue engineering, cartilage regeneration, gene delivery, drug delivery, bioadhesive, and others	[459, 463]
κ-carrageenan	Red seaweeds (Rhodophyceae)	Anti-inflammatory, antithrombotic, cytoprotective, antiviral, antitumour, antioxidant, antipathogenic, drugs delivery, wound dressing, and wound repair	[464, 465]
Silk fibroin	Silkworms, spiders, and other insects	wound healing, skin restoration, cellular adhesion, wound contraction, re-epithelialization, angiogenesis, collagen production, stimulation of cell migration, and inactivation of the apoptotic pathway	[466–468]

Extracellular vehicles (EVs)

Extracellular matrix (ECM), growth factors, and hormones are just a few of the biomaterials and composites that have recently undergone modifications to improve cell survival, motility, and proliferation. One of the most promising methods for wound healing is extracellular vehicles (EVs). EVs are released by a variety of cells and are crucial for the phases of wound healing (hemostasis, inflammation, proliferation, and remodelling) as well as intercellular communication that promotes regeneration. Additionally, by labelling EVs with certain surface proteins, they can be created to carry particular cargo and employed for targeted delivery [469–473]. Exosomes, which are created during endosomal sorting, microvesicles, which directly arise from plasma membranes, and apoptotic bodies, which are created following cell death, can all be classified as subpopulations of EVs [474–476]. The regulation of hemostasis and each stage of wound healing are considerably aided by EV. The most prevalent EVs in blood circulation are platelet-derived EVs (PEVs) [476–478]. The activated form of integrin IIb-3 mediates the role of PEVs from thrombin-stimulated platelets in the development of fibrin clots [479]. This form of integrin αIIbβ3 has a high affinity for fibrinogen. PEVs were also discovered to bind tissue factor (TF) and factor XII, as well as to stimulate the generation of thrombin, but only in the presence of factor VII and xii, indicating that they mediate both intrinsic and extrinsic coagulation pathways [480]. Neutrophil-derived EVs (NDEVs) have been shown to have pro- or anti-inflammatory effects by boosting the production of ROS and IL-8 [481]. EVs are also involved in inflammation. Additionally, EVs take part in the remodelling phase (starting fibroplast differentiation) and the proliferation phase (EVs from wound edge keratinocytes, or KCs-EVs) [482]. EVs can be made from plants, stem cells, or engineering [482, 483]. Membranous vesicles formed from plants are similar to mammalian exosomes but have different chemical

compositions and include fewer proteins and no cholesterol in the lipid layer. EVs made from plants are less harmful, safer, and expensive. Wheat, broccoli, ginger, grapefruit, grape, lemon, and EVs were also discovered in the xylem and phloem of woody plants, according to recent investigations [484, 485]. Several plant have been known in pharmacognosy and phytochemistry to own antibleeding and hemostatic properties such as *Rubia cordifolia*, *Alchornea cordifelia*, *Aspilia africana*, *Baphia nitida*, *Ageratum conyzoides*, *Chromolaena odorata, Jathropha curcas* , *Landolphia owariensis, Dalbergia sissoo*, *Aloe spesiosa*, *Beta vulgaris*, *Humulus lupulus*, *Salix alba*, and others [486–488]. Different studies demonstrated the effect of plant-derived EVs on wound healing; Perut et al. [489] isolated and purified plant-derived exosome-like nanovesicles (EPDENs) from strawberry juice of *Fragaria* x *ananassa* which is characterized of high anthocyanins, folic acid, flavonols, vitamin C, and short RNAs and miRNAs contents. The uptake of *Fragaria*-derived EPDENs by mesenchymal stromal cells (MSC) did not affect cell viability and prevented oxidative stress which may be due to the presence of vitamin C. Additionally, exosome-like nanovesicles isolated from Citrus limon L. (EXO-CLs) were investigated and examined in vitro on MSC by Baldini et al. [490]. The findings indicated that EXO-CLs contained short RNA sequences (20–30 bp), vitamin C, and citrate. EXO-CLs were taken up by MSC and had a significant antioxidant activity, according to in vitro tests. Ju et al. team also found that grape exosome-like nanoparticles (GELNs) have a protective effect against dextran sulphate sodium-induced colitis and facilitate intestinal tissue remodelling [491]. Similar outcomes were also observed with ginger-derived EVs, which promoted intestinal wound healing and are presently being investigated in clinical trials for inflammatory bowel disease and colon cancer [320, 482, 484]. Other plant-derived EVs, such as those from grapefruit and wheatgrass, improved wound healing by boosting cell viability and motility [485, 492]. The formation of hypertrophic scars and keloid lesions has also been demonstrated to be decreased by herbal extracts and active herbal compounds, including onion extract, epigallocatechin gallate from green tea, resveratrol obtained from peanuts, and others [493]. To retain EVs at the wound site and encourage longer, more effective results, they can be applied topically or encapsulated into scaffolds like hydrogels. Natural polymers like chitosan, alginate, and collagen are examples of hydrogels. Synthetic polymers like polyethene glycol, polyglycolic acid, and polyurethane are also possible [494–497]. A promising method to introduce EVs into the wound site with extended release involves encapsulating them in hydrogels [498]. To create naturally produced EVs for wound healing, further research on particular plant species is required.

Treatment of skin disorders

Diabetic foot ulcers (DFUs)

Patients with uncontrolled diabetes mellitus frequently develop diabetic foot ulcers (DFUs), which can be brought on by poor glycemic management, peripheral vascular disease, neuropathy, or inadequate foot care [499]. According to the International Diabetes Foundation [500], there are 40–60 million persons worldwide with DFUs. DFUs can be treated using several methods, including as gene therapy, stem cells, skin substitutes, and antibiotics. Due to the high cost of local debridement (removal of nonviable wound tissue) and negative pressure therapy, antibiotic resistance as a result of prolonged use, the ineffectiveness of growth factors to inhibit bacterial growth, and the existence of stem cell and gene therapies in the experimental stage, nonsurgical treatments for DFUs must be quick and inexpensive [501]. Due to their anti-inflammatory qualities, several herbal extracts have been utilized as traditional treatments to treat wounds. Liu et al. [501] designed an experiment to explore the effects of five herbal extracts on wound healing, *Bauhinia purpurea* (inhibit inflammation, and act as analgesic and antipyretic) [502], *Paeoniae rubrae* (ameliorating inflammation by inhibiting glycogen synthase kinase 3β (GSK3β)) [503], *Angelica dahurica* (accelerate wound healing by regulating inflammation) [504], *Acorus calamus* L (promote collagen maturation) [505], and *Radix Angelicae biseratae* (inhibit inflammation and regulate immune system in osteoarthritis) [506]. In order to determine the mechanisms of action for wound healing, the mixture of herbal plants was identified by Ultra-High-Performance Liquid Chromatography (UHPLC) and Quadrupole Exactive-Mass Spectrometer (QE-MS) and tested in vivo on a rat model with diabetic ulcer wound utilizing transcriptomics and proteomics. The mixture speeds up the healing of wounds by encouraging angiogenesis and the growth of M2 macrophages, according to the results. Specific miRNAs and proteins were found to be crucial for controlling wound healing by

transcriptomics and proteomics. Consequently, the herbal combination may offer a potential method to quicken the healing of diabetic wounds [501].

Atopic dermatitis (AD)

Atopic dermatitis (AD), a chronic and relapsing inflammatory skin condition that affects children and is characterized by itchy, eczematous skin lesions, is another skin condition [507, 508]. Intense itching results in skin damage that compromises tissue repair and allows microorganisms to infiltrate the skin [509, 510]. The absence of precise disease processes is a challenge for the development of successful AD therapeutics. Flavonoids, a type of secondary metabolite found in plants, exhibit a variety of antiallergic properties, including antioxidant, anti-inflammatory, antiangiogenic, antibacterial, and antiviral effects [511, 512]. In foods ingested as part of a daily diet, quercetin (Fig. 5) is an illustration of a flavonoid [513]. Quercetin inhibits the release of histamine, proinflammatory cytokines, and interleukin (IL)-4 and -13, among other antiallergic characteristics. Despite this, there have only been a few research on quercetin's effects on AD [514, 515]. Therefore, Beken et al. [516] studied the effect of quercetin on AD model of human keratinocyte and treated it with IL- 4, -13, and tumour necrosis factor-α (TNF-α) to mimic AD in vitro. Result showed that quercetin accelerated wound healing by reducing AD-inducing agents IL-1β, IL-6, IL-8, thymic stromal lymphopoietin, phosphorylation of extracellular signal-regulated kinase 1/2/mitogen-activated protein kinase (ERK1/2 MAPK), and nuclear factor-kappa B (NF-κB), while it upregulated the expression of IL-10, and antioxidant enzymes; glutathione peroxidase (GPx), superoxide dismutase-1 (SOD1), SOD2, and catalase (CAT), in addition to mRNA expression of Twist and Snail. Therefore, quercetin may act as a potential therapy for AD symptoms.

Conclusions and future recommendations

Extensive and inappropriate uses of antibiotics resulted in the development of antimicrobial resistance and the rise of bacterial strains that were resistant to multiple drugs (MDR) and multiple drugs extensively (XDR), which made the most powerful medications useless. Antimicrobial resistance is very concerning and urgent issue, and scientists are aware that the shelf life of antibiotics is finite. Natural product research is receiving a lot of interest internationally. Today, millions of people around the world turn to phytomedicine as one of their top options for the treatment of chronic illnesses. Medicinal plant extracts are crucial in the fight against infectious diseases that pose a threat to public health globally. Alkaloids, phenolics, polyphenols, terpenoids, essential oils, lectins, polypeptides, and polyacetylenes are a few examples of antimicrobial phytochemicals that can be utilized as adjuvants or substitutes against bacterial infections. Medicinal plants including St. John’s wort (*Hypericum perforatum*), Rosemary (*Rosmarinus officinalis*), Ginger (*Zingiber officinale*), and nopal cactus (*Opuntia ficus-indica* (*L.*)) are attracting the interest of researchers due to their high phytochemical contents. Additionally, probiotics, polysaccharides, polyphenols, fatty acids, and other bioactive substances can modulate the immune system and treat inflammatory skin diseases such atopic dermatitis (AD) and diabetic foot ulcers (DFUs). Natural anticarcinogenic, anti-inflammatory, and antioxidant substances can be employed to stop skin deterioration. Biopolymers derived from microorganisms, animals, and plants (cellulose, hyaluronic acid, collagen, alginate, and chitosan) and extracellular matrix (ECM) have bioactive properties that make them promising approaches for wound healing. These properties include antimicrobial, immune-modulatory, cell proliferative, and angiogenic effects.

It is essential to create innovative plans and tactics to deal with the issue of rising AMR. Medicinal plants contain unlimited source of bioactive compounds which has been used in the treatment of many diseases, especially against bacterial infections. Despite that fact, natural compounds have not yet been thoroughly investigated and many are still unexplored. Therefore, researchers should make efforts to isolate and identify new bioactive compounds from plant source to face antibacterial resistance and find new effective treatments. Researchers should focus on using appropriate extraction methods to isolate compounds from bioactive extracts, study the mechanism of action, test the compounds in vivo in animal models, and apply structural modification to improve pharmacodynamics and pharmacokinetics. Moreover, further studies should be done on the synergistic or additive interactions between plant compounds itself and with antibiotics to enhance the action of medications.

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Abbreviations

AMPs
Antimicrobial peptides
AD
Atopic dermatitis
BHT
Butylated hydroxytoluene
BHA
Butylated hydroxyanisole
CAT
Catalase
COPD
Chronic obstructive pulmonary disease
CNS
Coagulase-negative staphylococci
CHF
Congestive heart failure
CVNHs
Cyanovirin-N homologs
COX-2
Cyclooxygenase-2
CXCL
Chemokine
CRPs
Cysteine-rich peptides
DFUs
Diabetic foot ulcers
DCM
Dichloromethane
EAE
Enzyme-assisted extraction
SFE

Supercritical fluid extraction
EOs
Essential oils
EDA
Eucheuma denitculatum
ESA
Eucheuma serra
EXO-CLs
Exosome-like nanovesicles isolated from *Citrus limon* L.
ECM
Extracellular matrix
ERK1/2 MAPK
Extracellular signal-regulated kinase 1/2/mitogen-activated protein kinase
EVs
Extracellular vehicles
GNA-like
Galanthus nivalis agglutinin-like
GMA
Galaxaura marginate
GC
Gas chromatography
GPx
Glutathione peroxidase
GSK3β
Glycogen synthase kinase 3β
GELNs
Grape exosome-like nanoparticles
SD-GCMS
Headspace-gas chromatography/mass spectrometry
HTS
High-throughput screening
HPLC
High-performance liquid chromatography
HPTLC
High-performance thin-layer chromatography
HA
Hyaluronic acid
HPF
Hyperforin
Hyp
Hypericin
IR
Infrared spectroscopy
IFN-γ
Interferon
IL
Interleukin

IAs
Isoquinoline alkaloids
JRL
Jacalin-related lectin
LC–ESI–MS/MS
Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometric
LPS
Lipopolysaccharide
MS
Mass spectrometry
MSC
Mesenchymal stromal cells
MeOH
Methanol
MRSA
Methicillin-resistant *S. aureus*
MSCRAMM's
Microbial surface components recognizing adhesive matrix molecules
MICs
Minimum inhibitory concentrations
MDR/XDR
Multi- and extensively drug-resistant
NDEVs
Neutrophil-derived EVs
NLRP3
NOD-like receptor 3
NF-κB
Nuclear factor-kappa B
NMR
Nuclear magnetic resonance spectroscopy
OGEO
Ocimum gratissimum L. essential oil
OAAH
Oscillatoria agardhii agglutinin homolog
OA
Osteoarthritis
PC
Paper chromatography
PEVs
Platelet-derived EVs
TPA
Proanthocyanidin
PGE2
Prostaglandin E2
QE-MS
Quadrupole exactive-mass spectrometer
QS

Quorum sensing
ROS
Reactive oxygen species
STAT
Signal transducer and activator of transcription
SALT
Skin-associated lymphoid tissue
SPME-GC/MS
Solid-phase microextraction-gas chromatography/mass spectrometry
SJW
St. John’s wort
SOC
Standard of care
SAHA
Suberoylanilide hydroxamic acid
S-CO2
Supercritical carbon dioxide
SF
Supercritical fluid
SOD1
Superoxide dismutase-1
THC
Tetrahydrocannabinol
TLC
Thin-layer chromatography
TF
Tissue factor
TLR4
Toll-like receptor 4
TFC
Total flavonoid contents
TPC
Total phenolic
TCM
Traditional Chinese medicine
TB
Tuberculosis
TNF-α
Tumour necrosis factor-α
UHPLC
Ultra-high-performance liquid chromatography
UAE
Ultrasound-assisted extraction
UV
Ultraviolet spectroscopy
VRE
Vancomycin-resistant enterococci

VBNC
Viable but nonculturable cells
VVC
Vulvovaginal candidiasis
WHO
World Health Organization
ARs
α2-Adrenergic receptors

Publisher's Note

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Prevention of paclitaxel-induced peripheral neuropathy: literature review of potential pharmacological interventions

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ABSTRACT (ENGLISH)

Background

Paclitaxel administration is considered a keystone in the management of many types of cancers. However, paclitaxel chemotherapy often leads to peripheral neuropathy which is the most prominent adverse effect that reduces the patient's quality of life and demands dose reduction leading to decreased disease curing. Paclitaxel induces peripheral neuropathy through disruption of microtubules, distorted function of ion channels, axonal degeneration, and inflammatory events. So far, there is no standard medication to prevent the incidence of paclitaxel-induced peripheral neuropathy (PIPN).

Main body

Numerous preclinical studies in rats and rodents showed that several therapeutic agents have neuroprotective mechanisms and reduce the incidence of PIPN, proving their effectiveness in the prevention of PIPN in animal models. Different mechanisms, such as reduction of the expression of inflammatory mediators, quenching of reactive oxygen species, prevention of neuronal damage, and other mechanisms, have been explored. Moreover, many clinical trials have further established the neuroprotective effect of several investigational drugs on PIPN. Twenty preclinical studies of pharmacological interventions were reviewed for their preventive effect on neuropathy. These medications targeted cannabinoid receptors, oxidative stress, inflammatory response, and ion channels. Additionally, 25 clinical studies with pharmacological preventive interventions of PIPN have been reviewed, of which only 10 showed preventive action in PIPN.

Conclusion

Prevention of PIPN is currently considered an emergent field of research. This review highlights the potential interventions and presents recent findings from both preclinical and clinical studies on the significant prevention of PIPN to help in effective decision-making. However, further well-designed research is required to ascertain recommendations for clinical practice.

FULL TEXT

Background

Taxanes are considered first-line chemotherapeutic agents often used in several cancers including those of the breast, ovaries, prostate, gastric, head and neck, and non-small lung cancers [1]. Paclitaxel is one of the important and commonly used antineoplastic agents in this class due to its microtubule-stabilizing mechanism of action. It is commonly used in the management of several cancer types with extensive evidence proving its antimitotic effect [2, 3].

One of the prime adverse effects of taxanes including paclitaxel is peripheral neurotoxicity known as peripheral neuropathy [4]. Up to 97% of patients receiving paclitaxel will develop paclitaxel-induced peripheral neuropathy (PIPN), which turns into a chronic condition in more than 60% of cases [5]. It decreases the efficacy of the chemotherapy by causing patient discomfort and often resulting in dosage reduction or chemotherapy treatment termination. Significantly, those who suffer from chronic neuropathy have considerably poorer long-term quality of life [4].

Peripheral neuropathy is a neurological disorder affecting sensory, motor, and autonomic peripheral nerves [6]. Neuropathic pain resulting from paclitaxel administration develops because of somatosensory nerve damage. Mice administered with paclitaxel exhibit elevated activating transcription factor-3 levels in their large and medium dorsal root ganglion (DRG) neurons, which is indicative of neuronal damage [7]. In PIPN, axonal deterioration and depletion of intra-epidermal nerve fibers (IENFs) was reported, showing that DRG injury is the primary cause of taxane-induced nerve damage [8]. Taxane-induced peripheral neuropathy has been linked to several pathophysiological pathways, including oxidative stress, mitochondrial damage, and microtubule disruption [9].

These pathological pathways are demonstrated in Fig. 1.

Fig. 1 [Images not available. See PDF.]

Pathophysiology of paclitaxel-induced peripheral neuropathy (PIPN). *ATP* adenosine triphosphate, *C3* complement 3, *CX3CL1 C-X3-C motif chemokine ligand 1*, *IL* interleukin, *TNF* tumor necrosis factor, *ROS* reactive oxygen species

Paclitaxel alters microtubule dynamics which results in impairment in the passage of nutrients, organelles, and neurotransmitters across the neuronal axon leading to axonal degeneration or axonopathy [10]. Also, mitochondrial dysfunction such as morphological alterations, electrolyte imbalance, and reactive oxygen species (ROS) generation has been considered an important element in PIPN. Furthermore, PIPN in rats was linked to the emergence of behaviors caused by pain and mitochondrial disruption in myelinated fibers and C-fibers [11]. Oxidative stress and inflammatory mediators also have a critical importance in the pathophysiology of PIPN [11, 12]. The number of cycles, length of therapy, patient's age, use of other neurotoxic medications, as well as the presence of risk factors like diabetes, alcoholism, and previous neuropathy, have been linked to the development of PIPN. Additionally, genetic polymorphism in genes like CYP2C8 (cytochrome P450 family 2 subfamily C member 8) and KCNN3 (potassium calcium-activated channel subfamily N member 3) is also linked to the occurrence of PIPN [13].

The most common clinical presentation of PIPN patients is paranesthesia, tingling, and burning "stock and glove." In more extreme cases, however, it can lead to loss of sensation, motor deficiencies, and autonomic malfunction. Patients typically exhibit sensory symptoms with a "stocking and glove" description, affecting the extremities and extending toward the proximal body parts. Hyperalgesia and allodynia, due to tactile and heat stimuli, may be experienced [14, 15].

Because of the evolving pathophysiologic pathways mechanisms and the diversity of causes and risk factors, preventive interventions are desperately needed to lower the prevalence of PIPN [16]. Despite emerging evidence, there are no established pharmacological interventions for PIPN prevention. This review aims to map the existing literature on interventions evaluated for the prevention of PIPN. A summary and overview of the types of pharmacological interventions studied through preclinical and clinical trials and their effects on outcomes were reported in this review. Additionally, an insight into the effects of genetic polymorphism on the development of PIPN was also reviewed.

Literature search

We conducted a comprehensive search in several databases, including MEDLINE through PubMed, Web of Science, the Cochrane Library, EBSCOhost, and Scopus. Studies that were published in the English language from year 1999 to year 2023 are included in this review. The search keywords used were "prevention," "neuropathy," "paclitaxel," "neurotoxicity," "chemotherapy-induced peripheral neuropathy," "controlled clinical trials," and "preclinical studies."

Main text

Preclinical studies for the prevention of paclitaxel-induced peripheral neuropathy

Multiple preclinical studies in rodent models indicate that various pharmacologic agents may offer protective effects against peripheral neurotoxicity induced by paclitaxel.

One study in experimental rats showed that the antianginal trimetazidine reduced apoptosis, oxidative stress, and neuroinflammation which are all effects resulting in axonal degeneration and are linked to recurrent paclitaxel administration. The mode of action was shown to be due to the upregulation of Notch1 and progranulin [17]. Additionally, another study further confirmed PIPN reduction by trimetazidine through modulating toll-like receptor 4 (TLR4)/p38 and Klotho protein expression in Swiss albino mice [18].

The angiotensin-II receptor blocker (ARB) losartan demonstrated protective properties against PIPN in experimental rats. Its administration proved its anti-inflammatory effect on microglia in the central nervous system (CNS) through the reduced expression of inflammatory mediators such as tumor necrosis factor-alpha (TNFα) and interleukin-6 (IL-6) and stimulation of peroxisome proliferator-activated receptor gamma (PPARγ) [19]. Similarly, telmisartan, another

ARB, reduced PIPN in mice through its inhibition of cytochrome-p450-epoxygenase (CYP2J6) and the prevention of oxidized lipid synthesis [20]. The involvement of Angiotensin-2 was further demonstrated in the preclinical study where ramipril administration attenuated functional neuropathy secondary to paclitaxel in mouse models [21].
An animal study investigating the potential anti-inflammatory role of hesperidin in PIPN has shown positive results. Hesperidin administration reduced oxidative stress and inflammatory response in nerve tissue secondary to paclitaxel use. This is assumed to be because hesperidin's antioxidant nature allows it to scavenge reactive oxygen species (ROS) generated from mitochondria, subsequently preventing membrane damage caused by ROS [22].
The antioxidant effects of vitamin C and curcumin have been investigated in suppressing PIPN with demonstrated benefits in the reduction of TNFα and IL-6 levels in the DRG of rats [23, 24]. Melatonin was found to exhibit similar effects in reducing mitochondrial damage and attenuating PIPN in animal studies [25].
Furthermore, rosuvastatin showed a reduction in pro-inflammatory mediators and oxidative stress in paclitaxel-treated mice [26]. This is speculated to be due to possessing pleiotropic and anti-inflammatory properties, further confirming its anti-neuropathic potential in rat models [27].
Medications used in the treatment of neuropathic pain such as duloxetine, pregabalin, and amitriptyline were found to attenuate PIPN in animal models [28–30]. The preventive mechanism was found to be via downregulation of pro-inflammatory biomarkers such as IL-6 and TNFα as well as upregulation of the antioxidant capacity.
Metformin was studied in paclitaxel-treated mice, and the results indicate the effectiveness of metformin in attenuating hyperalgesia priming induced by paclitaxel through its stimulation of adenosine monophosphate-activated protein kinase (AMPK) [31].
On the other hand, the effect of alogliptin, a dipeptidyl peptidase 4 (DPP-4) inhibitor, on chemotherapy-induced peripheral neuropathy was investigated using mice, and its protective effect was demonstrated only against oxaliplatin-induced neurotoxicity but not that induced by paclitaxel nor bortezomib [32].
Moreover, co-treatment with the phosphodiesterase (PDE) inhibitor cilostazol halted the dedifferentiation of Schwann cells, secondary to paclitaxel administration, mediated by cyclic adenosine monophosphate (cAMP) signaling and demyelination in a mixed culture of Schwann cells and DRG neurons [33].
The role of glutamate neurotransmitter and the development of PIPN was confirmed in rat models where the co-administration of valproate suppressed glutamate accumulation and paclitaxel-induced mechanical allodynia [34].
It was previously shown that the acute administration of paclitaxel to neuroblastoma cells in culture increased the binding of the cytoplasmic calcium-binding protein neuronal calcium sensor 1 (NCS-1) to the inositol 1,4,5 tris-phosphate receptor (InsP3R) [35, 36]. A preclinical investigation confirmed that a single prophylactic injection of Ibudilast or lithium might inhibit PIPN in mice before they received paclitaxel therapy. These substances work by interfering with the way paclitaxel, NCS-1, and the InsP3R interact [37].

Clinical studies for the prevention of paclitaxel-induced peripheral neuropathy

Several clinical randomized studies have been reported in the literature demonstrating the use of investigational drugs in the prevention of PIPN showing promising evidence of their effectiveness. Table 1 summarizes the clinical studies investigating the potential role of different agents in the prevention of peripheral neuropathy (PN) secondary to paclitaxel chemotherapy.

Table 1. Clinical studies for the prevention of paclitaxel-induced peripheral neuropathy (PIPN)

Study	Population	Chemotherapy dosing	Number of patients	Intervention	Measured outcomes	Summary of findings

Aghili et al. [38]	Breast Cancer (BC) Patients	Paclitaxel 175 mg/m^2 every 3 weeks	40	1. Gabapentin 900 mg daily PO 2. Placebo	1. NCV 2. Neuropathy grading assessed by NCI-CTCAE version 4.0	1. Significant differences in the incidence of neuropathy 2. Significant differences in Nerve Conduction velocity
Pandey et al. [39]	Breast, Ovarian and Lung Cancer Patients	Paclitaxel 135–200 mg/m^2 every 3 weeks or 60–80 mg/m^2 weekly.124	136	1. Gabapentin 300 mg 2. Placebo	1. Neuropathy grading assessed by NCI-CTCAE version 5.0 2. NCV 3. QOL assessed by EORTC-QLQ-CIPN20 score	1. Incidence of neuropathy grade 2 was lower in Gabapentin group
Ghoreishi et al. [40]	BC Patients	Paclitaxel 175 mg/m^2 every 3 weeks	57	1. Omega 3 fatty acids 1920 mg daily PO 2. Placebo	1. Neuropathy grading assessed by rTNS 2. Nerve conduction Study 3. Serum levels of omega-3 fatty acids	1. Significant differences in neuropathy
Bakry et al. [41]	BC Patients	Paclitaxel 175 mg/m^2 biweekly	73	1. Metformin 850 mg twice daily PO 2. Placebo	1. Neuropathy grading assessed by NCI-CTCAE version 5.0 2. Quality of life (QOL) assessed by FACT-GOG-NTX subscale 3. BPI-SF 4. Serum NGF 5. Serum neurotensin NT	1. Significant differences in incidence of grade 2–3 neuropathy

Khalefa et al. [42]	BC Patients	Paclitaxel 80 mg/m^2 weekly for 12 weeks	75	1. N-acetylcysteine 1200 mg daily or twice daily PO 2. Control	1. Neuropathy grading assessed by NCI-CTCAE v4.0 and mTNS 2. QOL assessed by FACT-GOG-NTX subscale 3. Serum NGF 4. Serum MDA	1. Significant differences in neuropathy grade 2–3, mTNS, and QOL scores
2. Significant increase in serum NGF and decrease in serum MDA	Haroun et al. [43]	BC Patients	Paclitaxel 175 mg/m^2 biweekly	59	1. Cilostazol 100 mg Twice daily PO 2. Placebo	1. Neuropathy grading assessed by NCI-CTCAE version 4 2. QOL assessed by FACT-GOG-NTx subscale 3. Serum NGF 4. Serum NfL
1. Significant difference in neuropathy between the two groups 2. Significant increase in serum NGF in cilostazol group 3. No significant difference in NfL	Gelmon et al. [44]	Metastatic BC Patients	Paclitaxel 250 mg/m^2 every three weeks	37	1. Amifostine 910 mg/m2 IV 2. Control	1. Reduction of neurotoxicity associated with paclitaxel 2. Compare myelosuppression, myalgias, and response rate in two groups
1. No significant differences in neuropathy between the two groups	Loven et al. [45]	Ovarian Cancer Patients	Paclitaxel 175 mg/m^2	43	1. Glutamate 1500 mg daily PO 2. Placebo	1. Signs and symptoms of peripheral neuropathy

1. No significant differences in frequency of signs or symptoms	Leal et al. [46]	Cancer Patients	Paclitaxel 150–200 mg/m^2 and Carboplatin AUC = 5–7 every 21–28 days	185	1. Glutathione 1.5 mg/m^2 IV 2. Placebo control	1. QOL assessed by EORTC-QLQ-CIPN20 score 2. Neuropathy grading assessed by NCI-CTCAE v4.0 3. FACT-O questionnaire

1. No significant differences in acute pain score 2. No significant differences in in EORTC-QLQ- CIPN20 scores	Hershman et al. [47]	BC Patients	Paclitaxel 175 mg/m^2 biweekly, Paclitaxel 80 mg/m^2 weekly, Docetaxel 75 mg/m^2	409	1. Acetyl-L-carnitine 3000 mg daily PO 2. Placebo	1. Neurotoxicity scored by FACT-NTX score 2. FACT-Taxane-TOI 3. FACIT-Fatigue Scale
1. Significant deterioration in Neurotoxicity (NTX) scores	Pachman et al. [48]	BC Patients	Paclitaxel 80 mg/m^2 weekly	47	1. Minocycline 200 mg on day one followed by 100 mg daily 2. Placebo	1. Pain scores and other continuous variables converted to a 0 - 100 scale to assess the quality of life of the patient 2. Area under the curve of the entire course of treatment assessed 3. EORTC-QLQ-CIPN20 score

1. Significant difference in acute pain scores 2. No significant differences in sensory neuropathy score of the EORTC-QLQ-CIPN20 between the two groups	Shinde et al. [49]	Cancer Patients	Paclitaxel 80 mg/m^2 weekly	46	1. Pregabalin 150 mg daily PO 2. Placebo	1. EORTC-QLQ-CIPN20 score
1. No significant differences in acute pain score 2. No significant differences in in EORTC-QLQ- CIPN20 scores	Argyriou et al. [50]	Patients with solid or nonamyloid cancer malignancy	Paclitaxel-based regimens	32	1. Vitamin E 300 mg twice daily, 2. Control	1. PNP score
1. Significant differences in incidence of neuropathy and (PNP) scores	Argyriou et al. [51]	Patients with nonamyloid malignancies	Paclitaxel, Carboplatin, or their combinations	31	1. Vitamin E 600 mg daily 2. Control	1. PNP score

1. Significant differences in neuropathy scores between the two groups	Heiba et al. [52]	Patients with solid or nonamyloid malignancies	Taxane-Based Regimens	140	1. Vitamin E 400 mg twice daily 2. Control	1. Neuropathy grading assessed by NCI-CTCAE v. 5.0
1. Significant differences in duration and severity of neuropathy between the two groups	Aghabozorgi et al. [53]	BC Patients	Paclitaxel Based Regimens	40	1. Duloxetine 30 mg daily 2. Placebo	1. Neurotoxicity assessed by PNQ 2. Nerve Conduction Study

1. According to PNQ: 50% of the placebo group (n=20) had neurotoxicity, in the duloxetine group (n=20) two patients had mild neurotoxicity 2. Nerve Conduction tests show significant differences between the two groups related to the mean of median of sensory latency, Median Motor latency and Median motor velocity 3. No significant difference between the two groups related to the relative risk of polyneuropathy	Abbas et al. [54]	Patients with different malignancies	Paclitaxel 80 mg/m^2 weekly	50	1. Glutamine 15 gm daily 2. Control	1. Clinical Examination including vibrations, and grading of power in muscles, cranial nerve examination, and postural drop 2. NCI-CTC 3. Nerve conduction study

1. No significant difference in neuropathy between the two groups	Holotiuk et al. [55]	BC Patients	AT regimen (paclitaxel 175 mg/m^2 - doxorubicin 60 mg/m^2) or ET regimen (paclitaxel 175 mg/m^2, - epirubicin 90 mg/m^2	100	1. Alpha lipoic acid ALA 600 mg P.O. once daily and ipidacrine hydrochloride 20 mg P.O. three times a day 2. Control	1. Electroneuromyography Examinations

1. Significant reduction in the severity of nerve damage	Sánchez-Lara et al. [56]	Non-small cell lung cancer (NSCLC) Patients with stage IIIb and IV	Paclitaxel 175 mg/m^2 and Cisplatin 75 mg/m^2/Carboplatin AUC = 6	92	1. Eicosatetraenoic acid (EPA) 2. Control	1. Body weight, body composition, inflammatory parameters, and HROL
1. Significant difference in neuropathy between the two groups	Davis et al. [57]	Patients with solid tumors	Paclitaxel 175 mg/m^2 then carboplatin AUC = 6	117	1. Recombinant human LIF 2 or 4 μg/kg subcutaneous	1. CPNE Score

1. No significant differences in CPNE or any of the individual neurologic tests	2. Placebo	Schloss et al. [58]	Patients with a neoplastic disease	Regimens include Paclitaxel, Docetaxel, Oxaliplatin, or Vincristine	47	1. Vitamin B complex 2. Placebo
1. Neuropathy scored by TNS 2. Neurotoxicity assessed by PNQ 3. EORTC-QLQ-CIPN20 score	1. No significant difference in prevention of CIPN	Cho et al. [59]	Non-small cell lung cancer patients (NSCLC)	Paclitaxel Based Regimens	16	1. Lafutidine 10 mg twice daily 2. Placebo
1. Neuropathy grading assessed by NCI-CTCAE v5.0 2. PNQ 3. FACT-GOG-NTX subscale	1. No significance differences in prevention of CIPN	Kaku et al. [60]	Ovarian or Endometrial Cancer	Paclitaxel 175 mg/m^2-Carboplatin AUC = 5–6 (TC regimen)	29	1. Goshajinkigan (GJG) 7.5 gm/day (three times daily) 2. Control

1. CPT 2. Pain assessed by VAS 3. FACT-Taxane 4. Neuropathy grading assessed by NCI-CTCAE version 3.0	1. Significant Difference in the prevention of neuropathy favoring Goshajinkigan	Su et al. [61]	BC Patients	Regimens include Paclitaxel 175 mg/m^2, or Docetaxel 90 mg/m^2	183	1. Ganglioside monosialic acid (GM1) 80 mg once per day for 3 days (The day before start of Taxane therapy) 2. Placebo
1. FACT-NTX 2. Neuropathy grading assessed by NCI-CTCAE version 4.0 3. ENS	1. Significant difference in reduction of severity and incidence of Taxane-Induced Peripheral Neuropathy	Werida et al. [62]	BC Patients	Four cycles of doxorubicin plus cyclophosphamide every 21 days, followed by 12 cycles of paclitaxel weekly	64	1. Alpha Lipoic Acid 600 mg PO once daily 2. Placebo

BC breast cancer, *NCV* nerve conduction velocity, *NCI-CTCAE* national cancer institute common terminology criteria for adverse event, *rTNS* reduced total neuropathy score, *FACT-GOG-Ntx* functional assessment of cancer therapy/gynecologic oncology group- neurotoxicity subscale, *BPI-SF* brief pain inventory short form, *NGF* nerve growth factor, *NT* neurotensin, *mTNS* modified total neuropathy score, *MDA* malondialdehyde, *NfL* neurofilament light chain, *EORTC-QLQ-CIPN20 European Organization of Research and Treatment of Cancer Score*, *FACT-O* quality of life is assessed using the functional assessment of cancer therapy for patients with ovarian cancer questionnaire, *FACT-NTX* functional assessment of cancer therapy-taxane, *FACT-Taxane-TOI* FACT-taxane-trial outcome index, *FACIT* functional assessment of chronic illness, *PNP* modified peripheral neuropathy score, *PNQ* patient neurotoxicity questionnaire, *NCI-CTC* national cancer institute-common toxicity scale, *HROL* health-related quality of life, *CPNE* standardized composite peripheral neuropathy electrophysiology, *TNS* total neuropathy score, *CPT* current perception threshold, *VAS* visual analogue scale, *ENS* eastern cooperative oncology group neuropathy scale, *ALA* alpha lipoic acid, *BNP* brain natriuretic peptides

Anticonvulsants

Pregabalin and gabapentin are commonly reported to treat different neuropathic pain. A randomized double-blinded clinical pilot study on the use of pregabalin in preventing PIPN reported no significant difference in worst pain scores between the control arm and the pregabalin arm. Moreover, there were no differences across the arms in the worst, average, and least pain area under the curve (AUCs) throughout the first cycle of therapy ($p=0.48$, 0.62, 0.22, and 0.07, respectively) or the maximum of average pain. Additionally, the European Organization of Research and Treatment of Cancer Quality of Life Questionnaire (EORTC-QLQ-CIPN20) sensory subscale did not significantly differ between the two arms according to growth curve models or AUC analysis ($p=0.88$ and $p=0.46$, respectively) [49].

In another double-blinded, placebo-controlled study 40 breast cancer patients were randomly assigned to receive gabapentin or placebo. In all four cycles, the gabapentin group's neuropathy was primarily grade 1, with no reported cases of ≥ grade 3 neuropathy. In the gabapentin group, the rate of grade 2 and 3 neuropathy was considerably

lower (P<0.001) than in the placebo group. After four cycles of paclitaxel, the gabapentin group's Nerve Conduction Velocity (NCV) changed to be lower than that of the placebo group (17.7% vs. 61.0% reduction in NCV for sural nerve and 21.9% vs. 62.5% fall in NCV for peroneal nerve) [38].

Biguanides

A double-blinded randomized controlled trial (RCT) assessing the efficacy of metformin in the prevention of PIPN showed that the development of grade two or more peripheral neuropathy (PN) was significantly lower in the metformin group compared to placebo (p=0.001). Additionally, the time to develop PN was significantly longer in the metformin group. Furthermore, serum nerve growth factor (NGF) was significantly lower in the metformin group, and comparable levels of serum neurotensin were found in the two study groups [41].

Nutritional supplements

Omega 3 fatty acid protective effect in PIPN was assessed in a double-blinded RCT and showed a significant difference in PN occurrence (OR 0.3, 95% CI (0.10–0.88), p=0.029). The two study groups did not show a significant trend in terms of PIPN severity differences; nevertheless, the placebo group had greater frequencies of PN in all score categories (0.95% CI (−2.06 −0.02), p=0.054). [40]

Vitamin E neuroprotective effect was evaluated in three RCTs, where the incidence of PIPN and modified peripheral neuropathy (PNP) score were significantly lower with Vitamin E. [50] Also, neurotoxicity was more common in the control group than in the vitamin E-supplemented patients [51]. The frequency of ≥ grade 2 neuropathy, was comparable across the two arms; and only a non-significant difference in grade 3 neuropathy was reported. When comparing the two arms, there was a significant difference in the time length of neuropathy, which was measured from the onset of the first ≥ grade 2 or PN to the point at which it resolves to grade 1 neuropathy [52].

Vitamin B complex when used in conjunction with neurotoxic chemotherapy regimens did not prevent CIPN nor was it more effective than a placebo [58].

One RCT indicated the administration of alpha lipoic acid (ALA) in conjunction with the inhibitor of acetylcholinesterase ipidacrine hydrochloride (IPD) significantly reduced the extent of damage to the sural nerves (SNs) and superficial peroneal nerves (SPNs) caused by paclitaxel in patients prescribed for polychemotherapy (PCT). This was done by identifying significant differences in the electroneuromyography (ENMG) indicators of sensory nerve parameters between the studied groups [55]. Another double-blinded RCT showed that the percent of peripheral neuropathy grade 3 was significantly lower in the ALA group. Furthermore, the FACT-GOG-Ntx-12 questionnaire total score was significantly higher in the ALA group. Regarding the biomarkers, ALA group showed lower levels of brain natriuretic peptides (BNP), tumor necrosis factor-alpha (TNF-α), Malondialdehyde (MDA), and NT in comparison with the control group [62].

A controlled study assessed the oral nutritional supplement Eicosatetraenoic (ONS-EPA) acid effect in patients with advanced non-small cell lung cancer, compared to the control group. Patients in the ONS-EPA group showed significantly reduced neuropathy [56].

Another supplement, acetyl-L-carnitine (ALC) has been studied in PIPN. Over two years, CIPN was statistically significantly worse after twenty-four weeks of ALC therapy [47].

Goshajinkigan (GJG), a traditional Japanese herbal medicine, has been evaluated in a randomized controlled trial showing that (GJG) has a neuroprotective effect and nerve-repairing effect in CIPN [60].

Amino-acids and peptides

A randomized controlled study investigating glutamine in PIPN reported an overall frequency of neuropathy across all grades to be 78% at three months and 80% at six months. At six months, the incidences of grade 1 (48%), grade 2 (22%), and grade 3 (10%) neuropathy were recorded and (20%) did not experience neuropathy. Weekly paclitaxel-induced symptoms were mostly of grades 1 and 2, but not grade 4 symptoms. No significant differences were observed across the treatment groups in terms of the symptoms and weekly PIPN was not improved by glutamine [54].

Glutamate has been evaluated in a double-blinded RCT, showing that the selected dosage regimen was ineffective in the prevention of PIPN. Both groups' frequency of signs and symptoms was similar; however, the glutamate

group's neurotoxicity symptoms tended to manifest at lower severity levels. Additionally, there was similarity between the two groups in the frequency of aberrant electrodiagnostic findings [45].

A double-blinded RCT evaluated the use of glutathione for the prevention of paclitaxel/carboplatin-induced peripheral neuropathy. The results revealed no statistical significance difference in determining neurotoxicity with grade 2 using the National Cancer Institute Common Terminology Criteria for Adverse Event (NCI-CTCAE) v4.0 scale or in the time to development of neurotoxicity with grade 2. Moreover, no significant difference in neurotoxicity was measured by the EORTC-QLQ-CIPN20 [63].

Cytokines

Another double-blinded RCT showed that Recombinant Human Leukemia Inhibitory Factor (LIF) was not protective against carboplatin/paclitaxel-induced CIPN. There was a comparable standardized composite peripheral neuropathy electrophysiology (CPNE) score between the baseline and cycle 4, the last cycle, or the post-treatment assessment in both groups [57].

Mucolytic agent

N-acetylcysteine was evaluated for its effect on the prevention of PIPN because of its activity in reducing oxidative stress and elimination of ROS. RCT results demonstrated that when compared to the low-dosage group (61.9%) and the control group (100%), the high-dose group's occurrence of grades 2 to 3 PN was significantly reduced (28.6%). A significant improvement in QOL and modified total neuropathy score (mTNS) scores was recorded. Additionally, there were significant differences in serum MDA levels between the high-dosage and low-dose groups, as well as significantly greater levels of NGF in the high-dose group [42].

Phosphodiesterase inhibitors

Cilostazol has been evaluated for its preventive effect on PIPN, and when comparing the cilostazol group (40%) to the control group (86.7%), there was a significant difference in the occurrence of grade 2 and 3 peripheral neuropathies. The control group had a greater rate of clinically significant worsening in neuropathy-related quality of life compared to the cilostazol group. The cilostazol group showed a greater percent increase in serum NGF from baseline. At the end of the study, the circulation levels of the neurofilament light chain (NfL) were found to be comparable across the two arms [43].

Tetracycline antibiotics

Minocycline was studied in a multicentric, double-blinded, pilot trial and reported no significant dissimilarity during the first cycle of treatment. Still, there was a significant difference in the daily average area under the curve (AUC) pain score attributable to paclitaxel acute pain syndrome (P-APS), in favor of minocycline. Additionally, there was a tendency toward improvement in the daily worst pain AUC score across the 12 cycles. The overall EORTC-QLQ-CIPN20 sensory subscale did not significantly change between minocycline and placebo, despite the decrease in P-APS linked to minocycline usage [48].

Antidepressant

Over a long time, the antidepressant duloxetine has been evaluated for the management of CIPN, and recently, it was assessed in a double-blinded RCT to prevent PIPN. According to Patient Neurotoxicity Questionnaire (PNQ), 10 (50%) of the 20 participants in the placebo group experienced neurotoxicity (two mild cases, three moderate cases, four severe cases, and one disabled case). Nonetheless, two patients in the duloxetine group experienced moderate neurotoxicity. Median motor, sensory latency, and motor velocity were shown to have significant variations across the groups. The relative risk of polyneuropathy (relative risk: 1), however, was comparable between the two groups. According to the findings, an electrodiagnostic investigation supported the possibility that duloxetine could be a beneficial medication for breast cancer patients in reducing PIPN [53].

H2 antagonist

A small, randomized placebo-controlled study was conducted to evaluate lafutidine in the prevention of PIPN. Due to limited recruitment, the planned total of patients was not attained. Neuralgia of grade 2 or above affected 22.2% of the lafutidine group compared to 14.3% in the control group. In the lafutidine group, 100% of the participants had peripheral sensory neuropathy grade 2 or above, compared to 71.4% in the control group ($p=0.175$). In neither

group, there was evidence of PN of grade 3 or higher. The two groups' PNQ scores did not differ significantly from one another. Following the fourth cycle, there was a tendency for the lafutidine group to have lower FACT/GOG-Ntx scores than the control group. Between the two groups, there was no statistically significant difference in progression-free survival (PFS) [59].

Others

A randomized double-blinded placebo-controlled study was conducted to assess the effect of ganglioside monosialic acid (GM1) in the prevention of PIPN, which showed that the GM1 group had a lower incidence of PN grade 1 or higher in CTCAE v4.0 grading. Additionally, the GM1 group had a better significant difference in the FACT-NTX score. Moreover, a lower significant difference in the Eastern Cooperative Oncology Group Neuropathy scale (ENS) in the GM1 group was reported [61].

Assessment tools of PIPN including patient-reported outcome measurements (PROMs)

Patient-reported outcome measures (PROMs) are used more widely as a significant tool for the assessment of PIPN and are considered a valuable tool for collecting PN symptoms. PROMs are mostly used as endpoint measures in PIPN treatment and prevention clinical trials, as well as in research settings to characterize the natural history of neuropathy development and recovery. The most investigated PROMs were the EORTC-QLQ-CIPN20 and FACT-GOG-Ntx.

EORTC-QLQ-CIPN20

EORTC-QLQ-CIPN20 is a 12-item quality-of-life questionnaire that was developed to gather information on patients' experiences with CIPN-related symptoms and functional limitations. The CIPN20 comprises three subscales motor, sensory, and autonomic. Using a four-point rating system, patients indicate how much they have experienced each symptom (or "item") over the past seven days (1 = Not at all, 2 = A little bit, 3 = Quite a little, and 4 = Very much). Higher scores indicate a greater symptom burden. The three subscales are each calculated as the sum of component items, linearly transformed to a 0–100 scale [64].

FACT-GOG-Ntx

The FACT/GOG-Ntx was developed in cooperation between the Gynecologic Oncology Group (GOG) and the Functional Assessment of Chronic Illness Therapy group. The original 11-item FACT/GOG-Ntx11 questionnaire was created to assess the magnitude of CIPN and its effects on patients' quality of life in relation to motor, sensory, and auditory neuropathy and dysfunction. A five-point rating system is used for each item (0 = Not at all, 1 = A little bit, 2 = Somewhat, 3 = Quite a bit, and 4 = alot). The items' scores are reversed according to the FACIT groups' scoring convention, with greater total scores indicating better quality of life [65, 66].

Genetic single nucleotide polymorphisms associated with PIPN

Polymorphisms associated with paclitaxel metabolism

PIPN sensitivity may be raised by polymorphisms in genes related to the metabolism of the drug. Increased severity of PIPN has often been linked to single nucleotide polymorphisms (SNPs) in the ABCB1 gene [67–69]. In previous reports of patients with breast cancer receiving taxanes, SNPs in CYP2C8 and CYP3A4 were identified as causes of ≥ grade 2 CIPN [68, 70, 71].

Polymorphisms associated with microtubule function

Genes linked to microtubule function have been investigated to anticipate their relationship with PIPN since taxanes alter microtubule function and could contribute to PIPN pathogenesis. In 1303 European patients receiving paclitaxel, a SNP in the β tubulin IIb-encoding gene TUBB2A was linked to PIPN [72]. However, in 454 patients with ovarian cancer treated with paclitaxel and carboplatin, additive SNPs in MAPT and GSK3B were linked to reported neuropathy [73].

Polymorphisms associated with inherited neuropathies

The relationship between CIPN and genes linked to hereditary neuropathies has also been investigated. In African American patients receiving paclitaxel, SBF2, linked to Charcot-Marie-Tooth (CMT) disorder, was linked to CIPN [74]. Another study involving 58 individuals receiving paclitaxel discovered that FZD3 was linked to CIPN but not SBF2 [74]. Also, FGD4 was linked to CIPN in a trial investigating 219 breast cancer patients receiving taxanes [75].

In a larger cohort of 855 patients of European origin treated with paclitaxel, findings confirmed variation in FGD4 gene was linked to the reported sensory CIPN [76]. Another trial examining 269 cancer patients undergoing treatment in Alliance N08C1 found that ARHGEF10 was linked to CIPN when 49 CMT genes were examined in blood samples [77]. These results have been proved in 138 patients receiving paclitaxel in Alliance N08CA [78].

Polymorphisms associated with inflammatory pathways

An increasing corpus of research indicates that PIPN is influenced by inflammation, and changes in inflammatory pathways were linked to neuropathic pain [9, 79]. SNP in FCAMR, which encodes the FC receptor, led to a substantial relationship with CIPN in 3,431 breast cancer patients receiving paclitaxel treatment [80].

Polymorphisms associated with ion channels

Moreover, PIPN may potentially result from disruption of neuronal function via ion channels [9, 81] and SCN10A encode sodium channels located in the dorsal root ganglia, specifically Nav1.7 and Nav1.8. Among 186 Japanese patients with taxanes-treated breast and ovarian cancer, a SNP in the encoding gene SCN9A was linked to the development of≥grade 2 CIPN [82].

Polymorphisms associated with neuronal function

Genes related to cellular repair pathways and nervous system development and function have been connected to PIPN. In patients receiving taxanes, CIPN is linked to changes in the genes coding the Eph receptors (EPHA4, EPHA5, EPHA6, EPHA8), a class of tyrosine kinase receptors responsible for nerve growth and regulation [83–87]. Also, in 107 patients of gynecologic malignancies undergoing taxane or platinum chemotherapy, polymorphisms in the neural development-related genes SOX10 and GPX7 were linked to CIPN [88].

Future perspectives

Potential difficulties in many clinical trials investigating PIPN prevention include small sample sizes, the absence of placebo control groups, varying dosing and treatment regimens of investigational medications, non-standardized definitions and assessments of neuropathy, and inclusion of cancer patients receiving different chemotherapy treatment protocols. These factors limit the generalizability and interpretation of study findings. To manage these obstacles, future trials should ensure randomized placebo-controlled designs with adequate statistical power. Standardizing objective neuropathy assessments and definitions of clinically significant PIPN across studies would enable cross-trial comparisons. Additionally, narrowing eligibility criteria to specific chemotherapy protocols may yield more homogeneous cohorts for evaluating preventive interventions.

Conclusion

Preventing paclitaxel-induced neuropathy is a complex and evolving field. The literature on PIPN prevention is characterized by the heterogeneity of study designs, patient populations, and outcome measures, making conclusive evidence challenging. The literature suggests a variety of potential approaches such as pharmacological interventions; however, more high-quality research is needed to establish clear recommendations for clinical practice.

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Author contributions

AMASM collected, distributed, and organized the data sets and prepared the first draft of the manuscript. NOES contributed to the conception and design of the study. The final manuscript was revised by ES, NOES, and HA. All the authors approved the final version of the manuscript.

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Availability of data and materials

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Consent for publication

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Competing interests

The authors declare that they have no competing interests.

Abbreviations

ABCB1
Adenosine 5′ triphosphate binding cassette subfamily B member 1
ALA
Alpha lipoic acid
ALC
Acetyl-L-carnitine
AMPK
Adenosine monophosphate protein kinase
ARB
Angiotensin II receptor blocker
ARHGEF10
Rho guanine nucleotide exchange factor 10
AT
Paclitaxel-doxorubicin regimen
AUC
Area under the curve
BID
Twice daily
BPI-SF
Brief pain inventory
BNP
Brain natriuretic peptides
cAMP
Cyclic adenosine monophosphate
CI
Confidence interval
CIPN
Chemotherapy-induced peripheral neuropathy
CMT
Charcot-Marie-Tooth
CNS
Central nervous system
CPNE
Standardized composite peripheral neuropathy electrophysiology score
CPT
Current perception threshold
CTCAE
Common terminology criteria for adverse events
CYP2C8
Cytochrome P450 family 2 subfamily C member 8
CYP2C8

Cytochrome P450 family 2 subfamily C member 8
CYP2J6
Cytochrome P450 family 2 subfamily J member 6
CYP3A4
Cytochrome P450 family 3 subfamily A member 4
DPP-4
Dipeptidyl peptidase inhibitor-4
DRG
Dorsal root ganglia
ENMG
Electroneuromyography
ENS
Eastern cooperative oncology group neuropathy scale
EORTC-QLQ-CIPN20
European Organization of Research and Treatment of Cancer Quality of Life, chemotherapy-induced peripheral neuropathy 20
EPHA4
Ephrin Type A receptor 4
EPHA5,
Ephrin Type A receptor 5
EPHA6,
Ephrin Type A receptor 6
EPHA8
Ephrin Type A receptor 8
ET
Paclitaxel-epirubicin regimen
FACIT
Functional assessment of chronic illness therapy
FACT-GOG-NTX
Functional assessment of cancer therapy/gynecologic oncology group-neurotoxicity
FACT-NTX
Functional assessment of chemotherapy-taxane
FC
Fc alpha and mu receptor
FCAMR
Fc alpha and mu receptor
FDG3
Facio-genital dysplasia gene 3
FGD4
FYVE-RhoGEF and PH domain containing 4
FZD3
Frizzled class receptor 3
GJG
Goshajinkigan
GM1
Ganglioside monosialic acid
GPX7

Glutathione peroxidase 7
GSK3B
Glycogen synthase kinase 3 beta
HROL
Health related quality of life
IENF
Intra-epidermal nerve fibers
IL-6
Interlukin-6
InsP3R
Inositol 1,4,5 tris-phosphate receptor
IPD
Ipidacrine hydrochloride
IV
Intravenous
KCNN3
Potassium calcium-activated channel subfamily N member 3
LIF
Leukemia inhibitory factor
MAPT
Microtubule-associated protein tau
MDA
Malondialdehyde
mTNS
Modified total neuropathy score
NCI-CTCAE
National cancer institute’s common toxicity criteria for adverse event
NCS-1
Neuronal calcium sensor 1
NCV
Nerve conduction velocity
Nfl
Neurofilament chain light
NGF
Nerve growth factor
NSCLC
Non-small cell lung cancer
NT
Neurotensin
ONS-EPA
Oral nutritional supplement eicosatetraenoic
OR
Odds ratio
P-APS
Paclitaxel acute pain syndrome
PCT
Polychemotherapy

PDE
Phosphodiesterase
PIPN
Paclitaxel-induced peripheral neuropathy
PN
Peripheral neuropathy
PNP
Peripheral neuropathy score
PNQ
Patient neurotoxicity questionnaire
PNQ
Patient neurotoxicity questionnaire
PO
Per oral
PPARγ
Peroxisome proliferator-activated receptor gamma
PROM
Patient-reported outcome measurements
PTX
Paclitaxel
QLQ
Quality-of-life questionnaire
QOL
Quality of life
ROS
Reactive oxygen species
rTNS
Total neuropathy score
SCN10A
Sodium voltage-gated channel alpha subunit 10
SCN9A
Sodium voltage-gated channel alpha subunit 9
SN
Sural nerves
SNP
Single nucleotide polymorphism
SOX10
Sky box transcription factor 10
SPN
Superficial peroneal nerves
TLR4
Toll-like receptor 4
TNFα
Tumor necrosis factor α
TUBB2A
Tubulin beta 2A
VAS

Visual analogue scale

Publisher's Note

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Preclinical study on camellia sinensis extract-loaded nanophytosomes for enhancement of memory-boosting activity: optimization by central composite design

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ABSTRACT (ENGLISH)

Background

The purpose of the present study was to enhance the memory-boosting activity of the standardized hydroalcoholic *Camellia sinensis* extract (CSE) by the formation of nanophytosomes with Leciva S70 phospholipid. The central composite design was used to optimize the solvent evaporation method for the formulation of *C. sinesis* phytosomes (CSP).

Results

The optimized formulation had a mean particle size of 212.3 nm ± 0.39, PDI of 0.238 ± 0.0197, and zeta potential of −42.02 ± 0.995 mV. *C. sinensis* phytosome formation was confirmed by analytical techniques. The aqueous solubility of the developed CSP was 95.92 ± 0.31, which is 7.34 times greater than that of pure CSE (13.07 ± 0.19). CSP was found more effective than either pure CSE (26.42 ± 0.4654%) or the physical mixture (32.15 ± 0.4596%) in releasing the CSE from the formulation (72.16 ± 0.5248%). Acute toxicity study corroborated the safety of CSP in rats. CSP demonstrated a significant ($p<0.05$) reduction in escape and transferred latency on both days (15th and 16th) as compared to CSE, indicating the improvement of the memory-boosting activity. Furthermore, CSP-treated rats significantly improved acetylcholine (Ach) levels and brain tissue concentration compared with CSE. Moreover, the phytosomal formulation of CSP exhibited its rationality with an improvement of bioavailability by 3.21 folds compared with pure CSE.

Conclusion

The presence of phospholipids in the CSP formulation and the formation of smaller particles may aid in crossing the blood–brain barrier, increasing brain tissue concentration and bioavailability. This, in turn, leads to an increase in memory-boosting activity.

FULL TEXT

Background

Neurodegenerative diseases are chronic, debilitating conditions marked by significant cognitive impairments, gradual death of neurons, and secondary abnormalities in the white matter tract [1]. Alzheimer's disease (AD) is a progressive, neurodegenerative brain disease that affects memory and other cognitive abilities over time. It causes dementia, behavioral changes, memory and thinking skill impairments, personality disorders, and diffuse anatomical abnormalities in the brain [2]. AD is characterized by the deposition of β1 amyloid peptide plaque deposition, cholinergic cell loss, especially in the basal forebrain, and neurofibrillary tangles [3]. Acetylcholine (Ach), a neurotransmitter, is lost along with cholinergic cells. Dementia appears to be caused mainly by a decline in Ach in the brains of AD patients [4]. In 50 to 70% of cases, Alzheimer's dementia affects persons in their middle to late years. As people become older, the prevalence of AD doubles every five years after the age of 65.

Currently, individuals diagnosed with AD and other forms of dementia, such as transient ischemic episodes, stroke, organic brain disorders, mental retardation, and multi-infarct dementia, have limited pharmaceutical treatment alternatives at their disposal. The current therapies are essentially symptomatic; there has not been a noteworthy therapeutic breakthrough that prevents, modifies, or controls dementia and AD [5]. The most popular AChE inhibitors used to treat cholinergic deficiency in the brain are donepezil, rivastigmine, and galantamine; however, they have limitations such as a short duration of action, a low bioavailability, and a restricted therapeutic index [6]. Additionally, due to additional negative effects and non-specificity in the site of action, several synthetic drugs promoted as cognitive enhancers, including piracetam, amphetamine, pemoline, pyritinol, and others, are not safe for long-term use in humans. Their usage is also constrained by significant side effects such as hepatotoxicity [7]. Only one or two of every 10,000 of these chemical and synthetic compounds are clinically successful and safe enough for regulatory approval in the clinical process, despite the drawn-out development process. In reality, clinical trials for approximately half of all drug candidates fail. So considering these major drawbacks of synthetic molecules, many pharmaceutical firms are currently focusing on the development of plant-derived drug delivery systems [8]. Over the past decade, herbal and ayurvedic drugs from the plant kingdom have become a subject of world importance in neurodegenerative disorders with both medicinal and economic implications. Regular and widespread use of herbs throughout the world has increased serious concerns over their quality, safety, and efficacy. Thus, proper scientific evidence or assessment has become the criteria for the acceptance of herbal health claims [9]. However, the scientific literature supporting the efficacy of herbal therapies is incomplete. Few well-controlled studies support the efficacy of herbal remedies in the treatment and clinical improvement of patients with neurodegenerative disorders like dementia, AD, and Parkinson's disease. Available scientific evidence has not yet confirmed the validity of their popular role in the treatment of these diseases [10]. The use of herbal medicine becoming popular due to the toxicity and side effects of allopathic medicines. Medicinal plants play an important role in the development of potent therapeutic agents.

The herbs acting on the brain are called Nootropic herbs and their isolated constituents are referred to as smart drugs. These herbs enhance memory as well as increase blood circulation in the brain. Different plants have been used as memory enhancers in folkloric medicine [11]. Therefore, it is necessary to prepare herbal formulations by using some reputed herbal sources as a memory enhancer. However, water-soluble phytoconstituents (like flavonoids, tannins, glycoside &aglycones, etc.) are poorly absorbed either due to their large molecular size which cannot absorb by passive diffusion or due to their poor lipid solubility; severely limiting their ability to pass across the lipid-rich biological membranes, resulting poor bioavailability [12]. Lipid solubility and molecular size represent the primary limiting factors for drug molecules to traverse biological membranes and achieve systemic absorption following oral or topical administration. The particle size reduction is achieved by using two novel techniques phytosomes and nanonization to easily pass through the blood–brain barrier for maintaining their concentration at the site of action [13, 14]. Standardized plant extracts or mainly polar phytoconstituents like flavonoids, terpenoids, tannins, and xanthones when complexed with phospholipids like phosphatidylcholine give rise to a new drug delivery technology called phytosome (or herbosome) showing much better absorption profile following oral administration owing to improved lipid solubility which enables them to cross the biological membrane, resulting enhanced

bioavailability at lesser dose [15, 16].

The *Theaceae* family includes the *C. sinensis* plant, generally referred to as the tea plant. It is a prominent medicinal herb in many indigenous medical systems, including Ayurveda, Unani, and homeopathy, and is utilized extensively by tribal people in China and India. Polyphenols, including (but are not limited to) catechin, gallocatechin, epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate (EGCG), are found in *C. sinensis*, with EGCG having the highest concentration and being the most active phytoconstituents. It contains alkaloids (caffeine, theobromine, and theophylline), polysaccharides, vitamins, volatile oils, and minerals [17]. It has many biological activities, including stimulant, diuretic, astringent, antioxidant, anticancer, anti-inflammatory, anti-obesity, antistress, hepatoprotective, cardioprotective, antiviral, antidiabetic, neuroprotective, gastrointestinal tract problem relieving activity, anti-inflammatory, analgesic, antipyretic, antiallergic, and skeleton muscle system reducing activity. These factors contribute to its enormous popularity. Green tea's potent effect on creating or lowering neurotransmitters, including acetylcholine, dopamine, and serotonin, all of which are involved in memory and learning, contributes to its ability to improve these processes [18, 19]. Polyphenols may also aid in the prevention and treatment of dementia [20].

The main objective of the present investigation was to increase the water solubility, bioavailability, and memory-enhancing activity of the standardized hydroalcoholic *C. sinensis* extract by forming a vesicular complex with Leciva S70 phospholipid in the form of phytosomes.

Material and methods

Materials

The standardized hydroalcoholic *C. sinensis* leaf extract was provided by Arjuna Remedies in Kerala. Leciva S70 (Phospholipid) was provided by VAV Life Sciences Pvt. Ltd, Mumbai Sigma-Aldrich supplied Epigallocatechin 3 gallate (EGCG), scopolamine HBr (hydrobromide), and piracetam. The dialysis membrane 60 was provided by Analab Fine Chemicals. Ethanol, methanol, n-octanol, and chloroform were furnished by Sigma-Aldrich Corporation. The rest of the materials were of superb analytical quality.

Methods

Preparation of *C. sinensis* phytosomes

The formulations for the *C. sinensis* leaf extract-loaded phytosomes (CSP) were developed using solvent evaporation techniques. 250-ml round-bottom flasks were filled with various ratios of *C. sinensis* extract (CSE) and Leciva S70 (1:1, 1:2, and 1:3). Then, 50 ml of ethanol was added to this. The reaction was conducted by maintaining the RBF-containing reaction mixture at different temperatures (40, 50, and 60 °C) for a predetermined time (1/2, 3 h), following the CCD. During heating, the reaction mixture was stirred. The solution was then evaporated and stored in a vacuum desiccator for a night to remove any trace of solvent that might have been left over after the initial incubation. The dry mixture was held in an amber bottle for later use [20].

Experimental design and statistics

The effects of independent parameters like drug ratio (% w/w), temperature (^{o}c), and time (Hr), on entrapment efficiency (EE), were analyzed using central composite design (CCD), an optimization tool based on the response surface methodology. Using Design Expert 7.0.0 software, the experimental data were analyzed. Table 1 lists the levels of the considered independent variables and their experimental ranges for EE (% w/w).

Table 1. Experimental central composite design factors at three levels regions

Central composite design (CCD)	Coded factors	Levels		
−1	0	+1	*Independent variables*	

			Phospholipid: drug ratio (% w/w)	X
1	2	3	Temperature (°C)	Y
40	50	60	Time (Hr)	Z
1	2	3	*Dependent variable*: Entrapment efficiency in % (A)	

Entrapment Efficiency (EE)

The efficiency of the developed phytosomes was assessed through a solvent extraction method as described previously. One hundred mg of the formulation was weighed and evenly dispersed throughout 10 ml chloroform. The produced phytosomes and Leciva S70 were dissolved in chloroform making CSE insoluble. The resulting mixture was centrifuged (Remi, Mumbai) at 5000 RPM. The sediment was removed from the transparent supernatant solution. Alcohol (ethanol) was used as the solvent in a UV/VIS spectrophotometer (UV 3000+, LAB INDIA) for non-aggregated CSE which showed a peak at 274 nm. Finally, a calibration curve was used to estimate the Epigallocatechin 3 gallate concentration using the Y = 0.02275x + 0.017429 regression equation.

Particle size

Particle size distribution and mean particle size of the optimized batch of CSP were determined at a constant temperature of 25 °C using Malvern Instrument, Malvern, UK (Model: ZEN 3600). The water was used to distribute the particles evenly. The polydispersity index (PDI) was used to determine the extent to which size distributions varied [21].

Zeta potential determination

The zeta potential was analyzed using the Scientific SZ-100 HORIBA (for Windows [Z Type] Ver2.40) analyzer. The optimum CSP formulation was tested for stability by zeta potential analysis. The average zeta potential, charge, and mobility of optimized CSP were measured after 60 s of study [22].

FTIR spectroscopy

The FTIR spectra of CSE, physical mixture (PM), CSP, and Leciva S70 (phospholipid) were obtained using an FTIR-7600 (Lambda Scientific) spectrophotometer. All samples were dried in a hot air oven at 50 °C for two hours to remove moisture. Each sample (~1 mg) was evenly mixed with potassium bromide (approximately 100 mg), then compressed at a pressure of 10 Ton/nm^2 to form a disk-shaped pellet. The resulting pellet was placed inside the sample container and scanned from 4000 to 500 cm-1 at a resolution of 4 cm-1.

Powder X-ray diffraction (XRD)

The polymorphic states of CSE, Leciva S70, PM, and CSP were investigated utilizing a powder X-ray diffractometer (Model: D8 Advance, Bruker AXS, USA) equipped with a Bragg–Brentano geometry (θ/2) optical setup to record X-ray diffraction patterns. The samples were scanned between 3 and 60 degrees, with a setup angle range of between 0.2 and 2θ and a count time of 0.5 s.

Solubility analysis

The solubility of CSE, PM, and CSP was determined by using the method already described [23]. Briefly, the excessive amount of samples was dissolved in either water or n-octanol (10 ml) in airtight glass containers at room temperature (25 °C). The glass bottles containing the solutions were shaken with an orbital shaker (RIVOTEK) for 24 h and centrifuged for 20 min at 4000 RPM (REMI, India). The clear supernatant was isolated and passed through a membrane filter (0.45μ). 1 ml of this filtrate was then diluted appropriately and analyzed at 274 nm using a UV/ VIS spectrophotometer (UV 3000+, LAB INDIA).

In Vitro *release studies*

The in vitro release of CSE, PM, and CSP was performed using a dialysis membrane (MW cut off>12,000 KDa). Before filling the samples, the dialysis membrane was soaked in distilled water for 1 min. Fifty mg of CSE was dissolved in a buffer solution (4 ml) to form a suspension. Under the same conditions, an equivalent amount of CSP was dispersed into to buffer solution. The dialysis bag was filled with the prepared dispersions and tied from both ends. The dialysis membrane bag containing the sample was suspended in a mixture of phosphate-buffered saline (PBS) (200 ml, pH 7.4) and Tween 20 (1% w/v) in a glass beaker. The buffer solution was stirred under magnetic stirring at 100 RPM maintaining a temperature of 37 ± 0.5 °C. The samples (5 ml) were withdrawn at a predetermined time interval and replenished with an equal volume of freshly prepared buffer to maintain the sink condition. The aliquots were filtered using a 0.45-micron membrane filter before being measured at 274 nm (Telange et al. 2017) using a UV/VIS spectrophotometer (UV 3000+, LAB INDIA). In addition to these studies, we have performed Differential scanning calorimetry (DSC), Scanning electron microscopy (SEM), Proton Nuclear Magnetic Resonance and stability study. The detailed experimental methods of these studies is provided in Additional file 1.

Acute toxicity study

All animal handlings and experiments were approved by the Animal Ethical Committee of Crystal Biological Solution, Pune, with approval number CRY/2122/070. Acute toxicity testing was performed on healthy, non-pregnant, and nulliparous female Wistar rats. Four experimental groups of 12 female rats ($n=3$) were formed. Groups I and II were given CSP formulations at 300 mg/kg bw, while groups III and IV received 2000 mg/kg bw. The dose titration was done with the population from each group's safety in mind. The Wistar rats were starved for three hours before and after the dosage. Throughout the trial, water was available at all times. Individual animals were monitored for the first 30, 60, 120, 180, and 240 min following treatment and once a day for the next 14 days [24].

Dosing and sampling schedule

For the in vivo memory-enhancing activity investigation, 36 Wistar rats of both sexes (male and female) weighing 175–240 gm were employed. The animals were divided into six groups each containing 6 in each groups. As per CPCSEA guidelines, rats were provided with free access to food and water and kept in an animal house with standard laboratory conditions (constant room temperature (25 ± 2 °C), relative humidity (50–70% RH), and a 12-h light/dark cycle). Carboxymethyl cellulose (CMC) 0.5% (10 ml/kg bw) was administered to Group I (Control) for 15 days, (Negative control) Scopolamine HBr (0.4 mg/kg bw) was received to Group II (Negative control) by i.p. route on the 15th day. In Group III (the Positive control group), subjects were given piracetam orally at a regular dose (200 mg/kg body weight) for 15 days. Group IV received an oral dose of *C. sinensis* extract (CSE) (250 mg/kg bw) for 15 days, followed 45 min later by an i.p injection of scopolamine HBr (0.4 mg/kg bw). After 15 days of oral administration of CSP formulation (equal to 250 mg/kg bw of CSE), scopolamine HBr (0.4 mg/kg bw) was injected (i.p.) after 45 min of CSP formulation administration in Group V, 15 days of oral piracetam (200 mg/kg bw) were followed by 45 min of injection (i.p) of scopolamine HBr (0.4 mg/kg bw) in Group VI.

In 0.5% (w/v) CMC solution, CSE, CSP, and piracetam solutions were produced. All animals were treated according to the schedule and exposed to a behavioral analysis study. Simultaneously, the same groups were employed for three different models. Animals were pre-treated with MWMT, EPMT, and PCT on alternating days before the tests. On day 15, 90 min following the administration of the relevant dose, the transfer latency (TL)/ or the escape latency (EL) was determined. Both of these measurements took place. After 24 h had passed since the last session, the retention of all learned tasks was assessed [25].

In vivo *memory-enhancing/boosting activity*

In this study, the term “memory-enhancing/boosting activity” means to elevate the memory and cognitive functions which were determined using various behavioral tests including Morris Water Maze Test, Elevated Plus Maze Test, Pole Climbing Test, and the measured outcomes like escape latency, transfer latency, acetylcholine, serotonine, and dopamine levels. The memory-enhancing efficacy of optimized CSP in contrast to CSE was compared.

Elevated plus maze test

Using an exteroceptive behavioral paradigm, the EPMT was utilized to examine the effects of training on rat memory and learning (where the stimulus was external to the body). The apparatus had two bare arms (each 50 cm × 10 cm) and two covered arms (each 50 cm × 40 cm × 10 cm). Extending the arms from a central platform, the maze was raised to 50 cm from the floor (10 cm × 10 cm). On day one, the rats were placed one by one at the very tip of the open arm, with their backs to the platform in the middle. The rat’s opposite gender was identified in any covered areas to see if the test rat’s retentive memory caused it to go quicker toward that region. The TL was determined by recording the time it took the rat to move inside any of the covered arms containing the opposing gender while using all four of its legs. On the first day, each animal’s TL was noted. Within the first 90 s, the animal was allowed to enter one of the covered arms. If it did not, it was coaxed into gently entering one of the arms. A time limit of 90 s was imposed there. After the rat had explored the maze for ten more seconds, it was put back in its cage. The retention of this learned task was evaluated 24 h after the last dose or on day 16 of treatment. A considerable decrease in the TL value of retention showed memory improvement [26].

Morris water maze test

The MWMT was performed in a circular swimming pool of 100 cm in diameter and 50 cm in height. The circular pool was built with filling and draining facilities and installed on a framework with the water level at waist height. The circular tank’s floor was divided into four equal quadrants. Up to a depth of 30 cm of water was added. One of the pool’s four corners has a plastic platform dug 2 cm below the water’s surface (9 cm in diameter and 28 cm in height). The milk was used to make the pool opaque. The trial platform was consistently situated in the same spot.

The rats were trained to swim without a platform on the first day of the test. Then, after a brief introduction, the animals were released into the tank and given 10 s to explore the exhibit, including sitting on the secret platform. After 90 s, if the rat still had not found the platform, it was placed on it and left there for 10 s. On alternating days, rats were trained for 15 days. The trial was successful when the rat sat on the hidden platform within 90 s. If rats spend more than three minutes looking for the hidden platform, that is a mistake. Throughout the investigation, the experimental setting remained the same. Memory improvement was indicated by a considerable fall in the EL value [27].

Pole climbing test

The cognitive processes of learning and memory retention were studied using Cook’s pole climbing equipment. The apparatus consists of a soundproof experimental chamber (25 × 25 × 25 cm) with a floor grid. The chamber’s floor grid is made of stainless-steel rods. The chamber’s floor grid received a scrambled shock (6 mA). A hole at the room’s center top was used to suspend a pole with a diameter of around 2.5 cm. Wistar rats were placed in the chamber and given 45 s to investigate their surroundings. The buzzer served as the conditioned stimulus (CS), and the 45 s of electrical shock through the grid floor served as the unconditioned stimulus (US). Once the animal connected the sound of the buzzer to the impending foot shock, it could avoid the pain by climbing the pole after the signal was given. A record of the EL quantity was kept. The animals were initially screened using this paradigm, and only those who exhibited an escape response on at least one of the trials were included [26].

Estimation of acetylcholinesterase activity in rat’s brain

On the 16th day, after conducting MWMT, EPMT, and PCT, Five Wistar rats from each group were anesthetized by giving an intramuscular injection (Ketamine Hydrochloride IP) and decapitated. Instantaneously after removal, the brains were washed in icy saline and frozen at -80^{0}C for later use. 0.1 M phosphate buffer (pH 8.0) was used to homogenize the tissue after measuring its weight (0.1 gm of tissue per ml of phosphate buffer). Two and a half milliliters of buffer and one hundred microliters of DTNB 5,5-dithio bis (2-nitrobenzoic acid) were placed in a cuvette. Then, 4 ml aliquots of the homogenate were added. The resulting mixture was well mixed, and the absorbance was

measured in a photometric calorimeter at 412 nm. After recording the basal measurement, 20 ml of the substrate (i.e., acetylthiocholine) was added to the solution mentioned above when the absorbance reached a steady value. The change in absorbance was measured every two minutes for 10 min. [27].

Estimation of dopamine concentration in Rat's brain

DA GENLISA™ ELISA kit was used for the quantitative determination of dopamine in Wistar rat brain homogenate solution by sandwich ELISA technique. Fifty µl of prepared rat dopamine (DA) standard solutions of 0.3, 0.6, 1.2, 2.4, 4.8, and 9.6 ng/ml was added to respective standard wells. These standard solutions were used for the construction of the calibration curve. Forty µl of Wistar rat brain homogenate sample solution from groups I to VI was added to respective sample wells. Ten µl of biotinylated DA antibody was added to the respective sample wells. The biotinylated DA antibody was not added to standard wells because the standard solution contains the biotinylated antibody. Fifty µl of streptavidin-HRP conjugate was added to all sample wells. Mixed well. The plate was covered with a sealer and incubated for 60 min at 37 °C. The plate was aspirated, washed four times with diluted wash buffer (1X), and the residual buffer was blotted by firmly tapping the plate upside down on absorbent paper. Wipe out all liquid from the bottom outside of the microtiter wells, as any residue can interfere with the reading step. Fifty µl TMB Substrate A was added followed by 50 µl TMB Substrate B in all the wells. The plate was covered and incubated at 37 °C for 10 min. The wells had turned bluish. Fifty µl of stop solution was added to all wells. The wells were turned from blue to yellow. Absorbance was recorded at 450 nm with a microplate reader within 10–15 min after the addition of the stop solution [28].

Estimation of serotonin concentration in rat's brain

The Rat Serotonin, ST GENLISA™ ELISA kit was used for the quantitative determination of serotonin in Wistar rat brain-homogenated solution by sandwich ELISA technique. Fifty µl of prepared serotonin (ST) standard solutions of 7.5, 15, 30, 60, 120, and 240 ng/ml was added to respective standard wells. These standard solutions were used for the construction of the calibration curve. Forty µl of Wistar rat brain homogenate sample solution from groups I to VI were added to respective sample wells. Ten µl of biotinylated ST antibody was added to the respective sample wells. The biotinylated ST antibody was not added to standard wells because the standard solution contains the biotinylated antibody. Fifty µl of streptavidin-HRP conjugate was added to the respective sample wells and also the standard wells. The streptavidin-HRP conjugate was not added to the blank well. Mixed well. The plate was covered with a sealer and incubated for 60 min at 37 °C. The plate was aspirated and washed four times with diluted wash buffer (1X), and the residual buffer was blotted by firmly tapping the plate upside down on absorbent paper. Wipe out all liquid from the bottom outside of the microtiter wells, as any residue can interfere with the reading step. Fifty µl TMB Substrate A was added, followed by 50 µl TMB Substrate B in all the wells. The plate was covered and incubated at 37 °C for 10 min. The wells had turned bluish. Fifty µl of stop solution was added to all wells. The wells were turned from blue to yellow. Absorbance was recorded at 450 nm with a microplate reader within 10–15 min after the addition of the stop solution [29].

Histopathological study

One animal from each group received an intramuscular dose of Ketamine Hydrochloride IP at the end of the treatment session, rendering it unconscious so that its brain could be dissected. The removed brain was stored in a 10% (v/v) formalin solution. A hematoxylin and eosin reagent was used to stain between 3- and 5-µ-thick sections. The brain slices were studied with an optical microscope, and pictures were taken with the microscope's attached digital camera at a magnification of 400x.

Procedure for estimation of the concentration of CSE and CSP in brain tissue

The concentration of CSE and CSP in brain tissue was determined using a brain-homogenated solution (4 ml) from animal groups IV and V. The homogenized brain solutions were placed into two distinct 5-ml centrifuge tubes and spun for 15 min at 10,000 rpm. CSE and CSP clear supernatants were separated and used for HPLC analysis.

High-Performance liquid chromatography (HPLC)

Epigallocatechin 3 gallate was used as a marker to estimate the CSE and CSP in brain tissue. A bioanalytical HPLC (Model: Waters 2695 alliance) approach was developed for the Epigallocatechin 3 gallate marker. A Zorbax SB C18

5μ (4.6 × 150) mm column was utilized. This experiment was conducted using the chromatographic gradient technique. As a mobile phase, water with 0.1% (v/v) formic acid and acetonitrile (ACN) with 0.08% (v/v) formic acid were observed. Mobile phase filtration was performed using a 0.45-m millipore filter. The mobile phase flow rate was maintained at 1.0 ml/min, and the column temperature was held at 30°c. At 274 nm, a PDA-type detector was utilized [30]. (Regression Equation: *Y* = 44,929.71 X–4900.50 and Retention time: 7.417 Minutes).

Comparative pharmacokinetic study of CSP and CSE in the blood plasma compartment

18 Wistar albino rats (both sexes) weighing between 240 and 370 g were used in the pharmacokinetic study. Wistar albino rats were housed in a standard laboratory setting and provided free access to food and water as per CPCSEA guidelines. For the study, animals were fasted overnight until 2 h after medication and then given food. Animals were divided into three groups containing six in each group. Group I (Control): 0.5% (w/v) CMC solution (10 ml/kg bw), Group II: CSE (250 mg/kg bw and Group III: CSP formulation (equivalent to 250 mg/kg bw of CSE). CSP and CSE were dissolved individually in a 0.5% (w/v) CMC solution and given to the different groups orally. All Wistar rats were anesthetized with Ketamine Hydrochloride IP (intramuscular injection). The retro-orbital vein was punctured to collect blood samples (0.5–0.7 ml) and collected in Eppendorf tubes at intervals of 0, 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 h. Plasma was separated using centrifugation (Remi, Mumbai, India) at 10,000 rpm for 10 min, and the sample was then frozen at -40°c for further drug analysis [31].

Preparation of plasma samples for HPLC analysis

One ml of purified plasma and 2 ml of methanol were placed in the centrifuge tube. After 30 s of vigorous agitation, centrifugation at 10,000 rpm for 10 min, and collection of the clear supernatant, the tube was discarded. The protein-free solution was stored in a tube at -40°c in the freezer until HPLC analysis was done.

Estimation of pharmacokinetic parameters

A protein-free clear supernatant (20 μl) was put into the chromatographic system that had been designed. The calculated concentration at each time was based on the obtained peak area. Then, directly from plasma concentration–time data estimated by one extravascular compartment model using PK Solver, the pharmacokinetic parameters (maximum plasma concentration) C max, (area under the concentration–time curve) AUC, (corresponding time) t max, (half-life, clearance) CL/F, and (volume of distribution) V/F were calculated.

Results

Preparation of CSP and statistical analysis of EE

Table 2 summarizes all the formulation batches as per CCD with independent and dependent variables. Table 3 summarizes the model statistics suggesting a quadratic model.

Table 2. Three independent factors central composite design matrix and corresponding entrapment efficiencies from the experiment

CCD batches	Factor X	Factor Y	Factor Z	Response
Phospholipid: drug ratio (% w/w)	Temperature (°C)	Time (Hr)	Entrapment efficiency (%)	1
1	40	1	62.11 ± 0.95	2
3	40	1	83.14 ± 1.01	3
1	60	1	70.06 ± 1.36	4
3	60	1	94.41 ± 1.33	5
1	40	3	77.09 ± 0.97	6

3	40	3	87.50 ± 0.92	7
1	60	3	82.05 ± 0.98	8
3	60	3	95.81 ± 1.04	9
0.32	50	2	59.15 ± 1.06	10
3.68	50	2	93.90 ± 1.15	11
2	33.18	2	80.22 ± 0.99	12
2	66.82	2	93.51 ± 1.38	13
2	50	0.32	82.94 ± 1.06	14
2	50	3.68	91.48 ± 1.33	15
2	50	2	88.56 ± 1.44	16
2	50	2	86.68 ± 0.77	17
2	50	2	86.71 ± 0.96	18
2	50	2	85.02 ± 1.21	19
2	50	2	89.61 ± 0.86	20

The data are shown as the Mean ± Standard Deviation (n = 3)

Table 3. Summarized data from the model statistics

Source	SD	R^2	Adjusted R^2	Predicted R^2	PRESS	Comment
Linear	4.675979	0.818925	0.784973	0.696805	585.7707	
2FI	4.670834	0.8532	0.785446	0.612729	748.2049	
Quadratic	2.076719	0.977677	0.957587	0.881202	229.517	Suggested
Cubic	2.090348	0.98643	0.957028	-0.1948	2308.345	Aliased

Based on the proposed model, the following polynomial quadratic equation was predicted%EE=87.06+9.37 X+4.02 Y+3.45 Z+0.83 XY-2.65 XZ-0.74 YZ-4.08 X2-0.43 Y2-0.31 Z2

The positive sign suggested a favorable effect on the reaction, while the negative sign indicated the opposite. Table 4 displays the ANOVA results for the quadratic model of the response surface.

Table 4. Analysis of variance data revealing how different factors affect the EE

Source	Sum of squares	df	Mean square	*F* Value	p value Prob >F	Comment
Model	1888.865	9	209.8738	48.66342	<0.0001	Significant
X-Phospholipid: drug ratio (% w/w)	1199.546	1	1199.546	278.1387	<0.0001	
Y-Temperature	220.2219	1	220.2219	51.06282	<0.0001	
Z-Time	162.3877	1	162.3877	37.65281	0.0001	
XY	5.561113	1	5.561113	1.289454	0.2826	
XZ	56.23301	1	56.23301	13.03874	0.0048	
YZ	4.425312	1	4.425312	1.026097	0.335	
X^2	240.3307	1	240.3307	55.72545	<0.0001	
Y^2	2.639323	1	2.639323	0.611979	0.4522	
Z^2	1.349202	1	1.349202	0.312839	0.5882	
Residual	43.12764	10	4.312764			
Lack of fit	27.27864	5	5.455727	1.721158	0.2829	Not significant
Pure error	15.849	5	3.1698			
Cor total	1931.992	19				

The model *F* value was found to be 48.66, making it statistically significant. A model *F* value this large could only have occurred by chance 0.01% of the time. There is only a 0.01% chance that a model *F* value this large is due to noise. The likelihood of error value (prob>F) was then used to evaluate the significance of each model term. If " prob >*F*" is less than 0.0500, then the model terms are significant; if it is more than 0.1000, they are not significant. Based on the F values, all three independent factors substantially impacted the EE of the developed phytosomes. When

controlling for factors like temperature and time, the phospholipid-to-drug ratio (%w/w) was the most crucial factor in EE (%). It has also been found that the phospholipid: drug ratio and temperature positively affected EE (%). The lack-of-fit test indicated that independent variables have a considerable effect on the response if their value is insignificant. Nonsignificant lack of fit, however, is good. The lack-of-fit F score of 1.72 indicates that the lack of fit is not statistically significant compared to the total error. R^2 value, which measures how well a model fits data, was calculated to be 0.9777, indicating that the model has a good fit. The corrected R^2 value of 0.9576 agrees reasonably well with the expected value of 0.8812 for the same variable. The signal-to-noise ratio is the standard by which accuracy is judged. It is preferable if the ratio is higher than 4. In this scenario, the signal-to-noise ratio of 25.039 was satisfactory [32]. They also prove the model's utility for steering one's way through the design process. 3D response surface plots are shown in Fig. 1 and indicate a strong influence of the studied factors X, Y, and Z on the EE. Increasing levels of X, Y, and Z were found to be favorable conditions for obtaining higher EE.

Fig. 1 [Images not available. See PDF.]

Three-dimensional surface response plot showing the interactive effect of **a** Phospholipid: Drug ratio and Temperature, **b** Phospholipid: Drug and Time, and **c** Temperature and Time on EE

Validation of the Model

An additional batch of the CSP formulation was formulated and tested for model validation. The EE of the optimized batch was found to be 94.75 ± 1.06%, which is very near to the model predicted value, confirming the validity and applicability of the suggested model. The percentage bias was estimated using the following equation and was found to be 1.82, less than 3%, confirming the model's relative robustness [33]**.**Bias%=Predicted value-Observed value/Predicted value×100.

Particle size

The particle size of the optimized formulation was determined to be 212.3 nm ± 0.39 and is exhibited in Fig. 2a. The polydispersity index (PDI) for the optimized formulation was found to be 0.238 ± 0.0197.

Fig. 2 [Images not available. See PDF.]

a Particle size distribution and **b** Zeta potential of optimized CSP

Zeta potential

The zeta potential is another critical parameter used to evaluate the stability of the formulation and is shown in Fig. 2b. The optimized formulation obtained after preparation was measured to have a zeta potential of −42.02 ± 0.995 mV.

Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of CSE, Leciva S70, Physical mixture (PM) of CSE and Leciva S70, and CSP are shown in Fig. 3 (from a to d). In CSE spectra, (a) peak appeared at 3417.96 cm^{-1}, representing OH stretching vibration for intermolecular hydrogen bonding substituted on a cyclic ringed structure left to the peak at 2925.48 cm^{-1} due to C-H stretching in Alkanes. The presence of a peak due to C-O stretching at 1238.08 cm^{-1} because of conjugation of the oxygen with the ring, along with the presence of a peak at 1342.21 cm^{-1} due to O–H in planer bending absorption, confirmed the phenolic structure. The presence of a peak at 1629.55 cm^{-1} and 1452.14 cm^{-1} due to aromatic C = C stretching and 1693.19 cm^{-1} due to C = O stretching confirmed the presence of the carbonyl is in conjugation with the aryl group. Generally, the C = O group from ketone appears in the range of 1720–1708 cm^{-1} for simple aliphatic ketone. Still, this band shifted to a lower frequency (1700-1680 cm^{-1}) when the C = O group was in conjugation with the phenyl or aryl group. C–N stretching absorption occurred at 1093.44 cm^{-1} in CSE.

Fig. 3 [Images not available. See PDF.]

FTIR spectra of **a** CSE, **b** Leciva S70, **c** PM, and **d** CSP

FTIR spectra of pure Leciva S70 (b) revealed a peak at 3415.32 due to OH stretch. The peak appeared at 2925.48 cm^{-1} and 2856.06 cm^{-1} (C-H stretching present in the long fatty acid chain), 1743.33 cm^{-1} (C = O stretch in

the fatty acids), 1236.15 cm^{-1} (P=O stretch), 1093.44 cm^{-1} (P–O–C stretch) and 970.02 cm^{-1} ($-N^{+}(CH_2)_3$ stretch). PM FTIR spectra (c) showed peaks that were characteristics of CSE and Leciva S70 phospholipid at 3415.32, 3010.34, 2929.34, 2856.06, 1737.55, 1631.48, 1463.71, 1230.36, 1087.66, and 968.09 cm^{-1}.

The FTIR spectra of CSP formulation (d) exhibited a broad peak at 3403.03 cm^{-1} (intermolecular hydrogen bonded O–H stretch), 2925.48 cm^{-1} (intense peak related to CSE due to C-H stretch), 1743.33(C=O stretch), 1625.70 and 1461.78 (due to C=C stretching in the aromatic ring), 1193.72 (P=O stretching), 1093.44 cm^{-1} (P-O-C stretch) and 973.88 cm^{-1} ($-N^{+}(CH_2)_3$ stretch).

Powder X-ray diffraction

Figure 4 shows CSE, Leciva S70, PM, and CSP x-ray diffraction patterns. CSE was found to be crystalline because its diffraction pattern showed several strong, sharp peaks, and one relatively broad peak. The presence of several sharp peaks and one relatively broad peak in the diffraction pattern is indicative of a crystalline material, as described by Andersan et al. [34]. Peaks for crystallinity in CSE were observed at 2θ values of 9.5, 12.45, 14.29, 24.14, 30.79, 34.20, 35.98, and 38.83°. Leciva S70 phospholipid exhibited two small and relatively broad peaks at 2θ values of 5.6, 7.5, and 20.27°, respectively. The physical mixture (PM) diffractogram presented few crystalline peaks with lower intensity than CSE and two peaks associated with Leciva S70 at 2θ values of 7.6, 9.47, 12.48, 19.93, 30.76, and 34.23^{0}. Possible causes for the diminished crystalline peak strength include the in situ production of partial aggregates between CSE and Leciva S70, a decrease in the amount of CSE in the sample, and interference from the Leciva S70 molecule.

Fig. 4 [Images not available. See PDF.]

XRD overlay spectra of CSE, PM, Leciva S70, and CSP

Solubility analysis

The solubility tests were performed on CSE, PM, and CSP using water and octanol. The lipophilic character of CSE was demonstrated by its lower aqueous solubility (13.07±0.19 μg/ml) and comparatively higher n-octanol solubility (383±2.00 μg/ml). Compared to the CSE, the physical mixture (PM) had 1.74 times higher aqueous solubility but no significant difference in n-octanol solubility. On the other hand, CSP had a much higher aqueous solubility (7.34 times) than CSE ($p<0.05$).

In vitro *release study*

Results of in vitro release of CSE, PM, and CSP are shown in Fig. 5. The release profiles of CSE and PM were found to be identical and nonsignificant throughout the study period. The release profile of CSE after 12 h was found to be 26.42±0.4654%, and for PM, it was 32.15±0.4596%. CSP followed a similar release pattern as CSE and PM during the initial 20 min. However, CSE's release from CSP significantly increased and reached 72.16±0.5248% after 12 h. In addition to these results few study results are provided in additional file 1. Differential scanning calorimetry (DSC) result is presented in Fig. S1. Scanning electron microscopy (SEM) results is shown in Fig. S2 and Proton Nuclear Magnetic Resonance data presented in Fig. S3. Also, the stability study result of the CSP formulation is shown in Table S1.

Fig. 5 [Images not available. See PDF.]

In vitro drug release of CSE, PM, and CSP (The data are shown as the Mean±Standard Deviation=3)

Acute toxicity study

Female rats administered 300, and 2000 mg/kg bw of a CSP formulation showed no signs of death. Furthermore, all the animals did not show any clinical signs of toxicity immediately after dosing and appeared normal for up to 4 h.

In vivo *memory-enhancing activity*

EL/TL on the first day (i.e., the 15th day of drug treatment) reflected the acquisition of learning behavior, and the second day (i.e., the 16th day) reflected the retention of knowledge or memory in the animals. Evidence of memory enhancement was seen in a statistically significant ($p<0.05$) drop in EL and TL values. The findings of an in vivo investigation on memory enhancement can be seen in Fig. 6(a–c).

Fig. 6 [Images not available. See PDF.]

Effect of CSE and CSP on spatial learning and memory in the a) EPMT, b) MWMT, and c) PCT. Data are expressed as Mean ± SEM values ($n = 6$ in each group). **a** = compared to a vehicle-treated normal control group; **b** = compared to the scopolamine HBr-treated group; **c** = compared with the piracetam-treated group; and **d** = compared with CSE + scopolamine HBr-treated group. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$) (one-way ANOVA followed by Tukey's multiple comparisons test)

Elevated Plus Maze Test

TL parameter (in seconds) was measured in EPMT. The reduction in TL was considered to be a memory improvement. The results of EPMT are shown in Fig. 6a. The animal group administered with scopolamine HBr showed a significant increase in TL ($p < 0.05$) on the 15th and ($p < 0.001$) on the 16th day compared with the vehicle-treated control group. The piracetam, CSP + scopolamine HBr, and piracetam + scopolamine HBr-treated group revealed a significant reduction in TL ($p < 0.001$, $p < 0.0001$, and $p < 0.01$) on the 15th day and ($p < 0.001$, $p < 0.0001$ and $p < 0.001$) on 16th day respectively when compared to vehicle-treated group. A significant reduction in TL ($p < 0.0001$) on both days was found in piracetam, CSE + scopolamine HBr, CSP + scopolamine HBr, and piracetam + scopolamine HBr-treated groups when compared to scopolamine HBr-treated group. When CSE + scopolamine HBr was compared to the piracetam-treated group, an increase in TL ($p < 0.05$) was noticed. CSP + scopolamine HBr-treated group showed a significant decrease in TL ($p < 0.01$) on the 15th day and ($p < 0.001$) on the 16th day compared to the CSE + scopolamine HBr-administered group. Overall, there was a significant decrease in TL of CSP formulation administered in animals compared to all groups.

Morris water maze test

The results of MWMT are shown in Fig. 6b. On day 16, EL was higher in the scopolamine HBr group ($p < 0.05$) compared to the control group. On days 15 and 16, EL was considerably ($p < 0.0001$) lower in the piracetam and CSP + scopolamine HBr-treated animal group compared to the control and scopolamine HBr-administered animal group. The CSE + scopolamine HBr-administered group exhibited a decrease in EL ($p < 0.01$) on the 15th and ($p < 0.001$) on the 16th day as compared to the scopolamine HBr-treated group. When the CSE + scopolamine HBr group was compared with the piracetam-treated group, it was observed that EL ($p < 0.01$) significantly increased on both days, i.e., the 15th and 16th day. When the EL of the CSP + scopolamine HBr group was compared to that of the CSE + scopolamine HBr group, a statistically significant drop was found on day 15 ($p < 0.01$) and day 16 ($p < 0.001$). The group given piracetam + scopolamine HBr had a significantly lower EL when compared to the control, scopolamine HBr, and CSE + scopolamine HBr-treated groups ($p < 0.0001$, $p < 0.0001$, and $p < 0.05$) on the 15th day and ($p < 0.001$, $p < 0.0001$, and $p < 0.05$) on the 16th day, respectively. However, the CSP + scopolamine HBr-treated group reduced EL significantly compared with CSE + scopolamine HBr, piracetam, and piracetam + scopolamine HBr-administered groups [26].

Pole climbing test

In the Pole Climbing Test, EL is the amount of time it takes the animal to ascend to avoid a foot shock following the sound of a buzzer. The reduction in EL (in seconds) was considered a memory improvement. The results of PCT are shown in Fig. 6c and found that the scopolamine HBr-administered group increased EL on the 15th ($p < 0.05$) and ($p < 0.001$) on the 16th day when compared with a control-treated group. Compared to scopolamine HBr-treated rats, piracetam and CSP + scopolamine HBr showed a statistically significant reduction in EL ($p < 0.0001$) on both days. Both CSE + scopolamine HBr and piracetam + scopolamine HBr resulted in lower EL levels 15 days after administration ($p < 0.01$, $p < 0.001$) and 16 days after administration ($p < 0.001$, $p < 0.0001$). It was noticed that the CSP formulation-treated group revealed a significant reduction in EL as compared to pure extract. In the case of all treated groups except scopolamine HBr, no significant increase or decrease in EL was observed compared to the control group on both days [26].

Acetylcholine esterase activity

CSE and CSP were tested for their influence on brain Ach esterase activity in scopolamine HBr-induced Wistar rats.

The results are depicted in Fig. 7. For this study, we evaluated Ach esterase activity using one-way ANOVA, then Tukey's multiple comparison test. Compared to the control group that received the vehicle, the scopolamine HBr group showed a statistically significant (p<0.01) increase in Ach esterase activity. This leads to a decrease in Ach transmitter level due to the breakdown of Ach by activated Ach esterase enzyme. A significant (p<0.001) reduction in Ach esterase activity was seen in the CSP + scopolamine HBr-treated group compared to the control group. Compared to the vehicle-treated control group, Ach esterase activity was not significantly different in any treatment groups besides those receiving scopolamine HBr and CSP + scopolamine HBr. Treatment with piracetam, CSP + scopolamine HBr, piracetam + scopolamine HBr, or CSE + scopolamine HBr significantly reduced Ach esterase activity (p<0.0001, p<0.0001, p<0.0001, p<0.001) and increased Ach level at the cholinergic synapse compared to scopolamine HBr administration. Animals who were given CSP + scopolamine HBr showed a more significant reduction in acetylcholine esterase activity (p<0.01) than those given CSE + scopolamine HBr group.
Fig. 7 [Images not available. See PDF.]

Effect of CSE and CSP on brain Ach esterase activity in scopolamine-induced Wistar rats. Data are shown as the Mean ± SEM values (n = 5 in each group). **a** = compared to the vehicle-treated normal control group, **b** = compared to the scopolamine HBr-treated group and **c** = compared to the CSE + scopolamine HBr-treated group (*p <0.05, **p <0.01, ***p <0.001, ****p <0.0001) (one-way ANOVA followed by Tukey's multiple comparisons test)

Estimation of dopamine concentration in Rat's Brain

The brain dopamine estimation was carried out by sandwich ELISA, and one-way ANOVA followed by Tukey's multiple comparison tests was used for the evaluation of dopamine level. The brain dopamine concentration level is shown in Fig. 8. The brain dopamine concentration was significantly increased in the piracetam and CSP-scopolamine HBr-treated groups (p<0.01) when compared to the control. Piracetam and CSE-scopolamine HBr, CSP-scopolamine HBr, and piracetam-scopolamine HBr-treated groups showed significantly increased in dopamine level (p<0.0001, p<0.05, p<0.0001, p<0.05) compared to scopolamine HBr-treated group. When the CSP-scopolamine HBr-treated group was compared with the CSE-scopolamine HBr-treated group, we found a significantly increased dopamine level (p<0.05). The brain dopamine concentration was found to be 18.07 ± 1.790 and 11.60 ± 1.718 ng/ml in the CSP and pure extract-treated groups, respectively.
Fig. 8 [Images not available. See PDF.]

Brain dopamine concentration level. Data are expressed as Mean ± SEM values (n = 5 in each group). **a** = compared to a vehicle-treated normal control group; **b** = compared to the scopolamine HBr-treated group; **c** = compared with CSE + scopolamine HBr-treated group. (*p <0.05, **p <0.01, ***p <0.001, ****p <0.0001) (one-way ANOVA followed by Tukey's multiple comparisons test)

Estimation of serotonin concentration in rat's brain

The brain serotonin level was determined by sandwich ELISA techniques. The result is exhibited in Fig. 9. A one-way ANOVA followed by Tukey's multiple comparison tests was used for the evaluation of serotonin levels. There was a significantly increased level of serotonin in piracetam, CSE-scopolamine HBr, CSP-scopolamine HBr, and piracetam-scopolamine HBr (p<0.0001, p<0.01, p<0.0001, p<0.01) compared to the control group. Brain serotonin levels were significantly increased (p<0.0001) in all groups when compared to the scopolamine HBr-treated group. There has been a significantly increased level of serotonin (p<0.0001) in the CSP formulation-treated group when compared to the CSE-scopolamine HBr-treated group. The brain serotonin concentration was found to be 47.92 ± 1.690 and 36.99 ± 1.742 ng/ml in the CSP formulation and pure extract-treated groups respectively.
Fig. 9 [Images not available. See PDF.]

Brain serotonin concentration level. Data are expressed as Mean ± SEM values (n = 5 in each group). **a** = compared to a vehicle-treated normal control group; **b** = compared to the scopolamine HBr-treated group; **c** = compared with CSE + scopolamine HBr-treated group, d = compared to piracetam-treated group. (*p <0.05, **p <0.01, ***p <0.001, ****p

<0.0001) (one-way ANOVA followed by Tukey's multiple comparisons test)

Histopathological study

The results of the histopathological examination of the brain are shown in Fig. 10. The normal control group did not exhibit any toxicity in terms of neuronal and vascular degeneration or glial cell infiltration (Fig. 10a). Moderate vascular deterioration, cerebral neuronal degeneration, necrosis, and glial cell infiltration were all observed in the brain of the disease-control animal (Fig. 10b). However, other animals belonging to different treatment groups showed mild pathological changes when compared with disease control. A mild type of glial cell infiltration and neuronal and vascular degeneration was observed in the piracetam (Fig. 10c) and CSE (Fig. 10d) administered group. The brains of CSP-treated animals showed only mild neuronal and vascular degeneration and glial cell infiltration as compared to CSE-treated group (Fig. 10e).

Fig. 10 [Images not available. See PDF.]

Histopathology micrographs of rat brain **a**] Group I, **b** Group II, **c** Group III, **d** Group IV, **e**] Group V, and **f** Group VI. Yellow, white, and red colored arrow indicates vascular degeneration, glial cell infiltration, and neuronal degeneration, respectively

Estimation of the concentration of CSE and CSP in brain tissue

CSE and CSP concentrations were measured in given animals over 15 days and were found to be 1.143 ± 0.045 µg/ml and 2.683 ± 0.21 µg/ml, respectively.

Comparative pharmacokinetic study of CSE and CSP in the blood plasma compartment

Table 5 displays the pharmacokinetic parameters that were determined using the PK Solver software. The results of the research showed that C_{max}, T_{max}, and $AUC_{0-\infty}$ were significantly higher (*p<0.05*) in animals given CSP as compared to those given CSE. Additionally, a longer absorption half-life was found for CSP. The fact that the CL/F and V/F were significantly (*p<0.05*) lower in the CSP-administered group mice compared to the CSE-treated animals provided additional support for CSP's longer residency and duration of effect.

Table 5. Results of comparative bioavailability study after oral administration of CSE and CSP formulation

Pharmacokinetic parameters	Treatment	
CSE (250 mg/kg)	CSP (~ 250 mg/kg of CSE)	C_{max} (µg/ml)
0.404 ± 0.116	0.828 ± 0.134	T_{max} (hours)
1.71 ± 0.013	3.72 ± 0.018	AUC 0-t (µg/ml*h)
2.492 ± 0.164	8.666 ± 0.186	AUC 0-inf (µg/ml*h)
2.762 ± 0.119	8.864 ± 0.131	t1/2 ka (hours)
0.549 ± 0.123	1.867 ± 0.224	V/F (mg/kg)/(µg/ml)
867.41 ± 0.019	300.74 ± 0.039	CL/F (mg/kg)/(µg/ml)/h

*The information is shown as Mean ± SD values (*n* = 6)

Discussion

C. sinensis phytosomes were formulated by a solvent evaporation method using Leciva S70 as a phospholipid at varying phospholipid: drug ratio, temperature, and time. The effects of these independent variables were determined on the EE of *C. sinensis* extract. In the optimization study, the optimal criterion was the achievement of maximum

EE at specific concentrations of the three independent variables. The optimum response of EE was reported to be 96.51% at phospholipid: drug concentration of 3% w/w, at a temperature of 60 °C, and time of 3 h using the desirability function, with good desirability of 1. The particle size of the phytosomes is quite an important parameter that needs to be considered during the development of formulations. Particle size significantly influences the distribution of the formulation within tissues and organs. It has been noted that the average particle size for drug distribution to the brain and other organs ranges from approximately 100 to about 1000 nm, with the precise value contingent upon the dosage form and administration method. A PDI value of up to 0.3 is considered to be acceptable. It suggests a homogeneous population of phospholipid vesicles, which is desirable in lipid-based drug delivery applications like liposome and nanoliposome formulation [35]. Values of zeta potential greater than -30 mv are considered as satisfactory and indicative of solid physical stability. Phospholipid type and structure determine the zeta potential value. This indicated that the developed CSP formulation had good physical strength due to its nanometer particle size, low PDI, and moderate zeta potential value [36].

When FTIR spectra of CSP were compared with CSE and Leciva S70 phospholipid spectra, it was observed that the OH stretching frequency of CSE at 3417.96 cm^{-1} changed to 3403.03 cm^{-1} in the CSP formulation indicated the presence of weak intermolecular interactions. The CSE peaks at 1033.66, 1147.44, 1238.08, and 1342.21 cm^{-1} were found to be disappeared and appeared to be new broad doublet strong peak at 1093.44 (similar to the peak of Leciva S70 but more intense as compared to Leciva S70) and 1193.72 cm^{-1}. The absorption frequency and intensity of peak at 1629.55 cm^{-1} in CSE appeared too shortened along with a slight change in absorption frequency in CSP formulation and found at 1625.70 cm^{-1}. In CSE and Leciva S70 FTIR spectra, a tiny peak was observed at 1093.44 cm^{-1}, which appeared strong and intense in the CSP formulation and indicated the presence of interaction. The changes in absorption frequencies of peaks in CSP formulation compared to CSE and Leciva S70 revealed the presence of weak intermolecular interaction between CSE and Leciva S70 phospholipid may be the reason for the formation of CSP [37, 38]. X-ray diffraction study was used to identify the crystal structure of various solid compounds. It is used to determine the degree of crystallinity. Amorphous material scatters at all wavelengths and gives a scattered pattern (continuous background); however, the crystalline material includes a crystal structure and produces definite diffraction lines or spots. The diffraction pattern of CSP revealed only one broad peak at 2θ values from 18.83 to 27.14°, and maximum peat height was obtained at 22.97°. Most of the prominent crystalline peaks often seen in diffraction patterns of CSE were not present in those of CSP. As a result of CSE phospholipid complex formation, most of the crystalline peaks of CSE disappeared. Based on its diffraction pattern, CSP indicated that CSE in the Leciva S70 matrix might exist in either a molecularly distributed or amorphous form [39].

The optimized formulation followed a zero-order drug release kinetic model with a significance value of $P<0.05$. The higher wettability and solubility of CSE in the prepared CSP formulation may account for the considerable difference in CSP release rate from CSE and PM. In the CSP formulation, CSE was changed from a crystalline to an amorphous state, which extended both the rate and the extent of release over 12 h. The enhanced solubility of CSP was due to the amphiphilic nature of LECIVA S 70. The increased wettability and enhanced dispersion of CSP due to changes in the structural morphology of crystalline CSE into partial amorphization imparted due to the amphiphilic nature of LECIVA S 70 phospholipid [40]. There was no significant change in the CSP formulation during 6 months. However, a slight drop in EE and an increase in vitro release were detected throughout the storage period. This could be due to the release of drugs from the lipid matrix [20]. The temperature influences the lipid matrix over time, resulting in slender drug release from the interior and a modest increase in drug release. As the length of the storage period increased, slight increases in particle size and a slight decrease in zeta potential were observed. This could be attributed to the effect of temperature, which causes disturbances in the formed electrically double diffuse layer of formulation, but it could also be attributed to something else. Animals showed no clinical signs of intoxication at daily observations up to 14 days at a studied dose. Therefore, under the OECD 423 Guidelines and the specified laboratory circumstances, the LD50 value for CSP was determined to be >2000—5000 mg/kg body weight, placing it in GHS Category 5 and making it safe for use. CSP exhibited higher efficiency compared with CSE in MWMT, EPMT, and PCT. Administration of CSE, CSP, and piracetam for 15 consecutive days protected memory from

memory impairment to different extents induced by scopolamine HBr. Hence, there was no observed increase in EL/TL even after the administration of scopolamine HBr on the 16th day. Thus, there was no significant practical effect on memory retention on the 16th day. Piracetam was used as a Positive control and showed significant improvements in memory in terms of EL/TL, as expected. Smaller particle size and the interaction between phospholipid and CSE, which improves the overall hydrophilicity and solubility of CSE, may account for the relative absorption of CSP after oral administration. From this, we can infer that the EL/TL in MWT, EPMT, and PCT were significantly lower in the CSP formulation-administered group compared to the CSE-administered group. Administration of CSP dose equivalent to pure extract (CSE) significantly increased the Ach level and inhibited Ach esterase activity. Acetylcholine is essential in controlling cognitive function in experimental and clinical settings. So CSP was more effective in increasing the level of Ach than CSE by decreasing the AchE activity [41, 42]. Dopaminergic neurons projecting from the substantia nigra to the striatum play a critical role in motor functions, while dopaminergic neurons originating in the ventral tegmental area (VTA) and projecting to the nucleus accumbens, hippocampus, and other cortical structures regulate rewarding learning [43]. Dopamine is the principal neurotransmitter involved in both the central nervous system (CNS) and peripheral nervous systems and plays a critical role in learning and memory. This release of dopamine helps us learn to associate the rewarding stimulus with the behavior that led to it [44]. Dopamine also plays a role in the consolidation of memories. This is the process by which memories are stored in the long term. When we learn something new, dopamine helps to strengthen the connections between the neurons that represent that information. This makes it more likely that we will be able to remember the information in the future. Dopamine levels are also important for working memory. This is a type of short-term memory that allows us to hold information in mind while we are working on a task. Dopamine helps keep information in working memory by increasing the activity of neurons in the prefrontal cortex. This allows us to focus on the task at hand and avoid distractions [45]. The prepared phytosome formulation (CSP) protects memory from scopolamine-induced memory impairment and enhances cognitive function by increasing dopamine concentration. A decline in DA concentration or inhibition of its synthesis or metabolism rates in the CNS can result in impairment of critical neurologic functions, such as cognition, behavior, and fine movements. The neurodegenerative disease often affects mental performance, particularly memory processes, and pathological changes have been reported to occur in the cholinergic, glutamatergic, serotonergic, and noradrenergic transmitter systems [45]. From these findings, it appears that several neurotransmitters are in some way involved in the formation and retrieval of memory traces. Measurement of cognitive performance in rats incorporates modulatory functions such as sensory, attentional, motivational, emotional, and motor processes. Serotonergic activity has been linked to emotional processes and may play a particular role in emotional-related work. Serotonin, one of the most important neurotransmitters in the central nervous system, is synthesized by the amino acid tryptophan and is important in learning and memory. Indeed, 5-HT pathways, 5-HT reuptake site/transporter (SERT), and 5-HT receptors are present in brain areas implicated in learning and memory [46]. Serotonergic projections originate in the ascending raphe nuclei localized in the brain stem, where 5-HT synthesis, storage, and reuptake occur, and extend to almost all forebrain areas involved in learning and memory [47]. 5-HT exerts an influence via cholinergic and glutamatergic pathways over the transfer of information. Decreased brain serotonin levels impaired memory [48]. So, in this study, we found a significantly increased serotonin level in the CSP formulation-treated group as compared to the pure extract, which exhibited an increase in memory and learning. Acute and chronic central nervous system illnesses share the characteristics of neuroinflammation. Neuroinflammation is caused by glial cells like astrocytes and microglia being persistently activated in the brain and releasing inflammatory mediators such as cytokines, matrix metalloproteinases (MMPs), reactive oxygen species (ROS), and nitric oxide [10]. An unpaired t test was used to compare CSE and CSP results, and it was observed that the concentration of CSP was significantly (p<0.05) greater than that of CSE. This demonstrates how CSE was delivered to the brain more effectively when it was a phytosome formulation rather than a pure extract. This could be the result of phospholipid being present in phytosomes, which might have changed GI permeability and prevented an apically directed efflux mechanism. It was found that the bioavailability of the CSP formulation was 3.21 times higher than that of the CSE formulation.

Molecular aggregates and phospholipids work together to promote intestinal absorption and water solubility, which increases the plasma bioavailability of CSP after a single oral dosage.

Saima Rubab et al. also studied the neuropharmacological potential of various morphological parts of *C. sinensis* and it was observed that both leaves and seeds are active as neuropharmacological agents and can be used as memory-boosting agents. Neuropharmacological evaluation of seed and leaf extracts on mice revealed dose-dependent effects. Seed extract demonstrated significant stimulant activity, while leaf extract induced notable calmness and moderation. Tests including head dip, open field, and rearing showed increased motor function with both extracts, with leaf extract notably calming. Seed extract exhibited greater stimulant effects in cage cross, swimming, and traction tests. Leaf extract showed similar effects but induced more calmness at higher doses. Micro-morphological investigation of Camellia sinensis is suggested for accurate identification. These findings highlight differing effects of plant parts, with seed extract being more stimulating and leaf extract inducing calming effects [49]. Similarly, Saima Rubab et al. performed phytochemical and pharmacological potential of *C. sinensis*. both seed and leaf showed significant analgesic effects. However, compared to seed extract which showed highly significant (p <0.001) increase in concentration dependent manner, leaf extract displayed highly significant results even at low dose with better results at high dose compared with standard [50, 51].

Muhammet Emin Cam et al. performed Morris's Water Maze Test to determine the potential of *C. Sinensis* leaves hydroalcoholic extract in AD. It was observed that the AD group reached the target quadrant later than the control group (p<0.05) in all days and the total time spent in the quadrant decreased. In the MWM test, rats underwent spatial learning evaluation. Throughout training, all groups showed reduced time to locate the underwater platform, indicating learning. However, the Alzheimer's disease (AD) group exhibited prolonged training duration, indicating spatial memory impairment. Metformin treatment notably improved learning abilities, as evidenced by decreased training time compared to the AD group. Overall, the MWM test revealed enhanced spatial learning with metformin treatment, highlighting its potential therapeutic efficacy in cognitive decline associated with AD [52]. Similar observations were also noted in our experiments. The culmination of our investigation aligns with the outcomes observed by other research groups, affirming the potential efficacy of CSP formulation as a cognitive enhancer, surpassing the effects elicited by conventional CSE methodologies. This assertion is substantiated by a collective appraisal of research findings across various groups, suggesting a notable advantage conferred by CSP in enhancing memory functions.

Conclusion

Overall, the findings of this study suggest that the developed CSP formulation holds significant promise as a memory-enhancing agent, outperforming the effects of the conventional CSE. The observed improvements in memory, cognitive function, and bioavailability underscore the potential of nanophytosomes in enhancing the therapeutic properties of natural extracts for neurological applications. Further research and clinical studies are warranted to explore the translational potential of this CSP formulation in the context of cognitive disorders and related conditions.

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Author contributions

VM was involved in experimental work, writing/drafting manuscript. SK helped in design of experiments. HM and HT contributed to supervision of the project.

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Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All animal handlings and experiments were approved by the Animal Ethical Committee of Crystal Biological Solution, Pune, with approval number CRY/2122/070.

Consent for publication

All authors agreed to publish the article in this journal.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations

CSE
Camellia sinensis Extract
CSP
Camellia sinesis Phytosomes
Ach
Acetylcholine
AD
Alzheimer’s disease
EGCG
Epigallocatechin gallate
EE
Entrapment efficiency
CCD
Central composite design
PDI
Polydispersity index
DSC
Differential scanning calorimetry
PBS
Phosphate-buffered saline
EPMT
Elevated Plus Maze Test
MWMT
Morris Water Maze Test
CS
Conditioned stimulus
US
Unconditioned stimulus

Publisher's Note

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DETAILS

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A preliminary study on the impact of nutrient stress induction on drug cytotoxicity in glioblastoma cells and fibroblasts

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ABSTRACT (ENGLISH)

Background

Nutrient stress (NS), one of the hallmarks of the tumour microenvironment, can render cancer cells tolerant to cytotoxicity. Fibroblasts, on the other hand, have cancer cell-like traits, such as plasticity and resiliency. Hence, this study aimed to evaluate the cytotoxicity of the drug on reseeded human U87 glioblastoma (GBM) cells as well as on mouse L929 fibroblasts in the form of monolayer and colonies that grew after NS induction.

Results

No treatment for 48 h showed a statistically significant difference in U87 cell viability when compared to the 50% hypothetical value. However, temozolomide (TMZ) (151.0 µg/ml) and azithromycin (AZI) (92.0 µg/ml) significantly diminished the number of U87 cell colonies compared to the untreated control, and AZI also outperformed doxycycline (DOXY) (147.0 µg/ml). L929 fibroblasts survived NS, but the cytotoxicity of AZI, DOXY, and AZI + DOXY (92.0 + 147.0 µg/ml) substantially increased than in L929 fibroblasts without NS induction.

Conclusions

The present findings suggest that NS does not inevitably contribute to cytotoxic drug tolerance in GBM cells. In addition, although fibroblasts can withstand NS, they can also become susceptible to cytotoxic drug-induced death; nevertheless, the type of drug may play a role.

FULL TEXT

Background

High-grade glioma, notably glioblastoma (GBM), is one of the most challenging cancers to treat, partly due to its high degree of heterogeneity. Despite the discovery of drugs that can interfere with GBM growth, such as temozolomide (TMZ), most patients succumbed to death. The major issues are that not all patients respond to the same effective drugs, and even if they do, patients ultimately develop resistance. Although much work is currently being expended on immune-based therapies [1], alternative effective agents are in constant demand.

Azithromycin (AZI) and doxycycline (DOXY) are intriguing candidates of antibiotics actively being studied for their anticancer attributes. Both drugs have exhibited substantial anti-proliferative and pro-apoptotic effects in different human cancer cells [2–5]. They are also capable of modulating various therapeutic targets associated with cancer

hallmarks, including angiogenesis and pro-survival autophagy [2, 4, 6, 7]. Furthermore, they are effective against drug-tolerant persister cells of a wide range of origins, such as cancer stem-like cells, in part by inhibiting mitochondrial oxidative phosphorylation (OXPHOS) [3, 8–10].

The tumour microenvironment (TME) is composed of a complex network of cells (e.g., malignant/stromal cells) and non-cellular components (e.g., extracellular matrix proteins) that surround and interact with one another, which mutually contribute to tumour growth and development. The metabolism of tumours, including GBM, is malleable to factors in the environment in which they emerge. Metabolism is the chemical process that allows cells to use available nutrient resources to sustain their survival and propagation. The availability and quantity of nutrients, as well as other elements that enable metabolism, such as oxygen, and the presence of other cells (such as fibroblasts, endothelial and immune cells) that compete for or fuel such nutrients, are all intrinsic factors that can influence tumour behaviours [11].

Nutrient scarcity is one of the hallmarks of TME, and the Warburg effect (a glucose-dependent glycolytic aerobic process) is an atypical metabolic phenomenon of cancer cells. As a result, cancer cells not only reprogram their metabolism but also develop various mechanisms to meet their energy demands and sustain survival. For instance, they switch metabolism towards mitochondrial OXPHOS and phenotype to a quiescent state in response to glucose stress [12, 13]. Secondly, cancer cells secrete molecules that promote angiogenesis to improve blood flow and delivery of oxygen and nutrients, as well as attract other cells (e.g., fibroblasts) that can provide additional resources [14, 15]. Indeed, the ability of cancer cells to adapt, survive, and propagate under such an intricate milieu promotes evolution, driving them towards a more aggressive phenotype/behaviour and resulting in drug resistance [11, 13, 16, 17].

Aside from that, tumour behaviours are greatly influenced by activated fibroblasts, one of the most abundant stromal cells in the TME. They are renowned as cancer-associated fibroblasts and are heterogeneous, with distinct functions that include tumour growth, survival, and metabolism, as well as mediating drug resistance [14, 15]. Ironically, fibroblasts have cancer cell-like traits, such as plasticity and resiliency, and they are associated with all stages of tumour progression [15]. Tumours have even been dubbed “wounds that do not heal” [18].

To that end, it is of interest to expound the impact of nutrient stress (NS) on drug cytotoxicity in GBM cells and fibroblasts. Our earlier findings showed that the concentrations of TMZ, AZI, and DOXY that caused 50% of U87 GBM cell monolayer to be affected (IC_{50}) under nutrient-favourable conditions for 48 h were 151.0, 92.0, and 147.0 µg/ml, respectively [19]. This study investigated their effects on U87 cell viability and colonies, as well as L929 fibroblast viability after the induction of NS.

Methods

Drugs and reagents

TMZ (Sigma-Aldrich, St. Louis, MO, USA), AZI (BioVision, Milpitas, CA, USA), DOXY (Sigma-Aldrich, St. Louis, MO, USA), 1 × phosphate buffer saline (PBS) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), 10.0% neutral buffered formalin (NBF) (Sigma-Aldrich, St. Louis, MO, USA), and gram crystal violet reagent (GCC Diagnostics, UK). Note that AZI (Sigma-Aldrich, St. Louis, MO, USA) was used to treat NIH3T3 and human dermal fibroblasts (HDF), but the manufacturer discontinued the drug in the middle of the study.

Culture of GBM cells and fibroblasts

The human U87 GBM cell line (HTB-14) was obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA) and maintained at 37 °C with 5% CO_2 and 95% humidity in Dulbecco’s Modified Eagle Medium (DMEM) (4.5 g/l glucose with L-glutamine and sodium pyruvate) (Nacalai Tesque, Kyoto, Japan), supplemented with 10% foetal bovine serum (FBS) (Tico Europe, Amstelveen, Netherlands) and 1% non-essential amino acid (Nacalai Tesque, Kyoto, Japan). Mouse connective tissue L929 and embryonic NIH3T3 fibroblasts (ATCC), and HDF (RIKEN Cell Bank, Tsukuba, Ibaraki, Japan) were cultured in the same manner as GBM cells.

NS induction

To induce NS in GBM cells, U87 cells were seeded in T75 flasks at a density of 1.0×10^3 in a final volume of 12 ml

and maintained under standard growth conditions. In brief, the medium was refreshed on days 30 and 33, cells were then subcultured on day 36, and harvested on day 38, followed by seeding. To induce NS in normal cells, L929 fibroblasts were seeded in T75 flasks at a density of 1.0×10^3 in a final volume of 12 ml and maintained under standard growth conditions. In brief, the medium was refreshed on day 48, fibroblasts were then subcultured on day 54, and harvested on day 56, followed by seeding.

The cells were sustained in culture for a period until some adherent (spindle-shaped) cells were observed (Fig. 1), day 30 and day 48 for GBM cells and fibroblasts, respectively. GBM cells were fully repopulated on day 36, while fibroblasts were on day 54. This field is based on the principle that metabolic activities in cancer cells differ from normal cells, with cancer cells exhibiting rapid abnormal growth and higher metabolic demands.

Fig. 1 [Images not available. See PDF.]

Representative of **a** U87 cells and **b** L929 fibroblasts following nutrient stress (NS) induction (10 × magnification, scale bars = 100 µm)

Determination of U87 cell viability

A total of 1.0×10^3 cells in a final volume of 100 µl was seeded in 96-well plates and cultured for 72 h before being treated. After 48 h of cytotoxic drug treatments, the MTT assay was carried out in accordance with the manufacturer's protocol, and the percentage of cell viability was calculated using the following equation:Cellviability %=Meanabsorbanceoftreatedcells-MeanabsorbanceofblankMeanabsorbanceofuntreatedcells-Meanabsorbanceof blank×100

Determination of U87 cell colonies

Cells were seeded in 6-well plates at a density of 1.0×10^3 in a final volume of three ml and maintained under standard growth conditions. On day 21, the medium was refreshed, and cells were further incubated until day 25, after which they were treated for 48 h. On day 27, cells were fixed with two ml of 10.0% NBF for 20 min, rinsed once with two ml of 1 × PBS, and stained in two ml of 0.01% crystal violet for 20 min. In each well, eight regions were randomly captured under a visual field (5 ×) using a phase-contrast inverted microscope (Zeiss, Germany), and the number of colonies with at least 50 cells was counted using ImageJ software (NIH Image, Bethesda MD, USA).

Determination of L929 fibroblast viability

A total of 5.0×10^3 cells in a final volume of 100 µl was seeded in 96-well plates and cultured for 24 h before being treated, followed by an MTT assay. The effects of cytotoxic drugs on the viability of fibroblasts, such as L929, NIH3T3, and HDF without NS induction were also assessed after 48 h of treatment.

Statistical analysis

Data passed the Shapiro–Wilk normality test ($p > 0.05$). One-sample *t*-test with 50% as a standard value of interest or the hypothetical value was performed using the SPSS version 26 software (IBM Corporation, NY, USA). While one-way analysis of variance (ANOVA) with Tukey's post hoc test and two-way ANOVA with Sidak's post hoc test were computed using the GraphPad Prism version 6.01 software (San Diego, CA, USA). All analyses were two-tailed and *p* value less than 0.05 was considered significantly different.

Results

Drug cytotoxicity on U87 cell viability

None of the treatments showed a statistically significant difference in U87 cell viability (Table 1). In other words, the drug cytotoxicity does not substantially differ from the hypothesised value of 50%. Besides, one-way ANOVA revealed no statistically significant difference between TMZ (151.0 µg/ml) (positive control group), AZI (92.0 µg/ml), DOXY (147.0 µg/ml), and AZI + DOXY (92.0 + 147.0 µg/ml) treatments ($F(3, 8) = 0.726$, $p = 0.565$) (Fig. 2). All in all, there is insufficient evidence to reject the null hypothesis in favour of an alternative hypothesis. It could not be statistically corroborated that NS induction renders GBM cells tolerant to cytotoxic drugs under the present test conditions.

Table 1. Effect of nutrient stress (NS) induction on drug cytotoxicity in U87 cells

Treatment	Mean (SD)	*t* statistic (degrees of freedom)	*p* value (2-tailed)*	Mean difference (95% confidence interval)
TMZ	71.26 (11.75)	3.133 (2)	0.089	21.26 (−7.94, 50.46)
AZI	95.39 (28.83)	2.728 (2)	0.112	45.39 (−26.21, 117.00)
DOXY	76.56 (19.28)	2.386 (2)	0.140	26.56 (−21.33, 74.45)
AZI + DOXY	83.03 (21.18)	2.701 (2)	0.114	33.03 (−19.58, 85.64)

*One-sample *t*-test

Fig. 2 [Images not available. See PDF.]

Drug cytotoxicity on reseeded U87 cell monolayer after nutrient stress (NS) induction. Data are the mean (standard deviation, SD) of three technical replicates from three independent experiments ($n=3$)

Drug cytotoxicity on U87 cell colonies

The cytotoxicity of tested drugs was further evaluated on U87 colonies that grew after the NS induction. One-way ANOVA discovered a statistically significant difference between groups ($F(4, 40)=7.455$, $p=0.0001$) (NC (61.89 (7.66), TMZ (47.00 (9.19), AZI (38.44 (7.02), DOXY (57.89 (15.68), and AZI + DOXY (51.33 (8.59)). Based on Tukey's post hoc test, TMZ and AZI significantly diminished the number of U87 cell colonies compared to the untreated group or negative control (NC) (Fig. 3). AZI also markedly outperformed DOXY.

Fig. 3 [Images not available. See PDF.]

Drug cytotoxicity on U87 cell colonies that grew after nutrient stress (NS) induction. Data are the mean (SD) of a single replicate from nine independent experiments ($n=9$). One-way ANOVA, followed by Tukey's post hoc test; *$p<0.05$; **$p<0.01$; ***$p<0.001$

Drug cytotoxicity on L929 fibroblasts

To compare the cytotoxicity of studied drugs, fibroblasts were treated at the IC50 levels in GBM cells. Based on the comparison made to the 50% hypothetical value, only AZI + DOXY treatment significantly decreased the viability of L929 fibroblasts (Table 2). It is worth noting that the mean difference in L929 fibroblast viability across treatments was lower than the hypothetical value, contrasting to U87 cells (Table 1).

Table 2. Effect of nutrient stress (NS) induction on drug cytotoxicity in L929 fibroblasts

Treatment	Mean (SD)	*t* statistic (degrees of freedom)	*p* value (2-tailed)*	Mean difference (95% confidence interval)
TMZ	33.17 (12.86)	−2.267 (2)	0.152	−16.83 (−48.78, 15.11)
AZI	25.27 (12.33)	−3.475 (2)	0.074	−24.73 (−55.36, 5.89)

DOXY	24.70 (12.61)	−3.474 (2)	0.074	−25.30 (−56.64, 6.04)
AZI + DOXY	11.33 (2.60)	−25.73 (2)	0.002**	−38.67 (−45.13, −32.20)

*One-sample *t*-test; ***p*<0.01

Further to that, a two-way ANOVA showed a non-significant difference between treatment groups (F (3, 16) = 1.303, p = 0.308) and a significant difference between treatment groups before (TMZ (40.27 (2.42), AZI (52.43 (7.42), DOXY (46.97 (10.26), and AZI + DOXY (46.40 (6.24)) and after NS induction (F (1, 16) = 36.56, p<0.0001); however, the interaction between before and after NS was not significant (F (3, 16) = 2.418, p = 0.104). A Sidak's post hoc test demonstrated that AZI, DOXY, and AZI + DOXY treatments exhibited an increased cytotoxic effect on L929 fibroblasts after the NS induction (Fig. 4). These data indicate that the induction of NS altered their sensitivity to tested drugs, except for TMZ, which had no meaningful difference between before and after NS. In fact, the lack of interaction between before and after NS suggests that the NS induction does not affect the cytotoxicity of the drug in fibroblasts; nevertheless, the type of drug may play a role.

Fig. 4 [Images not available. See PDF.]

Drug cytotoxicity on L929 fibroblasts and on reseeded L929 fibroblasts after nutrient stress (NS) induction. Data are the mean (SD) of three technical replicates from three independent experiments (n = 3). Two-way ANOVA, followed by Sidak's post hoc test; *p<0.05; **p<0.01

Additionally, a two-way ANOVA showed a significant difference between treatment groups (F (3, 16) = 18.35, p <0.0001) and a significant difference between fibroblast types (NIH3T3 vs. HDF) (F (1, 16) = 59.28, p<0.0001) (TMZ (66.27 (11.23) vs. 99.17 (2.29), AZI (44.80 (2.12) vs. 56.83 (7.58), DOXY (48.40 (7.63) vs. 78.60 (3.26), and AZI + DOXY (61.23 (5.70) vs. 83.30 (13.55)); though, the interaction between fibroblast types was not significant (F (3, 16) = 2.21, p = 0.127). A Sidak's post hoc test demonstrated that drug cytotoxicity differed across fibroblast types (Fig. 5), except for AZI. Despite this, the lack of interaction between fibroblast types suggests that the drug types may affect the effects.

Fig. 5 [Images not available. See PDF.]

Drug cytotoxicity on NIH3T3 fibroblasts and human dermal fibroblasts (HDF). Data are the mean (SD) of three technical replicates from three independent experiments (n = 3). Two-way ANOVA, followed by Sidak's post hoc test; *p<0.05; ***p<0.001

Discussion

In a previous study, AZI and DOXY have shown promising anticancer effects in GBM cells [19]. Nevertheless, the role of NS in driving tumour cells to adaptive growth and survival, as well as its involvement in the development of drug resistance, is a matter of ongoing research.

The main finding herein is that NS-induced U87 cells essentially do not develop into a drug-tolerant phenotype. The mean differences in U87 cell viability across treatments were not statistically significant. Moreover, TMZ and AZI retained their effectiveness in reducing U87 cell colonies. In these situations, different drug mechanisms and targets that certainly influence the findings cannot be ruled out. Indeed, the probable divergence of biological activities and oncogenic signalling pathways between reseeded monolayer and grown aggregates warrants further exploration. In particular, NS promotes chemoresistance [13, 16]. As per Mondal and colleagues, NS induction leads to phenotypic reprogramming, driving U87 cells into a glioma stem cell-like phenotype. Explicitly, NS-induced U87 cells demonstrated increased drug efflux capacity, which is consistent with the MTT cell viability assay, as the cytotoxicity of TMZ, cisplatin, and paclitaxel decreased on these cells [16].

On the other hand, glucose deprivation caused a marked sensitisation of U87 cells to TMZ and carboplatin, nearly

doubling the rate of apoptosis [13]. Nevertheless, further analysis revealed that it drove U87 and U251 GBM cells to exit the cell cycle and enter quiescence, and augmented autophagy, allowing them to withstand TMZ- and carboplatin-induced cytotoxicity. Indeed, autophagy is responsible not only in chemoresistance but also tumour survival by acting as a self-degradation channel that maintains metabolism and homeostasis, as well as an intracellular recycling platform that supplies the nutrients needed for growth [13, 14]. Likewise, when glucose was substituted with galactose, PANC1 pancreatic cancer cells switched to autophagy-dependent mitochondrial OXPHOS [12].

In contrast to the aforementioned, we previously found that 21-day survived U87 cells without medium replenishment were completely susceptible to AZI and DOXY treatments [19]. Notably, AZI exhibited preferential cytotoxicity in CAL27 oral squamous and Detroit 562 pharyngeal cancer cells under amino acid-deprived conditions by blocking autophagic flux [2]. GBM, like other cancers, prioritises glucose to produce adenosine triphosphate via aerobic glycolysis, but switches to mitochondrial respiration when nutrients or glucose are scarce [12, 13, 20]. At its core, AZI and DOXY have superior cytotoxicity to nutritionally stressed cells probably owing, in part, to the fact that they mediate cell death through mitochondrial respiration [3, 8–10] and autophagy inhibition [2, 4].

In a different study, tetrathiomolybdate (TM) has been discovered to be more toxic to neuroblastoma cells in low-glucose conditions, regardless of normoxia and hypoxia [21]. This can be partly attributed to the role of TM, which increases glucose consumption and activates glycolysis, making them vulnerable to death in low-glucose settings. Onodera and colleagues, alternatively, delineated that the redox system inhibitors, including penicillic acid (PA), have preferential cytotoxicity to PANC-1 pancreatic cancer cells in a nutrient-deprived condition [22]. In this case, nutrient deprivation raises the reactive oxygen species, while PA form an adduct with glutathione (GSH) and a lack of precursor amino acids required for GSH synthesis drops its level, rendering them defenceless to oxidative stress-mediated apoptotic death.

Herein, it was also discovered that L929 fibroblasts that survived for 48 days in nutrient-deprived conditions (re-enter the cell cycle and proliferate after being subjected to nutrient-favourable conditions) were more susceptible to AZI-, DOXY-, and AZI + DOXY-induced cytotoxicity. In different studies, AZI shows preferential cytotoxicity to senescent fibroblasts [23, 24], and AZI and DOXY both have senotherapeutic potential [24, 25]. These facts likely, in part, explain why the fibroblasts were more susceptible to them after NS induction. Certainly, NS activates autophagy, while autophagy promotes senescence in cancer cells and fibroblasts [14, 26]. Ironically, cancer cells with senescence reversal exhibit more aggressive behaviours [27], whereas autophagic-senescent fibroblasts promote tumour growth and metastasis metabolically through paracrine production of high-energy mitochondrial fuels [14]. Although acknowledging the specificity of drugs of interest is beyond the scope of the present work since fibroblasts were cultured under the same conditions as GBM cells, which may have altered their typical behaviours, data shows that they are adaptable in nature. Fibroblasts are known to have a high degree of plasticity and can change their behaviour depending on the signals and cues from the microenvironment [15]. In a different study, the IC_{50} value of AZI was reported to be 115 (49) μg/ml in primary human connective tissue-derived fibroblasts cultured in alpha-MEM supplemented with 10% FBS and 1 mM glutamine [28]. DOXY had an IC_{50} value of 310 μM in primary human skin-derived fibroblasts cultured in DMEM containing growth ingredients, including GlutaMAX, 4.5 g/l glucose, 10% FBS, and 1 mM sodium pyruvate [3]. In the study by Lamb et al., DOXY over the range of 50 to 500 μM showed little or no cytotoxicity in hTERT-BJ1 human foreskin fibroblasts (culture conditions not specified); though at 50 μM, it was significantly cytotoxic against MCF7 and T47D mammospheres [10].

Future recommendations

NS possesses the promise of sensitising cancer cells to chemotherapeutic regimens, which is intricately linked to the mechanism of action of the cytotoxic drugs. To further demonstrate the usefulness of AZI and/or DOXY in the context of cytotoxic preference in NS circumstances, the next envisioned phase entails the utilisation of an in vivo animal model that incorporates dietary restriction, such as through short-term fasting.

Conclusions

In short, this study suggests that NS does not inevitably contribute to cytotoxic drug tolerance in GBM cells. On the

other hand, although fibroblasts can withstand NS, they can also become susceptible to cytotoxic drug-induced death; however, the type of drug may play a role.

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Availability of data and material

All data generated or analysed during this study are available from the corresponding author on reasonable request.

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The authors declare that they have no competing interests.

Abbreviations

MTT

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

ATCC

American type culture collection

AZI

Azithromycin

ANOVA

One-way analysis of variance

DOXY

Doxycycline

DMEM

Dulbecco’s modified eagle medium

FBS

Foetal bovine serum

GBM

Glioblastoma

GSH

Glutathione

HDF

Human dermal fibroblasts

IC_{50}

Half-maximal inhibitory concentration

NS

Nutrient stress

NBF

Neutral buffered formalin
OXPHOS
Oxidative phosphorylation
PA
Penicillic acid
TMZ
Temozolomide
TME
Tumour microenvironment
TM
Tetrathiomolybdate

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The role of LncRNAs and CircRNAs in osteoporosis: a focus on osteogenesis and osteoclastogenesis signaling pathways

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ABSTRACT (ENGLISH)

Background

Osteoporosis is a crucial health concern interconnected with physical disabilities as well as financial burdens. It arises from an imbalance between osteoblasts and osteoclasts, provoking the reduction of bone mass and the disturbances in bone structure with high fracture risk. Considerable efforts were done to prevent and mitigate this public health issue. Nonetheless, further understanding of the etiopathology of osteoporosis and the underlying genetic and epigenetic pathways is required.

Main body

Emerging evidence indicates that noncoding RNAs, including long noncoding RNAs (lncRNAs) and circular RNAs (circRNAs), play crucial roles as epigenetic regulators in various pathological processes, including osteoporosis. LncRNAs are RNA transcripts with higher structural complexity that are developed owing to their secondary and tertiary structures, which allow them to create different binding sites for other biomolecules, such as DNA, RNA, and proteins. Another class of noncoding RNAs is circRNAs, which have a covalently closed loop structure without the 5′ cap and 3′ polyA tail and are formed by back-splicing of pre-mRNAs. Because of their closed structure, circRNAs are largely stable, resistant to RNA-degrading nucleases, and possess substantially longer circulatory half-lives than linear RNAs. Interestingly, both lncRNAs and circRNAs serve as competing endogenous RNAs by sponging multiple miRNA binding sites as well as interact with RNA-binding proteins (RBPs), thereby controlling the expression of their target genes. Several studies indicated that altered expression of these regulators could influence many biological processes in bone cells.

Conclusion

The current review provides current opinions on the role and the underlying mechanisms by which lncRNAs and circRNAs affect osteoblastic and osteoclastic activities. The deep understanding of these noncoding RNAs in

osteoporosis offers distinctive avenues for innovative treatment strategies.

FULL TEXT

Background

Osteoporosis is a devasting metabolic bone disease with declined bone mass, loss of microarchitecture, fragility, and high susceptibility to fractures. Globally, it is one of the crucial reasons for the long-term physical impairments prevailing in an aging population. Therefore, osteoporosis represents a serious universal health issue that imposes a heavy load on society and healthcare systems [1].

Bone homeostasis is a constant state of formation and resorption through osteoblast and osteoclast activities, respectively. Osteoporosis occurs due to elevated osteoclastic activity without osteoblastic compensation [2]. Diverse molecules such as estrogen, parathyroid hormone, calcitonin, and vitamin D are implicated in the development and progression of the disease [3]. Earlier studies have also linked the development of osteoporosis to a battery of risk factors, such as modifiable and non-modifiable ones. Modifiable risk factors include weight, smoking, alcohol consumption, inactivity, calcium/vitamin D status, and the administration of corticosteroids. On the other hand, non-modifiable risk factors comprise advancing age, sex, race, and genetic characteristics [4]. Interestingly, the majority of these variables influence osteoporosis by modulating the activity of osteoblasts and osteoclasts. Nevertheless, genetic factors are regarded as one of the most significant predictors of osteoporosis [5].

Osteoporosis can be classified as either a primary or secondary disease. Primary osteoporosis includes juvenile, postmenopausal (type-I), and senile osteoporosis (type-II) [6]. Juvenile osteoporosis occurs in childhood and adolescence as a result of genetic mutations that could predispose to quantitative or qualitative disturbances in the connective tissue component of bone. Type-I osteoporosis, also named hormonal osteoporosis, is triggered by estrogen decrement during menopause; meanwhile, type-2 or senile osteoporosis, as the name implies, affects both genders and reaches peak incidence in the mid-seventies. Secondary osteoporosis occurs as a result of underlying medical conditions, such as renal disorders, malignancies, endocrine diseases, or therapeutic interventions [7].

Currently, dual-energy X-ray absorptiometry (DEXA) is the gold standard technique for diagnosing osteoporosis and predicting skeletal fracture risk. Despite its value, DEXA can only detect changes in bone structure that have already occurred, which can take a considerable time to become detectable, and it provides limited insights into disease progression and treatment effectiveness [8]. As well, since osteoporosis is recognized as a ‘silent disease’, it is frequently undiagnosed until a symptomatic fracture occurs [9]. Therefore, there has been a growing interest in exploring alternative approaches, such as epigenetic studies, for the early detection of osteoporosis before the occurrence of fractures.

The epigenetic landscape exhibits a fundamental role in osteoporosis pathogenesis. Intriguingly, epigenetics arises from the overlapping between genes, a non-modifiable risk factor, and the environment, as a modifiable risk factor for osteoporosis. Previous reports documented that epigenetics control a variety of biological processes, such as chromosome inactivation and cell differentiation [10]. Moreover, epigenetic regulations could manipulate the etiopathogenesis of osteoporosis via controlling osteoblasts and osteoclast cells [11], as shown in Fig. 1.

Fig. 1 [Images not available. See PDF.]

ncRNAs regulate osteogenesis and osteoclastogenesis signaling pathways involved in the development of osteoporosis

LncRNAs are noncoding RNA transcripts >200 nt that are not translated into proteins. LncRNAs are implicated in physiological and pathological cascades, so they are nominated as important regulators and possible biomarkers for a variety of human disorders, including osteoporosis [12]. lncRNAs exert their role in several human diseases via functioning as miRNA reservoirs, binding to messenger RNAs (mRNAs) or pre-mRNAs, and controlling post-transcriptional expression [13]. In osteoporosis, lncRNAs participate in bone proliferation, apoptosis, and the inflammatory response, resulting in the regulation of osteogenic and osteoclastogenic processes.

Circular RNA is a distinct form of endogenous ncRNA, with transcripts ranging in length from hundreds to thousands

of nucleotides. The reverse splicing that occurs between the splice acceptor at the 5′ end and the splice donor at the 3′ end of the pre-mRNA creates a covalently closed loop structure for circRNAs. The unique structure of circRNAs confers significant stability, rendering them resistant to RNA-degrading nucleases and resulting in significantly longer half-lives in the circulatory system compared to linear RNAs [14]. Recent findings indicate circRNAs have significant involvement in key cellular processes relevant to the progression of various diseases, such as cell differentiation and proliferation, thus serving as innovative biomarkers for diagnosis and prognosis. CircRNAs act as epigenetic regulators through controlling gene splicing or transcription, translating proteins, interacting with RNA-binding proteins (RBPs), and sponging miRNA [15]. Many circRNAs are differentially expressed in osteoporosis to influence the expression of certain genes that are implicated in osteoblastic and osteoclastic activities.

Based on that, the current review endeavors to combine these data and show the expression profiles of lncRNAs and circRNAs in osteoporosis. It also focuses on their regulatory roles as well as their clinical utility as promising therapeutic targets from the perspective of osteogenesis and osteoclastogenesis.

Main text

Osteogenic signaling pathways

Osteogenesis is the differentiation of committed mesenchymal stem cells (MSCs) into mature, active osteoblasts through several stages of precursors. Osteogenesis is achieved through interrelated cascades including hippo, Wnt, notch, and bone morphogenetic protein (BMP). These axes modulate diverse transcription factors, such as runt-related transcription factor 2 (RUNX2). RUNX2 participates in triggering the dedication of MSCs to the osteogenic lineage and functions upstream from the other transcription factors specific to osteoblasts, such as osterix (Osx) and distal-less homeobox 5 (Dlx5) [16]. The Wnt/β-catenin pathway plays a potential role in the stabilization of β-catenin that sequentially migrates to the nucleus and controls the expression of genes related to osteoblastogenesis [17]. Dickkopf-1 (DKK1) is mainly expressed by osteoblasts and bone marrow stromal cells (BMSCs) and antagonizes the Wnt pathway.

Another signaling pathway is BMP, which is one of the members of the transforming growth factor (TGF)-β superfamily and plays a crucial role in regenerating osteogenic differentiation in osteoblasts. Briefly, BMP receptors bind with BMP ligands, resulting in phosphorylation of Smad1/5/8, which then attaches to the co-Smad (Smad4) to form a complex. The resultant complex moves to the nucleus, where it regulates the genes that affect osteoblast development in conjunction with co-activators and corepressors [18].

The Hippo signaling cascade modulates osteoblastic activity and thereby participates in bone development and maintenance. Sometimes, the Hippo pathway is turned off by inactivating (dephosphorylating) mammalian STE20-like protein kinases 1 and 2 (MST1/2) and large tumor suppressors 1 and 2 (LATS1/2). This results in the activation of the yes-associated protein (YAP) and the transcriptional coactivator with the PDZ-binding motif (TAZ), allowing them to translocate into the nucleus. Subsequently, YAP and TAZ act as transcriptional co-activators, interacting with transcription factors to regulate the expression of target genes related to osteoblast cell proliferation, differentiation, and survival [19].

Notch signaling, a highly preserved intercellular pathway, governs cell differentiation and proliferation as well as determines cell fate via the activation of cell surface receptors (Notch 1-4) and their delta-like ligands (DLL) 1, 3, 4, and Jagged 1, 2. This pathway promotes the expression of downstream target genes involved in osteogenesis regulation [20].

LncRNAs involved in osteogenesis regulation

LncRNAs that promote osteogenesis

BMSCs have a high osteogenic capability, which is considered an effective practical strategy for hindering bone loss. Previous reports elucidated the involvement of the lncRNAs in BMSC proliferation and differentiation. Herein, we review the expression of potential lncRNAs in osteoporosis and their significant roles in either promoting or inhibiting osteogenic differentiation, as shown in Table 1 and Fig. 2, respectively.

Table 1. The expression of lncRNAs-related osteogenesis in osteoporosis

LncRNAs that promote osteogenesis			
LncRNA	Expression in osteoporosis	Samples	References
LncRNA HAGLR	Downregulated	Peripheral blood of PMOP patients and OVX mice	[21]
LncRNA RAD51-AS1	Downregulated	hBMSCs of osteoporotic patients	[23]
LncRNA KCNQ1OT1	Downregulated	Bone tissue samples of PMOP and OVX mice	[24]
GATA4-mediated lncRNA MALAT1	Downregulated	Bone tissues of PMOP patients and OVX mice	[26]
Quercetin-mediated lncRNA MALAT1	Downregulated	OVX mice	[29]
Prrx-2-mediated lncRNA MIR22HG	Downregulated	OVX mice	[30]
LncRNA TERC	Downregulated	DEX-induced osteoporosis in BMSCs	[31]
LncRNA SNHG14	Downregulated	OVX mice	[34]
LncRNAs that inhibit osteogenesis			
LINC00205	Upregulated	Bone tissue samples of osteoporotic patients	[35]
LINC01234	Upregulated	Plasma of osteoporotic patients	[37]
LncRNA PCBP1-AS1	Upregulated	Bone tissue samples of osteoporotic patients	[38]
LncRNA KCNMA1-AS1	Upregulated	Bone tissue samples of osteoporotic patients	[40]
LncRNA SNHG1	Upregulated	Serum of osteoporotic patients	[41]
IRF-1-mediated lncRNA XIST	Upregulated	OVX mice	[42]

Fig. 2 [Images not available. See PDF.]

Role and mechanistic insights of lncRNAs in osteogenesis. All shapes shaded in green denote biomarkers increased during osteogenesis. All shapes shaded in red denote biomarkers decreased during osteogenesis

LncRNA homeobox D gene cluster antisense growth-associated long noncoding RNA (HAGLR) exhibited a significant decrease in the peripheral blood of patients with postmenopausal osteoporosis (PMOP) compared to

healthy controls. Conversely, its expression increased during BMSC osteogenic differentiation. In OVX mice, the overexpression of HAGLR alleviated PMOP by upregulating homeobox protein A10 (Hoxa10) expression through inhibition of miR-182-5p [21]. Indeed, Hoxa10, a crucial transcription factor in the osteogenic process, plays a pivotal role in regulating the production of osteoblasts [22]. In parallel, the **lncRNA RAD51-Antisense RNA 1 (RAD51-AS1)** was remarkably downregulated in human bone marrow stem cells (hBMSCs) obtained from osteoporotic patients compared to those from controls. It was observed that RAD51-AS1 knockdown inhibited the proliferation and differentiation of hBMSCs and boosted their apoptosis. Mechanistically, RAD51-AS1 was determined to stimulate the osteogenic differentiation of hBMSCs by interacting with Y-Box Binding Protein 1 (YBX1) to generate mRNA-protein complexes with SMAD7 and Smad ubiquitin regulatory factor 2 (SMURF2), thus hampering their translation. Additionally, it could transcriptionally upregulate proliferating cell nuclear antigen (PCNA) and SIVA1 by binding to their promoter regions, resulting in activation of the TGF-β signaling pathway [23]. **LncRNA KCNQ1OT1** also showed a significant decrease in PMOP patients compared to control group. Its overexpression enhanced the osteogenic differentiation of MC3T3-E1 cells and alleviated osteoporosis in OVX mice through upregulation of mTOR via sponging miR-421-3p [24].

Besides, GATA-binding protein 4 (GATA4) is crucial in modulating osteoblastic differentiation, bone remodeling, and mineralization [25]. Huang et al. linked GATA4 with **lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1)** in BMSCs during osteogenesis [26]. The study found that GATA4 overexpression promoted BMSC osteoblastic differentiation through upregulating lncRNA MALAT1. Notably, the expression of both GATA4 and lncRNA MALAT1 was reduced in the bone tissues of both PMOP patients and ovariectomized (OVX) mice. It was confirmed that GATA4 activated the transcription of MALAT1, which in turn formed an RBP complex with K-homology splicing regulatory protein (KHSRP) to decay the stability of neuronal precursor cell-expressed developmentally downregulated 4 (NEDD4) mRNA, the one that is responsible for the promotion of RUNX1 degradation by ubiquitination [27]. In sum, GATA4-mediated lncRNA MALAT1 stimulated osteogenic differentiation of BMSCs via inhibiting RUNX1 degradation through modulation of the KHSRP/NEDD4 axis, ultimately driving bone formation in PMOP. Furthermore, another study has also investigated the role and molecular mechanism of MALAT1 in osteoporosis. Interestingly, several phytochemicals have been found to be effective therapeutics for osteoporosis. Among them is quercetin, which has a promoting effect on BMSC osteogenesis [28]. Feng et al. documented that quercetin treatment could attenuate tumor necrosis factor alpha (TNFα)-impaired BMSCs osteogenesis via upregulating MALAT1 expression [29]. In OVX mice, serum MALAT1 level was markedly decreased, while quercetin treatment restored its level to some extent. Additionally, MALAT1 was involved in the modulating effect of quercetin on TNFα-impaired BMSC osteogenesis. Its knockdown abolished the rescuing effect of quercetin; stimulation of β-catenin expression and mitigation of NF-κB p65 translocation. This, in turn, compromised quercetin's osteogenic ability and facilitated the progression of osteoporosis. In the same context, Paired-Related Homeobox Protein 2 (Prrx2) is a transcription factor that belongs to the paired-related homeobox protein family and has been found to be involved in the regulation of lncRNA expression. Li et al. reported that myoblast-derived exosomal Prrx2 directly attached to the **MIR22HG** promoter and enhanced its expression [30]. Relevantly, high levels of MIR22HG alleviated OVX-induced bone loss by accelerating BMSC osteogenic differentiation through inhibition of miR-128, which in turn stimulated YAP1 expression and thus activated the Hippo pathway.

Other factors have been implicated in the regulation of lncRNAs during osteogenesis, impacting the development of osteoporosis. Dexamethasone (DEX) is a synthetic glucocorticoid that has been reported to regulate MSC osteogenic lineages. **LncRNA TERC** is among the lncRNAs identified to alleviate DEX-induced osteoporosis. It was observed that TERC overexpression mitigated the inhibitory effect of DEX on BMSC osteogenic differentiation by increasing the level of the enhancer of zeste homolog 2 (EZH2) protein. This elevation, in turn, facilitated the histone modification of DKK1, thereby activating the Wnt signaling pathway. Thus, TERC overexpression by lessening DEX-induced osteoporosis could provide an additional therapeutic strategy for glucocorticoid-induced osteoporosis [31]. Also, Autophagy is a mechanism beneficial for cell survival and growth [32], known to maintain bone remodeling by

removing reactive oxygen species (ROS) [33]. Xue et al. have found that **lncRNA Small nucleolar RNA host gene 14 (SNHG14)** overexpression activated autophagy in BMSCs as well as raised bone mineral density (BMD) and bone trabecular number in OVX mice [34]. Mechanically, an elevated level of SNHG14 stimulated myocyte enhancer factor 2C (Mef2c)-mediated autophagy activation through inhibition of miR-493-5p, predisposing to enhancement of the osteogenic ability of BMSCs. Thus, SNHG14 could alleviate osteoporosis progression via activation of autophagy by regulating the miR-493-5p/Mef2c axis.

LncRNAs that inhibit osteoblastogenesis

Certain lncRNAs have been identified as inhibitors of osteogenesis, which impede the process of bone formation and trigger osteoporosis development. For example, **LINC00205** was upregulated in bone tissue samples of osteoporotic patients with or without a spinal fracture, while it was downregulated during the osteogenic differentiation of human mesenchymal stem cells (hMSCs). Precisely, LINC00205 inhibited osteogenic differentiation by targeting miR-26b-5p and enhancing lysine (K)-specific methyltransferase 2C (KMT2C) expression. This latter contributed to a decrease in the expression of osteogenic-related genes such as RUNX2, alkaline phosphatase (ALP), and osteocalcin (OCN), resulting in the progression of osteoporosis [35]. Likewise, **LINC01234** was highly expressed in the plasma of patients with osteoporosis while exhibiting a gradual decrease during osteogenic differentiation of hMSCs. In this study, it was observed that LINC01234 impeded hMSC osteogenic differentiation by augmenting aldehyde oxidase 1 (AOX1) via regression of miR-513a-5p. Notably, the AOX1 gene encodes the cytosolic enzyme that catalyzes the formation of superoxide [36]. Thus, targeting LINC01234 could promote osteogenesis and hinder osteoporosis development [37].

LncRNA PCBP1-AS1 was highly expressed in osteoporotic tissues and decreased during the development of hBMSCs into osteoblasts. The knockdown of PCBP1-AS1 promoted the osteogenic differentiation capacity of hMSCs, while its overexpression exhibited an opposite effect. Mechanistically, PCBP1-AS1 targeted Pak family member p21-activated kinase 2 (PAK2) by inhibiting miR-126-5p [38]. Remarkably, PAK2 is required for various cellular activities, such as cytoskeletal remodeling and chromatin modulation [39]. Additionally, **lncRNA KCNMA1-AS1** was elevated in the osteoporotic subjects. Its suppression upregulated miR-1303, which resulted in the downregulation of cochlin (COCH), a known suppressor of embryonic stem cell differentiation. This led to the promotion of osteogenic differentiation in hMSCs and the mitigation of the progression of osteoporosis [40].

The intricate interplay between lncRNAs and apoptosis reveals a novel aspect of the pathogenesis of osteoporosis. Pan et al. reported that **lncRNA SNHG1** could regulate osteogenic differentiation by influencing pyroptosis, an inflammatory type of apoptosis triggered by inflammasomes and caspase-1 [41]. SNHG1 was observed to be elevated in the serum of osteoporotic patients. Its overexpression could suppress BMSC osteogenic differentiation through interaction with HMGB1, enhancing its expression. This, in turn, activated pyroptosis-associated factors (caspase-1 p20 and gasdermin D-N (GSDMD)-N) and the production of inflammatory interleukins (IL-1β and IL-18), leading to the development of osteoporosis. In tandem, interferon regulatory factor-1 (IRF-1) also participated in controlling cellular apoptosis during osteogenic differentiation through the regulation of **lncRNA X-inactive-specific transcript (XIST)**. Elevated levels of lncRNA XIST were observed in OVX mice, and its knockdown not only alleviated osteoporosis symptoms but also promoted osteogenic differentiation in hBMSCs. Simultaneously, IRF-1 accelerated osteogenic differentiation by repressing the transcription of XIST, which in turn upregulated miR-450b and subsequently decreased F-box and WD repeat domain-containing 7 (FBXW7) expression [42]. FBXW7 is regarded as an efficient tumor suppressor due to its role in cellular apoptosis and has also been observed to be involved in the osteogenic differentiation of BMSCs [43, 44].

CircRNAs involved in osteogenesis regulation

CircRNAs that promote osteogenesis

Several studies, conducted in BMSC-induced osteogenic differentiation, focused on RUNX2 regulation through various circRNA/miRNA axes. For instance, **circ-3626**, an exonic circRNA that arises from the STAG1 gene, was significantly increased during osteogenic differentiation of BMSCs, whereas it was decreased in bone tissues derived from osteoporotic patients and BMSCs of aged mice. Moreover, its overexpression dramatically accelerated

the osteogenic capability of BMSCs. Circ-3626 increased RUNX2 regulation by inhibiting miR-338-3p and thereby upregulating many osteogenic-related genes [45]. Similarly, **circ-RBM23** acted as a suppressor of miR-338-3p, enhancing RUNX2 and regulating the switch between osteogenesis and adipogenesis in MSCs. The authors reported that circ-RBM23 was elevated in osteogenesis, whereas it was depressed during adipogenesis in MSCs, providing a crucial goal for diagnosing and alleviating osteoporosis [46]. Furthermore, **circ_0011269, circ-VANGL1, and circ_0076690** were notably decreased in the clinical samples of osteoporotic patients. In hMSCs, circ_0011269, circ-VANGL1, and circ_0076690 upregulated RUNX2 expression via sponging miR-122, miR-217, and miR-152, respectively. These findings pointed out that the overexpression of these circRNAs promoted osteogenic differentiation and could be implicated in the pathogenesis of osteoporosis [47–49].

Previous reports have revealed the functions of various circRNAs in influencing SMAD5 expression, making them potential targets for therapeutic interventions related to skeletal disorders. **Circ_0001825** is newly recognized to be significantly downregulated in osteoporotic patients. The suppression of circ_0001825 reduced hMSC viability and osteogenic differentiation. Circ_0001825 promoted osteogenesis via sponging miR-1270, resulting in SMAD5 overexpression, which signifies the potential contribution of circ_0001825 to osteoporosis [50]. Another circRNA that has been detected to regulate SMAD5 in osteoporosis is **CircGLIS2.** The expression of circGLIS2 was obviously decreased in osteoporotic patients, while increased in hBMSCs upon osteogenic differentiation. Regression of circGLIS2 notably inhibited osteogenesis-related genes, such as osteopontin (OPN), OCN, and ALP activity. The study reported that circGLIS2 sponged miR-214-3p and upregulated Smad5, which stimulated osteogenic differentiation of the hBMSCs [51]. **Circ_0062582** was also observed to lessen the development of osteoporosis by affecting SMAD5 expression. It was reported that circ_0062582 was remarkably decreased in osteoporosis. Meanwhile, circ_0062582 was increased in osteoblast medium-induced hBMSCs, where it sequestered miR-197-3p and resulted in an elevation of SMAD5, thereby reflecting hBMSC proliferation and osteogenic differentiation [52].

In vitro studies using MSCs revealed that circRNAs play a regulatory role on several genes related to the BMP pathway. Both **circRNA_0048211** and **circ_0016624** were suppressed in PMOP patients compared to healthy controls. During hMSC osteogenic differentiation, the upregulated circRNA_0048211 and circ_0016624 increased BMP2 regulation through targeting miRNA-93-5p and miR-98, respectively, so attenuated development of PMOP [53, 54].

Regarding the Notch pathway, **circRNA_0001795** and **circ-ITCH** promoted osteogenic differentiation of hBMSCs by targeting YAP1 through sponging their respective miRNAs, miR-339-5p and miR-214 [55, 56]. Notably, the expression levels of both circRNAs were notably reduced in osteoporosis, and their depletion correlated with a decrease in the expression of osteogenic-related genes. Additionally, in vivo experiments demonstrated that the upregulation of circ-ITCH enhanced osteogenesis in ovariectomized (OVX) mice.

It has been found that **hsa_circ_0114581** is negatively correlated with osteoporosis pathogenesis. The upregulated hsa_circ_0114581 enhanced the expression of heterogeneous nuclear ribonucleoprotein A3 (HNRNPA3) to encourage osteogenic differentiation by suppressing hsa-miR-155-5p. This study also proved that HNRNPA3 was directly associated with osteogenic-related proteins in BMSCs and femur samples of either human bone tissue or OVX mice, suggesting its relationship with osteogenesis and bone formation [57]. Moreover, low levels of **circ_0019693** were recognized in osteoporotic patients. Its expression was further enhanced during the stages of osteogenic differentiation in BMSC. Intriguingly, it was indicated that circ_0019693 could promote osteogenesis through inhibiting miR-942-5p and increasing purkinje cell protein 4 (PCP4) [58]. When PCP4 is bound to CaM-dependent protein kinase, the rate of Ca^{+2} dissociation from calmodulin (CaM) increases, promoting calcium deposition during mineral nodule formation, which is a valid predictor of bone-forming ability [59]. **Hsa_circ_0006215** is another circRNA coupled between osteogenesis and angiogenesis and involved in the pathogenesis of senile osteoporosis. It was significantly downregulated in the BMSCs of osteoporotic patients, while its overexpression promoted the osteogenic differentiation of BMSCs. In vivo, its overexpression induced the repair of the single cortical bone defect model, suggesting that it could promote bone defect repair. Furthermore, it has been demonstrated that hsa_circ_0006215 influences RUNX2 and VEGF expression in BMSCs through sponging miRNA-942-5p [60]. In

GIOP patients, the **hsa_circ_0006393** level was downregulated compared to control ones. Furthermore, overexpression of hsa_circ_0006393 boosted the expression of osteogenic genes in bone tissue samples obtained from male GIOP patients as well as a GIOP mouse model. It was suggested to have osteogenic influence through inhibiting miR-145-5p and increasing FOXO1 expression [61].

Numerous studies have been conducted on human adipose stem cells (ASCs). It was found that **CircFOXP1** was significantly downregulated in the bone tissues of osteoporotic patients. Indeed, the pro-osteogenic activity of circFOXP1 was evaluated in vivo and in vitro, where circFOXP1 was found to enhance hASC osteogenesis by sponging miR-33a-5p and targeting FOXP1 expression [62]. Thus, circFOXP1 could be regarded as a candidate target for hASC-based therapy of osteoporosis.

Accumulating data indicates that circRNAs have the ability to interact with RBPs and alter their functions [63]. **CircStag1** was remarkably suppressed in the BMSCs of osteoporotic rats and bone tissue samples isolated from osteoporotic patients. Briefly, circStag1 interacted with human antigen R (HuR) and promoted its translocation into the cytoplasm. Sequentially, cytoplasmic HuR led to the stimulation of the Wnt cascade by stabilizing low-density lipoprotein receptor-related protein 5/6 (Lrp5/6), co-receptors for Wnt ligands, and enhancing β-catenin expression. It was also observed that the induction of circStag1 fostered osteogenesis in OVX mice [64]. According to Yao et al., **circ-Plod2** is an exon-type circRNA found in the cytoplasm, where it destabilized the Mpo-dependent osteogenic differentiation of BMSCs while having no effect on adipogenic differentiation or chondrogenic differentiation of these cells [65]. The study also found that the expression of circ-Plod2 was remarkably downregulated in OVX rats BMSCs, and its overexpression effectively lessened osteoporosis among them. Circ-Plod2 mediated its effect by interacting with IGF2BP2 to form an RNA–protein complex, which in turn inhibited the expression of Mpo mRNA in BMSCs. Another circRNA that plays a role in osteoporosis by interacting with RBP is **circPVT1**. CircPVT1 stimulates osteogenesis by inhibiting miR-30d-5p to increase integrin beta-3 (ITGB3) expression [66]. ITGB3, a member of the integrin family, functions as a downstream gene of Homeobox D3, which triggers the Wnt/β-catenin signaling pathway through the involvement of β3 integrin.

CircRNAs that inhibit osteogenesis

Other circRNAs exert a pivotal regulatory role in impeding osteogenesis through modulation of essential pathways and gene expression networks, thereby influencing the overall process of bone formation. A recent study has highlighted the mechanistic insight of **hsa_circ_0006859** in vitro. It was recognized that the expression of hsa_circ_0006859 was augmented in OVX mouse-derived BMSCs but remained modest expressed during osteogenic differentiation. Hsa_circ_0006859 overexpression hindered osteogenesis of BMSCs in the human fetal osteoblast cell line by targeting both miR-642b-5p/ephrin A2 (EFNA2) and miR-483-3p/dedicator of cytokinesis 3 (DOCK3) axes, resulting in the inactivation of the Wnt-signaling pathway [67]. Additionally, it has been reported that **hsa_circ_0008842**, identified as **circZNF367**, was substantially upregulated in the bone tissue of osteoporotic patients and dramatically downregulated in hBMSCs during osteogenic differentiation. The overexpression of circZNF367 suppressed migration and osteogenic differentiation of hBMSCs, both in vitro and in vivo, while its knockdown exhibited opposite effects. CircZNF367 could reduce osteogenic differentiation of hBMSCs through interaction with HuR, which reduced LRP5 mRNA stability [68].

Circ_0006873 was upregulated in the sera of osteoporotic patients and decreased during osteoblastic differentiation. Circ_0006873 could suppress osteoblastic differentiation and favor osteoporosis by sponging miR-142-5p, which in turn enhances PTEN expression and regulates the Akt signaling pathway [69]. Another study has also reported that circ_0006873 was significantly elevated in both serum samples and bone tissue samples of osteoporotic patients. Its overexpression was associated with a significant reduction in osteogenic differentiation of hMSCs through the sequestration of miR-20a and targeting SMURF2 [70].

Lipopolysaccharide (LPS) is a potent inducer of bone loss, resulting in inflammation that exacerbates osteoporosis by disrupting regular bone homeostasis. **CircAtp9b** is LPS-inducible, and its knockdown alleviates the inflammation triggered by LPS [71]. Interestingly, circAtp9b was significantly upregulated in plasma samples and osteoblasts derived from osteoporotic patients. LPS-treated osteoblasts increased circAtp9b expression in a dose-dependent

manner, confirming that the circAtp9b overexpression is likely triggered by LPS. Also, it was shown that circAtp9b upregulation increased LPS-induced osteoblast apoptosis in osteoporosis by sponging premature miR-17-92a and suppressing its maturation, which in turn accelerated osteoporosis progression [72].

There is little known about the role of circRNAs in melatonin (MEL)-induced BMSC osteogenic differentiation and osteoporosis progression. MEL has been detected as a booster of osteoblast proliferation and differentiation, fostering bone formation and reducing bone destruction in osteoporotic mice [73]. In this regard, Wang et al. found that melatonin improved osteogenic differentiation and repressed osteoporosis development by hampering **circ_0003865** expression [74]. This circRNA acts as a sponge for miR-3653-3p, consequently boosting the expression of growth arrest-specific gene 1 (GAS1) while suppressing the expression of bone-forming genes. Similarly, the expression of **circ_0005753** was significantly reduced during the osteogenic differentiation of BMSCs triggered by MEL. Molecularly, circ_0005753 maintained the stability of TXNIP mRNA through the recruitment of PTBP1. This study pointed out that MEL enhanced BMSC osteogenic differentiation through the regulation of the circ_0005753/PTBP1/TXNIP axis, which could shed light on a new treatment pathway to prevent osteoporosis [75]. Zhi et al. determined that exosomal **hsa_circ_0006859** was obviously upregulated in the serum of PMOP, efficiently distinguishing osteoporotic and osteopenic patients from healthy controls [76]. As well, it discriminated between osteoporotic and osteopenic patients with high specificity and sensitivity. Besides, ectopic expression of circ_0006859 provoked adipogenic differentiation and impeded osteoblastic differentiation in BMSCs via sponging miR-431-5p to induce ROCK1 expression. Thus, hsa_circ_0006859 may be a potential biomarker for the diagnosis and prognosis of osteoporosis and could modulate the harmony between adipogenesis and osteogenesis in BMSCs. According to whole transcriptome sequencing, Zhang et al. determined the expression profile of circRNAs in the peripheral blood of male osteoporotic patients versus healthy controls, and found that a total of 398 circRNAs were differentially expressed [77]. **Hsa_circ_0042409** was among the top 10 upregulated circRNAs and was recognized to be involved in the development of osteoporosis through regulating the expression level of kinesin light chain 1 (KLC1) via sponging hsa-miR-195-5p. Additionally, another study observed a significant upregulation of **circ_0134944** in both PMOP patients and OVX mice. Its enhanced expression suppressed osteogenesis in BMSCs through targeting miR-127-5p, causing an increase in pancreatic and duodenal homeobox 1 (PDX1) and sphingosine kinase 1 (SPHK1). PDX1 is a well-established transcription factor that regulates the activity of multiple genes by binding to their promoter regions, such as SPHK1, which has an impact on the regulation of osteogenic differentiation [78]. Furthermore, **Hsa_circ_0002060** knockdown in the human fetal osteoblast cell line (hFOB1.19) reversed the effect of DEX, which decreased matrix metalloproteinases (MMP) and increased ROS. Hsa_circ_0002060 modulated the survival of hFOB 1.19 cells by targeting miR-198-5p, resulting in an elevation of Bax expression that in turn triggered the apoptosis of osteoblasts. Additionally, hsa_circ_0002060 knockdown alleviated the progression of osteoporosis in OVX mice through the Jun N-terminal kinase (JNK) signaling pathway [79].

Collectively, manipulating these interrelated molecules could be beneficial in the management of osteoporosis. Thus, we reviewed the expression and molecular mechanisms by which circRNAs mediate osteoblast differentiation (Table 2 and Fig. 3).

Table 2. The expression of circRNAs-related osteogenesis in osteoporosis

CircRNAs that promote osteogenesis			
circRNAs	Expression in osteoporosis	Samples	References
Circ-3626	Downregulated	Bone tissues of osteoporotic patients and aged mice	[45]

Circ-RBM23	Downregulated	Osteoporotic patients	[46]
Circ_0011269	Downregulated	Serum/plasma samples of osteoporotic patients	[47]
Circ-VANGL1	Downregulated	Serum samples of osteoporotic patients	[48]
Circ_0076690	Downregulated	Serum/plasma samples of osteoporotic patients	[49]
Circ_0001825	Downregulated	Bone marrow samples of PMOP	[50]
CircGLIS2	Downregulated	Osteoporotic patients	[51]
Circ_0062582	Downregulated	Osteoporotic patients	[52]
Circ_0048211	Downregulated	hBMSCs of PMOP patients	[53]
Circ_0016624	Downregulated	Serum/plasma samples of PMOP	[54]
Circ_0001795	Downregulated	Bone marrow samples of osteoporotic patients	[55]
Circ-ITCH	Downregulated	Bone marrow samples of PMOP and OVX mice	[56]
Hsa_circ_0114581	Downregulated	Femur tissues of patients undergoing hip replacement operation and OVX mice	[57]
Circ_0019693	Downregulated	Serum samples and bone tissues of osteoporotic patients	[58]
Hsa_circ_0006215	Downregulated	BMSCs of osteoporotic patients and femoral monocortical defect in Mouse	[60]
Hsa_circ_0006393	Downregulated	Bone tissue samples of GIOP patients and GIOP mouse model	[61]
CircFOXP1	Downregulated	Bone tissues of osteoporotic patients and heterotopic bone formation assay in nude mice	[62]
CircStag1	Downregulated	Bone tissues of PMOP and OVX mice	[64]
Circ-Plod2	Downregulated	OVX mice	[65]
CircPVT1	Downregulated	BMSCs of osteoporotic patients	[66]
CircRNAs that inhibit osteogenesis			

Hsa-circ-0006859	Upregulated	OVX mice	[67]
Hsa_circ_0008842 (circZNF367)	Upregulated	Osteoporotic patients	[68]
Circ_0006873	Upregulated	Serum samples of osteoporotic patients Serum samples and bone tissues of osteoporotic patients	[69] [70]
CircAtp9b	Upregulated	Osteoblasts and plasma samples of osteoporotic patients	[72]
Circ_0003865	Upregulated	OVX mice	[74]
Circ_0005753	Upregulated	OVX mice	[75]
Hsa_circ_0006859	Upregulated	Serum samples of PMOP	[76]
Hsa_circ_0042409	Upregulated	Peripheral blood of osteoporotic patients	[77]
Circ_0134944	Upregulated	Blood mononuclear cells and OVX mice	[78]
Hsa_circ_0002060	Upregulated	OVX mice	[79]

Fig. 3 [Images not available. See PDF.]

Role and mechanistic insights of circRNAs in osteogenesis. All shapes shaded in green denote biomarkers increased during osteogenesis. All shapes shaded in red denote biomarkers decreased during osteogenesis

Osteoclastogenic signaling pathways

Osteoclastogenesis is the fusion of osteoclast precursors, which originate from hematopoietic cells to form multinucleated, active osteoclasts. Macrophage colony-stimulating factor (M-CSF), also known as CSF-1, and receptor activator for nuclear factor κB Ligand (RANKL) are two crucial cytokines that bind to their respective receptors, colony-stimulating factor-1 receptor (c-Fms) and RANK, to stimulate osteoclastogenesis through the regulation of delicate signaling pathways [80]. M-CSF stimulates the production and survival of osteoclast precursor cells by activating extracellular signal-regulated kinase (ERK) and serine/threonine kinase 1 (Akt). Meanwhile, RANKL facilitates the development of osteoclast precursors into osteoclasts by attracting TRAF6 to the RANK receptor, which then activates various downstream targets, including NF-κB, JNK, ERK, p38, and PI3K/Akt. This ultimately leads to the activation of the key regulator of osteoclast differentiation, nuclear factor of activated T cells 1 (NFATc1). This latter controls several osteoclast-specific genes, including TRAP, cathepsin K, and osteoclast-associated receptor (OSCAR) via interaction with other osteoclastic transcription factors, such as microphthalmia-

associated transcription factor (MITF) and c-Fos [81]. Additionally, the RANK/RANKL/OPG pathway is crucial for the metabolism of bone tissue, as RANKL stimulates the production and differentiation of osteoclasts, while osteoprotegerin (OPG) inhibits its action through binding to it [82]. Here, we summarized the relevant research investigation of lncRNAs (Table 3) and circRNAs (Table 4) in order to offer novel insights for future osteoporosis studies and treatments, with a specific focus on osteoclasts, as depicted in Fig. 4.

Table 3. The expression of lncRNAs-related osteoclastogenesis in osteoporosis

LncRNAs that promote osteoclastogenesis			
LncRNA	Expression in osteoporosis	Samples	References
LncRNA AK077216	Upregulated	Bone marrow and spleen derived monocytes/macrophages of OVX mice	[83]
LncRNA GAS5	Upregulated	Plasma samples of osteoporotic patients	[84]
LncRNA CRNDE	Upregulated	Osteoclasts of PMOP	[86]
LncRNAs that inhibit osteoclastogenesis			
BGN-mediated lncRNA NRON	Downregulated	OVX mice	[87]
LncRNA FTX	Downregulated	Bone and serum samples of osteoporotic patients	[88]

Table 4. The expression of circRNAs-related osteoclastogenesis in osteoporosis

CircRNAs that promote osteoclastogenesis			
CircRNA	Expression in osteoporosis	Samples	References
CircZNF367	Upregulated	OVX mice	[89]
CircFam190a	Upregulated	PBMCs of PMOP and OVX mice	[90]
CircBBS9 (hsa_circ_0134188) (mmu_circ_0001757)	Upregulated	Spine cancellous bones of osteoporotic patients and OVX mice	[91]
CircRNA_28313	Upregulated	OVX mice	[92]
CircRNAs that promote osteoclastogenesis			

Hsa_circ_0021739	Downregulated	PBMCs of POMOP	[93]
Circ_0007059	Downregulated	Bone tissue of PMOP	[94]
CircHmbox1	Downregulated	OVX mice	[95]

Fig. 4 [Images not available. See PDF.]

Role and mechanistic insights of lncRNAs/circRNAs in osteoclastogenesis

LncRNAs that promote osteoclastogenesis

lncRNAs have been demonstrated to govern osteoclastogenesis by controlling the expression of certain mRNAs. **LncRNA AK077216** was considerably upregulated during osteoclastogenesis in the bone marrow and spleen tissues of OVX mice. LncAK077216 overexpression upregulated NFATc1 and accelerated RANKL-induced osteoclastogenesis by downregulating NIP45 [83]. Additionally, **lncRNA GAS5** was upregulated in the plasma of osteoporotic patients compared to healthy participants and effectively distinguished between them with high sensitivity and specificity. It has been found that GAS5 overexpression promoted osteoclast apoptosis by sponging miR-21, thus exhibiting a protective effect in osteoporosis [84]. A previous study demonstrated that miR-21 was involved in the pathogenesis of osteoporosis through modulation of reversion-inducing cysteine-rich proteins with Kazal motifs (RECK), which play a role in cell apoptosis, proliferation, and differentiation in TNF-α-treated MSCs [85]. **LncRNA CRNDE** was also highly expressed in osteoclasts obtained from PMOP compared with those from healthy controls. After CRNDE knockdown, the cell percentage was significantly increased in the G0/G1 phase but decreased in the S-phase, promoting osteoclast apoptosis rate. Moreover, CRNDE improved osteoclast proliferation by regulating the PI3K/AKT signaling pathway [86].

LncRNAs that inhibit osteoclastogenesis

Extracellular vesicles (EVs) released by MSC have significant immunoregulatory effects on bone healing in osteoporosis. The **lncRNA NRON** was rich inside BMSC-derived EVs that have been triggered by bioactive glass nanoparticles (BGN) in an osteoporosis model of OVX mice. NRON reduced osteoclast differentiation through interaction with the nuclear factor of activated T cell transcription factors and blocking the nuclear translocation of NFATc1 [87]. Likewise, **lncRNA FTX** was significantly suppressed in the serum and bone tissues of osteoporotic patients compared to controls ones. FTX overexpression reduced osteoclastgenesis through upregulation of Notch1 by sponging miR-137 in CD14 + peripheral blood mononuclear cells (PBMCs) [88].

CircRNAs involved in osteoclastogenesis regulation

CircRNA that promote osteoclastogenesis

CircZNF367 was found to be upregulated in an OVX mouse model of osteoporosis. CircZNF367 promoted osteoclast differentiation by interacting with fused in sarcoma (FUS), facilitating its translocation into the cytoplasm to trigger the enhancement of CRY2 mRNA stability [89]. C**ircFam190a** was significantly upregulated in vitro and in an OVX mouse osteoporosis model, where it induced significant bone loss. The study reported that circFam190a could promote osteoclast formation and function by enhancing the binding between AKT1 and HSP90β, thereby protecting AKT1 from proteasome-mediated degradation and augmenting its stability as well as its activity [90]. Unfortunately, the existing clinical drugs were reported to impede osteoclasts and interfere with normal bone turnover. However, targeting specific osteoclast precursors may be more effective in preserving bone homeostasis. **circBBS9 (mmu_circ_0001757),** a highly conserved circRNA, was substantially upregulated in mononucleated pre-osteoclasts versus bone marrow macrophage (BMM). Moreover, the **circBBS9** homolog in humans **(hsa_circ_0134188)** was augmented in osteoporotic human bone samples as well as in PBMC-derived osteoclasts. Knockdown of circBBS9 using siRNA-circBBS9-loaded nanoparticles inhibited BMM multinucleation in vitro. As well, these particles avoided bone loss in an OVX-induced mouse model. Molecularly, knockdown of circBBS9 in osteoclast precursors inhibited osteoclast multinucleation via the miR-423-3p/Traf6 axis, demonstrating that

circBBS9 is an efficient post-transcriptional regulator [91]. C**ircRNA_28313** knockdown significantly inhibited osteoclastic differentiation within BMM cells in vitro, while suppressed OVX mice stimulated bone resorption in vivo. CircRNA_28313 acted as ceRNA by binding to miR-195a, resulting in upregulation of CSF1 and the development of osteoclast differentiation [92].

CircRNA that inhibit osteoclastogenesis

Hsa_circ_0021739 was downregulated in PMOP, and its expression was associated with the lumbar vertebra, femur, and forearm T-scores. Overexpression of hsa_circ_0021739 has also been shown to reduce hsa-miR-502-5p levels and hinder osteoclast differentiation [93]. Besides, Liu et al. screened the differential expression of circRNAs in PMOP using RNA-sequencing and found that **circ_0007059** expression was decreased in patients and during the osteoclast differentiation of hBMSCs [94]. The authors also demonstrated that circ_0007059 overexpression lessened hBMSC differentiation into the osteoclasts. Molecularly, circ_0007059 sponged miR-378 expression, which resulted in an upregulation of BMP-2 expression that was accompanied by a decrease in osteoclast-specific gene expression and TRAP staining, thus attenuating osteoporosis development. Moreover, **circHmbox1** was notably decreased during the formation of osteoclasts triggered by TNF-α in vivo and in vitro. CircHmbox1 acted by inhibiting miR-1247-5p to activate Bcl6. Interestingly, Bcl6 is a transcriptional repressor required in osteoclast development and the preservation of bone homeostasis. The study also demonstrated that exosomes with low expression of circHmbox1 produced by TNF-α-induced osteoclasts could inhibit osteoblast differentiation. Indeed, circHmbox1 overexpression remarkably alleviated the osteoporotic phenotypes in OVX mice [95].

Other CircRNAs related to osteoporosis and osteoporotic fracture

Yang et al. have observed that osteoporosis and fracture were indirectly correlated to **circ_0076906** expression while directly correlated to that of **circ_0134944** in PBMCs of postmenopausal women [96]. In vitro, inhibiting circ_0076906 expression augmented the expression of miR-548i, which in turn reduced the expression of osteoglycin (OGN). This latter is a bone anabolic factor that has been linked to the mineralization and osteogenesis processes by adjusting the expression of osteogenesis-specific genes. The study also demonstrated that the circ_0134944 upregulation suppressed miR-630 and enhanced the expression of toll-like receptor 4 (TLR4). TLR4 is highly expressed in bone marrow, immune cells, adipocytes, and osteoblasts and is involved in osteoblastogenesis and osteoclastogenesis [97, 98]. Thus, circ_0076906 and circ_0134944 were associated with a risk of osteoporotic fracture, leading to osteoporosis [96]. Besides, Wen et al. observed that the expression level of **hsa_circ_0076906** was greatly decreased in both bone tissue and serum samples of the osteoporotic group compared to the control group, besides its gradual induction during osteogenic differentiation of hMSCs [99]. Circ_0076906 was found to promote MSC differentiation and relieve osteoporosis through upregulating OGN expression by inhibiting miR-1305. Additionally, **circHIPK3** was significantly upregulated in both tissue and serum samples from osteoporotic fracture patients compared with controls. CircHIPK3 was shown to sponge miR-378a-3p and to upregulate HDAC4, which is a histone deacetylase that controls osteoblast differentiation by inhibiting Runx2 transcriptional activity [100].

Conclusions

This article provides an overview of the expression patterns of lncRNAs and circRNAs in osteoporosis. It also discusses their involvement in the differentiating of osteoblasts and osteoclasts, as well as the underlying molecular mechanisms implicated in their effects. Many lncRNAs and circRNAs act by interacting with RBP or sponging miRNAs to control the expression of targeted genes, hence impacting many pathways involved in osteogenesis and osteoclastogenesis. Despite the fact that both lncRNAs and circRNAs possess great potential in the prediction, diagnosis, and prognosis of osteoporosis, their regulatory ability in targeting proteins is still largely unknown. More future studies are also warranted on circRNAs in osteoporosis since they are scanty in comparison with those on lncRNAs. Moreover, studying the interactions of lncRNAs and circRNAs is of interest for gaining a deeper understanding of the pathogenesis of osteoporosis and promoting the discovery of new therapeutics and drugs.

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Author contributions

SMI Designing and writing the original draft. MAA Writing—review &editing. MIS Supervision, Writing—review &editing, Visualization. HAD Supervision, Writing—review &editing, Visualization. MME Supervision, Writing—review &editing, Visualization. All authors read and approved the final manuscript.

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Abbreviations

AAV
Adeno-associated virus
AKT
Serine/threonine kinase 1
ALP
Alkaline phosphatase
AOX1
Aldehyde oxidase 1
AS1
Antisense RNA 1
ASCs
Adipose stem cells
BGN
Bioactive glass nanoparticles
BMM
Bone marrow macrophage
BMP
Bone morphogenetic protein
BMSCs
Bone marrow stromal cells
CaM
Calmodulin
c-Fms
Colony-stimulating factor-1 receptor
CircRNA
Circular RNAs
COCH
Cochlin
DEX
Dexamethasone
DKK1
Dickkopf-1

DLL
Delta-like ligands
Dlx5
Distal-less homeobox 5
DOCK3
Dedicator of cytokinesis 3
EFNA2
Ephrin A2
ERK
Extracellular signal-regulated kinase
EVs
Extracellular vesicles
EZH2
Enhancer of zeste homolog 2
FBXW7
F-box and WD repeat domain-containing 7
FUS
Fused in sarcoma
Fz
Frizzled
GAS1
Growth arrest-specific gene 1
GATA4
GATA-binding protein 4
GSDMD-N
Gasdermin D-N
HAGLR
Homeobox D gene cluster antisense growth-associated long noncoding RNA
HBMSCs
Human bone marrow stem cells
HFOB1.19
Human fetal osteoblast
Hh
Hedgehogg
HMGB1
High-mobility group box chromosomal protein-1
HMSCs
Human mesenchymal stem cells
HNRNPA3
Heterogeneous nuclear ribonucleoprotein A3
Hoxa10
Homeobox protein A10
HOXD3
Homeobox D3
HuR
Human antigen R
IRF-1

Interferon regulatory factor-1
ITGB3
Integrin beta-3
JNK
Jun N-terminal kinase
KHSRP
K-homology splicing regulatory protein
KLC1
Kinesin light chain 1
KMT2C
Lysine (K)-specific methyltransferase 2C
LATS1/2
Large Tumor Suppressor 1 and 2
LncRNA
Long noncoding RNA
LPS
Lipopolysaccharide
LRP5/6
Low-density lipoprotein receptor-related protein 5/6
MALAT1
Metastasis-associated lung adenocarcinoma transcript 1
M-CSF
Macrophage colony-stimulating factor
Mef2c
Myocyte enhancer factor 2C
MEL
Melatonin
MITF
Microphthalmia-associated transcription factor
MMP
Matrix metalloproteinases
mRNAs
Messenger RNAs
MST1/2
Mammalian STE20-like protein kinases 1 and 2
NEDD4
Neuronal precursor cell-expressed developmentally downregulated 4
NFATc1
Nuclear factor of activated T cells 1
NICD
Notch intracellular domain
OCN
Osteocalcin
OGN
Osteoglycin
OPG
Osteoprotegerin

OPN
Osteopontin
OSCAR
Osteoclast-associated receptor
Osx
Osterix
OVX
Ovariectomized
PAK2
P21-activated kinase 2
PBMCs
Peripheral blood mononuclear cells
PCNA
Proliferating cell nuclear antigen
PCP4
Purkinje cell protein 4
PDX1
Pancreatic and duodenal homeobox 1
PMOP
Postmenopausal osteoporosis
Prrx2
Paired-Related Homeobox Protein 2
RAD51-AS1
RAD51-Antisense RNA 1
RANKL
Receptor activator for nuclear factor κB Ligand
RBP
RNA-binding protein
RECK
Reversion-inducing Cysteine-rich proteins with Kazal motifs
ROS
Reactive oxygen species
RUNX2
Runt-related transcription factor 2
SMURF2
Smad ubiquitin regulatory factor 2
SNHG14
Small nucleolar RNA host gene 14
SPHK1
Sphingosine kinase 1
TAZ
Transcriptional coactivator with PDZ-binding motif
TGFβ
Transforming growth factor β
TLR4
Toll-like receptor 4
TNF-α

Tumor necrosis factor alpha
XIST
X-inactive-specific transcript
YAP
Yes-associated protein
YBX1
Y-Box Binding Protein 1

Publisher's Note

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DETAILS

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Hyaluronic acid: comprehensive review of a multifunctional biopolymer

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ABSTRACT (ENGLISH)

Background

Hyaluronic acid (HA) has a broad range of cosmetic and therapeutic applications due to its unique physicochemical properties and involvement in various essential biological processes, including cell signaling, wound reparation, and tissue regeneration.

Main body

In this review, we provide a comprehensive overview of HA, including its history, physicochemical properties, roles, molecular biology, and biochemistry (including occurrence, biosynthesis, and degradation), as well as its chemical modifications and conventional and emerging production methods. We also examine HA's medical, pharmaceutical, and cosmetic applications and its derivatives in arthrology, ophthalmology, wound healing, odontology, oncology, drug delivery, 3D bioprinting, and cosmetology. Finally, we discuss the potential role of HA in preventing Covid-19.

Conclusion

Hyaluronic acid, a naturally found substance, has shown immense potential in the clinic. Thus, it is imperative to highlight its applications in the diverse fields impacting the lives of patients and healthy individuals.

FULL TEXT

Background

Hyaluronic acid (HA) is a naturally occurring glycosaminoglycan found in vertebrate connective, epithelial, and nervous tissues. This versatile substance has a broad range of applications in the medical and cosmetic industries, such as dermal fillers, osteoarthritis treatment, ophthalmology, and vesicoureteral reflux. In 2018, the global HA market was valued at USD 8.3 billion, with a projected Compound Annual Growth Rate (CAGR) of 7.8% during the forecast period [1–3]. HA was first discovered in cow's eyes in 1934 and later identified in humans and other animals. It is primarily found in the extracellular matrix of connective tissue, synovial fluid, and vital tissues such as the eye's vitreous, cartilage, fascia, and umbilical cord. In 1979, pharmaceutical-grade HA was produced by extracting and purifying the polymer from rooster combs and human umbilical cords [2]. HA is abundant in soft connective tissues, including skin, lungs, kidneys, brain, and muscles. Its unique viscoelastic properties, biocompatibility, and non-immunogenicity make it an ideal substance for clinical applications and cosmetic purposes [2, 4]. Due to changing beauty standards and rising health awareness, there has been a significant increase in nonsurgical cosmetic procedures, with hyaluronic acid injectables being the second most frequently performed procedure after Botox, with a total spending of more than USD 5 billion in America in 2015 [5, 6]. This review explores HA's potential benefits and limitations in various applications, such as tissue engineering, drug delivery, and wound healing, by examining this substance's positive and negative aspects to provide a comprehensive overview of its use in medicine and cosmetics.

Main text

Physiological functions of hyaluronic acid

Hyaluronic acid (HA) is a macromolecule that plays a vital role in the human body. It is a high molecular weight glycosaminoglycan composed of glucuronic acid and *N*-acetylglucosamine linked together via glycosidic bonds. In the body, it exists in sodium hyaluronate and is present in various soft connective tissues, including the skin, lungs, kidneys, brain, and muscle tissues [7]. HA's biological functions are diverse and significant. It plays a crucial role in regulating tissue hydration and water transport, maintaining the elasto-viscosity of connective tissues, and facilitating the supramolecular assembly of proteoglycans in the extracellular matrix. HA also engages in numerous receptor-mediated roles, such as cell detachment, mitosis, migration, tumor development and metastasis, and inflammation [8]. When bound to water molecules, HA forms a hydrated gel and acts as a water-binding agent that lubricates movable body parts, such as joints and muscles. HA's properties and functions have led to a broad range of

applications in the medical field. For example, it is commonly used in dermal fillers for cosmetic purposes and is also used to treat osteoarthritis. The increasing demand for nonsurgical cosmetic procedures has led to a surge in using hyaluronic acid injectables [9] (Fig. 1).

Fig. 1 [Images not available. See PDF.]

Applications of hyaluronic acid (HA) in different fields. HA is a versatile biomaterial with various applications in various areas. This figure provides an overview of the diverse applications of HA, including drug delivery, tissue engineering, cosmetic procedures, and wound healing. The figure shows that HA can form nanoparticles for drug delivery, including oral drugs, micelles, and tumor-targeting nanoparticles. HA nanoparticles can also be used in ocular applications, enhancing drug delivery to the eye and improving ocular bioavailability. HA has also been used in tissue engineering, including cardiovascular tissue engineering, stem cell delivery, reconstructive and plastic surgery, and scaffold construction for knee replacement and cartilage regeneration. HA hydrogels, cryogels, and carbon nanotubes have enhanced tissue regeneration and repair. HA is commonly used as a filler for facial rejuvenation and volumization in cosmetic procedures. HA-based liposomes can also be used for targeted drug delivery in cosmetics. Other applications of HA include embryo implantation, wound healing, and microneedle patches. HA has been shown to improve embryo implantation rates and promote wound healing. Microneedle patches incorporating HA can enhance transdermal drug delivery and promote skin hydration [Figure generated using https://www.biorender.com/]

Molecular biology and biochemistry

Hyaluronan is a linear glycosaminoglycan comprising approximately 10,000 disaccharide units of d-glucuronic acid and *N*-acetyl-d-glucosamine (Fig. 2A). The synthesis of HA is carried out by hyaluronan synthases, which are membrane-bound enzymes forming functional dimers with six transmembrane segments. The polymer chain is expelled through the plasma membrane during hyaluronan synthesis. The active form of the hyaluronan synthase enzyme was isolated from streptococci as a complex and characterized as a 42 kDa protein through immunological cross-reaction with the streptococcal enzyme and affinity labeling techniques [10]. Three mammalian genes are responsible for hyaluronan synthesis (HAS1, HAS2, and HAS3), each contributing to hyaluronan production with different molecular weights. The biological effects of hyaluronan are distinct from other biologically active molecules and are influenced by its molecular weight (Mw) [11, 12].

Fig. 2 [Images not available. See PDF.]

A Structure of hyaluronic acid. **B** Schematic diagram illustrating the key steps involved in hyaluronic acid (HA) synthesis and degradation. The diagram shows that the synthesis of HA is regulated by various factors, such as growth factors, cytokines, kinases, and other proteins that modulate the activity of HA synthases (HAS1, HAS2, and HAS3). These enzymes are responsible for synthesizing HA chains, which are then bound by HAS protein complexes and extruded onto the cell surfaces and into the extracellular matrix (ECM). The biological actions of HA are tightly regulated by its degradation, which is carried out by several hyaluronidase enzymes, including HYAL-1, HYAL-2, HYAL-3, HYAL-4, PH20/SPAM1, HYAL-P1, and ROS. These enzymes cleave the HA chains into smaller fragments, which can be metabolized or eliminated from the body

There is a total of 15 g of hyaluronan (HA) in the human body, and about 30% of it undergoes degradation through two distinct mechanisms (Fig. 3). One mechanism involves specific enzymatic degradation mediated by hyaluronidases, while the other mechanism is nonspecific and occurs due to oxidative damage caused by reactive oxygen species (ROS) [13–15]. ROS encompass hydrogen peroxide, peroxynitrite, nitric oxide, superoxide, and hypohalous acids. These ROS are generated during inflammatory responses in conditions like sepsis, tissue inflammation, and ischemia–reperfusion injury. They can degrade hyaluronan, a process that can occur due to ROS. The human genome contains six identified gene sequences related to hyaluronidase: HYAL-1, HYAL-2, HYAL-3 genes, HYAL-4 and PH20/SPAM1 genes, and HYAL-P1 pseudogene. These genes are associated with the production of hyaluronidase enzymes, which are involved in the degradation of hyaluronan [16, 17]. The degradation

of HA occurs partially within the tissue itself, but a significant portion occurs in local lymph nodes and within the endothelial cells of the liver. The remaining 70% of HA undergoing systemic catabolism is transported by hyaluronan, primarily carried to the lymph nodes through the lymphatic system. Within the lymph nodes, hyaluronan is internalized and broken down by the endothelial cells of the lymphatic vessels. Additionally, a small fraction of HA enters the bloodstream and undergoes degradation by the endothelial cells in the liver [18–20]. Hyaluronidase-mediated degradation of HA plays a crucial role in various critical regulatory processes, including embryonic development and wound healing. The significance of HA degradation by hyaluronidases is evident in mucopolysaccharide hyaluronidase deficiency, a lysosomal storage disorder characterized by elevated levels of HA in the plasma due to a defect in hyaluronidase activity [21, 22]. HA exhibits one of the most rapid turnover rates among molecules in the mammalian body. It is estimated that approximately one-third of the 15 g of HA present in an average adult human is turned over daily (Fig. 4). The high turnover of HA in various tissues requires equally high rates of synthesis and degradation [23–25].

Fig. 3 [Images not available. See PDF.]

Summary of the production of HA. This figure provides an overview of the methods used to produce HA, including in vitro production, bacterial production, and extraction from animal tissues. The first method described is in vitro production, which involves using enzymes derived from *Streptococcus pyogenes* and *Pasteurella multocida* to synthesize HA in a controlled laboratory setting. This method allows for the production of HA with precise molecular weight and purity, making it ideal for pharmaceutical and biomedical applications. The second method described is bacterial production, which involves using various strains of bacteria, including *Streptococci, Enterococcus faecalis, Escherichia coli, Bacillus subtilis,* and *Lactococcus lactis*, to produce HA. This method is relatively inexpensive and scalable, making it suitable for large-scale production of HA for commercial and industrial purposes. The third method described is an extraction from animal tissues, which involves isolating HA from various animal sources, including rooster comb, human umbilical cord, bovine synovial fluid, and vitreous humor of cattle. This method is less commonly used due to the challenges associated with obtaining HA from animal tissues, but it remains an essential source of HA for specific applications

Fig. 4 [Images not available. See PDF.]

Schematic diagram illustrating the electrospun nanofibers and hydrogel scaffold composite for tissue engineering applications. The figure shows that electrospinning is used to fabricate nanofibers from a hyaluronic acid (HA) polymer, which is then cross-linked with 1 M NaOH and 0.5% CaCl2 to form a hydrogel scaffold. The resulting composite material provides a suitable substrate for cell proliferation and stretching, which can lead to the formation of functional tissue structures. The electrospun nanofibers provide a high surface area-to-volume ratio, facilitating cell adhesion and migration, while the hydrogel scaffold offers mechanical support and promotes cell proliferation. The composite material can also be further functionalized with growth factors or other bioactive molecules to enhance tissue regeneration [Figure generated using https://www.biorender.com/]

Isolation from biological sources and manufacturing by biotechnology

HA is a glycosaminoglycan that serves vital functions in tissue hydration and cellular processes. Within the body, HA is synthesized by attaching sugar molecules to the reducing end of the polymer. This synthesis occurs within the plasma membrane of various cells, including fibroblasts. The resulting HA molecule extends into the pericellular space, contributing to its important physiological roles [26]. Historically, hyaluronic acid was extracted from animal tissues such as rooster combs, human umbilical cords, or other vertebrate tissue. However, this process was found to be relatively complex and expensive [27, 28]. In recent years, hyaluronic acid has been obtained through in vitro production or extraction from the cell walls of bacteria of streptococcal origin. Two types of hyaluronic acid can be produced depending on the method: isolation-origin HA and fermentation-origin HA. Isolation-origin HA is obtained through a series of steps that include the removal of epithelium from the rooster comb, followed by grinding of the comb. Subsequently, the ground material is treated with acetone, ethanol, and sodium chloride to extract and purify

the hyaluronic acid [29]. In contrast, the production of fermentation-origin HA involves the continuous fermentation of Streptococcus in a controlled culture environment, such as a chemostat. However, it is essential to note that fermentation-origin HA often contains substantial amounts of endotoxins and elevated bacterial levels, necessitating the removal of these impurities through subsequent purification steps [30]. Therefore, additional purification steps are required to minimize the presence of bacterial proteins. Remarkably, hyaluronan has been discovered in the capsule of specific microbial pathogens, including Pasteurella multocida and certain strains of Streptococcus (Fig. 5). These microorganisms have developed enzymatic systems that resemble those found in vertebrate hosts to facilitate hyaluronan synthesis within their capsules [31, 32]. These microorganisms employ hyaluronan as a protective capsule around their cells, effectively evading the host's immune system and facilitating adhesion and colonization of the bacterial cells. This hyaluronan-based encapsulation serves as camouflage, allowing the microorganisms to bypass the animal defense mechanisms [21, 33]. Isolation-origin HA generated in biological systems is often associated with proteins and other glycosaminoglycans, necessitating thorough purification processes [34, 35]. Complex purification processes are essential to obtain a genuine product from traditional resources like rooster combs while minimizing the degradation of the molecular chains. However, even with sophisticated purification and sterilization methods, the final product's molecular weight will likely decrease, resulting in a lower molecular weight [36, 37]. Furthermore, the production of isolation-origin HA from traditional sources also poses a risk of viral contamination, necessitating complex purification procedures that can be costly [38–40].

Fig. 5 [Images not available. See PDF.]

Applications of hyaluronic acid (HA) and its derivatives in various fields. The figure shows that HA and its derivatives can be used as a drug delivery system, where they serve as carriers for different therapeutic agents, including small molecules, proteins, and nucleic acids. The biocompatibility and biodegradability of HA make it an ideal material for sustained drug release, enhancing the therapeutic efficacy of the delivered agent. In cancer therapy, HA and its derivatives have been used for targeted drug delivery, as well as for imaging and diagnosis. HA-based nanoparticles can selectively accumulate in tumor tissues, releasing the drug payload and effectively inhibiting tumor growth. HA, and its derivatives have also been used in soft tissue regeneration, including wound healing, cartilage repair, and bone regeneration. HA-based scaffolds and hydrogels can support cell adhesion, proliferation, and differentiation, forming functional tissue structures. HA and its derivatives are commonly used in skin care products in the cosmetic industry due to their moisturizing and anti-aging properties. HA-based fillers can also be used for facial rejuvenation and volumization. Other applications of HA and its derivatives include dietary supplements, urology, odontology, and wound treatment. HA-based materials can be used in urology for bladder augmentation and incontinence treatment. In odontology, HA-based materials can be used for tissue engineering and implantology. In wound treatment, HA-based dressings can promote healing and prevent infection [Figure generated using https://www.biorender.com/]

Modification of HA

HA possesses various functional groups, such as carboxylic acids, *N*-acetyl groups, and alcohols, that can be modified to alter the properties of resulting materials for enhanced hydrophobicity and biological activity [41, 42]. These modifications are commonly carried out through chemical cross-linking or radical polymerization, leading to hydrogels known as hylans. Although HA is highly hydrophilic and soluble in water, it is often required to have limited solubility or insolubility for its use in medical devices. It can be achieved by conjugating or cross-linking HA [43, 44]. Chemical modification of HA enables its transformation into diverse physical forms such as viscoelastic solutions, hydrogels with varying stiffness, electrospun fibers, flexible sheets, macroporous and fibrillar sponges, nonwoven meshes, and nanoparticulate fluids, which find applications in various clinical and preclinical settings [44–46]. This is achieved by targeting three functional groups: primary and secondary hydroxyl groups, carboxylic acid, glucuronic acid, and *N*-acetyl groups. Different approaches, such as addition/condensation chemistry or radical polymerization, can cross-link these groups [47]. However, the direct application of HA-based products in humans presents substantial challenges in their development (Fig. 6). The market for these products is expensive, and ongoing efforts are being made to create new formulations. The globalization of the industry has heightened the need for stringent

quality controls to guarantee the safety of cosmetic products [47, 48]. Consequently, there is an immediate requirement to advance the development of cost-effective and efficient techniques for identifying and detecting toxic components present as contaminants or impurities.

Fig. 6 [Images not available. See PDF.]

Application of hyaluronic acid (HA) hydrogel in the regeneration of dental pulp and cosmeceuticals. **A** shows that HA hydrogel can regenerate damaged dental pulp. Dental pulp stem cells are mixed with the HA hydrogel and injected into the damaged pulp using a syringe. The HA hydrogel provides a suitable microenvironment for the proliferation and differentiation of dental pulp stem cells, leading to functional dental pulp tissue regeneration. In addition to dental pulp regeneration. **B** HA hydrogel can be incorporated into skincare products, such as creams and serums, to improve skin hydration and reduce the appearance of fine lines and wrinkles. HA hydrogel can also be used as a filler in facial rejuvenation procedures, providing immediate volumization and contouring [Figure generated using https://www.biorender.com/].

Nanofibers and nanomicelles

Nanofiber scaffolds have a broad range of applications in fields such as tissue engineering, wound dressing, cosmetics, and drug delivery [49]. Biopolymers are ideal materials for these scaffolds due to their biodegradability and biocompatibility. However, the industrial development of such formulations is challenging due to modifying HA with toxic reagents during chemical processes, which are challenging to eliminate from the final product, making it unsuitable for pharmaceutical applications [50, 51]. Nanofibers based on photocurable ester derivatives of HA or its salt have been developed to overcome this issue. The skin barrier at the topmost layer, the stratum corneum, can prevent the penetration of drugs. However, nanosized colloidal systems, such as nanoparticles, liposomes, nanoemulsions, micelles, and polymeric suspensions, have demonstrated the ability to enhance drug penetration through this barrier [52, 53]. These systems have received significant attention for delivering cosmetic and pharmaceutical compounds topically for local or systemic administration [54, 55]. Research on polymer-based drug delivery has aimed at developing biodegradable polymer systems to reduce the risk of accumulating non-biodegradable particles in the body [56]. HA is an intriguing material as a topical drug delivery agent since it is a substantial part of the skin's extracellular matrix and can be found in both the epidermis and dermis [57].

Hydrogels

Hydrogels are intricate polymeric networks characterized by a three-dimensional architecture that enables them to absorb substantial quantities of water while preserving their structural integrity [58]. Due to its very important physiological and biological roles in maintaining homeostasis in the human body, hydrogels made from HA have been developed for several biomedical applications such as, drug delivery, tissue engineering and regeneration, as well as diagnostics, etc. [59, 60]. Market for HA-based hydrogels is continuously expanding and HA hydrogels are already being used in medicine as viscosupplements, dermal fillers, would dressings, etc.

Although HA can form molecular networks in the presence of a solvent due to its conformation and molecular weight, it cannot form a physical gel alone which further warrants for further chemical modifications such as covalent cross-linking and use of gelling agents to prepare HA hydrogels. Chemical cross-linking, with some limitations, has been a versatile method to obtain HA hydrogel with excellent mechanical, chemical, and thermal stability [61]. HA-based hydrogels can be prepared by several methods, such as polymerization, enzymatic cross-linking, condensation reactions, and click chemistry. HA hydrogels can be directly cross-linked with the help of cross-linking agents such as glutaraldehyde, divinyl sulfone, bisepoxide, and carbodiimide. [62, 63]. Using Diels Alder-based click reaction, HA-based hydrogels with tunable properties were developed by reacting furan modified HA with peptide derivatized with bismaleimide in order to mimic extracellular matrix (ECM) for breast cancer cells invasion [64]. Furthermore, by avoiding the use of cytotoxic copper as a catalyst, HA-PEG hydrogels were synthesized by reacting cyclooctyne modified HA with azide functionalized PEG. This hydrogel showed excellent mechanical properties, gelation time, and high stability [65, 66]. Recently, using another naturally occurring click chemistry between cyanobenzothiazole and cysteine, an in situ forming injectable HA hydrogel with encapsulated camptothecin

nanocrystals was prepared for long-term treatment of inflammatory arthritis [66].

On the other hand, non-covalent bonds and supramolecular interactions have been researched to prepare physical hydrogels with tunable properties by applying various cues like pH, light, temperature, etc. [67, 68]. Taking advantage of inclusion complexation properties of cyclodextrins, self-assembled HA hydrogel was formed by reacting β-cyclodextrin with adamantane functionalized HA which displayed excellent shear thinning properties [69]. Interestingly, using gelling agents such as Pluronic F-127, thermosensitive HA hydrogel was prepared by mixing HA in water with Pluronic F-127. Due to the hydrophobic interactions of acetyl groups of HA and methyl groups of Pluronic F-127, stable and mechanically stronger hydrogel was formed which avoided the typical burst release of drugs when only Pluronic F-127 was used in hydrogel preparation [70].

Films

HA films have several advantages over conventional formulations like gels, ointments, and solution as films can be stable, long-lasting, and can enhance patient compliance. There have been continued research on HA-based films for the treatment of diverse diseases by overcoming the drug delivery barriers of drug molecules as well as delivery system itself [71, 72]. HA films have been found to have limited medical applications that require extended stability in aqueous environments due to their fast dissolution in water, poor mechanical stability, and rapid in vivo degradation. However, these limitations can be overcome by implementing physical and chemical cross-linking techniques [57, 73, 74]. To be suitable for biomedical applications, films must possess specific properties, such as self-supporting, adequate mechanical strength when hydrated, biocompatibility, biodegradability, non-cytotoxicity, and the ability to adjust in vivo stability [75, 76]. A new type of water-insoluble film composed of palmitoyl esters of hyaluronan (pHA) was developed in 2016 to overcome the solubility limitations of hyaluronan films [77]. A new method was formed in 2019 for creating free-standing films from lauroyl derivatives of HA without the need for cross-linking agents, plasticizers, toxic solvents, and activators. This method involves an artless single-step solution casting process. The resulting films were homogeneous, exhibited good mechanical strength, and were flexible. Hydrophobized or cross-linkable hyaluronan derivatives exhibit higher resistance to biodegradation. They can serve as scaffolds for cell culture and matrices for controlled drug-related augmentation of soft tissues via viscosupplementation [78]. Conjugation of hyaluronan with drugs also provides an exciting approach for targeted drug delivery [79]. Significant attention has been given to the preparation of hyaluronic acid derivatives that can undergo cross-linking reactions under mild physiological conditions to broaden their applications.

Applications of hyaluronan

HA is a biocompatible polysaccharide with distinctive physicochemical characteristics. These properties render it highly versatile and applicable in numerous medical domains [8]. In the human body, the total quantity of HA is estimated to be around 15 g in a 70-kg adult [80]. While HA is predominantly present in the skin, constituting approximately 50% of the overall HA content in the body, it is also distributed throughout various other tissues and fluids. HA can be found in the vitreous humor of the eye, the umbilical cord, and synovial fluid, as well as in all tissues and bodily fluids. This includes skeletal tissues, heart valves, the lungs, the aorta, the prostate gland, and specific structures of the penis, such as the tunica albuginea, corpora cavernosa, and corpus spongiosum [80–85].

HA in arthrology

Autograft reconstruction is a commonly employed surgical technique for treating severe ligament injuries. However, this approach has limitations, including the risk of donor site morbidity. Tissue engineering techniques that involve culturing isolated fibroblasts on scaffold materials offer a promising alternative to autografts [86, 87]. Successful regeneration in ligament and tendon tissues has been demonstrated through various scaffold materials. These scaffolds encompass both naturally occurring substances and synthetic materials. An effective strategy for ligament tissue engineering involves incorporating glycosaminoglycans (GAGs) or GAG-like materials as essential scaffold components [88]. The principal constituent of GAGs, integral components of extracellular matrices, has been proven to promote tissue healing in diverse tissue types. It is achieved through several mechanisms, including enhanced delivery of growth factors, improved cellular adhesion and proliferation, and the facilitation of anti-inflammatory response [89–91]. HA's biological effects could play a critical role in promoting the regeneration of ligament tissues.

Moreover, the use of HA and hylans for intra-articular treatment has gained broader acceptance as a therapeutic approach for managing pain associated with osteoarthritis [92, 93]. HA plays a crucial role in maintaining the viscoelastic properties of synovial fluid in the knee. In osteoarthritic joints, HA concentration is typically lower than in healthy joints. Therapy aims to restore the lost viscoelastic properties of synovial fluid by introducing HA. This can help alleviate osteoarthritis pain by reducing nerve impulses and sensitivity associated with the condition [94–96].

HA for eye drops and ophthalmic surgery

Hyaluronan possesses distinctive characteristics, such as stabilization of the reduction of friction during blinking, tear film, and prevention of harmful substances from binding to the eye due to its various properties such as viscoelasticity and hydrophilicity, which greatly diminish the signs of dry eye [8, 97, 98]. Its viscoelasticity is mainly related to its cushioning and lubricating effect, as it is a component of the eye (aqueous humor) and synovial fluid. This unique rheological property is exploited in applying hyaluronan in ophthalmic surgery, where it is mainly used to establish and maintain a secure status to progress healing of the postsurgical area [30, 99]. The benefits of HA in ophthalmology extend to various aspects. HA aids in stabilizing the tear film, reducing healing time, minimizing adhesion risk, decreasing free radicals' formation, and normalizing intraocular pressure. The rheological properties of sodium hyaluronate have been examined for ophthalmic viscosurgical device (OVD) applications during cataract surgery. It has been concluded that the viscoelastic and flow properties of binary formulations consisting of sodium hyaluronate and HPMC (hydroxypropyl methylcellulose) are suitable for use as OVD. These formulations effectively maintain the ocular spaces and can be administered quickly [100, 101]. Furthermore, the adhesive properties of both sodium hyaluronate and HPMC in the binary formulation provide an additional advantage. These properties enable the formulation to effectively interact with the corneal endothelium, resulting in durable protection of ocular tissues. This interaction enhances the overall efficacy and safety of the formulation in maintaining ocular health during surgical procedures or therapeutic interventions [98, 102, 103].

HA in wound healing and tissue repair

CD44, the primary receptor for HA, is a versatile transmembrane glycoprotein expressed in various isoforms and found in nearly all human cell types. CD44 can interact with HA and various growth factors, cytokines, and extracellular proteins. This comprehensive interaction profile allows CD44 to participate in diverse cellular processes and signaling pathways involved in development, tissue homeostasis, inflammation, and cancer progression. The ability of CD44 to engage with multiple ligands highlights its significance as a critical regulator of cell adhesion, migration, proliferation, and signaling events within the extracellular microenvironment [104]. The interaction between HA and CD44 is implicated in many intracellular signaling pathways that govern various cell biological processes. These processes include receptor-mediated internalization and degradation of hyaluronan, angiogenesis (the formation of new blood vessels), cell migration, proliferation (cell growth and division), aggregation (cell clustering), and adhesion to extracellular matrix (ECM) components. The HA-CD44 interaction is a critical modulator of these cellular activities, contributing to tissue development, wound healing, immune response, and other physiological and pathological processes [105, 106]. CD44 emerges as a pivotal player in inflammation and wound healing, encompassing intricate biological processes to restore damaged tissue. Throughout all phases of tissue repair, including cellular migration, inflammation, angiogenesis (formation of new blood vessels), remodeling, and scar formation, extracellular matrix components, including HA, exert significant regulatory influence. CD44, through its interaction with HA and other molecules, exerts precise control over these sequential events, orchestrating the complex interplay required for effective tissue repair and regeneration [107]. HA is a fundamental component of the ECM and possesses distinctive properties contributing to its crucial role in tissue regeneration. Besides its structural support, HA can also function as part of a feedback loop, promoting cell proliferation and migration in actively growing tissues. This interaction between HA and cells helps regulate critical tissue development, repair, and regeneration processes. HA contributes to the dynamic balance required for effective tissue growth and remodeling by influencing cell behavior [108]. Furthermore, the role of HA in maintaining water homeostasis can contribute to tissue hydration, which in turn has a beneficial impact on the healing process. During periods of rapid tissue proliferation, regeneration, and repair, there is an increase in HA levels. This heightened presence of HA helps

retain moisture, providing a hydrated microenvironment that supports cellular activities and facilitates optimal conditions for tissue healing and recovery. The ability of HA to regulate water balance within tissues underscores its significance in promoting efficient healing processes [109, 110]. As HA is implied in every step of the wound healing procedure, exogenous application of HA can provide faster healing.

HA in odontology

In dentistry, biological materials such as HA have a broad range of applications, including regeneration and reconstruction of dentine, gingiva, dental pulp, cancellous bone, mucosal wound repair, and constructing a biophysical barrier between gingiva and jaw bones [111]. HA can act as a biocompatible scaffold or niche for mesenchymal stem cell (from apical papilla) differentiation, polarity, and a biophysical trigger or reservoir for the controlled release of various cytokines and chemokines for paracrine and autocrine signaling [112]. Additionally, HA can neutralize bacterial hyaluronate lyase enzymes, exerting a bacteriostatic effect.

Oral ulcer

Recurrent aphthous stomatitis (RAS), known as canker sores, is the most prevalent inflammatory ulcerative condition affecting the oral mucosa. However, the management of oral ulcers remains a challenge for clinicians. While topical corticosteroids, antibiotics, and antimicrobial agents are widely used, there are feeble proofs supporting the efficacy of any topical therapy. For these molecules to be effective, they should be easily applicable and preserved at the site of mucosal ulcer (MU) for an extended period [113]. Several studies have explored HA as a topical remedy for MU of the oral cavity. Notably, topical treatment of chronic aphthous MU with 0.2% HA gel for two weeks has promoted healing without side effects. Lee et al. demonstrated the effectiveness of topical 0.2% HA gel in treating oral MU in patients with RAS and Behçet's disease, suggesting improved symptoms [114, 115]. Hence, the primary activity of HA appears to be in tissue regeneration, performing a wide range of biological activities, including activating phlogistic responses, aiding cellular differentiation, proliferation, migration, and vasculogenesis, and reducing collagen deposition and scarring [116].

Gingivitis and periodontitis

Gingivitis is a highly prevalent disease that affects 82% of the population. Dental plaque has been identified as a crucial etiological factor in developing gingivitis and periodontitis [117]. Consequently, treating gingivitis and periodontitis aims to reduce dental plaque accumulation. In vitro studies have demonstrated that HA inhibits bacterial growth and interferes with bacterial morphology [118, 119]. Regarding clinical studies, it has been found that HA reduces plaque accumulation and inhibits gingival inflammation. A survey by Gizligoz et al. examined the plaque inhibitory impact of HA mouthwash compared to chlorhexidine. It was found that HA revealed an almost similar plaque inhibitory effect to chlorhexidine [120]. Jentsch et al. evaluated the effectiveness of the topical treatment of 0.2% HA. They concluded that it benefitted gingivitis by lowering the plaque indices and improving the papillary bleeding index (PBI) concerning gingival crevicular fluid (GCF) variables [121]. Similarly, Pistorius et al. proposed that the topical application of a HA reduced the PBI and sulcus bleeding index (SBI) [122]. Additionally, Sahayata et al. claimed that oral application of 0.2% HA gel in gingivitis, in addition to dental scaling and oral hygiene, offered a successful consequential response in the gingival index (GI) and PBI of placebo or control group (scaling plus placebo gel) and negative control group (scaling only) [123]. Dental scaling and root planning with topical HA are beneficial therapies for controlling gingivitis and probing depths (PDs) in individuals with chronic gum disease. Annsofi Johannsen et al. explained the beneficial effects of HA-based formulations in treating periodontitis [124]. The adjunctive application of hyaluronan gel could benefit periodontal health. The hyaluronan-based scale and root planning (SRP) protocol resulted in statistically significantly more significant reductions in abnormal dental bleeding in SRP control. Additionally, Hyaluronan has also been proven to induce bacteriostatic effects in vitro [124].

Surgery

In a comparative analysis, the health status of peri-implant mucositis and peri-implantitis during the recovery period of functional implants using HA or CHX gels. Their results demonstrated a reduced bleeding index in the HA group compared to the control group managed with CHX. Therefore, treating peri-implant mucositis and per-implantitis patients with 0.2% HA gel may be beneficial. Ballini et al. proposed combining autologous bone graft with the

esterified low-molecular HA formulation can accelerate bone regeneration in periodontal intrabone anomalies [125]. Additionally, the topical spray of 0.2% HA proved beneficial in managing inflammation and trismus during postoperative surgeries. Romeo et al. also demonstrated that the utility of essential amino acids with 1.33% HA solution could aid in secondary intention healing in laser-induced wounds during the total excisional biopsy of the gingiva and palate of the oral cavity [126]. Although it is not beneficial in pain perception, it can considerably expedite the repair processes [119].

HA in bioinks for 3D bioprinting

Manufacturing a three-dimensional (3D) object by layer-wise deposition or combination of materials, including plastics, metals, ceramics, powders, liquids, and living cells, is called 3D printing. When utilized in biomedical engineering and regenerative medicine to produce complex biological scaffolds or viable tissue structures that in vivo tissues and organs, 3D printing technology is referred to as 3D bioprinting; it holds immense potential for the fabrication, personalized prosthetics, precision implants, and histological models, and for pharmaceutical interventions such as controlled drug delivery, and microphysiological systems or organ-on-chip based drug discovery and development [127–129]. In 3D bioprinting, bioink is the main component, and different biomaterials are utilized as bioinks that are evaluated for crucial properties to ensure ease in the process [130]. It is imperative that bioinks possess high biocompatibility and physiological relevance to nurture viable cells, are mechanically sturdy after printing, and offer precise resolution during 3D printing. Therefore, biophysical characteristics, such as extrusion compatibility and mechanical properties, fluidic nature, viscosity, biodegradability, and cytotoxicity, must be evaluated [131]. Among the leading bioprinting materials used in 3D bioprinting to develop biological structures is HA, a natural ECM. HA is primarily employed because of its biological integrity, elasticity, mechanical and biodegradation properties, mimicking ECM composition, self-assembling ability, and yielding good resolution during printing [132]. To obtain increased stability and cell viability, HA can also be combined with different semi-synthetic or chemically defined polymers, such as hydrogel polymers, which exhibit stable rheology properties and excellent biocompatibility, resulting in gels that demonstrate printability in good shape. This development of biomaterials and cell biology has paved the way for bionic and regenerative medicine to become vital research fields with fast growth [133].

HA in cancer therapy

Cancer is a significant contributor to morbidity and mortality globally, with an estimated 18.1 million new cases and 9.6 million deaths reported in 2018 [140]. In recent decades, the progress of nanotechnology in medicine has offered new and promising solutions and insights for detecting, preventing, and treating cancer [143, 144]. HA plays a crucial role in various aspects of cancer cell behavior, primarily through its interactions with the stromal environment. The dysregulation of HA synthesis and the subsequent overproduction of HA often occur during the malignant transformation of cells. The impact of HA on tumor development can vary depending on the specific circumstances being evaluated, as it has the potential to either suppress or support tumor growth [146]. Extensive research has provided substantial evidence regarding the role of hyaluronan in promoting malignancies. It has been observed that increased invasion and dissemination of cancer cells can be attributed, at least in part, to the mesenchymal conversion facilitated by HA overexpression [147]. Experimental studies have demonstrated that various components of the hyaluronan signaling pathway, such as HA synthases, HA receptors, and HYAL-1 hyaluronidase, significantly promote tumor growth, metastasis, and angiogenesis. These findings highlight the potential of targeting each component as a therapeutic approach to cancer treatment [148]. The role of hyaluronan in cancer progression can vary depending on the expressed isoforms of HA synthases (HAS). Cancer cells at different stages may utilize the three HAS isoforms differently to enhance their survival. This suggests that the specific isoform of HAS expressed by cancer cells could influence their behavior and response to treatment, highlighting the importance of considering the isoform-specific effects of HA in cancer research and therapy [149]. Multiple strategies have been devised to target different HA (hemagglutinin) family members. These strategies encompass small-molecule inhibitors, antibody-based therapies, and vaccine-based interventions [150]. These treatment approaches aim to block the intracellular signaling mediated by HA, which is critical in promoting tumor cell proliferation, motility,

invasion, and the induction of endothelial cell functions. HA has been incorporated into nanoparticle formulations to achieve targeted delivery of chemotherapy drugs and other anticancer compounds to tumor cells. These preparations take advantage of the interaction between HA and cell-surface HA receptors, offering several advantages, such as being nontoxic, nonimmunogenic, and amenable to modifications for enhanced efficacy [148, 151]. The utilization of HA nanosystems shows great potential in facilitating the targeted and safe delivery of chemotherapeutic drugs and other anticancer compounds specifically to tumor cells. By leveraging the unique properties of HA and its interactions with cell-surface receptors, these nanosystems can enhance the specificity of drug delivery while minimizing potential adverse effects on healthy tissues. This targeted approach holds promise in improving the efficacy and safety of cancer treatments [152]. The utilization of HA nanoparticles offers several advantages in anticancer therapy. One such advantage is the ability to improve the half-life of anticancer agents and concentrate their delivery to cells that overexpress HA receptors. This targeted approach enables the potential for enhanced effectiveness at lower doses, leading to reduced drug-related toxicities. Many antineoplastic drugs have been successfully conjugated to hyaluronic acid, developing novel compounds with promising antitumor effects. For instance, HA-modified polycaprolactone nanoparticles encapsulating naringenin have demonstrated encouraging results. In vitro studies have shown enhanced drug uptake by cancer cells, indicating improved cellular internalization. Furthermore, in vivo experiments on rats with urethane-induced lung cancer revealed inhibited tumor growth following treatment with these nanoparticles. This highlights the potential of HA-based formulations to enhance therapeutic outcomes in cancer treatment [153]. Furthermore, it has been observed that HA-coated chitosan nanoparticles facilitate the delivery of 5-fluorouracil specifically to tumor cells that overexpress the CD44 receptor. The HA coating on chitosan nanoparticles enhances their affinity to CD44 receptors, enabling targeted drug delivery. This targeted approach improves the uptake of 5-fluorouracil by tumor cells and enhances its therapeutic efficacy against cancer. This finding underscores the potential of HA-coated chitosan nanoparticles as a promising strategy for improving drug delivery and enhancing the effectiveness of anticancer therapies [154, 155]. Paclitaxel, a widely studied compound, has demonstrated significant potential as an anticancer agent. However, its poor solubility in water has limited its therapeutic use. Recent research has focused on addressing this challenge by exploring novel approaches, such as utilizing unsaturated derivatives of HA and various HA-paclitaxel conjugates. These innovative strategies aim to enhance the aqueous solubility of paclitaxel and improve its delivery to target cancer cells. Researchers have sought to overcome its solubility limitations and enhance its therapeutic efficacy by conjugating paclitaxel to HA. Additionally, this approach can potentially reduce drug-related toxicities associated with conventional formulations. Furthermore, other anticancer agents are successfully linked to HA beyond paclitaxel, aiming to overcome toxicity and impart new physicochemical characteristics to the drug. These efforts seek to improve drug stability, enhance targeted delivery, and optimize therapeutic outcomes. These advancements in HA-based conjugates and derivatives showcase the potential of HA as a versatile platform for improving the delivery and efficacy of various anticancer agents. Such research holds promise for developing safer and more effective treatments for cancer patients [105, 156]. Eurand Pharmaceuticals implemented a similar strategy using methotrexate (MTX), an antimetabolite and folic acid analog commonly used as an antineoplastic drug. They developed an HA-MTX conjugate and conducted studies to evaluate its efficacy. The HA-MTX conjugate demonstrated significant activity in a liver metastasis tumor model, indicating its potential in treating metastatic liver tumors. Additionally, it exhibited activity in a mammary carcinoma model, demonstrating its effectiveness in combating breast cancer. These findings highlight the promising therapeutic potential of the HA-MTX conjugate in targeting and treating neoplastic conditions. By conjugating MTX with HA, the researchers aimed to enhance drug delivery, potentially improving the treatment outcomes and reducing adverse effects associated with conventional MTX formulations. This research demonstrates the valuable application of HA-based conjugates in expanding the therapeutic options for anticancer drugs like MTX, potentially offering more effective and targeted treatments for liver metastasis and mammary carcinoma [157]. Toxicology and pharmacokinetics analyses have displayed an extended half-life and amplified area under the curve (AUC) worth concerning free MTX [158]. Recent work has highlighted the importance of hyaluronan in oncology and should be further researched.

HA for skin

The skin serves as a homeostatic indicator of overall physical and emotional well-being. Alterations in dermal characteristics such as temperature, tone, muscle tension, and hydration reflect somatic and emotional changes that occur in an individual, with the latter being beyond conscious control [134]. Skin aging is a complex, progressive, and irreversible process marked by biochemical, morphological, and biophysical changes in the body. With the global population aging and the increasing aesthetic demands of patients, the desire to appear youthful and healthy is gaining momentum. In the past, surgical interventions were the primary option for rejuvenation [135]. However, novel noninvasive outpatient techniques have revolutionized aesthetic dermatology. Injectable fillers, in particular, have garnered considerable attention due to their efficiency and safety [136]. Wrinkle filling remains a primary indication, but restoring volume and contours to achieve a natural, balanced look is equally vital in contemporary aesthetics. Additionally, advanced techniques have been developed to correct chin/nose deformities, and it is preferable to use biodegradable agents for aesthetic dermatology instead of permanent ones [137, 138]. Complications arising from using permanent agents in aesthetic dermatology can be particularly challenging to treat compared to those associated with biodegradable agents. Fortunately, a range of skin treatments, including injectable hyaluronic acid-based fillers (HAFs), are available to address age-related changes [139]. Fillers constitute an effective tool in skin rejuvenation, and while bovine collagen was previously the primary filler used for wrinkles and lip augmentation, since 1996, HA has become the preferred choice. Modern HA is produced through bacterial fermentation, eliminating the risk of animal-derived contamination, and because it is not species-specific, skin testing is not required [137, 140]. Recent statistics suggest that over 85% of dermal filler surgeries utilize HA derivatives. This figure will rise in the future, as no other potential filling agent is currently available to counter HA's popularity [141]. HA's efficacy, ease of administration, low toxicity, and high safety profile have made it the gold standard compound among fillers, and the list of cosmetic dermal fillers available continues to expand rapidly [142]. As the aging population seeks inexpensive and safe options to revise the signs of aging without major surgery, the popularity of HA-based fillers is only expected to increase [142].

The strength of HA to cross the biological barrier is primarily determined by its molecular weight (MW). High-MW HA, with a weight exceeding 600 KDa, has poor skin permeability and typically forms a very thin protective hydration veneer on the epidermis [45]. In contrast, low-MW HA can penetrate through the deeper layers of the skin and permeate up to the hypodermis level. Thus, using HA enables a comprehensive rejuvenation of the face, as thin HAs can be administrated by mesotherapy to rehydrate the skin's surface. In contrast, high-MW HAs have been used to address wrinkles, nasolabial folds, dark circles, under-eye hollows, and lip augmentation. High-MW HAs are also employed for tissue volume increase, highlighting the versatility of this approach [19]. Due to its unique viscoelasticity, biocompatibility, biodegradability, and non-immunogenicity, HA has been extensively applied in dermatology for its biomedical benefits, including skin anti-aging, anti-wrinkle, anti-nasolabial folds, skin rejuvenation, and dermal hydration properties [143]. HA can be administered through various routes, including ophthalmic, nasal, parenteral, topical, and intravenous, and in clinical, nutraceutical, nutritional, and cosmetic industries. Topical delivery systems offer several advantages over oral or parenteral delivery modes, such as overcoming the hepatic first-pass metabolism, improved prognosis, excellent dermal barrier permeability, and minimizing possible toxicity-related clinical adverse or side effects [45]. HA has been utilized to formulate microparticles for controlled dermal release of caffeine to medicate cellulite, topical hydrogels containing nonsteroidal anti-inflammatory drug diclofenac to manage actinic keratosis, and for manufacturing, HA-derived liposomes for healing dermal and subcutaneous wounds [45]. HA has also been extensively utilized for preparing transdermal formulations through various approaches, such as chemical tempering to create conjugates or physiochemical methods to create microneedles, including OVA-HA conjugates for noninvasive vaccination and HA-based microneedles for controlled release of insulin to treat Type I diabetes [144]. In current cosmetic trends, HA is commonly found in moisturizers, creams, gels, and serums due to its hydrating properties, lipid barrier enhancement, fine lines and wrinkles reduction, and skin tightening effects. Moreover, sunblock derived from hyaluronan may assist in preserving spry skin and shielding it against the detrimental impact of ultraviolet radiations

attributed to HA's potential free radical scavenging effects. Overall, HA's diverse and promising applications in various fields of medicine and cosmetics have established it as a highly desirable and versatile biomaterial.

Covid-19 and hyaluronic acid

In modern times, the coronavirus disease 2019 (COVID-19) pandemic posed a severe threat to international biosecurity and public health. The etiology of this respiratory illness is a novel coronavirus known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Clinical investigations revealed that SARS-CoV-2 infection triggers a biphasic immune response. During the initial incubation period, a first-line defense-based protective phase is activated, which requires the activation of the adaptive immune system to intercept the virus replication and disease progression to severe stages. Therefore, strategies to enhance immune responses at this stage are paramount [145]. To establish an effective host immune response during the disease incubation period, the host must be in good physical condition, which can induce peculiar antiviral immunity. However, if the adaptive immune response is compromised, the infection will continue replicating, leading to massive tissue damage, particularly in tissues with high ACE2 expression [146]. This triggers an inflammation-driven damaging phase characterized by lung parenchymal tissue inflammation mediated primarily by alveolar macrophages and other granulocytes. Pneumonitis is the primary etiology of lethal upper and lower respiratory tract disorders during the severe stage of the disease. Hence, suppressing the proinflammatory system is critical to managing the clinical symptoms when severe damage to lung parenchyma occurs. SARS-CoV-2 infection is classified into three different stages: stage I is an incubation phase when the patient is usually asymptomatic and sometimes the virus cannot be detected in body specimens; stage II, a non-critical symptomatic phase with detectable viral immunogens; and stage III, an extreme respiratory or general symptomatic stage with elevated viral load in the body. Histopathological examinations of the tissues collected from COVID-19 patient atopies revealed edema and the presence of abnormal hyaline membrane pulmonary mesenchyme, forecasting the existence of acute respiratory distress syndrome (ARDS) [147].

Hyaluronan, a primary constituent of the lung extracellular matrix (ECM) in the lungs, is found in the pulmonary mesenchymal tissue. It is a key player in airway homeostasis by regulating cellular functions, growth factors, cytokine behavior, and biomechanical forces, among other aspects [148]. In various respiratory diseases, such as COPD, atypical asthma, idiopathic arterial pulmonary hypertension, and ARDS, airway hyaluronan levels are elevated and are associated with poor lung function [149, 150]. Furthermore, there is mounting evidence that hyaluronan and its degradation products are of critical significance in the pathophysiology of the respiratory tract. Aerosolized exogenous hyaluronan has been shown to exert beneficial effects against airway inflammation, protect against bronchial hyperreactivity and remodeling, and disrupt biofilms associated with chronic infections [151, 152]. Therefore, exogenous hyaluronan may serve as a novel therapeutic option in conjunction with conventional medical or surgical therapy for respiratory tract diseases involving inflammation, epithelial survival, remodeling, and the microbiome, such as rhinitis, asthma, COPD, cystic fibrosis, ARDS, and pulmonary hypertension, and should be considered for COVID-19 treatment [153, 154].

Hydroxychloroquine is currently one of the leading drugs being investigated worldwide for COVID-19 [155]. To mitigate its intrinsic toxicity, enhance its bioavailability, localization, and controlled release, and improve its efficacy, a proposal has been developed to conjugate it with HA to formulate a hyaluronic acid-hydroxychloroquine conjugate [156]. The ability of hyaluronic acid to form conjugates with pharmacologically active compounds offers an opportunity for this approach [157]. However, no clinically approved immunoglobulins or specific therapeutic drugs are available for COVID-19. Rigorous research is ongoing to screen potential therapeutic targets that may aid in developing effectual prevention and successful treatment strategies [158].

HA in drug delivery

Conjugating active ingredients to HA can create pro-drugs with efficient physicochemical features, improved shelf life, stability, and therapeutic potency and safety compared to free drugs [2]. Since hyaluronan possesses multiple physiochemical properties, HA-drug conjugates can exert their biological activities as such. Moreover, therapeutic actions can also be achieved upon drug release when the chemical bonds linking active ingredients and HA are catalyzed in the biological system, ideally at the peculiar target sites [48, 159]. A diverse range of active ingredients

can be compounded into HA for topical or intravenous application. HA is primarily utilized in controlled release or targeted drug delivery systems because of its excellent biocompatible gelation properties. One example is a polymer network created by gelating the adipic dihydrazide derivative of HA cross-linked with reagent poly (ethylene glycol)–propionaldehyde. This macromolecule gives rise to a hydrogel [72, 160]. Transdermal drug delivery using HA is possible, but the challenge lies in that HA, a high molecular weight compound, cannot cross the stratum corneum. To overcome this issue, nanoparticles of HA can be utilized, which can deliver the drug to the dermis. Moreover, bioavailability has always been a limitation in ocular drug delivery due to various barriers [161, 162]. However, coating chitosan-based nanoparticles with HA can increase the cornea's retention time, thereby enhancing dexamethasone's bioavailability by almost two times. These nanoparticles are also suitable for gene delivery, as they are highly compatible with the mucous and ensure efficient transfer without loss of cell viability [163]. During eye-related surgeries, HA is employed to equilibrate the morphology of the frontal chamber. HA-based nanoparticles (NPs) in polymeric thin films can also serve as a hybrid therapeutic system for the controlled release of vitamin E to manage skin wounds [164]. HA formulations with phospholipids can develop surface-modified liposomes before or after liposome formulation [165]. HA-modified liposomes have shown great promise as drug carriers. They enhance drug stability in the dynamic blood flow, extend drug half-life, lower toxicity, improve tissue absorption and barrier permeability, sustain prolonged or controlled active ingredient release, and enhance therapeutic efficacy through synergistic actions [166]. HA and its derivatives have a strong affinity for CD44 receptors, specific receptors in cancerous tumors [167]. This makes HA an ideal candidate for targeted and effective delivery of anticancer drugs, given its high biocompatibility, non-immunogenicity, and non-toxicity [105]. Many approaches, like nanotheranostics and nanocarriers such as carbon tubes, quantum dots, and graphene, are used in conjugation with HA to achieve an efficient delivery system. In addition to anticancer drugs, HA is also used to deliver genes and proteins. HA-based microspheres and microparticles have been investigated as potential combinational compounds to enhance the bioadhesive properties, control drug delivery, and improve the ointments' physical quality. For instance, spray-dried HA-based microspheres have shown precision delivery of ofloxacin to the pulmonary tissues via nasal inhalation. This leads to better pharmacological impact than free ofloxacin and intravenous or oral routes of administration. HA and its derivatives have been utilized alone or in conjugates to formulate pro-drugs, surface-modified liposomes, NPs, microparticles, hydrogels, and other controlled drug delivery carriers [168, 169]. All these drug delivery systems are subject to intensive pharmaceutical optimization for harnessing the maximum benefits. This extensive biomedical and clinical reach is still in its infancy, as most of the findings are based on in vitro experiments, and there is a long way to go for the industrialization of HA-based pharmaceutical products [170].

Conclusion

This paper comprehensively overviews the natural biopolymer HA and its unique physicochemical characteristics, including biodegradability, biocompatibility, efficacy, safety, and immunogenicity (Table 1). HA has been generally utilized and proven successful in various biomedical applications, including controlled drug delivery and release, osteoarthritis treatment, open-wound healing, ocular surgery, odontology, cosmetology, regenerative medicine, and biomedical engineering. Extensive research from academia and the biomedical industry has been carried out to understand the various derivatives of HA and their applications. The game-changing potential of HA has been a driving force for this research. The application of HA for 3D bioprinting has also been discussed, along with its proposed use in combating the current COVID-19 crisis. Overall, the versatility and potential of HA make it a promising candidate for numerous future biomedical applications, and continued research in this field will undoubtedly yield more significant findings.

Table 1. Summarizing different types of HA forms available, their properties, and potential applications

Type of HA	Molecular weight	Properties	Potential application	Reference

Nanofibers	15–150 kDa	High porosity Mechanical strength Flexibility compared to microfibers Large surface area-to-volume ratio	Wound dressing Scaffolds for tissue engineering Drug release delivery systems Serums for cosmetics Nano masks Coatings for medical devices	[171–175]
Microfibers	100–700 kDa	Solubility is adjustable Different textile technologies can weave it Sterilizable	Tissue regeneration Pre- or postsurgical use Drug delivery or another active ingredient delivery Controlled release delivery system	[176–178]
Staple fibers	350 kDa–2.7 M	It can be loaded with growth factors or MRI contrast agents It can be combined with other HA forms for multilayer applications	Drug delivery and drug release materials Active layers for wound healing devices 3D structures Antiseptics Hemostatic Pads Scaffolds with GFs	[179–182]
Hydrogels	60–1000 kDa	Fully biocompatible and biodegradable Possible to incorporate cells, fibers, micro or nanoparticles, or active substances	Scaffolds Regenerative medicine Viscosupplementation Postsurgical adhesion Reservoir drug release Wound healing Cartilage tissue engineering Bioprinting Contact lenses preparation Super porous hydrogels in hygiene products	[73, 183–185]

Thin Films	15–1000 kDa	Swelling, degradation rates, and mechanical properties can be controlled by the type of modification and the degree of substitution	Prevention of postoperative adhesions Tissue engineering (cell sheets) Controlled release of active substances or growth factors Soluble or insoluble options Controlled dissolution materials Transparent or colored	[78, 180, 186, 187]
Micelles	10–20 kDa	Self-assembly into polymeric micelles with distinctive core–shell structures Non-covalent encapsulation of poorly water-soluble drugs HA in the shell can be used as a targeting molecule	Dermatology Topical applications and carrier system Enhancing penetration of encapsulated compounds into skin, hair, and nails Drug delivery systems—Parenteral applications	[52, 188–191]

Future perspective

Looking ahead, the future perspective of HA and its formulations are propitious and diverse, with ongoing research indicating novel applications and opportunities in various fields. Genetic manipulation of the HAS synthase enzyme and isoenzymes in cancer therapy offers a new approach to combat cancer progression. At the same time, research into identifying cancer-associated HAS proteins presents new opportunities for cancer therapy. In regenerative medicine, HA derivatives have significant implications for immunomodulation, angiogenesis, nerve regeneration, and hybrid materials, suggesting new avenues for treating novel diseases such as COVID-19. In addition to cancer therapy and regenerative medicine, HA can potentially treat chronic inflammation, cardiovascular disease, and neurodegenerative disorders, with new and innovative applications emerging continually. For example, HA-based hydrogels are being explored for controlled drug delivery and drug release, biotechnology, and biosensors for detecting disease biomarkers. At the same time, HA is being investigated as an adjuvant in vaccines for infectious diseases.

Furthermore, 3D bioprinting using HA-based bioinks shows significant potential for tissue engineering and regenerative medicine. Advances in genetic engineering and biotechnology offer the production of tailored HA derivatives with enhanced properties and functionality, expanding the scope of its applications. Hyaluronic acid has also shown promising results in wound healing and skin regeneration, making it a popular ingredient in cosmetic products. At the same time, its potential in treating eye disorders and orthopedic applications is being actively researched. Its biocompatibility and ability to mimic natural ECM components make it an ideal candidate for bioengineering and implant coatings. At the same time, its presence and expression level can be correlated with disease severity and progression, making it a valuable tool for diagnosis and monitoring. As our understanding of HA and its properties continues to evolve, the possibilities for its applications and potential in biomedical research are vast. The future of HA and its derivatives looks bright, with continued research offering the potential for developing innovative therapies and treatments.

Author contributions

ARCS and HMUF were involved in data collection and analysis, manuscript writing. HA and PRK helped in manuscript writing, editing, coordination. SN assisted in supervision, manuscript editing. NM contributed to manuscript structure, conceptualization, administration, supervision. All authors have read and approved the manuscript.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare no competing interests.

Abbreviations

HA
Hyaluronic acid
CAGR
Compound annual growth rate
ROS
Reactive oxygen species
pHA
Palmitoyl esters of hyaluronan
GAGs
Glycosaminoglycans
OVD
Ophthalmic viscosurgical device
ECM
Extracellular matrix
HAS
HA synthases
MTX
Methotrexate
AUC
Area under the curve
RAS
Recurrent aphthous stomatitis
MU
Mucosal ulcer
PBI
Papillary bleeding index
GCF
Gingival crevicular fluid
SBI
Sulcus bleeding index
PDs
Probing depths
SRP
Scale and root planning
3D
Three dimensional

NPs
Nanoparticles
COVID-19
Coronavirus disease 2019
ARDS
Acute respiratory distress syndrome

Publisher's Note

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DETAILS

Subject:	Tissue engineering; Osteoarthritis; Polymers; Umbilical cord; Hyaluronic acid; Arthritis; Nanoparticles; Wound healing; Lymphatic system; Connective tissue; Extracellular matrix; Cartilage; Molecular weight; Skin; Cosmetics; Hydration; Enzymes; Kinases; Transdermal medication; Hydrogels; Dermal fillers; Composite materials
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A new gas chromatographic method for quantification of Metformin hydrochloride and Vildagliptin in bulk and pharmaceutical dosage form:

development and validation

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ABSTRACT (ENGLISH)

Background

Metformin, an antidiabetic drug, assists in reducing the creation of glucose inside the liver. Vildagliptin, a DPP-4 inhibitor, enhances insulin release from the pancreas and reduces the hormones that elevate blood sugar levels. The combined medications work synergistically to lower blood sugar levels. This study was designed to develop and validate a reliable method of simultaneous assessment of Metformin and Vildagliptin in bulk and pharmaceutical dosage forms. For the chromatographic separation, a Gs-Tek INNOWAX column was utilized. This column has a length of 30 m, an internal diameter of 0.25 mm, and a 1.8 µm film thickness. For the detection, a Flame Ionization Detector was utilized. The ideal conditions included an injection volume of 1 µL with a split mode of 10 to 1 ratio, a flow rate of 1 mL/minute for the nitrogen carrier gas, an injector temperature of 300 °C, a detector temperature of 250 °C, an initial oven temperature of 100 °C that was maintained for seven minutes and then programmed to climb at a rate of 10 °C per minute up to a temperature of 300 °C.

Results

A gas chromatographic method that is simple, precise, accurate, robust, and reliable has been developed and implemented for the simultaneous estimation of Metformin and Vildagliptin in the tablet dosage form. The retention time for Metformin and Vildagliptin was 10.203 and 22.021 min. respectively. Validation studies were performed on the method's Linearity, detection limit (LOD), and quantitation limit (LOQ), as well as its accuracy, precision, system suitability, and robustness, using the norms established by the International Conference on Harmonization (ICH). The mean recovery value for Metformin and Vildagliptin was 100.31% (% R.S.D. = 0.6743%) and 100.33% (% R.S.D. = 0.6900%). All the results are within the acceptable range.

Conclusion

Validation of the developed method revealed that all the results were within an acceptable range, and techniques can be employed to analyze these two medications in combined dosage forms. It is the first method used for simultaneous estimation of these two drugs.

FULL TEXT

Background

Today's worldwide disease, Type 2 Diabetes Mellitus (T2DM), is treated by using drugs like Metformin (MET) and Vildagliptin (VID) [1]. Metformin, an antidiabetic drug, assists in reducing the creation of glucose inside the liver. Vildagliptin, a DPP-4 inhibitor, enhances insulin release from the pancreas and reduces the hormones that elevate blood sugar levels. In Fig. 1, the chemical structures of drugs are shown.

Fig. 1 [Images not available. See PDF.]

Chemical structure of **a** Metformin and **b** Vildagliptin

The combined medications work synergistically to reduce blood sugar levels. Patients with type 2 diabetes (T2DM) who take Vildagliptin, an orally active, potent, and selective inhibitor of dipeptidyl peptidase-IV (DPP-4), see improvements in their glycemic control, primarily because of improved pancreatic islet function [2]. In summary, Vildagliptin has been reported to enhance insulin levels and reduce the elevated glucagon release in type 2 diabetes people when paired with Metformin, a thiazolidinedione, a sulfonylurea, or insulin. Metformin has been used to treat

diabetes for 50 years, and although it has a distinct mode of action and does not resolve ß-cell dysfunction, it is still the primary medication advised by all guidelines [3].

The multiple pathophysiological anomalies and progressive nature of T2DM need various intensification techniques over time. As per recent guidelines, patients need therapeutic combinations much earlier to reach and maintain the ever-stricter glycemic goals. Adverse effects like hypoglycemia can be avoided with careful medicine selection. Metformin and Vildagliptin have merits over conventionally used combinations because they do not raise the probability of weight gain or induce hypoglycemia [4–6]. Compared to other diabetic drugs, the safety and tolerability profiles of the combination of Vildagliptin and Metformin are exceptional, and they appear to have positive effects on beta-cell activity [7, 8].

Various analytical methods were used to analyze Metformin when present alone or combined with other medications, including U.V. [9–12], HPLC [13–16], HPTLC [17] and GC techniques [18]. The analysis of the relevant literature showed that Vildagliptin, either on its own or in combination with other pharmaceuticals, has been evaluated by U.V. [18], HPLC [19], HPTLC [20, 21], and GC–MS techniques [22]. The literature study also suggests that only a few analytical methods are established for the determination of MET and VID in combined dosage forms, such as RP-HPLC [23], HPLC–MS/MS [24], and HPTLC [25]. This pharmacological combination has not yet been investigated using GC, and no attempts have been made to do so. This study proposes and validates a new gas chromatographic approach for the simultaneous detection of MET and VID in both their bulk form and their pharmaceutical dosage form.

Methods

Instrument

The Star Chromatography Workstation version 6.41 software was used to record the data, and the HSGC—Shimadzu 2010 I GC system, which relates to a flame ionization detector (FID), was used for all experiments.

Gas chromatographic settings

The Gs-Tek INNOWAX column, which has a 30 m length, 0.25 mm I.D., and 1.8 µm df, was used for the separation. FID was used to carry out the detection. Injection volume 1 µL in split mode 10:1, nitrogen used as carrier gas at a constant flow of 1 mL/min, injector temperature 300 °C, detector temperature 250 °C, beginning oven temperature 100 °C kept for 7 min, then scheduled to climb at a rate of 10 °C/min up to 300 °C, were the optimal circumstances.

Preparation of standard stock solutions

The standard stock solution was prepared by dissolving accurately weighed 500 mg of MET and 50 mg of VID, in ethanol in a 100 mL volumetric flask. It is further diluted by taking 1 mL stock solution with 10 mL ethanol to get containing 500 µg/mL MET and 50 µg/mL VID respectively.

Assay procedure for tablet formulation

Twenty commercially available Galvus Met Novartis formulation tablets were ground adequately into powder using mortar and pestle. In a 100 mL volumetric flask, we weighed out powder equal to 500.0 mg of MET and 50.0 mg of VID, added roughly 50 mL ethanol, sonicated for 2 min to ensure appropriate dissolution, and then brought the volume up to the mark using the same solvent. Whatman No.41 filter paper was used to filter the sample solution and 5 mL of the filtrate was placed in a 50 mL volumetric flask, with the remainder of the volume filled with ethanol. The method is developed and validated with the help of this stock solution.

Validation of method

The results of the analysis, which included Linearity, precision, accuracy, detection limit (LOD), quantitation limit (LOQ), system suitability, and robustness, were statistically validated and performed following ICH guidelines Q2 (R1) [26].

System suitability

Three injections of a solution containing 500 µg/mL MET and 50 µg/mL VID were used to evaluate the system's suitability. The subsequent factors like Retention time values, peak areas, tailing factor, theoretical plates, and resolution are considered.

Linearity and range

The stock solution was diluted with ethanol to create standard working solutions with concentrations ranging from 125.0 to 750.0 µg/mL for MET and 12.5 to 75.0 µg/mL for VID. Five different concentrations of each chemical were selected using the previously established gas chromatographic conditions, and three injections were performed for each dilution. Regression equations and correlation coefficients were obtained using calibration curves to develop the Linearity of the suggested method.

Study of LOD and LOQ

The ICH guideline describes multiple methods for estimating detection limits and quantitation limits. The LOD and LOQ This study determined the proposed approach's values using Eqs. 1 and 2.

1

LOD=3.3xσ/s

2

LOQ=10xσ/swhere *σ*the standard deviation of the *Y*-intercept. s the slope of the calibration curves.

Precision

Six sample solutions (*n*=6) were produced and analyzed using gas chromatography, with concentrations of 500.0 µg/mL for MET and 50.0 µg/mL for VID, respectively, to ensure intraday accuracy. The same solution was analyzed over three days to evaluate the Interday precision.

Accuracy

Analysis of standard drug additions at three levels, i.e., multiple-level recovery experiments, were used to determine the reliability of the approach. A predetermined amount of the sample was mixed with a reference standard of varying concentrations (80%, 100%, and 120%) before being evaluated for the substance.

Robustness

The method robustness was determined by measuring the percentage relative standard deviation (R.S.D.) of retention time and peak area after the oven starting temperature and flow rate were changed by 2.0 °C and 0.1 mL/sec, respectively.

Results

Chromatography

The INNOWAX column effectively separated drugs. As shown in Fig. 2, the retention times for MET and VID were 10.203 and 22.021 min. respectively.

Fig. 2 [Images not available. See PDF.]

Gas Chromatogram showing the presence of MET (500 µg/mL) and VID (50 µg/mL) in a standard solution

System suitability

It was decided to inject five separate injections for the system suitability study. The acceptance limitations were satisfied for all the system suitability criteria, including the number of theoretical plates, the tailing factor, and the resolution. Also, the relative standard deviation (R.S.D.) of retention time values (Rt) and peak area (drug) for five separate injections does not exceed 2%, indicating that the GC system is suitable for the analysis of MET and VID combinations. Table 1 provides a concise summary of the findings.

Table 1. System suitability parameters

Criterion	MET	VID
% R.S.D. of Rt	0.0247	0.0316
% R.S.D. of area	0.9673	0.8590

NTP	25,381	18,468
Tailing factor	1.03	1.09
Resolution	11.2	

Linearity and range

The parameters of the GC were discussed before they were used, and then the calibration curves for MET and VID were developed by plotting the detector's response against the concentration of the medications. The findings show a strong connection between the detector's response and the drug concentration. The concentration ranges and the slope, y-intercept, and correlation coefficient values for both drugs for MET and VID are provided in Figs. 3 and 4.

Fig. 3 [Images not available. See PDF.]

Calibration curve for MET for 125, 250, 375, 500, 625 and 750 ppm

Fig. 4 [Images not available. See PDF.]

Calibration curve for VID for 12.5, 25.0, 37.5, 50.0, 62.5 and 75.0 ppm

LOD and LOQ value

The Detection limit was found to be 12.14 µg/mL for MET and 2.44 µg/mL for VID, while the Quantitation limit was found to be 36.80 µg/mL for MET and 7.37 µg/mL for VID.

Precision

Table 2 presents the intraday and Interday precision % R.S.D. values, which all came in at less than 2.0%. These results indicate that the approach has an appropriate level of precision.

Table 2. Study of precision

Drug	% R. S. D Intra-day	% R. S. D Inter-day
MET	0.9673	0.9663
VID	0.8590	0.8606

Study of accuracy

Multiple-level recovery experiments were conducted to conduct the three-level recovery test, consisting of conventional standard additions. The mean recovery value for MET and VID was 100.31% (% R.S.D. = 0.6743%) and 100.33% (% R.S.D. = 0.6900%), respectively. Table 3 shows the findings of the accuracy of the developed method.

Table 3. Accuracy for the determination of M.E.T. and VID

Level of % Recovery	% Recovery*		% RSD*	
MET	VID	MET	VID	80
100.77	99.98	0.6576	0.8466	100
99.94	100.37	0.7350	0.6625	120

100.20	100.62	0.6305	0.5610	Mean

*Mean of three estimations (n=3)

Robustness

The procedure's reliability was analyzed by purposefully tinkering with the oven's beginning temperature (2.0 °C) and the flow rate (0.1 mL/min.). According to the data presented in Table 4, these adjustments did not substantially impact the retention times or peak areas.

Table 4. Robustness

Parameter changes		Retention time (Min)		Peak Area	
MET	VID	MET	VID	The initial temperature of the oven	98 °C
10.225	22.045	5,486,025	605,845	100 °C	10.204
22.021	5,475,845	603,654	102 °C	10.189	21.995
5,474,554	602,965	%RSD		0.1772	0.1136
0.1147	0.2489	Flow rate (mL/min)	0.9	10.225	22.052
5,485,445	605,868	1.0	10.205	22.018	5,475,841
603,674	1.1	10.155	22.001	5,467,828	601,964
%RSD		0.3537	0.1179	0.1611	0.3241

Analysis of commercial tablet dosage form

The chromatographic approach assessed the two drugs using their combined tablet formulation (500 mg MET, 50 mg VID) as shown in Fig. 5. The analysis was carried out five times with the tablet formulation. The findings obtained using the suggested strategy are presented in Table 5. The assay results demonstrated good accuracy and precision, and the chromatograms of the tablets exhibited no interfering peaks at any point in the analysis.

Fig. 5 [Images not available. See PDF.]

Gas Chromatogram showing the presence of MET (500 µg/mL) and VID (50 µg/mL) in a sample solution

Table 5. Result of analysis of marketed formulation

Drug	Label claim (mg/tab)	The amount found (mg/tab)	Drug Found * (%)	R.S.D. (%)
MET	500	496.8	99.35	0.0964

VID	50	49.71	99.42	0.1685

*Mean of six estimations (n=6)

Discussion

In this investigation, a novel gas chromatographic method employing the INNOWAX column was developed to simultaneously detect MET and VID in bulk and pharmaceutical dosage forms. In earlier experiments, the individual GC determination of MET and VID in a variety of samples was shown to be possible. How these two analytes are evaluated simultaneously employing GC in a pharmaceutical binary combination has not yet been documented. The established chromatographic approach mentioned here has greater specificity than methods like spectrophotometry, which does not segregate the chemicals being tested for. The HPLC techniques have a disadvantage. They require a significant amount of organic solvents, making them unfeasible for routine analysis in the pharmaceutical industry because of their high cost.

The primary purpose of this research was to establish a method that could make this determination in a quick, dependable, and direct manner with a minimum amount of sample preparation. This method does not involve any complexation or pretreatment of the medications that are the focus of the research, which contributes to its ease of use and cost-effectiveness. Also, this method supports the goal of the work that was done. The developed analytical technique has successfully passed validation regarding Linearity, range, LOD, LOQ, accuracy, and precision. The robustness of the developed analytical method was also verified.

Conclusion

The current study developed a simple, linear, precise, accurate, and robust gas chromatographic technique to evaluate MET and VID in bulk and pharmaceutical formulations. The resolution between the drugs was improved by the developed method. The procedure's performance was validated statistically per the ICH requirements, and the findings were deemed satisfactory. Also, this is the first instance that the gas chromatography technique has been invented and validated for estimating MET and VID in combination dosage form, to the greatest of our knowledge.

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Author contributions

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Competing interests

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Studies involving plants

Not applicable.

Abbreviations

MET

Metformin
VID
Vildagliptin
ICH
International conference of Harmonization
LoQ
Limit of detection
LoD
Limit of quantification
HPLC
High performance liquid chromatography
HPTLC
High performance thin layer chromatography
LC/MS
Liquid chromatography/Mass spectroscopy
LC–MS/MS
Liquid chromatography–Mass spectroscopy/Mass spectroscopy
R.S.D.
Relative standard deviation
U.S.P.
United Sate pharmacopoeia

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GCMS-based phytochemical profiling and in vitro pharmacological activities of plant Alangium salviifolium (L.f) Wang

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ABSTRACT (ENGLISH)

Background

There is an urge for traditional herbal remedies as an alternative to modern medicine in treating several diseases. A significant number of modern pharmaceutical drugs are based on or derived from medicinal plants or their extracts. These drugs derived from the plant origin have various antimicrobial, antioxidant, anticancer, anti-inflammatory

activities. *Alangium salviifolium* belongs to Cornaceae family and is well known for its medicinal properties. The present study was carried out to evaluate the antibacterial, antioxidant effect and possible bioactive components present in the chloroform, acetone, ethanol, methanol and aqueous extract of *Alangium salviifolium* leaves.

Methodology

Dried leaves of *Alangium salviifolium* were subjected to serial solvent extraction using increasing polarity of solvents, i.e., chloroform, acetone, methanol, ethanol, and distilled water. Crude extracts were further tested for qualitative analysis of phytochemicals using standard procedure, while GCMS analysis was performed to identify the probable phytocompounds. Antibacterial activity was performed against bacterial pathogens using agar well method, whereas antioxidant activity was performed using in vitro PM, DPPH and FRAP assays.

Results

Phytochemical analysis of the extracts revealed the presence of key phytochemical classes. Using gas chromatography-mass spectrometry, several high and low molecular weight chemical compound kinds were discovered. These chemical substances are regarded as having significant biological and pharmacological effects. All crude extracts had considerable and comparable in vitro antioxidant and antibacterial properties.

Conclusions

According to the findings of this study, *Alangium salviifolium* leaves are a rich source of phytoconstituents that are crucial in stopping the advancement of numerous disorders.

FULL TEXT

Background

Numerous metabolites that are more useful in the field of medicine are naturally produced by plants. More than 80% of the world's population, according to the World Health Organization (WHO), relies on traditional medicine for their primary healthcare requirements [1]. 21,000 plants are known to be used as medicines worldwide, according to the WHO [2]. As plants are the primary source of medicines in Siddha, Unani, and Ayurveda systems of medicine, India has a rich cultural history of ancient medicines [3]. Many of the chemicals produced by these medicinal plants have been shown to have therapeutic benefits [4]. More research works on ethnopharmacognosy were increased due to the result of the emergence of negative effects and microbial resistance to the chemically synthesized medications [5].

Secondary metabolites including tannins, steroids, phenolic compounds, and alkaloids, sometimes known as phytochemicals or phytocompounds, are what give plants their therapeutic potential. Some plant secondary metabolites with medicinal promise include morphine, vincristine, vinblastine, taxol and quinine [6]. Due to fewer side effects as compared to synthetic pharmaceutical chemicals, these phytochemicals have recently gained importance throughout the world and are used in the pharmaceutical industry for the drug development and treatment of major diseases like asthma, arthritis, cancer, and diabetes [7, 8]. These medicines have different antimicrobial, antioxidant, anticancer, and anti-inflammatory effects because they are derived from plants [9]. Antioxidants are substances that slow down or stop oxidative reactions that catalyze free radicals. The presence of phenolic substances such as flavonoids, phenolic acids, tannins, and phenolic diterpenes [10, 11] is primarily responsible for the antioxidant activity of plant products. Plants are protected from oxidative assault by antioxidants like BHT (Butylated Hydroxy-Toluene) and BHA (Butylated Hydroxy-Anisol), which bind oxidative damage to metallic ions, break down peroxides, or destroy free radicals [12]. According to Lobo et al. [13] and the rise in pathogen antimicrobial resistance, infectious diseases account for almost 50% of all deaths [14]. As a need of the hour researchers have created new, efficient antibacterial medications from natural phytochemicals [15, 16] demonstrated the potential of numerous herbs as sources of pharmaceuticals with lower toxicity. By screening a wide range of plant groups, the search for new antibacterial compounds continues. Higher plants are a possible source of novel antibiotic prototypes, according to research on the antibacterial activity of plant extracts and plant products [17, 18]. There is a need for more research into traditional health systems because certain traditional medicines have already developed substances that are effective against bacteria strains that are resistant to antibiotics [19, 20].

Alangium salviifolium, also known as sage leaved alangium or ankola, was chosen for the current study. It is a

member of the Cornaceae family, which is indigenous to China, India, Bangladesh, and the Philippines [21]. This family of plants often grows in arid or hotter climates. It is used to treat a variety of illnesses. According to numerous studies [22–25], it is widely used as a treatment for skin conditions, leprosy, asthma, epilepsy, hepatitis, scabies, and as an antidote for snake and dog bites. According to numerous studies [26–28], roots can be used to cure diarrhea, paralysis, piles, and vomiting. Although *Alangium salviifolium* leaves are a storehouse of many nutrients and bioactive chemicals, the systematic analysis of these leaves is still not sufficient in terms of the specific biological activity of their chemical constituents. The potential benefits of *A. salviifolium* for treating different medical issues should be further investigated. Thus, the objective of the current study was to identify the phytochemical components, antioxidant, and antibacterial properties of various solvent extracts of *A. salviifolium* leaves. Additionally, utilizing GC–MS and FTIR analysis, the bioactive elements of the extracts and the functional groups of the compounds were also found.

Methods

Collection of plant material

The plant *Alangium salviifolium* (L.f) Wang's leaves were collected in the month of February 2022 in Ankola, Uttar Kannada District, Karnataka, India. Dr. K. Kotresha, Professor, Department of Botany, Karnatak Science College, Dharwad, Fresh plant leaves were gathered, cleaned under running water, dried in the shade, and then blended to a coarse powder. For future use, the powder was kept in sealed containers.

Solvent extraction

Alangium salviifolium dry leaves were coarsely pulverized, and then, serial solvent extractions were performed using a Soxhlet apparatus. In increasing order of polarity, the following solvents were used for the extraction: chloroform, acetone, methanol, ethanol, and distilled water. Using a Rota-evaporator, the extracts were further concentrated. The airtight containers used to hold the concentrated extracts were chilled until use.

Phytochemical analysis

The presence of various phytochemical components, including alkaloids, tannins, phenols, sterols, terpenoids, glycosides, saponins, flavonoids, and carbohydrates, were screened for in the crude extracts of *Alangium salviifolium* plant leaves using a standard procedure [29].

Total phenol content (TPC) estimation

Utilizing 1.5 ml of the Folin–Ciocalteu (FC) reagent and 7.5% sodium carbonate (Na_2CO_3) solution, the plant extract of known concentration, i.e., 1 mg/1 ml of respective solvent, was subjected to oxidation. The absorbance reading at 750 nm was obtained during an hour of incubation at room temperature. The experiment was performed in triplicate, and results were expressed as mean ± standard deviation. The quantity was determined using the calibration curve for gallic acid. Gallic acid equivalent (GAE) mg/100 ml of sample was used to express the results [30].

Total flavonoid content (TFC) estimation

The 10% aluminum chloride ($AlCl_3$) and 1 M sodium acetate were combined with the known quantity of plant extract, i.e., 1 mg/1 ml of respective solvent. Following a 45-min incubation in the dark, the absorbance was measured at 415 nm. Using the Quercetin calibration curve, the quantity was determined. Quercetin equivalent (QE) mg/100 ml of sample was used to express the results [31]. The experiment was performed in triplicate, and results were expressed as mean ± standard deviation.

FTIR analysis

Utilizing a Perkin Elmer Spectrophotometer system, an FTIR study of *Alangium salviifolium* was carried out in order to identify the distinctive peaks between 400 and 4000 cm^{-1} and their functional groups. The FTIR's peak values were noted.

GC–MS profiling

The different solvent extracts of *A. salviifolium* were subjected to a GC–MS analysis with instrument model GCMS-QP 2010 Plus, Shimadzu). At an ionization voltage of 70 eV, injector temperature of 250 °C and injector mode was split with linear velocity 36.5 cm/s and pressure was 57.5 kPa. The instrument was run in electron impact mode. Approximately 1 µL of the sample was injected into mobile phase consisting of helium (99.9% purity) at a flow rate of

1 mL/min. The oven's temperature was first set to 60 °C for 2 min of isothermal operation before being raised to 100 °C at a rate of 10 °C per minute and then to 280 °C at a rate of 5 °C per minute for 9 min. The GC ran for 34 min in total. Comparing each component's average peak area to the total areas allowed us to determine the proportional percentage amount of each component. With the help of The National Institute of Standard and Technology-5 (NIST-5), a comparison was made between the spectra of the unknown component and the spectrum of the known components including the compound's name, chemical formula, molecular weight, and structure, which were identified.

In vitro antioxidant activity of *A. salviifolium's* leaves

Phosphomolybdenum assay

Using a standardized process, the phosphomolybdenum method was used to assess the antioxidant activity of the plant extracts [32] and ascorbic acid was used as reference standard. *Alangium salviifolium* extracts of concentration 1 mg/ml were added to each test tube separately along with 3 ml of distilled water and 1 ml of the phosphomolybdate (PM) reagent in varying concentrations ranging from 100 to 500µL. For 90 min, the tubes were incubated in a water bath at 95 °C. Following incubation, these tubes were cooled to room temperature, and the reaction mixture's absorbance was assessed at 695 nm.

2, 2 Diphenyl-2-picryl hydrazyl (DPPH) radical scavenging assay

A. salviifolium plant extracts were subjected to the Rice-Evans et al. [33] method for the DPPH radical scavenging assay. The known concentration of test samples (100 µg) was combined in various concentrations with 100 µl of a DPPH solution made in methanol (40 mM). The combination was incubated for 30 min at room temperature and in the dark to produce the desired color, which was measured at 517 nm along with ascorbic acid as a standard. Each sample’s DPPH scavenging activity was estimated using the formula below:Scavengingactivity%ofDPPH=100 ×Ac-At/Acwhere At is the absorbance of the test sample and Ac is the absorbance of the control reaction (100 µL of methanol plus 100 µL of DPPH solution).

Ferric ion reducing power (FRAP) assay

With a few minor modifications, the Oyaizu [34] method was used to assess the reducing power of ferric ions. The extracts of *A. salviifolium* (1 mg/ml) were pipetted into mixtures containing 2.5 ml of 0.2 M phosphate buffer, 2.5 ml (1% w/v) potassium ferricyanide, and various concentrations ranging from 100 to 500 µL. The mixture was then incubated at 50 °C for 20 min. After cooling the mixture, 2.5 ml of 10% w/v trichloroacetic acid and 0.5 ml of 0.1% w/v ferric chloride were added, and the combination was left at room temperature for 10 min to form a complex that was green in color. The absorbance was calculated using a spectrophotometer at 700 nm. The reference standard used was ascorbic acid.

Antibacterial activity

The antibacterial activity was screened using the agar well diffusion method [35] using *Pseudomonas aeruginosa* and *Staphylococcus aureus* cultures as the test organisms. 100µL of the uniformly diluted saline suspension were swabbed over the sterile agar plates. Along with the control drug Ciprofloxacin (30 µg), various concentrations of the extract (1 mg/ml) (30, 60, 90, and 120 µL/well) were added to the medium. The plates were incubated for 24 h at 37 °C. The diameter of the inhibition zones that formed around the wells after the incubation time was measured in millimeters.

Statistical analysis

The experiments were performed in the triplicate, and the results were expressed as mean ± standard deviation using IBM SPSS Statistics 20.0.

Results

Plant chemical analysis

The qualitative phytochemical analysis of five different solvent extracts of *Alangium salviifolium* revealed the presence of flavonoids, reducing sugar, and carbohydrates in chloroform, acetone, methanol, ethanol, and distilled water; in addition, glycosides, saponins, and tannins were present in aqueous extract. All five extracts contained alkaloids and phenols. The other four extracts, with the exception of methanol, all included lignin The results of

phytochemical analysis was shown in Table 1.

Table 1. Preliminary phytochemical analysis of the leaves extract of *Alangium salviifolium*

Tests	Inference				
Chloroform	Acetone	Ethanol	Methanol	Aqueous	1. Alkaloids
+	+	+	+	+	2. Flavonoids
+	+	+	+	+	3. Glycosides
–	–	+	–	+	4. Phenols
+	+	+	+	+	5. Lignin
+	+	+	–	+	6. Sterols
–	–	–	–	–	7. Saponins
–	–	–	–	+	8. Tannins
–	–	+	+	+	9.Terpenoids
–	–	–	–	–	10.Reducing sugar
+	–	+	–	–	11. Carbohydrate
+	–	+	+	+	12. Proteins and amino acids

'+' indicates Present; '–'indicates Absent

Total crude extraction

The phytochemical examination of *A. salviifolium* leaves revealed a significant amount of the plant secondary metabolites. Using the solvents chloroform, acetone, ethanol, methanol, and distilled water, the total yield of crude extracts from *A. salviifolium* leaves was determined to be 1.8%, 2.1%, 3.8%, 2.4%, and 2.8% (w/w), respectively.

Total phenol and flavonoid content

In terms of phenolic content, ethanol extract had the highest level (82.86 ± 0.04) mg/g GAE, followed by methanol extract (59.13 ± 0.02) mg/g GAE, acetone extract (47.61 ± 0.01) mg/g GAE, chloroform extract (12.51 ± 0.02) mg/g GAE, and aqueous extract (7.92 ± 0.02) mg/g GAE. In case of flavonoid content of all the extracts, chloroform extract had the highest amount of flavonoid content (71.86 ± 0.03) mg/g QE, followed by methanol extract (33.33 ± 0.05) mg/g QE, acetone extract (30.07 ± 0.04) mg/g QE, ethanol extract (16.3 ± 0.02) mg/g QE, and aqueous extract (5.04 ± 0.04) mg/g QE. Total phenolic and total flavonoid concentration in each extract showed a wide range of variance (Table 2).

Table 2. Total phenol and total flavonoid content from *A. salviifolium* (leaf extract)

Extract	Total phenolic content (mg)	Total flavonoid content (mg)
Chloroform	12.51 ± 0.029	71.86 ± 0.037
Acetone	47.61 ± 0.018	30.07 ± 0.046
Ethanol	82.86 ± 0.049	16.3 ± 0.012
Methanol	59.13 ± 0.021	33.33 ± 0.051
Aqueous	7.92 ± 0.020	5.04 ± 0.004

The results are expressed as Mean ± standard deviation

Fourier transform infrared spectroscopy analysis

The FTIR spectra of the all extracts revealed the presence of halogen and nitrogen compounds in common. The chloroform, acetone, and ethanol extracts revealed the presence of alkane and alcohol functional groups; the chloroform, acetone, methanol, and ethanol extracts revealed carbon dioxide and alkene groups; the chloroform extracts revealed the presence of aldehyde and ketone groups; the chloroform, methanol, and acetone extracts revealed amine groups; the acetone and ethanol extracts revealed esters groups; acetone, ethanol, methanol, aqueous extract showed sulfoxide group; carboxylic acid group was found in methanol and aqueous extracts; vinyl ether was found in methanol and aqueous extract revealed sulfonyl chloride groups. The results of FTIR are shown in Tables 3, 4, 5, 6, and 7 (Additional file 1: Figs. S1–S6).

Table 3. FTIR Interpretation of compounds of leaf chloroform extract of *A. salviifolium*

Sl. no.	Frequency	Functional group	Bond strength	Nature of bond
1	2916.92	Alkane	C–H Stretching	Covalent bond
2	2849.04	Alkane	C–H Stretching	Covalent bond
3	2360.24	Carbon-di-oxide	O=C=O stretching	Covalent bond

4	1735.51	Aldehyde	C=O stretching	Neutral
5	1667.49	Conjugated ketone	C=O stretching	Neutral
6	1545.36	Nitro compound	N–O stretching	Covalent bond
7	1510.94	Nitro compound	N–O stretching	Covalent bond
8	1451.37	Alkane (methyl group)	C–H bending	Covalent bond
9	1375.46	Alcohol	O–H bending	Covalent bond
10	1157.46	Amine	C–N stretching	Basic
11	1121.29	Amine	C–N stretching	Basic
12	1036.56	Amine	C–N stretching	Basic
13	916.96	Alkene	C=C bending	Nonpolar
14	838.03	Halo compound	C–Cl stretching	Polar
15	796.52	Alkene	C=C bending	Nonpolar
16	731.49	Alkene	C=C bending	Nonpolar
17	669.30	Alkene	C=C bending	Nonpolar
18	595.74	Halo compound	C–Br stretching	Polar covalent bond
19	565.05	Halo compound	C–I stretching	Nonpolar
20	522.19	Halo compound	C–I stretching	Nonpolar

Table 4. FTIR Interpretation of compounds of leaf acetone extract of *A. salviifolium*

Sl. no.	Frequency	Functional group	Bond strength	Nature of bond
1	3348.98	Aliphatic 1°–amine	N–H stretching	Nonpolar
2	2921.48	Alkane	C–H stretching	Nonpolar
3	2851.67	Alkane	C–H stretching	Nonpolar
4	2358.39	Carbon dioxide	O=C=O stretching	Covalent bond

5	2338.99	Carbon dioxide	O=C=O stretching	Covalent bond
6	1735.95	Esters (δ-lactone)	C=O stretching	Polar covalent bond
7	1554.43	Nitro compound	N–O stretching	Covalent bond
8	1510.98	Nitro compound	N–O stretching	Covalent bond
9	1457.26	Alkane	C–H bending	Covalent bond
10	1376.13	Alcohol	O–H bending	Covalent bond
11	1163.83	Ester	C–O stretching	Polar covalent bond
12	1033.49	Sulfoxide	S=O stretching	Covalent bond
13	885.42	Alkene	C=C bending	Nonpolar
14	834.26	Alkene	C=C bending	Nonpolar
15	720.53	Alkene	C=C bending	Nonpolar
16	667.77	Alkene	C=C bending	Nonpolar
17	607.39	Halo compound	C–I stretching	Nonpolar
18	579.89	Halo compound	C–Cl stretching	Polar
19	521.74	Halo compound	C–I stretching	Nonpolar
20	504.55	Halo compound	C–I stretching	Nonpolar

Table 5. FTIR interpretation of compounds of leaf methanol extract of *A. salviifolium*

Sl. no.	Frequency	Functional group	Bond strength	Nature of bond
1	3262.95	Alkyne	C–H stretching	Nonpolar
2	2924.46	Amine salt	N–H stretching	Nonpolar
3	2360.71	Carbon dioxide	O=C=O stretching	Covalent bond
4	1603.96	Conjugated alkene	C=C stretching	Nonpolar
5	1511.84	Nitro compound	N–O stretching	Covalent bond

6	1432.41	Carboxylic acid	O–H bending	Covalent bond
7	1215.22	Vinyl ether	C–O stretching	Polar covalent bond
8	1034.24	Sulfoxide	S=O stretching	Covalent bond
9	871.67	1,2,4-Trisubstituted	C–H bending	Nonpolar covalent bond
10	842.51	Halo compound	C–Cl stretching	Polar
11	803.25	Alkene	C=C bending	Nonpolar
12	645.09	Alkene	C=C bending	Nonpolar
13	588.99	Halo compound	C–Br stretching	Polar covalent bond
14	562.54	Halo compound	C–Br stretching	Polar covalent bond
15	546.82	Halo compound	C–Br stretching	Polar covalent bond
16	520.47	Halo compound	C–Br stretching	Polar covalent bond
17	491.11	Halo compound	C–I stretching	Nonpolar

Table 6. FTIR Interpretation of compounds of leaf ethanol extract of *A. salviifolium*

Sl. no.	Frequency	Functional group	Bond strength	Nature of bond
1	3261.48	Alcohol	O–H stretching	Covalent bond
2	2921.73	Alkane	C–H stretching	Nonpolar covalent bond
3	2851.76	Alkane	C–H stretching	Nonpolar covalent bond
4	2363.57	Carbon di oxide	O=C=O stretching	Covalent bond
5	1650.10	*δ*-Lactam	C=O stretching	Polar
6	1604.99	Conjugated alkene	C=C stretching	Nonpolar
7	1512.11	Nitro compound	N–O stretching	Covalent bond
8	1455.11	Alkane	C–H bending	Nonpolar covalent bond
9	1194.75	Ester	C–O stretching	Polar covalent bond

10	1034.20	Sulfoxide	S=O stretching	Covalent bond
11	889.42	Alkene	C=C bending	Nonpolar
12	667.43	Alkene	C=C bending	Nonpolar
13	596.40	Halo compound	C–I stretching	Nonpolar
14	565.05	Halo compound	C–I stretching	Nonpolar
15	546.93	Halo compound	C–Br stretching	Polar covalent bond
16	516.77	Halo compound	C–Br stretching	Polar covalent bond
17	503.04	Halo compound	C–I stretching	Nonpolar

Table 7. FTIR Interpretation of compounds of leaf aqueous extract of *A. salviifolium*

Sl. no.	Frequency	Functional group	Bond strength	Nature of bond
1	3281.10	Carboxylic acid	O–H stretching	Covalent bond
2	1556.67	Nitro compound	N–O stretching	Covalent bond
3	1405.05	Sulfonyl chloride	S=O stretching	Covalent bond
4	1039.62	Sulfoxide	S=O stretching	Covalent bond
5	550.72	Halo compound	C–Cl stretching	Polar
6	511.71	Halo compound	C–I stretching	Nonpolar

GC–MS analysis

The compounds from the GC–MS analysis of the chloroform, acetone, methanol, ethanol, and aqueous extracts of *A. salviifolium* leaves are shown in Tables 8, 9, 10, 11, and 12. In case of the chloroform extract, the compound Androst-5-ene-3, 17-diol, 17-methyl-, dipropan was found to be major compound, in case of acetone extract the compound Phytol was found to be major compound, for methanol extract the compound ethyl (dimethyl) isopropoxysilane seems to be major compound, in case of ethanol extract diethyl phthalate was found to be major compound, and for aqueous extract benzofuran, 2, 3-dihydro compound was found to be major compound. Overall, the GC–MS results of chloroform, acetone, ethanol, methanol, and aqueous extract showed the presence of 24, 53, 23, 31, and 6 compounds, respectively, and the results are shown in Tables 8, 9, 10, 11, and 12. Additional file 1: Figs. S7–S11 show the GCMS chromatogram for all the extracts.

Table 8. Compound identified in the chloroform extract of *A. salviifolium* using GCMS

Sl. no.	Compound name	Molecular formula	Molecular weight	Retention time	Peak area	Compound nature	Uses
1	3-Dodecene, (E)-	$C_{12}H_{24}$	168.32	7.615	0.85		
2	3-Hexadecene, (Z)-	$C_{16}H_{32}$	224.42	10.323	2.46	Unsaturated hydrocarbons	
3	Phenol, 3,5-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	206.32	11.945	8.77		
4	3-Hexadecene, (Z)-	$C_{16}H_{32}$	224.42	12.797	4.31	Unsaturated hydrocarbons	
5	2-Propenoic acid, octyl ester	$C_{11}H_{20}O_2$	184.27	13.992	0.72		
6	9-Eicosene, (E)-	$C_{20}H_{40}$	280.5	15.031	7.40	Alkene	Hypotensive effect, antimicrobial property
7	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296.5	15.534	7.13	Terpene alcohol	Catechol-O-Methyl-Transfearse inhibitor, antimicrobial, anti-inflammatory
8	9-Eicosene, (E)-	$C_{20}H_{40}$	280.5	17.058	8.09	Alkene	Hypotensive effect, antimicrobial property
9	Phytol	$C_{20}H_{40}O$	296.5	18.259	6.29	Diterpene	Antimicrobial, Anti-inflammatory, antiallergic, Anticancer, Diuretic, antidiabetic, Cytotoxicity, antiproliferative, cancer preventive
10	9-Tricosene, (Z)-	$C_{23}H_{46}$	322.6	18.907	9.13		
11	Pentane-2,4-dione, 3-(5-phenyl-2-furfurylidene	$C_{16}H_{14}O_3$	254.28	19.914	2.31		
12	1H-Cyclopenta[b]indol-3(2H)-one, 7-cyclohex	$C_{11}H_9NO$	171.19	20.014	3.83		

13	9-Tricosene, (Z)-	$C_{23}H_{46}$	322.6	20.606	6.69		
14	Dichloroacetic acid, heptadecyl ester	$C_{19}H_{36}Cl_2O_2$	367.4	23.631	2.37		
15	9-Tricosene, (Z)-	$C_{23}H_{46}$	322.6	25.093	1.56		
16	2-Methyl-Z-4-tetradecene	$C_{15}H_{30}$	210.40	25.973	1.75		Catechol-O-methyltransferase inhibitor
17	Vitamin E acetate	$C_{31}H_{52}O_3$	472.7	26.667	0.95	Ester of acetic acid and α-tocopherol	Antioxidants, stabilize membranes, affect eicosanoid signaling and cellular proliferation, and modulate immune responses
18	10,12-Octadecadienoic acid, 9-oxo- $$ (10E,12			27.421	0.87		
19	L-Ascorbic acid, 6-octadecanoate $$ L-Ascorbi	$C_{24}H_{42}O_7$	442.6	28.106	1.54		
20	Stigmasterol	$C_{29}H_{48}O$	412.7	28.403	14.10	Steroid	Anti-infammatory, antioxidant, antimicrobial, sedative activity, Anticancer, Diuretic, hypoglycemic and thyroid inhibiter, antiarthritic, antiasthama
21	Pregnan-20-one, 3-(acetyloxy)-17-hydroxy-, (3	$C_{23}H_{36}O_4$	376.5	29.247	1.62		
22	3-Cyclohexen-2-on-1-carboxylic acid, 1-methy	$C_8H_{12}O_2$	140.18	29.741	1.04	Esters	
23	Acetamide, 2-(3-methyl-2-oxo-2,3-dihydrobenz			31.819	0.87		
24	Androst-5-ene-3,17-diol, 17-methyl-, dipropan	$C_{19}H_{30}O_2$	290.4	33.859	5.34		

Table 9. Compound identified in the acetone extract of *A. salviifolium* using GCMS

Sl. no.	Compound name	Molecular formula	Molecular weight	Retention time	Peak area	Compound nature	Uses
1	2-Pentanone, 4-hydroxy-4-methyl-	$C_6H_{12}O_2$ or $(CH_3)_2C(OH)CH_2COCH_3$	116.16	3.145	6.81	Alcohol	Antifreeze and brake fluids
2	(3H) Indazole, 3,3-dimethyl-	$C_9H_{10}N_2$	146.19	4.835	1.22	Aromatic	
3	Oxetane, 2,2,4-trimethyl-	$C_6H_{12}O$	100.16	7.835	0.68		
4	3-Dimethylsilyloxytridecane	$C_{15}H_{33}OSi$	257.51	8.619	0.36		
5	9-Octadecene, (E)-	$C_{18}H_{36}$	252.5	12.797	4.28	Unsaturated fatty acid	
6	Benzene, (1-butylheptyl)-	$C_{16}H_{26}$	218.38	13.372	0.88	Aromatic	
7	Benzene, (1-propyloctyl)-	$C_{17}H_{28}$	232.4	13.501	0.47	Aromatic	
8	Cyclohexane, decyl-	$C_{16}H_{32}$	224.42	13.607	0.32		
9	Benzene, (1-ethylnonyl)-	$C_{17}H_{28}$	232.4	13.749	0.51		
10	Benzene, (1-methyldecyl)-	$C_{17}H_{28}$	232.4	14.167	0.70		
11	Benzene, (1-pentylheptyl)-	$C_{18}H_{30}$	246.4	14.430	0.36		
12	Benzene, (1-butyloctyl)-	$C_{18}H_{30}$	246.4	14.481	0.41		

13	Benzene, (1-propylnonyl)-	$C_{18}H_{30}$	246.4	14.620	0.54		
14	Benzeneacetic acid, 4-pentadecyl ester	$C_{23}H_{38}O_2$	346.54	14.874	1.50		
15	9-Eicosene, (E)-	$C_{20}H_{40}$	280.5	15.032	9.31	Alkene	Hypotensive effect, antimicrobial property
16	Benzene, (1-methylundecyl)-	$C_{18}H_{30}$	246.4	15.284	0.81		
17	Benzene, (1-pentyloctyl)-	$C_{19}H_{32}$	260.5	15.478	0.33		
18	9-Eicosyne	$C_{20}H_{38}$	278.5	15.540	1.46	Unsaturated hydrocarbon	
19	1,19-Eicosadiene	$C_{20}H_{38}$	278.5	15.793	0.36	Unsaturated hydrocarbon	
20	9-Heptadecanone	$C_{17}H_{34}O$	254.5	15.933	3.18		
21	Benzene, (1-methyldodecyl)-	$C_{19}H_{32}$	260.5	16.345	0.37		
22	1,2-Benzenedicarboxylic acid, butyl octyl ester	$C_{20}H_{30}O_4$	334.4	16.448	0.34	Dicarboxylic acid ester	Antimicrobial, antifouling
23	Heptadecanoic acid, heptadecyl ester	$C_{34}H_{68}O_2$	508.9	16.818	1.02	Fatty acid	Acidifier, acidulant, arachidonic acid inhibitor, inhibit production of uric acid
24	Phthalic acid, bis(7-methyloctyl) ester	$C_{26}H_{42}O_4$	418.6	16.929	1.02	Plasticizer compound	Antimicrobial antifouling
25	1-Nonadecene	$C_{19}H_{38}$	266.5	17.060	8.22	Unsaturated fatty acid	Antituberculosis, anticancer, antioxidant, antimicrobial, antifungal

26	n-Pentadecylcyclohexane	$C_{22}H_{42}$	294.6	17.884	1.12		Antitumor, neurodepressant, neurogenic
27	Phytol	$C_{20}H_{40}O$	296.5	18.261	21.70	Diterpene	Antimicrobial, anti-inflammatory, antiallergic, anticancer, Diuretic, antidiabetic, cytotoxicity, antiproliferative, cancer preventive
28	Tetracontane, 3,5,24-trimethyl-	$C_{43}H_{88}$	605.2	18.466	0.27		
29	Octadecane, 1-(ethenyloxy)-	$C_{20}H_{40}O$	296.5	18.675	0.41	Alkane	
30	1-Nonadecene	$C_{19}H_{38}$	266.5	18.910	5.50	Unsaturated fatty acid	Antituberculosis, anticancer, antioxidant, antimicrobial, antifungal
31	1,19-Eicosadiene	$C_{20}H_{38}$	278.5	19.703	1.19	Unsaturated hydrocarbon	
32	Tetrapentacontane, 1,54-dibromo-	$C_{54}H_{108}Br_2$	917.2	19.894	1.23	Hydrocarbon	
33	1-Pentacontanol	$C_{50}H_{102}O$	719.3	20.106	0.49		
34	1-Decanol, 2-hexyl-	$C_{16}H_{34}O$	242.44	20.396	1.03		Antimicrobial
35	1-Tricosene	$C_{23}H_{46}$	322.6	20.611	3.04	Alkene	Anticancer, anti-inflammatory
36	Tetrapentacontane, 1,54-dibromo-	$C_{54}H_{108}Br_2$	917.2	21.494	2.15	Hydrocarbon	
37	1-Octanol, 2-butyl-	$C_{12}H_{26}O$	186.33	21.695	0.29	Alcohol	Antimicrobial
38	Di-n-octyl phthalate	$C_{24}H_{38}O_4$	390.6	21.967	1.91	Plasticizer compound	Antimicrobial antifouling

39	Cyclooctacosane	$C_{28}H_{56}$	392.7	22.183	1.28	Saturated fatty acid	
40	Cyclononasiloxane, octadecamethyl-	$C_{18}H_{54}O_9Si_9$	667.4	22.840	0.32		Antimicrobial
41	17-Pentatriacontene	$C_{35}H_{70}$	490.9	22.979	2.41		Anti-inflammatory, anticancer, antibacterial, antiarthritic
42	Tetrapentacontane, 1,54-dibromo-	$C_{54}H_{108}Br_2$	917.2	23.178	0.38	Hydrocarbon	
43	Octadecane, 1,1'-[1,3-propanediylbis(oxy)]bis-	$C_{39}H_{80}O_2$	581.1	23.462	0.30		
44	Tetrapentacontane, 1,54-dibromo-	$C_{54}H_{108}Br_2$	917.2	23.648	0.40	Hydrocarbon	
45	2,6,10,14,18-Pentamethyl-2,6,10,14,18-eicosap	$C_{25}H_{52}$	352.7	23.975	0.60		
46	Trimethyl[4-(2-methyl-4-oxo-2-pentyl) phenoxy	$C_{15}H_{24}O_2Si$	264.43	24.234	0.27		
47	Tetrapentacontane, 1,54-dibromo-	$C_{54}H_{108}Br_2$	917.2	24.370	0.66	Hydrocarbon	
48	Dodecane, 1,12-dibromo-	$C_{12}H_{24}Br_2$	328.13	24.911	0.47		
49	1-Heptene, 1,3-diphenyl-1-(trimethylsilyloxy)-	$C_{22}H_{30}OSi$	338.6	25.131	0.39		
50	Pentatriacontane	$C_{35}H_{72}$	492.9	25.981	1.23		
51	Silicic acid, diethyl bis(trimethylsilyl) ester	$C_{10}H_{28}O_4Si_3$	296.58	26.695	0.51	Aliphatic esters	

52	Sulfurous acid, butyl undecyl ester	$C_{15}H_{32}O_3S$	292.5	28.115	0.99		
53	Stigmasterol	$C_{29}H_{48}O$	412.7	28.430	4.01	Steroid	Anti-infammatory, antioxidant, antimicrobial, sedative activity, anticancer, Diuretic, hypoglycemic and thyroid inhibiter, antiarthritic, antiasthama

Table 10. Compound identified in the methanol extract of *A. salviifolium* using GCMS

Sl. no.	Compound name	Molecular formula	Molecular weight	Retention time	Peak area	Compound nature	Uses
1	4-Propyl [1,3] oxathiane 3-oxide	$C_7H_{14}OS_2$	162.25	5.951	1.52		
2	Undecane	$C_{11}H_{24}$	156.31	6.297	1.24	Alkane	Lubricants and lubricant additives
3	Ethyl(dimethyl)isopropoxysilane	$C_7H_{18}OSi$	146.30	8.419	20.92		
4	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150.17	9.526	6.52	Phenolic	Antimicrobial, antioxidant, anti-inflammatory, analgesic, anti-germination
5	1-(3,6,6-Trimethyl-1,6,7,7a-tetrahydrocyclopen	$C_{13}H_{18}O_2$	206.28	10.575	1.26		
6	Alpha-L-rhamnopyranose	$C_6H_{12}O_5$	164.16	10.922	1.32		Antidote, antitumor, larvicide, antioxidant, anticancer
7	1,6-Anhydro-beta-D-glucopyranose (levoglucosan)	$C_6H_{10}O_5$	162.14	11.880	3.53	Carbohydrate	
8	7-Oxabicyclo[4.1.0]heptane, 1-methyl-4-(2-methyloxiranyl)-	$C_{10}H_{16}O_2$	168.23	13.115	1.87		
9	1-Hexadecanol	$C_{16}H_{34}O$	242.44	13.811	2.05	Alcohol	Antioxidant

10	5-Eicosene, (E)-	$C_{20}H_{40}$	280.5	15.021	1.56	Fatty acid	Antimicrobial and antifungal
11	7-Oxabicyclo[4.1.0]heptane, 1-methyl-4-(2-methyloxiranyl)-	$C_{10}H_{16}O_2$	168.23	15.162	2.90		
12	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296.5	15.524	2.44	Terpene alcohol	Catechol-O-Methyl-Transfearse inhibitor, antimicrobial, anti-inflammatory
13	2-Pentadecanone, 6,10,14-trimethyl-	$C_{18}H_{36}O$	268.5	15.614	1.31		Hypocholesterolemic, antioxidant, and lubrication
14	Pentadecanoic acid, 14-methyl-, methyl ester	$C_{17}H_{34}O_2$	270.5	16.417	2.35	Fatty acid	biomarker for rheumatoid arthritis, antioxidant, antifungal, antimicrobial
15	alpha-D-Galactopyranose, 6-O-(trimethylsilyl)	$C_{17}H_{34}B_2O_6Si$	384.2	16.555	4.89		Antidote, anticancer
16	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	16.791	5.15	Carboxylic acid	Antioxidant, hypocholesterolemic nematicide, pesticide, lubricant, antibiotic, antiandrogenic, flavor, hemolytic, 5-alphareductase inhibitor, cosmetics
17	3-Imino-6-phenylimino-cyclohexa-1,4-diene	$C_{12}H_{10}N_2$	182.22	17.537	4.45		
18	Oxiraneundecanoic acid, 3-pentyl-, methyl ester	$C_{19}H_{36}O_3$	312.5	18.117	1.92	Ester	
19	Phytol	$C_{20}H_{40}O$	296.5	18.248	4.32	Diterpene	Antimicrobial, anti-inflammatory, anticancer, diuretic, cytotoxicity, antiproliferative, cancer preventive, diuretic
20	Z-7-Tetradecenal	$C_{14}H_{26}O$	210.36	18.507	1.30		Anti-inflammatory
21	Octadecanoic acid	$C_{18}H_{36}O_2$	284.5	18.677	1.45	Saturated fatty acid	Antifungal, antitumor activity, antibacterial, lubricants, inhibit production of uric acid
22	10-Formamido-10,11-dihydro-2,3-dimethoxydi			19.919	1.37		

23	1H-Cyclopenta[b]indol-3(2H)-one, 7-cyclohex			20.012	8.26		
24	Cyclohexane, decyl-	$C_{16}H_{32}$	224.42	20.978	1.13		
25	Hexanoic acid, 2-ethyl-, hexadecyl ester	$C_{24}H_{48}O_2$	368.6	21.277	1.58		
26	Dinonanoin monocaprylin	$C_{29}H_{54}O_6$	498.7	23.842	3.41		
27	Methyltris(trimethylsiloxy)silane	$C_{10}H_{30}O_3Si_4$	310.68	24.916	1.24	Organosilicon compound	
28	Trimethyl[4-(2-methyl-4-oxo-2-pentyl)phenoxy	$C_{15}H_{24}O_2Si$	264.43	25.961	1.12	Organosilane	Antioxidant, antibacteria1, anti-inflammatory
29	1,2-Bis(trimethylsilyl)benzene	$C_{12}H_{22}Si_2$	222.47	26.786	1.75	Aromatic hydrcarbon	
30	Trimethyl[4-(1,1,3,3,-tetramethylbutyl)phenoxy	$C_{17}H_{30}OSi$	278.5	27.258	1.07		
31	Ergosta-7,22-dien-3-ol, (3.beta.,22E)- $$ Ergos			28.420	4.82		

Table 11. Compound identified in the ethanol extract of *A. salviifolium* using GCMS

Sl.no.	Compound name	Molecular formula	Molecular weight	Retention time	Peak area	Compound nature	Uses
1	2-Propenoic acid, 3-ethoxy-, ethyl ester, (E)-	$C_7H_{12}O_3$	144.17	3.927	0.33		
2	Cyclohexanol, 4-methyl-1-(1-methylethyl)-	$C_{10}H_{20}O$	156.26	5.066	0.10		
3	Benzyl chloride $$ Benzene, (chloromethyl)- $$	C_7H_7Cl	126.58	5.372	0.29		

4	Propane, 1,1,3-triethoxy-	$C_9H_{20}O_3$	176.25	6.020	0.12	Alkane	Antibacterial
5	Propane, 1,1-diethoxy-2-methyl-	$C_8H_{18}O$	146.23	6.370	3.20		Flavoring agent, Anti-malarial activity
6	Dodecane	$C_{12}H_{26}$	170.33	7.716	0.31		Activators of the heat-shock response signaling pathway, food additives, flavoring agents, lubricants, antifungal and antibacterial acitivity
7	Pentadecane	$C_{15}H_{32}$	212.41	10.419	0.37		Antibacterial
8	Pentadecane	$C_{15}H_{32}$	212.41	12.879	0.23		Antibacterial
9	Diethyl Phthalate	$C_{12}H_{14}O_4$	222.24	13.018	82.23		Antimicrobial, antioxidant, plasticizer, estrogenic
10	Phthalic acid, di-(1-hexen-5-yl) ester	$C_{20}H_{26}O_4$	330.4	13.249	4.47	Ester	Antifungal
11	Diethyl Phthalate	$C_{12}H_{14}O_4$	222.24	14.056	1.29		Antimicrobial, antioxidant, plasticizer, estrogenic
12	Phthalic acid, ethyl pentyl ester	$C_{15}H_{20}O_4$	264.32	14.594	0.28	Ester	
13	Pentadecane	$C_{15}H_{32}$	212.41	15.100	0.12		
14	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296.5	15.539	0.16	Terpene alcohol	Catechol-O-Methyl-Transfearse inhibitor, antimicrobial, anti-inflammatory
15	2-Octylcyclopropene-1-heptanol	$C_{18}H_{34}O$	266.5	15.990	0.15	alcohol	Antibacterial activity
16	Acetamide, 2-(diethylamino)-N-(2,6-dimethylph	$C_{14}H_{23}ClN_2O$	270.80	16.493	2.36		Antiplasmodial activity
17	Hexadecane, 2,6,10,14-tetramethyl-	$C_{20}H_{42}$	282.5	17.105	0.14		Antifungal, antibacterial, antitumor, and cytotoxic effects

18	4-(3,5-Di-tert-butyl-4-hydroxyphenyl)butyl acrylate	$C_{21}H_{32}O_3$	332.5	17.320	0.12	Phenols	Antimicrobial
19	Phytol	$C_{20}H_{40}O$	296.5	18.266	1.71	Diterpene	Antimicrobial, anti-inflammatory, antiallergic, anticancer, diuretic, antidiabetic, cytotoxicity, antiproliferative, cancer preventive
20	Acetic acid, trifluoro-, hexadecyl ester $$ Trifluoroacetoxy hexadecane	$C_{18}H_{33}F_3O_2$	338.4	20.104	0.11		
21	Benzyldiethyl-(2,6-xylylcarbamoylmethyl)-amm			22.021	1.61		
22	3-Butoxy-1,1,1,5,5,5-hexamethyl-3-(trimethylsiloxy	$C_{13}H_{36}O_4Si_4$	368.76	23.873	0.13		
23	3.beta-Acetoxy-bisnor-5-cholenamide	$C_{24}H_{37}NO_3$	387.6	28.448	0.17		B-galactosidase and β-glucuronidase inhibitor

Table 12. Compound identified in the aqueous extract of *A. salviifolium* using GCMS

Sl. no.	Compound name	Molecular formula	Molecular weight	Retention time	Peak area %	Compound nature	Uses
1	Benzofuran, 2,3-dihydro-	C_8H_8O	120.15	8.186	53.56	Heterocyclic aromatic	Anti-inflammatory and antifungal activity
2	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150.17	9.452	11.01		Antimicrobial, antioxidant, anti-inflammatory, analgesic, anti-germination
3	2-(2′,4′,4′,6′,6′,8′,8′-Heptamethyltetrasiloxan-2'-yl			11.548	6.44		
4	2-(3-Chloropropyl)-1,3-dioxolane	$C_6H_{11}ClO_2$	150.6	13.565	5.85		
5	Squalene	$C_{30}H_{50}$	410.7	23.954	17.84	Triterpene	Squalene-monooxygenase-inhibitor, chemo preventive, antibacterial, antioxidant, antitumor, cancer preventive, immunostimulant, lipoxygenase-inhibitor, Pesticide

6	Benzene,1-nitro-4-diphenylmethylazino-	$C_{20}H_{15}N_3O_2$	329.4	25.476	5.30		

A. salviifolium's antioxidant activity in vitro

Phosphomolybdenum (PM) assay

On comparison between the all extracts, i.e., for acetone, chloroform, ethanol, methanol, and aqueous extracts in the phosphomolybdenum (PM) assay, aqueous extract has shown the highest activity with higher absorbance, i.e., 0.991 ± 0.004 for 500 µg concentration, whereas standard ascorbic acid has shown 1.078 ± 0.003. The results of all test samples are shown in Table 13 and Additional file 1: Fig. S12.

Table 13. Phosphomolybdenum (PM) assay for *A. salviifolium* leaf extract

Concentration (µg/ml)	Standard ascorbic acid	Acetone	Chloroform	Ethanol	Methanol	Aqueous
100	0.347 ± 0.003	0.178 ± 0.009	0.139 ± 0.004	0.124 ± 0.001	0.291 ± 0.006	0.279 ± 0.004
200	0.513 ± 0.002	0.284 ± 0.004	0.244 ± 0.009	0.219 ± 0.006	0.375 ± 0.004	0.427 ± 0.004
300	0.681 ± 0.001	0.411 ± 0.002	0.398 ± 0.002	0.323 ± 0.004	0.526 ± 0.001	0.699 ± 0.002
400	0.887 ± 0.001	0.511 ± 0.005	0.429 ± 0.002	0.419 ± 0.006	0.717 ± 0.002	0.845 ± 0.002
500	1.078 ± 0.003	0.599 ± 0.007	0.523 ± 0.006	0.487 ± 0.003	0.871 ± 0.001	0.991 ± 0.004

The results are represented as mean ± standard deviation

Radical scavenging assay using 2, 2 diphenyl-2-picryl hydrazyl (DPPH)

In case of DPPH assay among the selected chloroform, acetone, ethanol, methanol, and aqueous extracts, aqueous extract has exhibited prominent activity with a higher percentage of inhibition, i.e., 84.892% and standard ascorbic acid shown around 86.271%. The study found that when aqueous extract was compared to regular ascorbic acid, it had the comparable antioxidant activity than the remaining extracts (Table 14).

Table 14. DPPH assay for *A. salviifolium* leaf extract

	Concentration (µg)	Percentage of inhibition	IC_{50} in µg
Standard ascorbic acid	10	86.271 ± 0.1016	5.788
Acetone	100	59.111 ± 0.2329	84.53
Chloroform	100	56.649 ± 0.3073	88.77
Ethanol	100	66.808 ± 0.4417	74.93

Methanol	100	76.703±0.3206	65.49
Aqueous	100	84.492±0.3556	58.89

The results are represented as mean±standard deviation

Ferric ion reducing power (FRAP) assay

Using standard ascorbic acid, the ferric ion reducing power (FRAP) assay was carried out on extracts of acetone, chloroform, ethanol, methanol, and distilled water. According to the study's findings (Table 15, Additional file 1: Fig. S13), aqueous extract had the highest antioxidant activity with higher absorbance OD value when compared to the other four extracts.

Table 15. FRAP assay for *A. salviifolium* leaf extract

Concentration (µg/ml)	Ascorbic acid	Chloroform	Acetone	Ethanol	Methanol	Aqueous
100	0.566±0.006	0.104±0.003	0.146±0.001	0.086±0.004	0.198±0.002	0.251±0.003
200	1.012±0.006	0.246±0.003	0.254±0.004	0.145±0.004	0.245±0.003	0.406±0.001
300	1.432±0.004	0.344±0.004	0.463±0.007	0.25±0.002	0.485±0.003	0.652±0.001
400	1.735±0.004	0.466±0.004	0.564±0.004	0.326±0.004	0.556±0.004	0.816±0.004
500	1.987±0.004	0.574±0.002	0.762±0.004	0.411±0.004	0.605±0.003	0.997±0.002

The results are represented as mean±standard deviation

Antibacterial activity of *Alangium salviifolium* leaves extract

Aqueous extract from the leaves of *A. salviifolium* had the greatest zone of inhibition against *Pseudomonas aeruginosa* and *Staphylococcus aureus* of all the leaf extracts tested for antibacterial activity. With a maximum inhibitory zone of 10 mm, leaf aqueous extract was found to be more effective to *Pseudomonas aeruginosa* than ethanol, acetone, and chloroform (5 mm) or methanol extract (3 mm). With a maximum inhibitory zone of 11 mm, *Staphylococcus aureus* was found to be more sensitive to the aqueous extract than to acetone, chloroform, ethanol, or methanol. Table 16 and Additional file 1: Figs. S14 and S15 display the measured zone of inhibition for leaf extracts produced from various solvents.

Table 16. Zone of inhibition (in mm) for different solvent extracts of leaves of *A. salviifolium*

Type of extract	Inhibition zone (mm)							
Pseudomonas aeruginosa				*Staphylococcus aureus*				30 µg
60 µg	90 µg	120 µg	30 µg	60 µg	90 µg	120 µg	Chloroform	3
4	5	5	1	2	4	5	Acetone	1

2	4	5	1	4	6	7	Methanol	0
1	3	3	0	1	2	2	Ethanol	1
3	4	5	1	2	2	3	Aqueous	5

Discussion

Numerous chemicals found in plants that are known to be biologically active and to have a variety of pharmacological effects [36, 37]. These plant secondary metabolites include several important natural antioxidant sources that are safer and more efficient than synthetic antioxidants [38]. The most prevalent phenolic molecules that act as natural antioxidants in plants include ascorbic acid, carotenoids, and flavonoids [39].

The phytochemicals found in the plant *A. salviifolium* were extracted in the current study utilizing a variety of increasing polarity solvents, including chloroform, acetone, ethanol, methanol, and distilled water. Different phytochemical tests were used to identify the phytochemicals, which included the presence of reducing sugar, alkaloids, flavonoids, phenols, lignin, glycosides, and tannins. Plants use alkaloids, the majority of which have a severe bitter taste and are very toxic, to protect themselves from herbivory, pathogenic microbial attack, and invertebrate pests. Numerous studies on phenolic compounds have demonstrated the significance of these compounds in demonstrating biologically active properties like anti-inflammatory, antidiabetic, antioxidant, antibacterial, anticancer, etc. [38]. Because of this, the total phenolic and total flavonoid contents of various extracts of *A. salviifolium* leaves were determined, as well as their antioxidant potential by in vitro phosphomolybdenum, DPPH, and ferric ion reducing power (FRAP) assay methods. In the current investigation, chloroform extract (71.86 mg/g QE) and ethanol extract (82.86 mg/g GAE) were shown to have the greatest concentrations of total flavonoid and phenol, respectively. It can be expected that the biological activity of the leaves of *A. salviifolium* may be caused by the presence of flavonoids and phenolic compounds in it based on the measurement of the total phenol and flavonoid content in them.

Based on the peak value ratio, the functional groups of the plant extracts are identified using the FTIR spectrum. Aldehyde, ketones, phenol, alkanes, alkenes, alcohol, aromatic, aliphatic amines, and amine compounds, as well as nitrogen and halogen compounds, were all confirmed to be present by FTIR analysis. For the examination of non-polar components and volatile essential oils, fatty acids, and lipids in the majority of medicinal plants, gas chromatography and mass spectroscopy (GCMS) investigations have become more and more helpful [40]. The existence of diverse bioactive components in all of the *A. salviifolium* extracts was confirmed by GC–MS analysis of the various solvent extracts used in the current study. Stigmasterol, (E)-9-Eicosene, 3, 7, 11, and 15-Tetramethyl-2-hexadecen-1-ol, Phytol were the main compounds identified in the chloroform extract; 9-Eicosene, 1-Nonadecene, 2- 4-hydroxy-4-methyl- Pentanone, and 9-Octadecene in acetone, whereas diethyl phthalate, di-(1-hexen-5-yl) ester phthalic acid in ethanol. Aqueous extract revealed the presence of 2,3-dihydro-Benzofuran and Squalene, as well as 7,1-cyclohex-1H-cyclopenta[b]indol-3(2H)-one, 2-Methoxy-4-vinylphenol, n-Hexadecanoic acid, alpha-D-Galactopyranose, 6-O-(trimethylsilyl) in methanol. Among the identified compounds, most of them are known to possess several biological activities such as stigmasterol was known to have anti-infammatory, antioxidant, antimicrobial, anticancer, antiarthritic, and antiasthama activity [41, 42]; (E)-9-Eicosene was known to have antimicrobial property [43]; 3,7,11,15-Tetramethyl-2-hexadecen-1-ol was known to have antimicrobial and anti-inflammatory property [44], and phytol was known to have antimicrobial, anti-inflammatory, antiallergic, anticancer, diuretic, antidiabetic, cytotoxicity, antiproliferative, cancer preventive properties [41, 45–47], 1-Nonadecene was proven to have antituberculosis, anticancer, antioxidant, antimicrobial and antifungal activities [48–50], and squalene was reported to have antibacterial, antioxidant, antitumor, cancer preventive and immunostimulant property [46, 51]. According to Neha et al. [52], an antioxidant is a chemical that can inhibit or block the oxidation of lipids or other

molecules by avoiding the onset of oxidative chain reactions. As a result, it can stop or undo the harm that oxygen does to the body's cells. Natural antioxidants are more popular these days because of their potential to improve health and fend off diseases. In the current work, three assay methods—the Phosphomolybdenum (PM), DPPH, and ferric ion reducing power (FRAP) assay methods—were used to determine the antioxidant activity of *Alangium salviifolium* leaves extract. Different crude extracts of *Alangium salviifolium* leaves underwent PM, DPPH, and FRAP assays, and the results were compared to ascorbic acid standard. The phosphate-Mo (V) complex is a bluish green color, and its synthesis results in the reduction of molybdate ions, which is evaluated spectrophotometrically in the phosphomolybdenum test [32]. It is a method that is often used in laboratories to evaluate the overall antioxidant activity of plant extracts. Aqueous extract had the highest activity in the current investigation (0.991 ± 0.004) (Table 13, Additional file 1: Fig. S12).

The assessed antioxidant's potential to scavenge free radicals is revealed by the decrease in DPPH solution absorbance during the reaction. *Alangium salviifolium* plant secondary metabolites such as alkaloids, flavonoids, tannins, phenols, and glycosides are abundant in the plant's crude extracts. By contributing a hydrogen molecule, each of these bioactive compounds has the ability to oxidize the DPPH solution [53]. Using chloroform, acetone, ethanol, methanol, and aqueous extract, the antioxidant activity of *Alangium salviifolium* was assessed in the current study and compared to that of conventional ascorbic acid. According to the results (IC_{50} value: 58.89 µg/ml) (Table 14), aqueous extract had the highest level of scavenging activity compared to all other extracts.

The ferric ion reducing power (FRAP) assay is a method that examines how antioxidants in an acidic media reduce ferric ion (Fe3+)-ligand complex to the strikingly blue ferrous (Fe2+) complex. According to this approach, absorption is inversely related to reducing potential; the greater the absorbance, the greater the antioxidants' capacity to reduce [54]. In the current analysis, aqueous extract was proven to be significant than chloroform, acetone, ethanol and methanol extracts in terms of antioxidant activity (0.997 ± 0.002) (Table 15, Additional file 1: Fig. S13). The investigated extracts' antioxidant activity varied greatly among the different solvent extracts, and the results concluded that on comparison among the tested extracts aqueous extract demonstrated a greater overall antioxidant capacity with noticeably superior outcomes. According to research by Shravya et al. [1], the antioxidant activity of *Alangium salviifolium* leaves was found to be superior than that of the plant's roots. However, *Alangium salviifolium* leaf extract demonstrated significant antioxidant activity against the DPPH radical and was comparable to earlier findings for this plant, but there was no relationship between antioxidant activity and TPC for *Alangium salviifolium* leaves [55].

Over the past three decades, pharmaceutical companies have created a variety of innovative antibiotic treatments, but bacteria have grown more resistant to these medications. Plant extracts are a fantastic source of pathogen-fighting antibacterial compounds. They can therefore be utilized to treat a variety of infectious disorders brought on by virulent microorganisms. *Staphylococcus aureus* and *Pseudomonas aeruginosa* were used in this work to test the plant extract from *Alangium salviifolium* for antibacterial activity. In both test organisms (Zone of Inhibition-19 mm and 22 mm), aqueous extract stood out among the extracts for its strong antibacterial activity (Table 16, Additional file 1: Figs. S14 and S15). However, *Alangium salviifolium* stem bark and flower extract have been found in the past to have strong antibacterial activity against a variety of bacteria [56, 57]. Additionally, there are not many reports on the antibacterial properties of *Alangium salviifolium* leaf extract. The results of the current investigation make it abundantly evident that water extract proven to be have significant antibacterial activity, whereas acetone, chloroform, ethanol, and methanol have noticeable antibacterial properties. Our study's findings indicate that *Alangium salviifolium* leaves can act as a natural antioxidant to stop the onset and spread of a variety of ailments. To isolate and purify the plant chemicals for this antioxidant and antibacterial properties, more research is required.

Conclusions

In the present study, the plant *Alangium salviifolium* was selected and using phytochemical and GCMS analysis the different solvent extracts of the plant have shown the presence of several metabolites such as phenols and alkaloids, GC–MS analysis has shown the presence of several compounds which are having industry and medicinal applications. Among the different solvent extracts, ethanol and chloroform extract have shown the presence of

highest phenolic and flavonoid content, respectively. Further, all selected extracts were screened for biological activities such as antioxidant and antibacterial activity. The results concluded that aqueous extract of plant *Alangium salviifolium* proven to be having potent properties in all performed assay. Hence, in future the molecular level of studies and animal model can be studied to understand its pathway studies.

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Author contributions

All authors were involved in concept, design, and collection of data, interpretation, writing and critically revising the article. All authors approve final version of the article.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable because the present work doesn't involve any humans or animal study. The present study involves the plant materials and As per the guidelines of the university, the plant was identified and verified and its herbarium specimen (No BT was submitted to the Dept. of Botany, Karnataka Science College, Dharwad. The authenticate certificate for the plant identification was taken and uploaded in supplementary section.

Competing interests

The authors declare no competing interests.

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Unveiling geniposide from Paederia foetida as a potential antihypertensive treatment: an integrated approach involving in vivo and computational methods

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ABSTRACT (ENGLISH)

Background

Hypertension is one of the burning topics in today's world. Natural product can open a new window in the treatment as they are lesser side effect compared to synthetic compounds. *Paederia foetida* a naturally occurring plant has proven its biological importance in many aspects. In this present study, the ethanolic extract of *Paederia foetida* has effectively proven its antihypertensive activity against Amphetamine-induced hypertension.

Results

The study was carried out for 4 weeks with five different groups where the groups receiving *Paederia foetida* (400 mg/kg) for 4 weeks result in decrease in blood pressure and was found helpful in maintaining the sodium and potassium balance in rat's serum. Amphetamine induces decreasing sodium level that can be countered by *Paederia foetida* whole plant extract. Geniposide, an active ingredient present in this plant, is having

antihypertensive activity, so it was docked against different PDB IDs (3OLL, 3OLS, 5DX3, 5DXE &6PIT), to find its anti-hypertension effectiveness through computational chemistry. The docking investigations identified that estrogen receptor (PDB ID: 3OLS) exhibited the highest possibility to be the site of action. Docking score of Geniposide with 3OLS was -8.91 which is quit comparable with the internal ligand Estradiol.

Conclusion

To assess the binding affinity of Geniposide with the estrogen receptor, an additional molecular dynamics simulation was conducted. The result strongly suggests that Geniposide has the potential to function as an activator of estrogen receptor through of β-ligand binding. This key finding reveals that Geniposide may serve as a potential in the treatment of hypertension by modulating the activity of the estrogen receptor.

FULL TEXT

Background

A chronic medical condition known as hypertension causes raised blood pressure in the arteries, also known as arterial hypertension. Above 140/90 mmHg of systolic and diastolic blood pressure is often considered as hypertension. Since high blood pressure is one of the major risk factors for coronary artery disease and other consequences, including heart failure, stroke, kidney disease, and diabetes, it is one of the main causes of mortality, globally [1]. According to the Global Burden of Disease study, hypertension is also regarded as the main risk factor for disability-adjusted life years, globally [2]. According to the World Health Organization, by 2025, 1.5 billion people will have hypertension, and the condition is projected to be a factor for more than 7 million fatalities annually [3]. Numerous research are focused on development of novel antihypertensive medicines and new therapy options because of the massive prevalence of hypertension, worldwide [4, 5]. There are different classes of antihypertensive medications, including angiotensin-converting enzyme (ACE) inhibitors, beta-blockers, and calcium channel blockers used in management of blood pressure, through the control of cardiac output, peripheral vascular resistance, and circulating blood volume [6, 7]. These antihypertensive medications are widely used to treat hypertension and associated cardiovascular disorders; however, these drugs also have undesirable side effects [6]. On account of the fact that natural herbal products made from medicinal plants are thought to have lesser adverse effects, thereby can be an alternative treatments and can be preferred over the existing medication [5]. Recently, it has been revealed that medicinal herbs can reduce hypertension and can be used empirically as antihypertensive medicines [8, 9]. Oxidative stress can be considered as an early warning sign for both hypertension and cardiovascular disorders so plants as an natural antioxidant can have the capability to enhance their antihypertensive effects [10, 11]. Diuretics have a significant role in the clinical management of hypertension. These medications influence the renal tubular segments' salt re-absorption to boost kidney urine production [12, 13]. NCC (Sodium chloride co-transporters) and NKCC2 (Na–K-2Cl co-transporter) are the main targets of diuretics. Recent research has demonstrated that the NKCC2-oxidative stress-responsive kinase 1 (OSR1)-with-no-lysine kinase (WNK) signaling pathway is crucial for controlling blood pressure [14]. WNK may control sodium chloride co-transporters (NCC) through the intermediate kinases OSR1 [15, 16]. A Rubiaceae family member *Paederia foetida*, also known as Prasariniin in Sanskrit, is a climbing plant with a strong foetid odor. Its Hindi name is Gandhaprasarini, and its English name is Chinese Flower [17]. Numerous scientific studies have been conducted to examine the activity of *Paederia foetida* in the management of rheumatic diseases, anti-inflammatory, hepatoprotective, and chemolithotripsy encourages sexual energy, boosts physical strength, semen production, and creates a young glow [18–25]. *Paederia foetida* possesses paederolone, paederone, sitosterol, paederoside, and asperuloside, along with their glycosides it has been identified through phytochemical studies [26–28]. The leaves of the plant are a good source of vitamin C, alkaloids, ketone alcohol, and beta-carotene [29]. Important constituents such asasperuloside, beta-sitosterol, and lupeol are well quantified in leaves, too [30]. Asperuloside, scandoside, and paederoside are iridoid glucosides that are present in the plant's aerial portions [31]. Friedelin, campesterol, ursolic acid, hentriacontane, hentriacontanol, ceryl alcohol, palmitic acid, and methyl mercaptan ellagic acids are other components of *P. foetida*. [32]. Another Iridoid glycosides are also found in roots of this plant known as Geniposide [33]. Our focus of this study is on Geniposide.

Yang Fu et al. [14] demonstrated the ability of Geniposide to increase urine production and the excretion of salt and chloride ions. They further revealed that the antihypertensive effects of Geniposide were considered due to their inhibition of the activation of the WNK signaling pathway, which is mediated by the estrogen receptor. So, considering this key finding we performed molecular docking and molecular dynamics (MD) simulations studies of Geniposide and various PDB IDs based on the structure of estrogen receptor.

Molecular docking, a computer-based method, can be employed to predict the non-covalent interactions between a macromolecule and a small molecule [34]. Based on the scoring method molecular docking analyses, the energy and binding strength of the complexes are observed [35]. This technique is frequently employed to conduct preliminary screening of small compounds by analyzing their binding characteristics to drug target. This creates the necessary foundation for rational drug design of novel drugs that can have higher specificity and greater efficacy [36]. Molecular dynamics (MD) simulations rely on general physics model guiding inter-atomic interactions and predict the motion of each molecule in a molecular system. Molecular dynamic simulations can determine the stability and dynamic properties of these protein–ligand complexes by analyzing the statistical parameters of these simulations [37, 38].

The present study evaluates the antihypertensive activity of the ethanolic extract of *Paederia foetida* against Amphetamine-induced hypertension in Wistar rats. Subsequently, an *in silico* molecular docking and dynamic simulation studies were conducted using the key phytochemical constituent, Geniposide to evaluate the potential of Geniposide and to consider it as a medication to lower blood pressure in the near future.

Materials and Methods

All the chemicals and reagents used were of analytical grade. Amphetamine and Hydralazine were purchased from Sigma-Aldrich, Mumbai, India. All drug solutions were freshly prepared in saline water before each experiment. Concentrated sulfuric acid, potassium bismuth iodide, Wagner reagent, Hagers reagents, sodium hydroxide, copper sulfate, hydrochloric acid, hydrogen peroxide, 4-aminoantipyrine, 4-chlorophenol, phosphotungstic acid, sodium dihydrogen phosphate, citric acid, potassium sulfate, and trichloracetic acid were purchased from Deshpande Laboratories Pvt Ltd, Bhopal, India.

Collection and authentication of plant material

The whole plant of *Paederia foetida* was collected and dried in 2022 from Bankura, West Bengal, India, with the GPS coordinates of 22°59′51.7′′N 87°OO’39.4′′E. The plant was authenticated by department of Botany, Bankura Christian College, West Bengal, India. The whole plant was shade-dried under room temperature and grinned to a fine powder.

Preparation of extraction

A whole plant powder (100 g) of *Paederia foetida* was extracted by maceration in 400 mL of ethanol for 14 days with frequent agitation. The mixture was filtered through clean muslin cloth followed by double filtration with Whatman No. 1 filter paper and the filtrate was concentrated by rotary evaporator with the vacuum at 50 ºC, poured into glass Petri dishes, and brought to dryness at 60ºC oven.

Phytochemical screening of different extract

Preliminary phytochemical screening was performed to identify the phytoconstituents present in the extracts. Phytochemical tests, such as Molish test, Dragendroff test, Mayers test, Wagner test, Hager test, Biuret test, Million test, ferric chloride test, lead acetate test, gelatin solution test, Shinoda test, alkaline reagent test, Legal test, Bajlets test, and Liebermann-Burchard test, were performed to determine the carbohydrates, alkaloids, proteins, tannins, flavonoids, glycosides, saponin, and steroids from ethanolic extract of *Paederia foetida.*

Procurement of animals

The experiments were carried out according to the guidelines of the CPCSEA, New Delhi, India. The study protocol was approved by the Institutional Animal Ethical Committee of Aditya Bangalore Institute of Pharmacy Education and Research Bangalore, Karnataka, India (approval number: 64/1611/CPCSEA). Female Wistar rats (Wistar strain) weighing between 150 and 200 g were obtained from local vendor, Bangalore, Karnataka. Animals were housed into five groups under standard laboratory conditions, i.e., 25 °C ± 1 °C/45–55% RH and 12/12 h light and

dark conditions in the animal house of Aditya Bangalore Institute of Pharmacy Education and Research, Bangalore, Karnataka, India. The rats are kept with free access to food (Hindustan Lever, India).

Antihypertensive activity

The rats were divided into five groups and each group contain six rats (n = 6). Group I was for positive control, received 0.9% saline p.o, group II marked for negative control, and received Amphetamine 5 mg/kg/day, (i.p.). Groups III and IV were allocated as test 1 and test 2 and received Amphetamine (5 mg/kg/day, i.p.) along with *Paederia foetida* (200 mg/kg/day, p.o.) and Amphetamine (5 mg/kg/day, i.p.) along with *Paederia foetida* (400 mg/kg/day, p.o.) for 4 weeks, respectively. Group V was marked as standard and received Amphetamine (5 mg/kg/day, i.p.) and Hydralazine (25 mg/kg., i.p.).

Blood pressure monitoring

Blood pressure was determined by placing an animal in the supplied restrainer. The tail-cuff warming chamber was attached and selected the number of test cycles and then pressed auto-calibrate to run. The tail-cuff method required the minimum amount of heat to measure blood pressure.

Noninvasive method of blood pressure determination

The cuff consists of latex balloon measuring 5 cm × 2 cm with 0.5 mm thickness. This balloon was placed in a circular plastic case having a diameter of 23 mm with a central hole of 12 mm diameter. The balloon was kept in such a manner that it remains in contact with the inner surface of the plastic case around the central hole so that this balloon encircles the tail. One end of the triway was connected with the balloon (tail-cuff) and the other two ends were connected to an inflating–deflating pump and sphygmomanometer. The system measured the systolic BP by determining the cuff pressure (reflected on the sphygmomanometer) and the blood flow (pulse) to the tail was eliminated. This elimination of blood flow (pulse) was recorded by a pulse transducer connected to a single-channel physiograph through a suitable coupler. The animals were acclimatized and kept in restrainers. The tail was passed through the hole of the newly designed cuff and the pulse transducer was tied around the tail distal to the cuff. As the system was switched on and the pulse was recorded on the physiographic paper. The cuff was inflated by the pump and the pressure in the cuff was raised until the pulse was eliminated. The pressure at which the pulse was eliminated, and noted as the systolic BP of the animal. The systolic BP and pulse were also recorded by the NIBP system of ADI for comparison.

Estimation of electrolytes

Method for assessment of serum electrolyte

Wistar rats were used for the biochemical analysis of serum. The rats were provided with the usual diet and the blood samples were collected for the determination of the amount of electrolytes present in blood (Na^+, K^+).

Sodium and potassium ion estimation

Sodium and potassium determination was performed in an automated Roche 9180 electrolyte analyzer (Basel, Switzerland). The 2 ml of blood samples was collected from rats through retro-orbital plexus after anesthetizing through a diethyl ether inhaler. The blood samples were kept for 30 min then centrifuged in a cooling centrifuge at 5000 rpm for 10 min to obtain the serum. The calibration of the instrument was performed, then run a control sample as mentioned. Finally, the serum sample was run to obtain the results.

In silico analysis

Ligand and protein preparations

The selected compound Geniposide and Estradiol (Internal ligand) were utilized for the molecular docking analysis for estrogen receptors. The three-dimensional structures of Geniposide and Estradiol were acquired from the PubChem database (https://pubchem.ncbi.nlm.nih.gov) (accessed on 20 April 2023) in SDF MOL format. The SDF MOL format was converted into PDB format by using OpenBableGUI 3.1.1 software. Three-dimensional structures (mainly crystal) of different PDB ID like 3OLL [40], 3OLS [40], 5DX3 [41], 5DXE [41], and 6PIT [42] were chosen for the investigation acquired from RCSB Protein Data Bank (PDB). 3OLL-Crystal structure of phosphorylated estrogen receptor beta ligand binding domain, 3OLS-Crystal structure of phosphorylated estrogen receptor beta ligand binding domain, 5DXE-Estrogen Receptor Alpha Ligand Binding Domain Y537S Mutant in Complex with Stapled

Peptide SRC2-P4 and Estradiol, 5DX3-Estrogen Receptor Alpha Ligand Binding Domain Y537S Mutant in Complex with Stapled Peptide SRC2-P3 and Estradiol &6PIT-Estrogen Receptor Alpha Ligand Binding Domain Y537S Mutant in Complex with SRC2 Stapled Peptide 41A and Estradiol. The process of getting ready the proteins involved accessing the PDB ID to retrieve the protein from the server, uploading the molecule, adding hydrogens, applying specific turning to residues, examining interactions and geometry for all atoms, and acquiring the finished protein file. Estradiol was isolated from the protein's binding pocket as an internal ligand to reveal the grid coordinates throughout the active region. The stabilized structure was stored after being downloaded from the server in PDBQT format for the purpose of protein–ligand docking.

Molecular docking of protein and ligand

The Autodock-4.2.6 program (ADP) was used to carry out molecular docking of Geniposide &Estradiol with estrogen receptors. ADP tools were used to prepare the protein and ligands. The coordinate values used in grid settings were acquired from re-docking studies, and the dimensions of the grid box were 60 × 60x60 in the x, y, and z directions. In each case, the spacing of the grid point was 0.375". Auto grid-4.2 was utilized to create the map files. For search criteria, a genetic algorithm (GA) was employed. Autogrid and Autodock operation were the last step of docking analysis and were performed in Autodock-4.2.6. The molecular docking of individual ligands on the appropriate protein was carried out using Autodock-4.2 and Autogrid-4.2, respectively. Interactions between molecules and binding energy (kcal/mol) were measured and analyzed.

ADME profile

The Swiss ADME online server (http://www.swissadme.ch/index.php) was used to assess the drug-likeness patterns and pharmacokinetic characteristics of the investigated compounds, where SMILES format of the compounds was uploaded to the webpage and the assessment procedure was completed.

Toxicity profile

The ProTox-II online server (https://tox-new.charite.de/protox_II/index.php) was used to predict the toxicities of the investigated compounds, where SMILES format of the compounds was uploaded to the webpage and the assessment procedure was completed.

Molecular dynamic simulation

Molecular dynamics (MD) simulation was carried out using GROMACS 2022.2. The following steps were utilized.

(a) Preparation of enzyme

The 3-dimensional (3D) models of the Estrogen receptor in complex with Geniposide were exported to PDB format using Pymol. The dynamic behavior of the complex was evaluated using molecular dynamic (MD) simulation in the GROMACS package program (version 2022.2) [43–45]. Protein topology was constructed by pdb2gmx with the CHARMM27 force field [46], and ligand topology was generated using the SwissParam server [47].

(b) Setting up a system for simulation

After applying the force field, the complex was inserted into the system. It was solvated with the TIP3P water model [48] in a cubic box greater than 1 nm from the edge of the protein with periodic boundary conditions. The system was neutralized by adding Na + ions, and energy minimization was done for 50,000 steps using the steepest descent algorithm. It was then followed by 100 ps of NVT simulation at 300 K and 100 ps of NPT simulations to equilibrate the whole system. The Leapfrog algorithm was employed in the constant-temperature, constant-pressure (NPT) ensemble to separately couple each component like protein, ligand, water molecules, and ions [49]. The Berendsen temperature and pressure coupling constants were set to 1 and 2, respectively, to keep the system in a stable environment (300 K temperature and 1 bar pressure) [50]. Finally, MD simulation for 100 nswas performed in isothermal and isobaric condition ensemble at 300 K. The pressure coupling with time-constant was set at 1 ps to maintain pressure constant at 1 bar, and the LINCS algorithm [51] was used to constrain the bond lengths. The Van der Waals and Coulomb interactions were truncated at 1.2 nm, and the PME algorithm [52] inbuilt into GROMACS was used to minimize the error from truncation.

(c) Visualization and analysis of simulation

The trajectory file was visualized through VMD (Visual Molecular Dynamics) 1.9.2 [53] and analyzed by the tool Hero

MD Analysis [54, 55] and Xmgrace 5.1.25 [56, 57].

Results

Preparation of extract and phytochemical screening

The crude ethanolic extract was prepared by maceration process for a period of 14 days and finally the yield was calculated. Nearly 100 gm of the whole plant was extracted and the yield was found to be 12.16 g. An appropriate concentration of the extracts was made in distilled water for further studies. Preliminary phytochemical screening of ethanolic extract of *Paederia foetida* was performed and revealed the presence of different phytoconstituents, proteins, amino acids, alkaloids, phenolic compounds, carbohydrates, alkaloids, tannins, flavonoids, and glycosides. The results of all these tests are summarized in Table 1.

Table 1. Phytochemical screening of *Paederiafoetida*

Serial no	Test	Result
1	Proteins and amino acids	+
2	Alkaloids	+
3	Phenolic compounds	+
4	Carbohydrates	+
5	Glycosides	+
6	Saponins	−
7	Flavonoids	+
8	Fixed oils and fats	−
9	Terpenoids	−
10	Sterols	−

The symbol (+) denotes presence and (−) denotes absence of phytoconstituents

Measurement of blood pressure by noninvasive (indirect) method after administration of Paederia foetida whole plant extract

The drug-induced hypertension in rats was acquired by administration of Amphetamine for 4 weeks (negative control) in rats, and produced a significant elevation in the systolic blood pressure (SBP) as measured by tail-cuff method on 1st, 2nd, 3rd, and 4th week, respectively, when compared to the control rats. Rats that received *Paederia foetida* whole plant extract (200 and 400 mg/kg/day, p.o., test 1 &2) for 4th weeks along with Amphetamine was found to reduce the SBP significantly in 3rd and 4th week when compared to Amphetamine-induced hypertensive rats, thus implying an antihypertensive activity. The detailed antihypertensive study is summarized in Table 2 and Additional file 1: Figure S1.

Table 2. SBP of Amphetamine-induced rat along with *Paederiafoetida* and Hydralazine

Treatment groups (mg/kg)	Mean SBP (mm/Hg)				
0 week	1st week	2nd week	3rd week	4th week	Control
108.32 ± 2.34*	109.43 ± 2.10*	114.12 ± 1.19*	112.23 ± 1.03*	110.15 ± 0.34*	Amphetamine (5)
108.21 ± 1.21*	116.14 ± 1.90*	131.01 ± 1.02*	139.57 ± 1.21*	148.07 ± 1.04*	Amphetamine (5) + *Paederiafoetida* whole plant extract (200)
109.10 ± 0.19*	114.27 ± 1.54	125.32 ± 1.23*	122.27 ± 0.91*	119.37 ± 0.56*	Amphetamine (5) + *Paederiafoetida*(P.F.) whole plant extract (400)
108.45 ± 1.11*	112.65 ± 0.65*	120.54 ± 0.45*	119.68 ± 1.02*	116.67 ± 0.34*	Amphetamine (5) + Hydralazine (25)

Values are expressed as mean ± std, n = 6. Differences were considered significant at *P value of < 0.05. For numerical results, analysis of variance (ANOVA) was performed using GraphPad InStat version 3

*indicates level of significance

Estimation of sodium and potassium ion in serum of rats

The blood sample from different groups was collected and serum was separated to carry out the estimation of sodium and potassium levels in the serum of rats. The normal control (Group I) test 1, (Group III), test 2 (Group IV) &standard (Group V) exhibited normal values of sodium, whereas the negative control group (administered with Amphetamine) showed a relative decrease in Na^+ level in blood serum for 4 weeks in rats. Our findings also satisfy the literature survey as it was mentioned by Suzanne et al. 2002 [39] that Amphetamine toxicity can relatively decrease the Na^+ level than the normal value in blood serum. The result of the study is represented in Table 3 and Additional file 1: Figure S2. In the case of the serum potassium level in rats, no significant changes were noticed in all the four groups (Table 4 and Additional file 1: Figure S3).

Table 3. Effect of *Paederiafoetida* whole plant extract (200, 400 mg/kg/day, p.o., for 4 weeks) on serum sodium value, in Amphetamine-induced hypertensive rats and taking Hydralazine as a standard

Treatment groups(mg/kg)	Serum Na + (mMol/ml)

1st week	2nd week	3rd week	4th week	Control
152.23±0.18*	152.47±0.19*	153±0.27*	153.42±0.44*	Amphetamine (5)
151.40±0.48*	150.85±0.44*	149.85±0.97*	148.83±0.46*	Amphetamine (5)+ *Paederiafoetida* (P.F.) (200)
151.31±0.30*	151.55±0.48*	151.81±0.22*	151.29±0.37*	Amphetamine (5)+ *Paederiafoetida* (P.F.) (400)
151.39±0.89*	151.66±0.55*	151.96±0.87*	152.09±0.17*	Amphetamine (5)+ Hydralazine (25)

Values are expressed as mean±std, n=6. Differences were considered significant at *P value of<0.05. For numerical results, analysis of variance (ANOVA) was performed using GraphPad InStat version 3
*indicates level of significance

Table 4. Effect of *Paederiafoetida* whole plant extract (200, 400 mg/kg/day, p.o., for 4 weeks) on serum potassium value, in Amphetamine-induced hypertensive rats and taking Hydralazine as a standard

Serum K^+(mMol/ml)				
Treatment groups (mg/kg)	1st week	2nd week	3rd week	4th week
Normal control	5.15±0.17**	5.29±0.09**	5.31±0.09**	5.26±0.13**
Amphetamine (5)	5.29±0.16**	5.38±0.07**	5.49±0.10**	5.36±0.10**
Amphetamine (5)+*Paederiafoetida*(P.F.)(200)	5.26±0.11**	5.33±0.13**	5.39±0.03**	5.36±0.10**
Amphetamine (5)+*Paederiafoetida*(P.F.)(400)	5.23±0.17**	5.42±0.14**	5.36±0.15**	5.32±0.09**
Amphetamine (5)+Hydralazine(25)	5.20±0.03**	5.27±0.04**	5.23±0.06**	5.24±0.00**

Values are expressed as mean±std, n=6. Differences were considered significant at *P value of<0.05. For numerical results, analysis of variance (ANOVA) was performed using GraphPad InStat version 3
**indicates level of significance of ANOVA result

In silico activity of Geniposide and Estradiol against estrogen receptor

Molecular docking of Geniposide and Estradiol

The literature survey reveals that Geniposide is an active ingredient which can effectively reduce the blood pressure by inhibiting the activation of WNK signaling pathway mediated by the estrogen receptor [14]. The goal of the

molecular docking study was to examine at the binding energy and molecular interactions of Geniposide and Estradiol in the active site of the estrogen receptor, to achieve a strong rational correlation through computational studies. In addition, each compound's binding mechanism was examined for molecular interactions. The results showed that Geniposide had very close significant binding energy compared to the internal ligand Estradiol (Table 5). Among all the interactions (3OLS, 3OLL, 5DX3, 5DXE, 6PIT), PDB ID: 3OLS, Geniposide was found to have a very good docking score. The result of the binding and molecular interaction between PDB ID: 3OLS &Geniposide (Fig. 1c) revealed that Geniposide formed hydrogen bonds with HIS475, GLU3O5, LEU298, LEU339, and ARG346 through oxygen groups having bond lengths of 3.10, 2.76, 2.46, 3.33 &3.07 Å, respectively. In the case of internal ligand Estradiol, it showed hydrogen bond interaction between HIS475 &ARG346 through oxygen atoms having bond lengths of 3.10 &3.17 Å, respectively (Fig. 1d). All other interactions are shown in Fig. 2. 2D molecular interactions of Geniposide and Estradiol with respect to all PDB ID are given in Additional file 1 (Figure S4- Figure S8).

Table 5. Binding energy of Geniposide and Estradiol with estrogen receptor

Compounds	PDB ID	S(Kcal mol^{-1})	Amino acids	Interaction	Length	Figure No
Geniposide	3OLL	-7.67	ARG346 LEU298 GLU305 HIS475	(Lig.) O…..H (ARG346) (Lig.) O…..H (LEU298) (Lig.) O…..H (GLU305) (Lig.) O…..H (HIS475)	2.95 2.66 2.52 3.18	1(a)
Estradiol	-9.93	HIS475 ARG346	(Lig.) O…..H (HIS 475) (Lig.) O…..H (ARG 346)	3.03 3.09	1(b)	Geniposide
3OLS	-8.91	HIS475 LEU339 GLU305 ARG346 LEU298	(Lig.) O…..H (HIS 475) (Lig.) H…..O (LEU 339) (Lig.) H…..O (GLU 305) (Lig.) H…..O (GLU 305) (Lig.) O…..H (ARG 346) (Lig.) O…..H (LEU 298)	3.10 3.33 2.76 2.34 3.07 2.46	1(c)	Estradiol

-10.91	HIS475 ARG346	(Lig.) O….. H (HIS 475) (Lig.) O….. H (ARG 346)	3.10 3.17	1(d)	Geniposide	5DX3
-7.33	HIS524 LEU346 ALA350 GLU353 LEU387 ARG394	(Lig.) O….. H (HIS 524) (Lig.) O….. H (LEU 346) (Lig.) O….. H (ALA 350) (Lig.) O….. H (GLU 353) (Lig.) O….. H (GLU 353) (Lig.) O….. H (LEU 387) (Lig.) O….. H (ARG 394)	3.13 2.37 3.07 3.17 2.17 3.14 2.93	2(a)	Estradiol	-10.61

HIS524 ARG394	(Lig.)O….H(HIS524) (Lig.)O….H(ARG394)	3.11 2.98	2(b)	Geniposide	5DXE	-8.39

HIS524 LEU346 GLU353 LEU387 ARG394	(Lig.)O….H(HIS524) (Lig.)O….H(LEU346) (Lig.)O….H(GLU353) (Lig.)O….H(LEU387) (Lig.)H….O(ARG394)	3.06 2.38 2.39 2.90 2.94	2(c)	Estradiol	-10.88	HIS524 ARG394

(Lig.) O….. H (HIS 524) (Lig.) O….. H (ARG 394)	3.07 2.98	2(d)	Geniposide	6PIT	-8.21	HIS524 LEU 346 GLU 353 ARG 394 LEU 387
(Lig.) O….. H (HIS 524) (Lig.) O….. H (LEU 346) (Lig.) O….. H (GLU 353) (Lig.) O….. H (GLU 353) (Lig.) O….. H (ARG 394) (Lig.) O….. H (LEU 387)	3.08 2.41 2.29 3.31 2.92 2.95	2(e)	Estradiol	-10.53	ARG394	(Lig.) O…. .H (ARG 394)

Fig. 1 [Images not available. See PDF.]

3D Molecular interaction of: **a** Geniposide and **b** Estradiol with 3OLL and **c** Geniposide and **d** Estradiol with 3OLS

Fig. 2 [Images not available. See PDF.]

3D Molecular interaction of: **a** Geniposide and **b** Estradiol with 5DX3, **c** Geniposide and **d** Estradiol with 5DXE and **e** Geniposide and **f** Estradiol with 6PIT

ADME profile

The pharmacokinetics, physicochemical, and drug-likeness parameters of the compounds Geniposide (Plant derived) &Estradiol (Internal Ligand) were evaluated using the freely available online server Swiss ADME (http://www.swissadme.ch/index.php). The overall results of Swiss ADME prediction (Table 6) elucidated that the examined compounds showed a predicted log Po/w value in the range of -1.25 to 3.40 and disclosed no alerts for Pan Assay Interfering Substances (PAINS). Geniposide has poor solubility in water, but Estradiol was practically insoluble in water. Geniposide does not show BBB permeability, but Estradiol shows BBB permeability. This parameter was well understood from the boiled egg chart (Fig. 3). Both of these compounds follow Lipinski rule of five. Estradiol was found to be a CYP2D6 inhibitor, whereas Geniposide does not inhibit the enzyme CYP2D6 that suggests that Geniposide does not interfere with the metabolism of other drugs associated with CYP2D6. Lastly, the oral bioavailability radar charts (Fig. 4) showed good oral bioavailability as it is polar in nature, whereas Estradiol is nonpolar in nature. This result reflects Geniposide a favorable compound for good pharmacokinetic features. Along with this features, Geniposides also possess greater flexibility, lesser lipophilicity, and higher solubility when compared to Estradiol. This proves that Geniposide is having better ADME profile than Estradiol.

Table 6. ADME properties of the selected compounds predicted using SwissADME web server

Property	Geniposide	Estradiol
MW	388.37	272.38
Consensus LogPo/w	-1.25	3.40
Log *S* (ESOL)	Very soluble	Moderately soluble
#Rotatable bonds	6	0
#H-bond acceptors	10	2
#H-bond donors	5	2
MR	86.89	81.03
TPSA	155.14	40.46
Lipinski violations	0	0
Ghose violations	1	0
Veber violations	1	0

Egan violations	1	0
Muegge violations	2	0
Bioavailability score	0.11	0.55
PAINS alerts	0	0
Brenk alerts	1	0
Leadlikeness violations	1	1
GI absorption	Low	High
BBB permeant	No	Yes
P-gp substrate	No	Yes
CYP1A2 inhibitor	No	No
CYP2C19 inhibitor	No	No
CYP2C9 inhibitor	No	No
CYP2D6 inhibitor	No	Yes
CYP3A4 inhibitor	No	No

Fig. 3 [Images not available. See PDF.]

The boiled egg chart of the compounds: Geniposide (Molecule 1) and Estradiol (Molecule 2)
Fig. 4 [Images not available. See PDF.]

Bioavailability radar chart of the compounds; **a** Geniposide and **b** Estradiol. The pink area demonstrates the range of the optimal property values for oral bioavailability and the red line is the compounds' predicted properties. Saturation (INSATU), size (SIZE), polarity (POLAR), solubility (INSOLU), lipophilicity (LIPO), and flexibility (FLEX)

Toxicity profile

The toxicity parameters of Geniposide (Plant derived) &Estradiol (Internal Ligand) were evaluated using the online server ProTox-II (https://tox.new.charite.de/protox_II/index.php) and the result is depicted in Tables 7 and 8. It was observed that Geniposide is safer in comparison with Estradiol and it is clearly understood from the toxicity radar chart and toxicity table that all the toxicity parameters were inactive except immunotoxicity in case of Geniposide, whereas several toxicity parameters were found active in Estradiol. The immunotoxicity of Geniposide was found to be 0.52, whereas that of Estradiol was 0.99. This refers that Geniposide is safer option than that of Estradiol. Geniposide is much safer compare to Estradiol for the target androgen receptor, from the toxicity profile study of both these drugs depleted in Additional file 1: Figure S9.

Table 7. Toxicity model report of Geniposide

Classification	Target	Prediction	Probability
Organ toxicity	Hepatotoxicity	Inactive	0.84
Toxicity end points	Carcinogenicity	Inactive	0.85
Toxicity end points	Immunotoxicity	Active	0.52
Toxicity end points	Mutagenicity	Inactive	0.63
Toxicity end points	Cytotoxicity	Inactive	0.66
Tox21-Nuclear receptor signaling pathways	Aryl hydrocarbon receptor (AhR)	Inactive	0.96
Tox21-Nuclear receptor signaling pathways	Androgen receptor (AR)	Inactive	0.94
Tox21-Nuclear receptor signaling pathways	Androgen receptor ligand binding domain (AR-LBD)	Inactive	0.9
Tox21-Nuclear receptor signaling pathways	Aromatase	Inactive	0.9
Tox21-Nuclear receptor signaling pathways	Estrogen receptor alpha (ER)	Inactive	0.68
Tox21-Nuclear receptor signaling pathways	Estrogen receptor ligand binding domain (ER-LBD)	Inactive	0.97
Tox21-Nuclear receptor signaling pathways	Peroxisome proliferator activated receptor gamma (PPAR-Gamma)	Inactive	0.94
Tox21-Stress response pathways	Nuclear factor (erythroid-derived 2)-like 2/antioxidant responsive element (nrf2/ARE)	Inactive	0.96
Tox21-Stress response pathways	Heat shock factor response element (HSE)	Inactive	0.96
Tox21-Stress response pathways	Mitochondrial membrane potential (MMP)	Inactive	0.79
Tox21-Stress response pathways	Phosphoprotein (tumor suppressor) p53	Inactive	0.9

Tox21-Stress response pathways	ATPase family AAA domain-containing protein 5 (ATAD5)	Inactive	0.96

Table 8. Toxicity model report of Estradiol

Classification	Target	Prediction	Probability
Organ toxicity	Hepatotoxicity	Inactive	0.72
Toxicity end points	Carcinogenicity	Inactive	0.84
Toxicity end points	Immunotoxicity	Active	0.99
Toxicity end points	Mutagenicity	Inactive	0.98
Toxicity end points	Cytotoxicity	Inactive	0.87
Tox21-Nuclear receptor signaling pathways	Aryl hydrocarbon receptor (AhR)	Inactive	1
Tox21-Nuclear receptor signaling pathways	Androgen receptor (AR)	Active	1
Tox21-Nuclear receptor signaling pathways	Androgen receptor ligand binding domain (AR-LBD)	Active	1
Tox21-Nuclear receptor signaling pathways	Aromatase	Inactive	0.98
Tox21-Nuclear receptor signaling pathways	Estrogen receptor alpha (ER)	Active	1
Tox21-Nuclear receptor signaling pathways	Estrogen receptor ligand binding domain (ER-LBD)	Active	1
Tox21-Nuclear receptor signaling pathways	Peroxisome proliferator activated receptor gamma (PPAR-Gamma)	Inactive	0.99
Tox21-Stress response pathways	Nuclear factor (erythroid-derived 2)-like 2/antioxidant responsive element (nrf2/ARE)	Inactive	0.98
Tox21-Stress response pathways	Heat shock factor response element (HSE)	Inactive	0.98

Tox21-Stress response pathways	Mitochondrial membrane potential (MMP)	Active	1
Tox21-Stress response pathways	Phosphoprotein (tumor suppressor) p53	Active	1
Tox21-Stress response pathways	ATPase family AAA domain-containing protein 5 (ATAD5)	Inactive	1

Molecular dynamic simulations of estrogen receptorin Apoprotein form and in complex with molecules, Geniposide and Estradiol

In order to evaluate the binding of molecule, Geniposide and Estradiol inside the β-ligand binding domain of Estrogen receptor, we have carried out MD simulations for a period of 100 ns for the three models, A) Apoprotein, B) Geniposide-3OLS, and C) Estradiol-3OLS complex (Fig. 5). Their simulations were evaluated using various statistical parameters including: root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF), h-bond interactions, and its %occupancies over the time.

Fig. 5 [Images not available. See PDF.]

Graphical representation of **A** Apoprotein form, **B** Geniposide and **C** Estradiol bound inside the active site of Estrogen receptor, where protein is shown in cartoon representation and the ligand is shown in CPK representation

RMSD analysis

Analyzing the protein-RMSD can give insights into any structural conformation that protein undergoes during the simulation. The multiplot for protein Cα and ligand RMSD versus time is shown in Figs. 6 and 7, respectively. The protein CαRMSD plot indicates that the Apoprotein and the complexes (protein and ligand) have thoroughly followed the equilibrium RMSD value of around 0.17 nm.

Fig. 6 [Images not available. See PDF.]

Graphical representation of the plots showing protein Cα RMSD (nm) versus time (100 ns) for different models

Fig. 7 [Images not available. See PDF.]

Graphical representation of the plots showing ligand RMSD (nm) versus time (100 ns) for Geniposide-3OLS and Estradiol-3OLS complex

The ligand RMSD plot revealed the stability of both Geniposide and Estradiol in their binding to the estrogen receptor during the entire simulation. Geniposide and Estradiol achieved RMSD values of approximately 0.1 nm and 0.025 nm, respectively.

RMSF analysis

Protein RMSF proves valuable for assessing local changes in the protein chain. The multiplot for protein RMSF (nm) versus residue number index is depicted in Fig. 8. Encouragingly, the plot indicates fluctuations of less than 0.15 nm for most of the protein sequence, with the exception of the loop region between ASN407-SER422 in all three cases—Apoprotein, Geniposide-3OLS, and Estradiol-3OLS. Although this loop region exhibits fluctuations of around 0.35 nm, it does not impact ligand binding, as no residue in this region contributes to the ligand's binding cavity. The residues within the binding cavity remain stable with fluctuations of significantly lower magnitude.

Fig. 8 [Images not available. See PDF.]

Graphical representation of the plots showing protein RMSF (nm) versus residue index number of protein for different models

H-bond interaction

Molecular interactions, especially h-bond interactions, are sensitive to distance and angle variations, susceptible to disruption under dynamic conditions. In this study, we scrutinized the ligand–protein interactions in both complexes. Figure 9 presents the plot of the number of hydrogen bonds versus time. Notably, Geniposide exhibited up to three stable h-bond contacts during the simulation, while Estradiol displayed up to two stable contacts.

Fig. 9 [Images not available. See PDF.]

Pictorial representation of the number of h-bond contacts formed by Geniposide and Estradiol in complex with Estrogen receptor (3OLS)

Figure 10 illustrates the %occupancies histogram of h-bond contacts formed by the ligand Geniposide in complex with the Estrogen receptor. The most stable interaction was observed with residue GLU305, exhibiting an occupancy of 114.98%. This indicates that the ligand established multiple stable h-bond contacts with this residue (see Fig. 11), while Geniposide weakly interacted with other residues, including LEU298, LEU339, GLY472, and HIS475, with %occupancies of 3.55, 4.63, 2.57, and 2.16%, respectively. In comparison, the ligand Estradiol interacted with residues HIS475 and GLU305, with occupancies of 72.26% and 42.27%, respectively. Other residues, such as MET295, LEU298, and GLY472, exhibited interactions with occupancies of 2.4%, 2.06%, and 1.01%, respectively. In summary, similar to Estradiol, Geniposide efficiently activates the Estrogen receptor, demonstrating effective binding within the β-ligand domain. Estrogen receptor activation is known to promote vasodilation and exert antihypertensive effects by modulating the renin–angiotensin–aldosterone system [58].

Fig. 10 [Images not available. See PDF.]

Histogram representation of %occupancies of the h-bond protein ligand contacts of Estradiol and Geniposide in complex with Estrogen receptor (3OLS)

Fig. 11 [Images not available. See PDF.]

Representation of multiple h-bond contact by Geniposide with residue GLU305 of Estrogen receptor

Total energy

The total energy profiles obtained from the MD simulations of the Geniposide-3OLS and Estradiol-3OLS complexes offer valuable insights into their stability and energetic characteristics. Across the 100 ns simulation period, both complexes displayed minor fluctuations in total energy, indicating the attainment of a relatively stable equilibrium state (Fig. 12). These fluctuations remained within acceptable ranges, indicative of a well-equilibrated simulation that faithfully captured the dynamic behavior of the complexes.

Fig. 12 [Images not available. See PDF.]

Graphical representation of the total energy (KJ/mol) versus time (100 ns) for 3OLS-Geniposide and 3OLS-Estradiol complex

Discussion

Hypertension is also regarded as the main risk factor for disability-adjusted life years globally, according to the Global Burden of Disease study [2]. Numerous research are focused on development of novel antihypertensive medicines and new therapy options because of the massive prevalence of hypertension, worldwide [4, 5]. Recently, it has been revealed that medicinal herbs can reduce hypertension and can be used empirically as antihypertensive medicines [8, 9]. Diuretics have a significant role in the clinical management of hypertension. These medications influence the renal tubular segments' salt re-absorption to boost kidney urine production [12, 13]. NCC (Sodium chloride co-transporters) and NKCC2 (Na–K-2Cl co-transporter) are the main targets of diuretics. Recent research has demonstrated that the NKCC2-oxidative stress-responsive kinase 1 (OSR1)-with-no-lysine kinase (WNK) signaling pathway is crucial for controlling blood pressure [14]. A Rubiaceae family member *Paederia foetida*, also known as Prasariniin Sanskrit, is a climbing plant with a strong foetid odor. Its Hindi name is Gandhaprasarini, and its English name is Chinese Flower [17]. Various types of phytochemicals are present in the *Paederia foetida*,

among these one Iridoid glycosides known as Geniposide present in the root of this plant [33]. Yang Fu et al. [14] demonstrated that the ability of Geniposide to increase urine production and the excretion of salt and chloride ions. They further revealed that the antihypertensive effects of Geniposide was considered due to their inhibition of the activation of the WNK signaling pathway, which is mediated by the estrogen receptor. So, considering this key finding we performed molecular docking and molecular dynamics (MD) simulations studies of Geniposide and various PDB IDs based on the structure of estrogen receptor. *Paederia foetida* whole plant extract has an antihypertensive action in Amphetamine-induced hypertensive rats which is evident by a considerable decrease in blood pressure. Amphetamine induces decreasing sodium level that can be countered by *Paederia foetida* whole plant extract, whereas sodium &potassium level remains to be same in all the four groups of rats. The goal of the molecular docking study was to examine the binding energy and molecular interactions of Geniposide and Estradiol in the active site of the estrogen receptor, to achieve a strong rational correlation through computational studies. In addition, each compound's binding mechanism was examined for molecular interactions. The results showed that Geniposide had very close significant binding energy compared to the internal ligand Estradiol. The result of ADME study reflects that Geniposide is a favorable compound for good pharmacokinetic features. Along with these features, Geniposides also possess greater flexibility, lesser lipophilicity, and higher solubility when compared to Estradiol. This proved that Geniposide is having better ADME profile than Estradiol. Geniposide is safer in comparison with Estradiol and it is clearly understood from the toxicity radar chart and toxicity table that all the toxicity parameters were inactive except immunotoxicity in case of Geniposide, whereas several toxicity parameters were found active in Estradiol. The molecular dynamics revealed that Geniposide forms stable interactions with key amino acid, GLU305 of β-ligand binding domain of estrogen receptor, suggesting its potential as an activator of estrogen receptor. After overall evaluation of all the data, we can say that Geniposide is a potential drug candidate for the treatment hypertension through the modulation of estrogen receptor.

Conclusion

The present work concludes, *Paederia foetida* whole plant extract has an antihypertensive action in Amphetamine-induced hypertensive rats which is evident by a considerable decrease in blood pressure. Amphetamine induces decreasing sodium level that can be countered by *Paederia foetida* whole plant extract, whereas sodium &potassium level remains to be same in all the four groups of rats. According to the OECD (Organization of economic co-operation and development 425 guidelines) *Paederia foetida*, whole plant extract was safe for administration at 200 and 400 mg/kg. Among the two dosages tested for *Paederia foetida*, whole plant extract 400 mg/kg body weight exhibited the highest considerable antihypertensive effect. Geniposide, a natural moiety found in this plant, is having antihypertensive activity. Docking study was carried out on different PDB ID to find out the potency of the compound as an antihypertensive. Geniposide, an antihypertensive agent present in this plant, is used for in silico study to define its potential as an antihypertensive agent. The ligand structure was docked with different PDB IDs (3OLL, 3OLS, 5DX3, 5DXE &6PIT). The docking studies revlealed the PDB ID: 3OLS to be most suitable in terms of its comparison with the internal ligand, Estradiol. Further molecular dynamics simulation study was carried out to evaluate the binding between Geniposide and estrogen receptor. The findings indicate that Geniposide forms stable interactions with key amino acid, GLU305 of β-ligand binding domain of estrogen receptor, suggesting its potential as an activator of estrogen receptor. Thus, Geniposide can be a useful compound for the treatment of hypertension through the modulation of estrogen receptor.

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Author contributions

Conceptualization, supervision, formal analysis, writing, review and editing were done by Suddhasattya Dey and Arijit Mondal; Ravi Rawat, Dibya Lochan Mohanty, Deeparani Urolagin, Ameeduzzafar Zafar, Padma Charan

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate

The study protocol was approved by the Institutional Animal Ethical Committee of Aditya Bangalore Institute of Pharmacy Education and Research Bangalore, Karnataka, India (approval number: 64/1611/CPCSEA). Female Wistar rats (Wistar strain) weighing between 150 and 200 g were obtained from local vendor, Bangalore, Karnataka. Animals were housed into five groups under standard laboratory conditions, i.e., 25 °C ± 1 °C/45–55% RH and 12/12 h light and dark conditions in the animal house of Aditya Bangalore Institute of Pharmacy Education and Research, Bangalore, Karnataka, India. The rats are kept with free access to food (Hindustan Lever, India).

Consent for publication

Not Applicable.

Competing interests

The authors declare that they have no known competing interests or personal relationship that could have appeared to influence the work report in this paper.

Abbreviations

PDB ID
Protein data bank identifier
SDF
Structure data file
WNK
With-no-lysine kinase
Pdb2gmx
GROMACS program
CHARMM27
Chemistry at Harvard Molecular Mechanics
TIP3P
Transferable intermolecular mechanics potential with 3 points
LINCS
Linear constraint solver
PME
Particle Mesh Ewald
ADME
Absorption distribution metabolism elimination
CPK
Corey–Pauling–Koltun
NPT isobaric
Isothermal ensemble
NVT
Constant volume ensemble
PDBQT
Protein data bank, partial charge (Q), and atom type (T) format

OPLS
Optimized potentials for liquid simulations
BBB
Blood–brain barrier
GI
Gastrointestinal
MR
Molar refractivity
PAINS
Pan Assay Interfering Substances
TPSA
Topological polar surface area
VMD
Visual Molecular Dynamics
ps
Pico seconds
ns
Nanoseconds
K
Kelvin
MD
Molecular dynamics
RMSD
Root-mean-square deviation
RMSF
Root-mean-square fluctuation
nm
Nano meter
p.o.
Orally
Å
Amstrong

Publisher's Note

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DETAILS

Subject:	Simulation; Chloride; Pharmacy; Diuretics; Blood pressure; Phytochemicals; Sodium; Hypertension; Laboratories; Cardiovascular disease; Antihypertensives; Potassium; Estrogens; Kinases; Amphetamines; Oxidative stress
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Investigation of the antidiabetic and probiotic properties of lactic acid bacteria isolated from some ethnic fermented foods of Darjeeling District

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ABSTRACT (ENGLISH)

Background

Indigenous communities residing in the Darjeeling Himalayan region and its adjacent hilly areas have a deeply rooted cultural tradition of consuming a diverse range of vegetable and milk-based fermented products, believed to confer various health advantages. With this traditional knowledge, lactic acid bacteria (LAB) were isolated from popular fermented foods such as Chhurpi (derived from *Bos grunniens* milk), Gundruk (made from *Brassica juncea* leaves), Sinki (derived from *Raphanus sativus* taproots), and Kinema (produced from *Glycine max* beans). This study aimed to investigate the probiotic properties of the prevalent LABs, including aggregation properties, bile salt hydrolase activities, survival under gastro-inhibitory conditions, safety evaluations, and their potential health-promoting attributes, with a specific focus on inhibiting α-amylase and α-glucosidase enzymes.

Results

Five of the LAB isolates demonstrated notable viability rates exceeding 85% when exposed to gastro-inhibitory challenges. Based on 16S rRNA gene sequencing, these isolates were identified as *Pediococcus pentosaceus* (isolate GAD), *Lactobacillus plantarum* (isolates KAD and CAD), *Lactobacillus brevis* (isolate SAD), and *Lactiplantibacillus plantarum* (isolate CMD). These LAB isolates exhibited versatile carbon source utilization, significant auto- and co-aggregation, and bile salt hydrolase (BSH) properties. Auto-aggregation capacity notably increased over time, ranging from 30 to 150 min, with percentage increments from 4.83 ± 1.92% to 67.60 ± 5.93%. *L. brevis* SAD displayed the highest co-aggregation increment (%) against *Staphylococcus aureus*, while *L. plantarum* KAD demonstrated potent antimicrobial activity. *In vitro* analyses postulated potential health benefits related to antidiabetic properties, particularly inhibiting α-amylase and α-glucosidase enzymes. *L. brevis* SAD exhibited the highest α-glucosidase inhibitory activity, while *L. plantarum* KAD displayed the most potent α-amylase inhibitory activity. Comprehensive safety assessments, including antibiotic susceptibility profiling, hemolytic activity evaluation, and *in vivo* acute toxicity studies, confirmed the suitability of these LAB isolates for human consumption.

Conclusions

The isolates show promising probiotic characteristics and significant potential in addressing metabolic health. These results carry substantial scientific implications, suggesting the pharmaceutical-based applications of these traditional fermented foods. Further *in vivo* investigation is recommended to fully elucidate and exploit the health benefits of these LAB isolates, opening avenues for potential therapeutic interventions and the development of functional foods.

FULL TEXT

Background

The beneficial effect associated with consuming fermented food products containing probiotic microorganisms is conventionally recognized. This has intrigued researchers to isolate and characterize novel probiotics from diverse sources. Fermented foods function as rich repositories of probiotics, rendering them valuable as dietary supplements for health benefits. Certain ancient societies have a longstanding tradition of incorporating fermented foods into their diets, and some are still produced through traditional methods [1, 2]. The microbial fermentation of raw ingredients in these foods facilitates the production of highly nutritious components, including bacterial metabolites, which are believed to confer health-promoting effects [3].

Research on identifying and characterizing new probiotic isolates and developing probiotic supplements with health-enhancing properties has gained increasing significance, especially in metabolic disorders such as diabetes. One strategy to mitigate the glucose load involves the delayed metabolism of carbohydrates, leading to a more balanced glucose profile. Studies have shown that inhibiting α-glucosidase and α-amylase enzymes can delay glucose absorption, resulting in a gradual increase in postprandial blood glucose levels [4, 5]. Notably, certain *Lactobacillus* spp. have demonstrated effective inhibition of α-glucosidase and α-amylase enzyme activity in vitro [6].

In the sub-Himalayan regions of West Bengal, India, particularly in the Darjeeling district, various fermented dishes

are regularly consumed [7]. Several communities residing in the Darjeeling district and neighboring hilly areas of northern West Bengal traditionally prepare and consume different fermented milk and vegetable-based dishes. Examples include “Sinki” which is fermented radish (*Raphanus sativus*) taproot, “Gundruk” which is fermented leaves of certain *Brassica* species (*Brassica juncea*), “Kinema” which is fermented soybean (*Glycine max*) seeds, and “Chhurpi” which is a fermented cheese product made from the milk of Himalayan *Bos grunniens*. These preparations are reported to foster the growth of LAB isolates [8, 9]. However, further research is necessary to elucidate the antidiabetic, probiotic, and safety attributes of the LABs.

In this study, LAB strains from some popular ethnic fermented foods of Darjeeling have been isolated, and their *in vitro* antidiabetic and probiotic potentials, along with safety aspects, have been investigated.

Methods

Sample collection, processing, and isolation of LAB

Fermented food samples were collected from two different villages in the Darjeeling district of West Bengal, India, during March and April 2022. Chhurpi, a dairy-based fermented product, was collected from Tukdah Forest village, while soft Chhurpi and vegetable-based fermented preparations, namely Gundruk, Kinema, and Sinki, were collected from Bijanbari village. The samples were collected separately in sterile 50mL centrifuge tubes and transported to the laboratory in thermally insulated ice boxes to maintain their integrity. Standard procedure was followed for the collection of the samples [10].

Similar to the traditional processing for consumption, Kinema, Gundruk, and Sinki were mixed with distilled water and incubated at 37 ℃ for 20 min. Then, 200µL sample from these liquid mixtures was taken and added to separate De-Man, Rogosa, and Sharpe (MRS) (Hi-Media, India) broth and incubated at 37 ℃ for 48 h. Hard and soft Chhurpi was directly used as inoculum for MRS broth and incubated following the same process. After 48 h of incubation, all the inoculated broths were serially diluted up to 10^{-7} in phosphate buffer saline (PBS) (SRL, India) and spread on MRS agar plates separately. These plates were then incubated at 37 ℃ for 48 h. Plates with a suitable number of colonies (neither too many nor too few) were selected, and their colony numbers were counted in terms of colony-forming units (CFU). Bacterial load of all the food samples was calculated in CFU per milliliter (mL) or CFU per gram (g) unit. Colonies with distinct physical characteristics were then selected and streaked onto fresh MRS plates to make single colonies and to isolate pure cultures.

The isolated pure colonies were then transferred to MRS agar slants for experimental use and stocked in 40% glycerol for storage. The isolates were named based on the source (C for Chhurpi, G for Gundruk, K for Kinema, and S for Sinki), the month of collection (M for March, A for April), and the district of collection (D for Darjeeling), followed by sequential numbers to represent each isolate from each source. The slants were stored at 4 ℃ and sub-cultured every 15 days, not exceeding 2 passages (the transfer of organisms from an established culture to a fresh medium), while glycerol stocks were stored at – 20 ℃ and restocked every 2 months (not exceeding 5 passages) [11].

Preliminary screening of the isolates based on gastro-inhibition tolerance activity

In order to identify the most resilient LAB from the initial isolates, the isolates were exposed to conditions resembling the harsh environment of the human gastrointestinal (GI) tract. These conditions included lysozyme-mediated degradation, acidic pH, and high bile concentration [12]. After the preliminary screening, tolerance to alkaline pH was also evaluated for the selected strains.

To assess lysozyme tolerance, a standard protocol was followed [13]. Briefly, overnight-grown bacterial cells were subjected to lysozyme tolerance testing. The cells were harvested, washed, and resuspended in Ringer's solution. A suspension of 10 µL was inoculated into a sterile electrolyte solution containing lysozyme at a concentration of 100 mg/L (SRL, India). A control broth without lysozyme was also prepared. The samples were then incubated at 37 °C, and after 2 h, the viable cell count was determined as a measure of lysozyme tolerance (in terms of Log CFU/mL).

For the analysis of acid and bile tolerance, standard protocols with certain experimental adjustments were followed [14, 15]. To assess acid tolerance, the isolates were cultured in acidic MRS broths (Test) adjusted to pH 3.0 with

1 M hydrochloric acid, alongside control MRS broth with normal pH. Here, a direct endpoint method was employed. This involved determining the survival percentage by comparing the bacterial count (expressed as log CFU/mL) in the control broth (not subjected to acidification) with that in the test broth (acidified to pH 3.0). For the experimental assessment of bile, acid, and lysozyme tolerance, *L. plantarum* MCC 2156 served as the positive reference strain [16]. The assessment was performed after a fixed incubation time of 3 h, utilizing an overnight-grown culture as the inoculum at a concentration of 1% V/V. Similarly, for the bile tolerance assay, a test broth containing 2% (w/v) deoxycholic acid (SRL, India) was used, and again, Log CFU/mL values of the 'Test' broth were compared with those of the 'Control' broth involving the same incubation parameters. The survivability of the isolates was calculated by comparing the Log CFU/mL values of the 'Test' and the 'Control' broths, and the survivability for each isolate was expressed as 'Percent Survival' (% Survival) using the following formula:PercentSurvival(%Survival)=LogCFUpermL ofTextLogCFUpermLofControl×100

Isolates showing survival values≥85% for the GI conditions (lysozyme, acid, and bile tolerance) were selected for further analysis. The chosen cut-off value of "85%" was selected because scoring more than 85% of all three parameters creates a probability of success (85%×85%×85%) and reinforces the strains' potential to thrive in the harsh GI environment, making them promising candidates for probiotic use.

After preliminary screening, survival of the selected isolates in alkaline pH was further assessed, taking alkaline-tolerant *Escherichia coli* K12 strain as control. Briefly, broths with pH levels of 8.5, reflecting the maximum pH detected in the human gastrointestinal tract [17], were prepared alongside control broths with unaltered pH. Inoculums were incubated for 12 h at 37 °C, and the % OD change of the individual broths was measured at 600 nm in a spectrophotometer (Shimadzu UV-1900I, Japan) indicating the extent of growth inhibition due to alkaline pH.

Molecular identification of the LAB isolates

The selected LAB isolates were identified by analyzing the partial sequences of their 16S rRNA genes through molecular phylogeny. The genomic DNA of each isolate was separately extracted following standard protocols outlined by De et al. [18]. The partial 16S rRNA gene was then amplified using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′) as described in a previous study by Frank et al. [19]. For amplification, reaction mixture was prepared using reagents from Promega (USA) and Eurofins Genomics, India (primers) following the manufacturer's instructions. Thermal cycling was carried out in a Mastercycler® Nexus GX2 thermal cycler (Eppendorf instrumente gmbh, Germany). The PCR products were visualized on an ethidium bromide-agarose gel under a UV transilluminator (Hi-Media, India). The PCR products were purified and sequenced using Sanger's method at Heredity Biosciences, India. The resulting sequences were aligned in the NCBI-BLAST (https://blast.ncbi.nlm.nih.gov/blast.cgi) for similarity analysis and to infer functional and evolutionary relationships between sequences. The raw sequences obtained were trimmed and aligned using the MEGA 11 version 11.0.13 package with similar sequences retrieved from the database for drawing the phylogenetic relationship [20]. Finally, the curated sequences were deposited in the GenBank (https://www.ncbi.nlm.nih.gov/websub/) for public access. Phylogenetic tree and evolutionary relationships were constructed using the neighbor-joining method in the MEGA 11 (v. 11.0.13) package, with *Leuconostoc gasicomitatum* strain Tb1-10 serving as the outgroup [20]. To assess the accuracy of the tree topologies, bootstrap analysis with 100 repetitions was performed [21].

Assessment of probiotics properties

Carbohydrate fermentation profiling and esculin hydrolysis

A good probiotic strain should have the ability to utilize multiple carbohydrate sources, which ensures its survival in the host's digestive tract [22]. To identify the patterns of sugar fermentation, and esculin hydrolysis of the LAB isolates, a standard carbohydrate fermentation kit (Hi-Lacto Identification Kit, KB020-10KT, Hi-Media, India) was used. For the assessment, 50 µL of overnight-grown cultures was aseptically applied to each of the specific designated wells of the kit by surface inoculation method, and then, the kits were again sealed off and incubated at 37 °C for 24 h, and after the incubation, the results were interpreted as per the instruction manual (as positive, + or negative, −).

Auto-aggregation assay

Modified version of the auto-aggregation assay developed by Del Re et al. [23] was used to test the capacity of the isolated LAB isolates to auto-aggregate. Briefly, 2 mL of bacterial suspension from overnight-grown cultures was vortexed, and then, the mixture was allowed to stand in static condition at 37 °C, and at regular intervals (30 min, 60 min, 90 min, 120 min, and 150 min) 100 µL of liquid from the upper surface from each bacterial culture was taken and combined with 900 µL of PBS (0.1 M), and then, the absorbance was measured using a microplate reader (SPECTROstarNano, BMG Labtech, Germany) at 600 nm (A_{600}). The auto-aggregation (%) of all the LAB isolates was then calculated by the formula:Auto-aggregation(%)=A0-ATA0×100where A_0 is A_{600} at 0 h and A_T represents the A_{600} of cell suspension at different time intervals (30 min, 60 min, 90 min, 120 min and 150 min). For comparison and analysis, the percentage value of each time frame was compared with the previous time frame for individual LAB isolates.

Co-aggregation assay

For co-aggregation assay, four commonly used laboratory strains were used as control. These were *Escherichia coli* K12 ATCC® 29425™, *Pseudomonas aeruginosa* CCEB-481 ATCC® 10145™, *Staphylococcus aureus* NCTC-8532 ATCC® 12600™, *Bacillus cereus* CCM-2010 ATCC® 14579™. For assessing the co-aggregation properties, standard protocols were followed with some modifications [24]. For this assay, overnight cultures of LAB isolates were mixed with the overnight cultures of the above-mentioned laboratory strains in equal volumes to form a cell suspension of a definite bacterial count (10^9 CFU/mL). The mixed suspensions were then incubated at 37 °C for 2 and 8 h. 2 mL of pure bacterial suspensions (for each bacterium) was taken as control. After the defined time duration, the optical density was measured at 600 nm. The following formula calculated the percentage values of co-aggregation of all the LAB isolates:Co - aggregation(%)=100×ALS+ALAB2-AmixALS+ALAB2where A_{LS} and A_{LAB} stand for the OD_{600} of the pure cultures of the laboratory strains (LS) and LAB (LAB) isolates, respectively. A_{mix} is the OD of the mixed suspension different LAB and LS. Percent co-aggregation of the 8th h was compared with that of the 4th h (for each LAB to each LS).

For the auto- and co-aggregation assays too, *L. plantarum* MCC 2156 served as the reference strain [16] for comparing the performance of the isolated LABs.

Bile salt hydrolase activity (BSH)

One of the most potent health-promoting features of any LAB is the BSH activity, which determines its ability to break down conjugated bile salts [25]. For the assessment of BSH activity, one of the standard protocols laid down by Hernández-gómez et al. [26] was used with few modifications. Briefly, bile salt (Oxbile, Hi-Media) and $CaCl_2$ were added to MRS agar at 0.5% (w/v) and 0.37 g/L, respectively. Sterile disks made from filter paper were spot inoculated with 10 µl of overnight-grown LAB cultures in MRS broth, and finally, the disks were laid over modified MRS agar plates and then incubated at 37 °C for 48 h (to give ample amount of time for the deposition of detectable amount of precipitate). It was considered positive when bile acid precipitations started to form surrounding the disk in a diffused manner.

Cell surface enzyme characteristics

Extracellular enzyme activities like proteolytic, lipolytic, and amylolytic properties were evaluated following standard protocol [27].

For proteolytic property assessment, skim milk powder (SRL, India) was dissolved in 100 mL of bacteriological agar media to create skim milk agar plates. Then fresh overnight cultures of LAB isolates were inoculated onto skim milk agar plates, which were then incubated for 24 h at 37 ℃. The bacteria showing translucent haloes around the colonies were thought to have proteolytic properties.

Tributyrin supplement (SRL, India) was employed as a source of lipids to assess extracellular lipase activity. Tributyrin was autoclaved at 120 ℃ for 15 min. It was then diluted by 1/100 mL and added to Tributyrin Supplement Agar (TSA; SRL, India). The resultant colloidal medium was plated, allowed to dry, streaked with the LAB isolates, and then incubated for 24 h at 37 °C. Positive result was interpreted by the presence of a clear zone and the emergence of broken lipid droplets around the colonies.

For amylolytic activity assessment, 1 g starch is added in 100 mL of sterile nutrient agar media (SRL, India) and autoclaved at 121 ℃ for 15 min. The starch agar plates were streaked with cultures of the LAB isolates, incubated for 24 h at 37 °C, and after that, the plates were flooded with 1% iodine solution. Clear zones around the streaked lines indicated the presence of extracellular amylase enzyme, while the absence of such zones indicated negative results.

Antimicrobial properties

In order to check the antimicrobial activity, indicator strains, viz. *E. coli* K12ATCC® 29425™, *E. coli* HB101 ATCC® 33694™, *P. aeruginosa* CCEB-481 ATCC® 10145™, *S. aureus* NCTC-8532 ATCC® 12600™, *B. cereus* CCM-2010 ATCC® 14579™, were used and the experiment was conducted following a standard protocol [15] with experimental modifications. The overnight cultures of LAB isolates were centrifuged for 5 min at 8000×*g* (Eppendorf 5430R, Eppendorf Instrumente GmbH, Germany), the cells were pelleted out, and the cell-free supernatant was utilized to assess the antibacterial activity. A sterile borer was used to make 6-mm-diameter wells in Muller Hinton Agar (MHA) plates (SRL, India) that had been surface inoculated with overnight-grown culture suspensions of the indicator organisms (100 mL). Each well received 150 mL of cell-free supernatant of all LAB isolates, after which the plates were kept at 37 °C for a day and checked for zones of inhibition (ZOI). Results were interpreted as no inhibition = −, ‘+’ = ZOI: 1–3 mm; ‘++’ = ZOI: 3–8 mm; ‘+++’ = ZOI: ≥ 8 mm (the ZOI was calculated after subtracting the well diameter size, i.e., 6 mm).

Cell surface hydrophobicity (CSH) assessment

One of the most commonly used methods to evaluate CSH activity is to find the hydrocarbon attachment propensities of the bacteria in question. CSH of the LAB isolates was determined using a standard protocol [28] with certain modifications. Briefly, after 24 h of incubation bacterial cultures were washed twice in PBS and then resuspended to a concentration of ~ 10^9 CFU/mL, and the initial absorbance at 600 nm value was determined (A_0). A two-phase system was then created by adding 3 mL of cell suspension with 1 mL of solvent (Xylene, Ethyl acetate, Acetone). It was then vortexed for 2 min. After 20 min of incubation, the aqueous phase was removed, and its absorbance at 600 nm was measured (A_1). CSH or ‘% adhesion’ or hydrophobicity was calculated according to following formula:CSHorAdhesion(%)=A0-AaA0×100where A_0 is the OD_{600} of bacterial culture before solvent mixing and A_1 is the OD_{600} of bacterial culture after solvent mixing.

In vitro antidiabetic assessment

Partial or complete inhibition of carbohydrate-hydrolyzing enzymes determines the glucose-lowering activity of any LAB isolates because it reduces the amount of available free glucose in peripheral circulation.

α -amylase inhibitory activity

The ability of the isolated LAB strains to inhibit α-amylase was assessed following standard protocol [29]. Briefly, 250 µL of cell-free supernatant of each overnight LAB culture was mixed with 250 µL of 0.5 mg/mL α-amylase (Merck, India) solution and then incubated for 10 min at 25 °C. After that, 250 µL of starch solution (1% w/v in 0.02 M sodium phosphate buffer) was added to the reaction mixture and again incubated at 25 °C for 10 min. The reaction was then terminated with the addition of 500 µL of 3,5-dinitrosalicylic acid (DNS) color reagent (Merck, India) (96 mM DNS and 5.31 M sodium potassium tartrate in 2 M sodium hydroxide solution). The reaction mixture was heated for 5 min, cooled at room temperature, and diluted four times, and then, the absorbance was measured at 540 nm. The reaction mixture without bacterial supernatant was taken as a control. The inhibition propensity (%) was calculated according to the following formula:where A_C is the absorbance of the control reaction mixture and A_B is the absorbance of the reaction mixture with bacterial supernatant.α-amylaseinhibitoryactivity(%)=(AC-AB)AC×100

α -glucosidase inhibitory activity

The α-glucosidase inhibition activity was carried out following standard protocol [30]. Briefly, 25 µL of cell-free supernatant from each overnight LAB culture was mixed with 150 µL 0.01 M of potassium phosphate buffer (pH 6.8) and incubated for 10 min. To this mixture, 50 µL of α -glucosidase (Merck, India) enzyme was added and again incubated for 15 min at 37 °C. Then to this reaction mixture, 75 µL of 5 mM p-nitrophenol-D-glucopyranoside (pNPG) (Merck, India) substrate was added and incubated for 30 min at 37 °C; the enzymatic reaction was stopped with the

addition of 1 mL of 0.1 M Na_2CO_3. The absorbance of the reaction mixture was measured at 540 nm. The reaction mixture without bacterial supernatant was taken as a control. The inhibition propensity (%) was calculated according to the following formula: α-glucosidaseinhibitoryactivity(%)=1-ABAC×100 where A_C is the absorbance of the control reaction mixture and A_B is the absorbance of the reaction mixture with bacterial supernatant.

Several strains *of L. plantarum* have been documented for their significant anti-diabetic properties, alongside notable *in vitro* inhibition of α -amylase and α -glucosidase enzymes. Hence, *L. plantarum* MCC 2156 has been employed as a reference strain to validate the experimental protocols and to assess the performance of the isolated LABs in these assays [31, 32].

Safety assessment

Antibiotic susceptibility profiling

Antibiotic susceptibility of a potent probiotic is linked with host safety. Antibiotic susceptibility of the selected isolates was carried out on MHA plates using antibiotic discs (Hi-Media Combi IV Octadisc, Hi-Media, India) of standard antibiotics, ampicillin (amp) 10 µg, cephalothin (cep) 30 µg, chloramphenicol (c) 30 µg, clindamycin (cd) 2 µg, erythromycin (e) 15 µg, gentamicin (gen) 10 µg, oxacillin (ox) 1 µg, vancomycin (va) 30 µg, streptomycin (str) 10 µg, kanamycin (kana) 15 µg, and tetracyclin (tet) 20 µg standard antibiotics. The results obtained were interpreted according the standard methods [33]. Briefly, the obtained ZOI diameter values were grouped into 2 subsection, "+" (having ZOI≤8) and "−" (having ZOI≥8) and the result was interpreted in a table.

Hemolytic activity

The hemolytic activity of bacteria is its capacity to degrade red blood cells and release hemoglobin, and it is required for the safety assessment of a putative probiotic. For this assay, standard protocol with slight modification was used [34]. Briefly, overnight cultures of LAB isolates were spread onto Blood Agar Plates (tryptone soy agar plate containing 5% human blood) and incubated for 24 h. The plates were then examined for patterns of hemolysis that is whether complete (beta), partial (alpha), or absence of visible (gamma) destruction of RBCs indicated [35] by a clear zone around the colonies. *E. coli* K12 was taken as positive hemolytic indicator strain to define the experiment's accuracy.

In vivo acute toxicity test

To ensure the safety of the LAB (probiotic isolates), acute oral toxicity study was conducted on Swiss albino mice (20±2 g) aged 6–8 weeks. The mice were sourced from a licensed animal dealer (Chakraborty Enterprise, Kolkata, India; Regd. No. 1443/PO/Bt/s/11/CPCSEA) and housed in controlled conditions with specific temperature, humidity, and light–dark cycle. They were acclimatized for two weeks in the animal house keeping them in cages and with free access to pellet diet and water. The study was approved by the Institutional Animal Ethical Committee (IAEC) of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) of the University as per rules, under the animal ethical approval number IAEC/NBU/2022/27. The mice were randomly divided into six groups a vehicle group and five groups with dietary LAB supplementation. Each group received a single dose of bacterial suspension (6×10^9 CFU/Kg body weight). Observations of various parameters such as aggression, food and drink intake, sedation, diarrhea, fur loss, and lethargy were recorded at regular intervals over 2 weeks. Particular attention was given to identifying any late signs of toxicological effects within the first four hours of each day. The investigation followed the OECD guideline for acute oral toxicity testing [36, 37].

Statistical analysis and graphical interpretation

All *in vitro* experiments were repeated three times. The data were represented by the replicate value's mean and standard deviation. GraphPad prism software package Version 8.0.2 was used for statistical analysis for data normality (Shapiro–Wilk test), statistical differences (ANOVA), multiple-comparison post hoc testing (Dunnet's or Tukey's test for significance test), and data visualization. $p \leq 0.05$ were considered significant.

Results

Isolation and screening of the isolates for tolerance to gastro-inhibitory conditions

Colony counts with respect to the weight of the source sample led to the enumeration of bacterial load in the food samples. In Gundruk it was 3.83×10^7 CFU/g, in Kinema it was 6.52×10^8 CFU/g, in Soft Chhurpi it was 1.65×10^7

CFU/g, in Hard Chhurpi it was 1.51×10^7 CFU/g, and in Sinki it was 6.03×10^8 CFU/g. A total number of 20 isolates were obtained from different food sources. From Gundruk, 4 isolates, from Kinema 4 isolates, from Soft Chhurpi 6 isolates, from Sinki 4 isolates, and from Hard Chhurpi 2 isolates were acquired. All the 20 isolates were subjected to screening based on their gastro resistant properties. The results are depicted in Fig. 1.

Fig. 1 [Images not available. See PDF.]

Lysozyme, acid, and bile tolerance (in terms of % survival) of 20 bacterial isolates from fermented foods of Darjeeling

Isolates GAD1, KAD3, CAD3, SAD1, and CMD1 showed ≥ 85% survival in all the tolerance tests involving lysozyme, acid, and bile. These five isolates have no significant differences in comparison with the control strain for the gastro-inhibitory (bile, acid and lysozyme tolerance) tests and their influence on host body weight (refer Additional file 1: Fig. S1 and S2 for the results of gastro-inhibitory parameters and influence on host body weight respectively). For tolerance to alkaline pH, four of these isolates except CAD3 showed noteworthy reduction in survival rates compared to the reference *E. coli* K12 strain (refer Additional file 1: Fig. S3 for the results of alkaline tolerance of the isolates). These five isolates, subjected to further analysis were subsequently named as GAD, KAD, CAD, SAD, and CMD for easy understanding.

Molecular identification and phylogenetic analysis

The concentration of the isolated DNA from the five isolates was 44 µg/mL. 16S rRNA gene amplicons of the five isolates were compared to those of the strains listed in GenBank using NCBI-BLAST. Isolate GAD (Acc. No. OQ306562) showed highest percent identity (99.05%) with *Pediococcus pentosaceus* isolate 4412 (Acc. No. MT544941.1); isolate KAD (Acc. No. OQ306564) showed highest percent identity (98.18%) with *Lactobacillus plantarum* strain 6415 (Acc. No. MT515856.1); isolate CAD (Acc. No. OQ306566) showed highest percent identity (99.39%) with *L. plantarum* strain 1887 (Acc. No. MT597711.1); isolate SAD (Acc. No. OQ306549) showed highest percent identity (99.45%) with *L. brevis* isolate gp71 (Acc. No. KM495920.1); and isolate CMD (Acc. No. OQ306547) showed highest percent identity (99.52%) with *Lactiplantibacillus plantarum* strain thankcomeLP1 (Acc. No. MZ045749.1).

A phylogenetic tree constructed using the neighbor-joining approach with a bootstrap test (100 repetitions) depicts the proportion of duplicate trees in which the linked taxa clustered together (Fig. 2).

Fig. 2 [Images not available. See PDF.]

Phylogenetic tree demonstrating the evolutionary relationship of isolated five LAB strains from fermented foods of Darjeeling, as inferred by the neighbor-joining approach based on the 16S rRNA gene sequences in MEGA11. The evolutionary distances were computed using the Maximum Composite Likelihood method. This analysis involved 37 nucleotide sequences and 1551 positions

Probiotic assessment

Carbohydrate fermentation pattern and esculin hydrolysis

All the isolates could ferment carbohydrate sources present in the kit, viz. xylose, cellobiose, arabinose, maltose, galactose, mannose, mellibiose, raffinose, sucrose, and trehalose. For esculin hydrolysis, all showed positive properties.

Auto-aggregation assay

Auto-aggregation values of all five LAB isolates increased significantly starting from 30 min up to 150 min (from 4.83 ± 1.92 to 67.60 ± 5.93%). Most isolates tended not to collapse after forming clumps during the incubation period. Longer incubation times resulted in higher and significant auto-aggregation percentages (Fig. 3a). *L. plantarum* strain KAD showed highest percent aggregation amongst these five isolates. Overall, it can be seen that auto-aggregation values of all the LAB isolates increased significantly in each successive time frame after initial dissociation.

Fig. 3 [Images not available. See PDF.]

Aggregation properties of isolated five LAB strains. **a** Auto-aggregation measured at successive intervals of 30 min reveal significant increase in all strains (adjusted *p* value ≤ 0.0001). **b** Heatmap showing co-aggregation properties of the strains against known intestinal commensals. Key: A, GAD; B, KAD; C, CAD; D, SAD; E, CMD; 1, *E. coli* K12; 2, *P. aeruginosa*; 3, *S. aureus*; 4, *B. cereus*

Co-aggregation assay

All the five LAB isolates have shown the ability to co-aggregate with the intestinal commensal strains tested. Highest percent co-aggregation at 2nd hr was seen in the case of CMD (39.55 ± 1.16%) with *E. coli* K12, and the least was seen in the case of SAD (9.35 ± 4.80%) with *S. aureus*., whereas in the case of the 4th hr, the isolate SAD showed the highest co-aggregation percentage (84.76 ± 0.44%) with *E. coli* K12, and during the same time frame, the least percent co-aggregation value was seen in the case of CMD (23.50 ± 2.22%) with *B. cereus*. Maximum percent co-aggregation increment (2nd–4th h) is seen in association with *S. aureus* with the isolates KAD (18.96 ± 6.46–66.74 ± 0.37%), CAD (9.97 ± 0.74–70.26 ± 1.05%) and SAD (9.35 ± 4.80–71.21 ± 1.82%) (Fig. 3b).

The five LABs exhibited no significant overall variances compared to the positive strain for the auto-aggregation and co-aggregation properties (Additional file 1: Fig. S1). However, in three specific instances (% auto-aggregation at 90, 120, and 150 min), positive strain yielded higher values.

Bile salt hydrolase (BSH) activity

All five LAB isolates displayed BSH-positive results, which was consistent with the isolates' earlier documented bile salt tolerance results. SAD showed the highest precipitation zone (6.66 ± 2.51 mm), while CMD showed the lowest (2 ± 1 mm). All of the other isolates displayed values that are more or less in the middle, falling between 2.33 mm and 3.66 mm (Fig. 4a, b).

Fig. 4 [Images not available. See PDF.]

Bile Salt Hydrolysis (BSH) assay of isolated five LAB strains **a** MRS medium containing bile salt showing BSH activity of the LAB strains as evidenced by the development of a precipitation zone around the colony. **b** Graphical representation of the BSH activity of isolated five LAB strains. The diameters of the precipitation zones are given in millimeters (excluding the diameter of the disc)

Cell surface enzyme characteristics

The five LAB isolates displayed cell surface enzyme features to varied degrees (Table 1). GAD displayed the highest levels of proteolytic activity; CAD and SAD showed highest lipolytic activities; GAD and KAD showed highest amylolytic activities. None of the LAB isolates showed positive results in all the three parameters.

Table 1. Cell surface enzyme characteristics and antimicrobial profiling of isolated five LAB isolates

Isolates	Cell surface enzyme characteristics			Antimicrobial profiling against pathogens				
Proteolytic activity	Lipolytic activity	Amylolytic activity	*E. coli* K12	*E. coli* HB101	*P. aeruginosa*	*S. aureus*	*B. cereus*	GAD
+++	−	++	++	++	−	++	++	KAD
++	−	++	+	++	+	++	++	CAD

−	++	−	+	++	+	+	++	SAD
−	++	−	−	+++	+	++	++	CMD

[a]'+++' = highly positive; '++' = moderately positive; '+' = weekly positive; '−' = negative

[b]'−' = no inhibition; '+' = ZOI: 1–3 mm; '++' = ZOI: 3–8 mm; '+++' = ZOI: ≥ 8 mm

Antimicrobial properties

In the case of antimicrobial properties, it was seen that all the five LAB isolates showed the highest antimicrobial properties against *E. coli* HB101, whereas the least antimicrobial activity against *P. aeruginosa.* Among LAB isolates, KAD and CAD showed antimicrobial activity against all the strains tested. The complete absence of antimicrobial activity was seen in the case of GAD (against *P. aeruginosa*), SAD (against *E. coli* K12), and CMD against (*P. aeruginosa*) (Table 1).

Cell surface hydrophobicity (CSH) assessment

Microbial adhesion to solvents (MATS) serves as an illustration of the association between surface hydrophobicity and adhesion nature. The highest hydrophobicity (adhesion/CSH %) for all the LAB isolates was seen in the case of ethyl acetate, followed by acetone and then by xylene. On the other hand, among all the isolates tested with all the solvents, isolate KAD showed the highest adhesion/CSH % (75.15 ± 0.57%) with ethyl acetate, whereas the lowest was seen in the case of CMD (4.53 ± 3.64) with ethyl acetate. Overall, on the basis of solvents, SAD showed the highest adhesion/CSH % with xylene (28.97 ± 13.96%), isolate KAD with ethyl acetate (75.15 ± 0.574%), and isolate GAD with acetone (41.87 ± 3.56) (Fig. 5).

Fig. 5 [Images not available. See PDF.]

Graph showing cell surface hydrophobicity (Adhesion/CSH %) of isolated five LAB strains with various solvents

In vitro antidiabetic assessment

α-amylase and α -glucosidase inhibitory activity

In the present study, it was found that the α-amylase inhibitory activity for all five isolates ranged from 53 to 63%. Where KAD showed highest inhibition (63.16 ± 1.91%) followed by isolate SAD (62.45 ± 0.53). Isolates GAD and CAD showed similar inhibitory pattern (58.42 ± 0.41% and 58.38 ± 1.64%, respectively). The least inhibition was shown by isolate CMD (53.37 ± 1.47%) (Fig. 6a). In the case of α -glucosidase inhibitory activity, it was seen that the values ranged from 34 to 39%. The highest inhibition was seen in the case of isolate SAD (39.75 ± 2.78%) followed by isolate CMD (37.30 ± 2.40%); isolate GAD and KAD showed similar pattern of inhibition (36.54 ± 3.50% and 35.47 ± 2.06%, respectively). The least activity was seen in the case of isolate CAD (34.86 ± 2.78%) (Fig. 6b).

Fig. 6 [Images not available. See PDF.]

α-Amylase and α -glucosidase inhibitory activity of isolated five LAB strains

All the five LABs exhibited negligible variances in the % inhibition in α-amylase and α-glucosidase activities compared to the positive strain (Additional File 1: Fig. S1).

Safety assessment

Antibiotic susceptibility profiling and hemolytic activity

All the selected LAB isolates showed varying degrees of susceptibility against a panel of standard antibiotics. Isolate-wise resistance was observed against certain antibiotics. Isolate GAD showed resistance against gentamicin, vancomycin, streptomycin, and kanamycin; isolate KAD showed resistance against erythromycin, oxacillin, vancomycin, streptomycin, kanamycin; isolate CAD showed resistance against all antibiotics except oxacillin and tetracycline; isolate SAD showed resistance against chloramphenicol, clindamycin, streptomycin, and tetracycline; isolate CMD showed resistance against ampicillin, cephalothin, oxacillin, vancomycin, kanamycin, and tetracycline

(Table 2; Additional file 1: Table S1 for the antibiotic susceptibility assessment of LABs in terms of zone of inhibition).

Table 2. Antibiotic susceptibility assessment of the isolated five LAB isolates

Isolate	Antibiotics[a,b]										
Amp	Cep	C	CD	E	Gen	Ox	Va	Str	Kana	Tet	GAD
−	−	−	−	−	+	−	+	+	+	−	KAD
−	−	−	−	+	−	+	+	+	+	−	CAD
+	+	+	+	+	+	−	+	+	+	−	SAD
−	−	+	+	−	−	−	−	+	−	+	CMD

[a] *Amp* ampicillin; *Cep* cephalothin; *C* chloramphenicol; *CD* clindamycin; *E* erythromycin; *Gen* gentamicin; *Ox* oxacillin; *Va* vancomycin; *Str* streptomycin; *Kana* kanamycin; *Tet* tetracycline

[b] "+" denotes resistance &"−" denotes susceptibility

In case of hemolytic activity, all the five LAB isolates displayed a pattern of gamma hemolysis which indicates lack of hemolysis as in this case there should be no reaction in the surrounding medium, whereas the *E. coli* K12 strain showed beta hemolysis (Fig. 7).

Fig. 7 [Images not available. See PDF.]

Hemolytic activity assessment of isolated five LAB strains (compared with hemolysis-positive *E. coli* K12)

In vivo acute toxicity test

After oral treatment of a predetermined high dosage of bacterial suspension (6×10^9 CFU/Kg body weight) of each isolate to separate male and female mice, there was no mortality or side effects noted during the first 24 h. During the subsequent 14-day observation period in each group, there was no mortality or deviation in the way the animals appeared or behaved or changes in their feeding habits or body weight gain (Additional file 1: Fig. S2).

Discussion

All five LAB isolates have primary probiotic attributes like gastro-tolerant activities, tolerance to alkaline pH which may enable them to survive in the harsh intestinal microenvironment [38]. Their ability to utilize multiple carbon sources enhances their persistence in conditions like food deprivation or dietary changes. Furthermore, the capacity of LAB isolates to auto and co-aggregate with similar and other bacterial strains provides protection against external pressures, including host immune attacks, ensuring prolonged persistence in the GI tract [39]. In the present study, all LAB isolates showed BSH-positive results, indicating their capability to degrade conjugated bile salts, contributing to the lowering of serum cholesterol levels [24]. Notably, the LABs' production of deconjugated bile salts suggests reduced reabsorption and increased excretion of free bile acids in the stool. Additionally, all the LAB isolates exhibited cell surface enzyme characteristics needed for the breakdown of proteins, lipids, and carbohydrates. This may be linked to improved food digestibility and nutritional quality associated with health benefits of consuming these fermented foods.

Phylogenetic analysis of these isolates revealed that they group with the members of the family *Lactobacillaceae*. Such bacteria are known for producing short-chain fatty acids (SCFAs) which are reported to have beneficial effects on physiological and metabolic homeostasis of mammals [40–43]. These LAB isolates also demonstrated potent antimicrobial properties against common pathogens, producing organic acids, bacteriocins, and hydrogen peroxide to resist the growth of pathogenic microbes [44–46]. The ability of LAB isolates to bind to the intestinal mucosa

enhances their survival and growth in the GI tract. Higher CSH% (cell surface hydrophobicity) indicates strong adhesion abilities and competitive exclusion of other microbes, fostering interactions with bacterial communities in the GI tract [47]. Moreover, CSH activity determines the ability of cells to attach and separate from surfaces. A probiotic strain's intestinal colonization, or adherence and persistence, is measured by the hydrophobicity of its cell surface. High hydrophobicity indicates more colonization capability [48]. The LAB isolates also fulfil these criteria by showing higher CSH% values. In respect with the of the antidiabetic activity, the isolated LAB displayed significant inhibitory activity against α-glucosidase and α-amylase enzymes, which can potentially slowdown glucose absorption and reduce postprandial blood sugar levels. This highlights their potential as therapeutic hypoglycemic agents as well as hyperglycemia-preventive supplements. Regarding antibiotic resistance, some LAB isolates showed resistance to vancomycin, streptomycin, and kanamycin [49]. Moreover, lack of hemolytic activity of the isolates demonstrated their safety. The results from acute toxicity tests reaffirmed the safety of these LAB isolates, establishing their status as "Generally Recognized as Safe" [50].

Conclusion

This investigation highlights the probiotic attributes of five LAB isolates, namely *P. pentosaceus* isolate GAD, *L. plantarum* isolate KAD, *L. plantarum* isolate CAD, *L. brevis* isolate SAD, and *L. plantarum* isolate CMD. All the five isolates show potential for probiotic applications and as naturally derived pharmaceutical agents, owing to their gastro-tolerance, metabolic activities, adhesion capabilities, antimicrobial properties, and hypoglycaemic effects. These findings support their safety as health-beneficial LABs. However, further *in vivo* investigations are essential to validate and establish their suitability to be used as dietary supplements.

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Author contributions

TS conceptualized and supervised the project. AJG and SG performed the experiments, and prepared the first draft of the original manuscript. TS, MDC, AJG, and SG analyzed and interpreted the data and curated the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The information created and analyzed during the current investigation is available upon reasonable request from the corresponding author.

Declarations

Ethics approval and consent to participate

The *in vivo* animal experimentation was approved by the Institutional Animal Ethical Committee (IAEC) of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) of the University of North Bengal in West Bengal, India (Vide ref no. IAEC/NBU/2022/27 Dated. 23.09.2022).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

BSH
Bile salt hydrolase
CLSI
Clinical and Laboratory Standards Institute
CSH
Cell surface hydrophobicity
GI
Gastrointestinal
LAB
Lactic acid bacteria
LS
Laboratory strains
MATS
Microbial adhesion to solvents
MHA
Mueller Hinton Agar
MRS
De-Man, Rogosa, and Sharpe
OD
Optical density
OECD
Organization for Economic Co-operation and Development
PBS
Phosphate buffer saline
SCFA
Short-chain fatty acid

Publisher's Note

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Evaluating the effect of artificial intelligence on pharmaceutical product and drug discovery in China

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ABSTRACT (ENGLISH)

The pharmaceutical sector has recently witnessed a transformative improvement and shift toward artificial intelligence (AI) in its drug and pharmaceutical delivery process and procedures. Hence, this research delves into the benefits and obstacles pharmaceutical firms face in utilizing AI in China. Globally, China is recognized as a dominant pillar in research and development in the pharmaceutical industry. The country has incorporated AI approaches and technologies to improve the drug industry's cost, efficiency and development. Therefore, this study applies the case study method and evaluation of prior studies to assess AI's potential benefits and challenges in the drug and pharmaceutical enterprises. The research provided an in-depth evaluation of AI in the various phases of the drug discovery process. The research outcome indicated that AI's benefits include drug repurposing, target identification, clinical trial optimization, quality assurance, and control and efficient drug distribution method. However, the analysis revealed that China faces several challenges that impact the pace and extent of integration of AI in its pharmaceutical industry. These challenges include a lack of standardized data, a shortage of skilled labor or professionals, and data and privacy concerns. In addition, the research provides three case studies that focused on f XtalPi-AI-Enhanced Drug Discover, BioMap: Accelerating Drug Development Through AI and iCarbonX: AI-Driven Precision Medicine and provided a comprehensive analysis of how these firms have used AI to stimulate their drug discovery process. The study also provides policies that can help improve the integration of AI in the pharmaceutical and drug delivery process.

FULL TEXT

Background

In recent years, integrating artificial intelligence (AI) into various industries has catalyzed transformative changes, with the pharmaceutical sector no exception [25]. This study delves into the profound implications of AI in drug discovery and development, with a specific focus on pharmaceutical companies operating in China. Sufyan, Shokat, and Ashfaq [44] asserted that as technological advancements continue redefining traditional methodologies, it becomes imperative to assess the comparative strategies employed by pharmaceutical enterprises in harnessing AI to enhance drug discovery and development processes. Lengthy and resource-intensive drug development pipelines have long characterized the pharmaceutical industry. The introduction of AI promises to expedite these processes, potentially revolutionizing the landscape of drug discovery [29]. However, the adoption and implementation of AI technologies vary among pharmaceutical companies, and a comparative analysis provides an understanding of the diverse approaches employed by industry players in China [29]. This research seeks to unravel the intricacies of AI utilization, evaluating the impact on efficiency, cost-effectiveness, and overall success rates in drug discovery and development. Thus, this research provides a novel approach that can help understand the opportunities and challenges faced by pharmaceutical in China. Moreover, as indicated by Kim et al. [20], China has become a center for the advancement of drugs. As AI applications advance in sophistication, it will become strategically imperative for firms looking to gain a competitive advantage to integrate them into their drug development processes.

Moreover, implementing AI tools, including machine learning and data analytical approaches, is revolutionizing drug and pharmaceutical enterprises to identify and initiate a new therapeutic in contemporary times [19]. Compared to the traditional methods used for drug discovery and pharmaceutical process, Sultana and Rangareddy [46] recounted that AI has offered firms the ability to process huge amounts of clinical, biological, and chemical data in a timely and sustainable manner. As the demand for novel and effective therapeutics continues to rise, so does the pressure on pharmaceutical companies to innovate and streamline their drug discovery and development pipelines. AI presents a unique opportunity to augment human capabilities, offering the potential to uncover hidden patterns in data, predict potential drug candidates, and optimize clinical trial designs [3]. This exploration into the utilization of AI in drug discovery and development processes among pharmaceutical companies sheds light on the current state of the industry but also lays the groundwork for understanding the future trajectory of pharmaceutical innovation in the era of artificial intelligence [3].

The research problem at the core of this investigation revolves around identifying the key determinants that influence

the adoption and efficacy of AI in drug discovery among pharmaceutical companies in China. By conducting a comparative analysis, we aim to uncover patterns, trends, and success factors that distinguish the strategies of various enterprises. Moreover, this study seeks to address potential challenges and ethical considerations associated with implementing AI in drug development, contributing to a comprehensive understanding of the evolving landscape in the context of the Chinese pharmaceutical industry. As AI technologies evolve rapidly, staying abreast of the dynamic relationship between pharmaceutical companies and these innovations is paramount [5]. The findings of this research endeavor are poised to offer valuable insights not only for industry stakeholders but also for policymakers, researchers, and the broader scientific community. Ultimately, by scrutinizing the impact of AI in drug discovery and development within the unique context of Chinese pharmaceutical companies, this study contributes to the ongoing discourse on the role of artificial intelligence in shaping the future of healthcare and innovation.

This research significantly contributes to the evolving field of pharmaceuticals and artificial intelligence. By focusing specifically on China, the study provides valuable insights into the utilization of AI in drug discovery and development processes within the Chinese pharmaceutical industry. This geographical focus is crucial, as China has emerged as a major player in the global pharmaceutical market, and understanding how AI is integrated into its drug development practices is essential for academic and industry stakeholders [51]. The novelty of this research lies in its comparative analysis, which allows for a comprehensive understanding of the different approaches adopted by pharmaceutical companies in China regarding AI in drug discovery. The study explores various AI techniques, algorithms, and technologies these companies employ, shedding light on the diversity of strategies within the Chinese pharmaceutical sector. This comparative aspect contributes to the existing body of knowledge by highlighting variations in AI adoption and implementation across different companies, offering a nuanced perspective on AI integration in drug development.

The practical significance of the research is evident in its potential to inform policymakers, industry professionals, and researchers about the current landscape of AI in drug discovery in China. The findings can guide decision-makers in developing targeted policies and strategies to foster AI's responsible and effective use in the pharmaceutical sector. Moreover, pharmaceutical companies can benefit from the insights by understanding their counterparts' successful AI applications and challenges, potentially optimizing their drug discovery and development processes. This research holds practical implications for advancing the intersection of artificial intelligence and pharmaceuticals, with potential global implications given China's growing influence in the industry.

Literature review

AI: a transformative tool for advancing pharmaceutical product lifecycle

AI has emerged as a transformative force in advancing the pharmaceutical product life cycle across various stages, from drug discovery to post-market surveillance [53]. One notable contribution of AI is in drug discovery, which accelerates the identification of potential drug candidates. By leveraging machine learning algorithms, AI can analyze vast datasets, including genomic information and chemical properties, to predict how different compounds might behave and interact [9]. This expedites the discovery process, significantly reducing the time and costs traditionally associated with bringing a new pharmaceutical product to market. In the development phase, AI is crucial in optimizing clinical trials [2]. Machine learning algorithms can analyze patient data to identify suitable trial candidates, predict potential adverse effects, and optimize the trial design. This enhances the efficiency of clinical trials and improves patient outcomes by ensuring that the right individuals are enrolled, leading to more robust and reliable results [33]. Additionally, AI can assist in the monitoring and analysis of real-world evidence, providing insights into the long-term efficacy and safety of pharmaceutical products [43].

Once a drug is approved and enters the market, AI contributes to its life cycle by enhancing manufacturing processes [17]. AI-powered systems can monitor and optimize production, ensuring consistency and quality while minimizing waste. Predictive maintenance powered by AI can also reduce downtime in manufacturing facilities, improving overall efficiency [40]. These manufacturing advancements benefit pharmaceutical companies and contribute to a more reliable and accessible supply of medications for patients. In the post-market phase, AI facilitates pharmacovigilance and drug safety monitoring. According to Deng et al. [12], AI can promptly identify

possible adverse responses and new security vulnerabilities by examining vast amounts of data, such as online communities and medical records.

AI can quickly detect potential adverse reactions and emerging safety concerns by analyzing large-scale data, including electronic health records and social media [12]. This makes it possible for pharmaceutical firms and regulatory organizations to act quickly, protecting the welfare of patients and upholding the general public's trust in pharmaceuticals. Over every phase of the life cycle, a more proactive and instantaneous policy regarding pharmaceutical security is made possible by AI's constant surveillance features. [7]. Floresta et al. [14] revealed that AI helps provide personalized medicine by evaluating the patient data to recognize specific biomarkers and genetic markers that may affect the therapy responses. This enables the improvement of customized or targeted medicines that optimize efficacy while minimizing the adverse effects.

Furthermore, AI aids in personalized medicine by analyzing patient data to identify specific genetic markers and biomarkers that can influence drug response. This allows for the development of targeted therapies tailored to individual patients, maximizing efficacy while minimizing adverse effects [14]. A substantial advancement in the life progression of medical products, tailoring therapy regimes based on a patient's genetic profile, represents an essential turning point in the medical field. According to Gupta et al. [15], AI can greatly accelerate the lifespan of pharmaceutical products. Knake [21] advances the discussion by indicating that AI has an enormous effect on every stage of the product life cycle, from accelerating the discovery of new drugs to streamlining clinical trials, boosting post-market monitoring and facilitating targeted therapy. As technology evolves, the pharmaceutical industry is poised to benefit from further innovations and improvements, ultimately leading to more efficient, effective, and patient-centric healthcare solutions [42]. In Fig. 1, Paul et al. [36] identified various AI networks and decision tree algorithm that helps improve pharmaceutical products.

Fig. 1 [Images not available. See PDF.]

AI algorithm in drug development.

Source: Paul et al. [36]

Artificial intelligence: a sustainable approach to drug discovery

AI has emerged as a sustainable and revolutionary approach to drug discovery, offering unprecedented opportunities to streamline and enhance the traditionally time-consuming and resource-intensive process [48]. Hence, Bhattamisra et al. [6] mentioned that AI's important enhancement to drug development is its speedy digestion of enormous amounts of medical and scientific information. Thus, AI enables health practitioners to identify prospective drug candidates more accurately and efficiently than they could using the conventional approach when they employ ML. Thus, complex compositions can be examined and explored using ML. AI's capacity to substantially reduce the cost and time connected with bridging new drugs to the market demonstrates the sustainability of this kind of drug delivery process. Therefore, AI expedites the initial drug discovery phase by promptly recognizing novel compounds and forecasting their physiological impact on patients. AI's capacity to significantly reduce the time and cost associated with bringing new drugs to market demonstrates the sustainability of this kind of drug development. AI expedites the initial phases of drug discovery by promptly recognizing novel compounds and forecasting their physiological effects. This increases the likelihood of successful and noteworthy pharmaceutical product launches and helps pharmaceutical businesses allocate resources more wisely, both of which support stability in the healthcare sector.

The sustainable aspect of AI in drug discovery is evident in its potential to significantly reduce the cost and time required to bring new drugs to market. By expediting the identification of promising compounds and predicting their biological activity, AI accelerates the initial phases of drug discovery. This allows pharmaceutical companies to allocate resources more efficiently and increases the likelihood of bringing successful and impactful drugs to market, promoting sustainability within the pharmaceutical industry [47]. Furthermore, AI contributes to sustainability by facilitating a more targeted and personalized approach to drug development. Machine learning models can analyze patient data, identifying specific genetic markers and biomarkers influencing individual medication responses [35].

This personalized medicine approach not only enhances the effectiveness of treatments but also minimizes adverse effects, reducing the overall environmental impact associated with the production and disposal of pharmaceuticals. In addition to its role in the early stages of drug discovery, AI supports sustainability in optimizing clinical trials [32]. By analyzing patient data, AI can help identify suitable trial candidates, predict potential adverse effects, and optimize trial designs. This ensures that clinical trials are more efficient, with reduced resource requirements and a higher likelihood of success. Streamlining clinical trials contributes to the overall sustainability of drug development processes [4].

Hence, the integration of AI in drug discovery also aligns with sustainable practices by improving the efficiency of drug manufacturing. AI-powered systems can monitor and optimize production processes, reducing waste and ensuring consistent product quality [4]. Predictive maintenance, enabled by AI, minimizes downtime in manufacturing facilities, contributing to a more sustainable and resource-efficient pharmaceutical production ecosystem [24]. Moreover, AI enhances sustainability in drug discovery through its contribution to pharmacovigilance and drug safety. By continuously analyzing real-world data, including electronic health records and social media, AI can quickly detect and assess potential adverse reactions and safety concerns [49]. This proactive monitoring ensures patient safety and builds trust in pharmaceutical products, promoting the industry's long-term sustainability. Accordingly, integrating AI in drug discovery represents a sustainable approach that addresses various challenges in the pharmaceutical industry [24]. AI's impact is multifaceted, from expediting drug discovery and optimizing clinical trials to supporting personalized medicine and improving manufacturing efficiency. As the field continues to evolve, the sustainable application of AI in drug discovery holds the promise of transforming the pharmaceutical industry toward more efficient, effective, and environmentally conscious practices [45]—Table 1 provides a list of some prominent AI tools used in drug discovery.

Table 1. Examples of AI tools in drug discovery

AI tools in drug discovery	Explanation	References
IBM Watson for drug discovery	IBM Watson for Drug Discovery leverages AI to analyze biomedical literature, clinical trial data, and other relevant information to help researchers identify potential drug candidates and biomarkers	Chen et al. [10] and Hatz et al. [18]
Atomwise	Atomwise uses deep learning for the virtual screening of potential drug compounds. It analyzes molecular structures to predict their binding affinity with target proteins, expediting the identification of potential drug candidates	Carpenter and Huang [8]
DeepChem	DeepChem is an open-source platform that provides a collection of deep-learning tools for drug discovery	Korshunova et al. [23]
In silico medicine	In silico medicines aims to provide aging research and medicine discovery by applying AI. It also utilizes biological data and seeks promising medication options that focus on sickness, which comprises neurological conditions and cancer	Shaker et al. [41]

Recursion pharmaceuticals	Recursion Pharmaceuticals screens and analyzes biological pictures with artificial intelligence (AI) to find possible medication prospects. Their approach enables rapid screening of biological abnormalities by combining machine learning and computer vision	Malandraki-Miller and Riley [31]
OpenEye scientific software	OpenEye provides a suite of AI tools for cheminformatics research and structural architecture. Their software helps with drug candidate optimization, biological attribute forecasting and chemical-based dataset assessment	Cox and Gupta [11] and Rifaioglu et al. [38]
Schrodinger	Schrodinger offers a system for molecular interaction modeling, digital screening, and discovery of medicines package that integrates AI. It helps scientists optimize tiny compounds to create medications and estimate binding capacities	Adelusi et al. [1]

Advancing pharmaceutical product and drug discovery in China: the role of AI

AI is transforming China's life science and healthcare enterprises as it has become essential for creating medication and pharmaceutical products. Given China's growing pharmaceutical industry, it has recognized the potential of AI to revolutionize conventional drug research methods, leading to improved creativity and efficiency [45]. AI has significantly improved China's drug discovery process by speedily and accurately analyzing large amounts of data, accelerating the recognition of promising potential medicines.

According to Deshmukh [13], in the drug discovery process, AI algorithms can be used to evaluate complex chemical information and biological analysis comprising proteomics, genomics and chemical composition. Chinese medicinal product companies use AI to improve the accuracy of target authentication, which raises the effectiveness rate of pharmaceutical research initiatives [28]. The application of AI in this phase is particularly crucial for addressing diseases prevalent in China and globally, providing a tailored and efficient approach to drug development. Furthermore, AI is optimizing the clinical trial process in China, another critical phase in pharmaceutical product development. Machine learning algorithms are employed to analyze patient data, identify suitable clinical trial participants, and predict potential adverse effects [28]. This personalized approach to clinical trials accelerates the testing process. It ensures that drugs are tailored to diverse genetic profiles, contributing to developing more effective and safer pharmaceutical products [30].

China's commitment to AI in pharmaceuticals extends to manufacturing processes. AI-powered systems are being integrated into production facilities to monitor and optimize manufacturing, ensuring consistency, quality, and efficiency [39]. Predictive AI-powered maintenance minimizes downtime, contributing to China's more reliable and sustainable pharmaceutical manufacturing ecosystem. These advancements benefit local pharmaceutical companies and position China as a global leader in adopting cutting-edge technologies for drug production [39]. AI can quickly identify potential adverse reactions and safety concerns by analyzing real-world data from diverse sources, including electronic health records and social media. This continuous monitoring enhances patient safety and regulatory compliance, contributing to the sustainable growth of the pharmaceutical industry in China [36].

Moreover, AI is fostering international collaborations and partnerships in drug discovery. Chinese pharmaceutical companies are increasingly collaborating with global AI and biotech firms to access cutting-edge technologies and expertise [22]. These collaborations facilitate knowledge exchange, talent development, and the integration of diverse perspectives, ultimately contributing to a more robust and globally connected pharmaceutical ecosystem in China [16]. Therefore, the integral role of AI in advancing drug discovery and pharmaceutical products in China underscores the nation's commitment to embracing innovative technologies [24]. AI is reshaping the entire

pharmaceutical product life cycle, from accelerating drug discovery and optimizing clinical trials to improving manufacturing processes and ensuring post-market safety [34]. As China continues to invest in research, development, and collaborations, the synergy between AI and pharmaceuticals is poised to drive transformative changes in healthcare, benefiting both the nation and the global community [26].

A case study on the utilization of AI in drug discovery and development processes among pharmaceutical companies

Case of iCarbonX: AI-driven precision medicine

iCarbonX, a prominent entity in the Chinese healthcare sector, specializes in AI-driven precision medicine, leveraging advanced technologies to analyze diverse datasets, including genomics, lifestyle factors, and clinical records. By forming strategic alliances with academic institutions, iCarbonX collects large amounts of genomic data and uses AI algorithms for predictive modeling to find individual treatment plans. Machine learning is then used to detect biomarkers and help create targeted medicines. A global pioneer in precision medicine, iCarbonX can now produce medicines with more efficacy and fewer side effects thanks to the successful integration of AI. This has led to advancements in personalized medicine. But the business also has to deal with issues like data protection, moral dilemmas, and the need for smooth communication between AI specialists and healthcare professionals. Constant endeavors center on guaranteeing adherence to regulations and fostering public confidence. [26]. Figure 2 shows IsoLight, a precision medical tool invented by iCarbonX for profiling the cells of single-cell chips and software products.

Fig. 2 [Images not available. See PDF.]

IsoLight precision medical tool

Case of BioMap: accelerating drug development through AI

The dynamic biotechnology startup BioMap was founded in China and is dedicated to utilizing state-of-the-art AI-driven methods to hasten medication development. With a focus on efficiency enhancement, the company uses artificial intelligence (AI) to assess complicated biological datasets, including pharmaceutical interactions, proteomics, and genomes. BioMap's strategy approach combines AI algorithms for lead optimization and target discovery with real-time data analytics and predictive modeling to speed up drug development. Collaborations between AI firms and academic institutions expand their technological capabilities. Because of this, BioMap's implementation of AI has significantly decreased the time and expenses related to drug development, allowing for the quicker identification of viable drug candidates and creating a more effective development pipeline. The firm has successfully brought innovative treatments to market in a record time. Notwithstanding these achievements, BioMap still has issues with its AI infrastructure's scalability and integration of various datasets. To keep personnel updated on the latest developments in AI technology, ongoing efforts are being made to solve these issues and provide them with ongoing education and training. A sample picture of the BioMap tool used for accelerating the drug development process is captured in Fig. 3.

Fig. 3 [Images not available. See PDF.]

BioMap AI tool for drug delivery

Case of XtalPi: AI-enhanced drug discovery

XtalPi, a pioneering pharmaceutical technology company in China, specializes in advancing drug discovery through AI-enhanced methodologies. Their strategic focus is leveraging computational approaches to optimize drug formulations and accurately predict properties. XtalPi's distinctive approach integrates quantum mechanics and AI algorithms, enabling the modeling of molecular interactions, prediction of drug solubility, and optimization of drug formulations. Through partnerships with global pharmaceutical firms, XtalPi enhances its access to diverse datasets, facilitating virtual screening of compounds and expediting the lead optimization process. Moreover, Xtalpi has listed these objectives as their mandate in improving health care and drug delivery systems, and they include:

1. Target characterization and identification of binding modes
2. Ideation and exploration of novel chemical space
3. Accurate forecasting of potency and properties
4. Formulation, synthesizing and evaluation strategies
5. AI-enhanced physics based on computational modeling

The incorporation of AI at XtalPi has transformed drug discovery, markedly reduced preclinical development timelines, and identified more stable and effective drug formulations. Despite these successes, XtalPi grapples with challenges related to the intricate nature of quantum mechanics simulations and the continual validation of AI models. Ongoing efforts are directed at ensuring seamless integration with existing pharmaceutical workflows [37]. Figure 4 indicates the collaborative approach adopted by Xtalpi with partners to power macromolecule programs that can create custom-based solutions integrated into their AI machine called the ID4 platform.

Fig. 4 [Images not available. See PDF.]

AI machine-ID4 platform

Evaluating the benefits and challenges of AI in the drug discovery and pharmaceutical product

Potential benefits of AI in drug discovery and pharmaceutical product

Drug repurposing

AI is essential to drug repurposing because it provides a strong and creative method for finding novel therapeutic applications for already-approved medications. AI's capacity to evaluate large and varied historical data, comprising genetic, surgical, and pharmacologic statistics, is one of the primary benefits of medication repurposing [52]. AI algorithms can find possible interconnections and links through examination that might not be readily evident using more conventional techniques. AI can quickly treat existing medications with an opportunity to alleviate various ailments by employing ML models to go through complex data and unearth novel findings [52]. Specialists can bypass some phases of expansion, such as assurance of safety, which was already completed after the drug's first acceptance, by using AI to repurpose existing pharmaceuticals [20]. To meet patients' immediate healthcare demands, it is very important to streamline the clinical trial process because it enables clients to receive therapies more quickly.

Sufyan et al. [44] denoted that AI systems can track subtle patterns and connections in data that may escape human scrutiny. Thus, the ability to make coincidental discoveries broadens the scope of repurposing medication, revealing possible remedies for ailments other than those for which they were originally designed. Moreover, AI-driven medication repurposing multidisciplinary perspective facilitates a more comprehensive comprehension of the biological causes of disease, opening the door to novel and unanticipated methods for therapy [19]. In summary, AI is essential for drug repurposing because it can transform the drug development process by providing quicker, more efficient and creative measures to find new therapeutic uses for already-approved medications. This has the potential to find novel answers to urgent medical challenges and expedite the delivery of therapies to patients.

Target identification

The technique of finding a medicine involves a critical called pinpointing the target. Because of its numerous advantages, AI plays a significant role in the success and efficacy of this procedure. Since AI can sift through vast amounts of complex biological information and data, it is essential for target identification [22]. AI algorithms can find

possible treatment targets more speedily than conventional techniques since they can evaluate proteomics, genomes and other omics data. Moreover, AI helps find fresh pathways and interconnections in information essential to the target assessment process. ML algorithms recognize patterns and correlations that may be challenging for human researchers to identify [28]. This capability allows AI to reveal potential targets that may have been overlooked using conventional approaches. By simultaneously considering many factors, AI enhances the comprehensiveness and accuracy of target identification, opening up new avenues for developing targeted and effective therapeutic interventions.

The essence of AI in target identification is further underscored by its role in personalized medicine. AI can analyze large-scale patient data, including genetic information, to identify specific disease-associated biomarkers [26]. This personalized approach allows for identifying effective targets in treating a particular disease and is tailored to individual patient profiles, potentially improving treatment outcomes and minimizing adverse effects [27]. In essence, AI in target identification brings a level of precision and efficiency that has the potential to revolutionize the drug discovery process and pave the way for more effective and personalized therapeutic interventions [50].

Clinical trial optimization

ML algorithms can analyze diverse data sources, including electronic health records and real-world data, to identify suitable candidates for clinical trials. By streamlining the recruitment process and matching patients with specific trial criteria, AI accelerates the enrolment phase, reducing delays and ensuring that trials progress more swiftly [16, 22, 36]. Another critical role of AI in clinical trial optimization is in the design and management of trials. AI systems can assess historical data, enhance trial protocols and predict medical challenges confronting patients. AI also help to maximize client satisfaction and participation in clinical studies. AI provided the platform to evaluate patient input from various reports, including patient forums and social media platforms, using sentiment analysis and processing of natural language. With these insightful data on patient experience, trial administrators will be better equipped to quickly resolve issues, strengthen their interaction plans and increase participant engagement. By providing a data-driven, patient-centered approach, the integration of AI in clinical trial optimization marks a paradigm shift and the ability to expedite procedures, lower costs and more quickly bring novel therapies to the marketplace [24, 26, 34].

Quality control and assurance through AI

Mahato [30] revealed that with its enhanced ability to improve efficiency, precision, and the overall quality of the product, AI is becoming a more vital component of quality assurance and control throughout a wide range of industries. AI ensures that products satisfy strict requirements by automating quality control operations in production. Large production of data sets can be evaluated by ML algorithms to locate abnormalities and trends, enabling real-time prompt corrections and monitoring. This predictive capability helps improve the overall quality of the production process by assisting in the detection and prevention of possible flaws [52]. In pharmaceuticals and healthcare, AI transforms quality assurance by streamlining processes and ensuring compliance with rigorous standards. Large volumes of data about pharmaceutical production, testing in labs, and experimental treatments can be analyzed by AI-powered systems, which can then spot variations and discrepancies from forecasted outcomes. This lowers the potency that client will receive defective medicals by enabling prompt identification of such issues [20]. Additionally, AI contributes to regulatory compliance by automating documentation processes, facilitating easier traceability, and ensuring that every step in the production and testing phases aligns with established quality standards.

Kulkov [25] reported that using AI software to control and quality assurance medical products is a common practice. Testing tools engineered by AI can automatically find vulnerabilities in the software, security issues, and operational problems. Thus, software development cycles can be accelerated while upholding rigorous standards of quality because of these technologies' ability to carry out complicated test situations more quickly than conventional

approaches. Likewise, Hasselgren and Oprea [17] espoused that through ML, these systems can learn from previous testing situations and improve their ability to recognize possible problems with every iteration. This improves the effectiveness of the testing procedure while also adding to the general resilience and dependability of software offerings.

AI in drug distribution

AI is instrumental in route optimization and logistics planning. Through advanced algorithms and machine learning models, AI can analyze various factors such as traffic conditions, weather, and transportation costs to optimize the delivery routes of pharmaceutical products. This ensures timely and cost-effective distribution and minimizes the environmental impact associated with transportation. AI-driven route optimization can lead to more sustainable drug distribution practices, aligning with broader efforts to reduce carbon footprints in the pharmaceutical industry [9]. Additionally, AI improves the accuracy and efficiency of order fulfillment processes in drug distribution. AI-powered automated systems can handle order processing, packing, and labeling with high precision, reducing the likelihood of errors and enhancing overall operational efficiency [2]. These AI-driven systems can adapt to fluctuating demand, prioritize critical orders, and provide real-time tracking information, ensuring that healthcare providers and patients receive the proper medications at the right time. Integrating AI in drug distribution is thus instrumental in achieving precision, reliability, and sustainability in the pharmaceutical supply chain [33].

Ongoing challenges facing the implementation of AI among pharmaceutical industries in China

While promising, the adoption of artificial intelligence (AI) in the pharmaceutical industry in China faces several challenges that impact the pace and extent of integration. One primary challenge is the *lack of standardized data*. The pharmaceutical sector has vast and diverse datasets, including clinical trial data, patient records, and genomic information. The absence of standardized formats and interoperability issues among these datasets can impede the seamless integration of AI solutions, making it challenging for pharmaceutical companies to harness the full potential of AI in data-driven decision-making processes [17, 40, 43].

Moreover, the *demand for experts proficient in pharmaceutical sciences* and AI technologies exceeds the current supply [12]. The development, application, and upkeep of AI-driven approaches in drug research, clinical trials, and other pharmaceutical industries are hampered by this shortage. It will take targeted educational initiatives and training programs to close this talent gap and give experts the multidisciplinary skills necessary for sustainable AI integration [7]. Regulatory issues further hamper the extensive use of AI in the Chinese pharmaceutical sector. The healthcare industry's legal framework is always changing, and pharmaceutical companies need to maneuver through intricate procedures to maintain conformance. Adoption may be slowed by the absence of precise and uniform criteria, which could lead to ambiguities and delays when applying for regulatory clearances for AI-driven technologies. [14].

Privacy and data security concerns also pose a big obstacle to using AI in healthcare. There is a greater emphasis on guaranteeing data privacy and protection since healthcare data, such as genetic knowledge and records of patients, are delicate [14]. To alleviate these concerns and promote the broad adoption of AI in pharmaceutical operations, businesses need to invest in cybersecurity safeguards and build confidence with all relevant parties, including clients and regulatory authorities. [15]. Therefore, even if AI has a lot of prospective advantages for the Chinese pharmaceutical business, its wide-ranging implementation will need to address issues, including data harmonization, talent shortages, regulatory complications, and data security concerns [48].

Research conclusion and policy directions

Conclusions

Exploring the influence of AI in drug discovery and the development of pharmaceutical enterprises in China has

revealed a substantial shift toward using innovative approaches to promote and enhance the health sector in the country. The current analysis has provided a comprehensive understanding of the potential transformative of applying AI tools in reducing cost, enhancing efficiency and streamlining drug development pipeline policies. The current assessment has revealed that the strategic application and adoption of AI reflects an expanding recognition of its capacity to expedite the invention of new therapeutics, thereby improving and advancing health care and medical science. In addition, this research has shed light on the assumption that China's drug and pharmaceutical industry is at the forefront of outlining cutting-edge tools for research and development which can foster a culture of collaboration and innovation. The report emphasizes the tremendous steps Chinese pharmaceutical businesses have taken in incorporating AI. Still, it also emphasizes the necessity for continued research, international cooperation, and regulatory frameworks to address issues and guarantee AI's moral and responsible application in drug creation. Amidst the paradigm shift in the pharmaceutical industry, stakeholders need to exercise caution when navigating the ethical, legal, and societal ramifications of incorporating artificial intelligence (AI) into the complex drug development processes. In the end, the comparative research indicates that the pharmaceutical business could undergo a revolution if AI technologies are successfully incorporated, with China establishing itself as a key participant in this revolutionary process.

Policy directions

Based on the study's outcome, the research proposes that certain policy recommendations must be implemented to optimize AI's impact on drug discovery. Firstly, fostering collaboration between pharmaceutical companies and research institutions is crucial. Establishing a framework for data sharing and joint projects can leverage collective knowledge and resources, accelerating the pace of drug discovery.

Secondly, there is a need for standardized regulatory guidelines governing the ethical use of AI in pharmaceutical research. Developing a comprehensive set of regulations ensures the responsible and transparent deployment of AI technologies, addressing concerns related to data privacy, bias, and the potential misuse of AI-generated insights. Establishing clear ethical boundaries allows trust in AI applications within the drug development process to be cultivated among industry stakeholders and the public. Furthermore, incentivizing AI research and development through government policies can stimulate innovation. Offering tax breaks or research grants to pharmaceutical companies investing in AI technologies encourages the industry to explore and implement cutting-edge solutions. This bolsters the competitive edge of Chinese pharmaceutical companies in the global market and positions the nation as a leader in AI-driven drug discovery.

Moreover, educational initiatives should be prioritized to bridge the gap between AI developers and pharmaceutical researchers. Promoting interdisciplinary programs that combine expertise in both AI and pharmaceutical sciences can cultivate a new generation of professionals equipped to harness the full potential of AI in drug discovery. Collaboration between academic institutions and industry players can facilitate knowledge exchange, fostering an environment conducive to continuous learning and adaptation. In addition, creating a centralized AI platform that aggregates and anonymizes data from various pharmaceutical companies can enhance the industry's collective intelligence. This platform could serve as a hub for shared insights, best practices, and AI algorithms, using advancements in drug discovery on a broader scale. Such a collaborative approach mitigates the fragmentation of efforts and maximizes the benefits of AI technologies across the entire pharmaceutical ecosystem.

Lastly, ongoing monitoring and evaluation mechanisms should be established to assess the impact of AI interventions in drug discovery and development. Regular audits can ensure compliance with ethical standards, measure implemented policies' effectiveness, and identify improvement areas. This adaptive approach enables the refinement of policies based on real-world outcomes, ensuring that the integration of AI in pharmaceutical processes

aligns with societal expectations and ethical considerations. In conclusion, a comprehensive and collaborative approach, anchored by robust regulations and incentives, is essential to harness the full potential of AI in drug discovery among pharmaceutical companies in China.

Limitations and future research directions

Integrating AI in drug discovery and development has undeniably revolutionized the pharmaceutical landscape, particularly in China. However, this technological advancement is not without its limitations. One major challenge lies in the availability and quality of data for training AI models. The success of AI algorithms in drug discovery heavily depends on access to comprehensive and reliable datasets, which can be a bottleneck in regions where data sharing and standardization practices may vary. Additionally, the interpretability and explainability of AI-generated insights remain significant concerns, especially in the highly regulated field of pharmaceuticals. Future research in this domain should address these limitations by establishing robust data-sharing frameworks, improving the transparency of AI algorithms, and enhancing the collaboration between pharmaceutical companies, research institutions, and regulatory bodies. Moreover, exploring the potential ethical implications of AI-driven drug discovery and development is crucial to ensuring responsible and equitable deployment of these technologies in advancing healthcare solutions.

Author contributions

AKS did conceptualization and data curation; FN performed formal analysis and funding acquisition.

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The manuscript has no associated data.

Declarations

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DETAILS

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Bioanalytical method development and validation for quantification of amivantamab in rat plasma by LC-MS/MS

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ABSTRACT (ENGLISH)

Background

Amivantamab is a monoclonal bispecific anti-EGFR-MET antibody used to treat non-small cell lung cancer. There were no published methods using a liquid chromatographic—tandem mass spectrometric approach to develop and validate a feasible, novel, and thoroughly validated method for quantifying amivantamab in rat plasma.

Results

The liquid–liquid extraction method was used to extract the analyte from rat plasma. The analyte was separated using acetonitrile–ammonium formate buffer (40:60) as a mobile phase on waters, alliance e-2695 model high-pressure liquid chromatographic system having Agilent eclipse C_{18}, 150 mm × 4.6 mm, 3.5 µm column. The overall runtime was 6 min at a 1.0 ml/min flow rate. The method showed significant sensitivity and acceptable linearity over the 5.00–100.00 ng/ml concentration range. Accuracy was proved by mean percent recovery ranging from 98.03 to 99.99%. The intraday precision coefficient of variation (%) ranged between 0.31 and 5.43. Also, the findings such as C_{max}, t_{max}, AUC_{0-t}, $AUC_{0-\infty}$, and half-life values of amivantamab showed that the technique was helpful for pharmacokinetic studies.

Conclusions

All the validated parameters were found to be within the acceptable range. The validated method was found to be simple, accurate, precise, and reproducible and hence can be used for the routine analysis of amivantamab, such as in-process quality control by liquid chromatographic—tandem mass spectrometry.

FULL TEXT

Background

One of the most frequent malignancies linked to occupational exposures is lung cancer. Mesothelioma and lung cancer incidence have been related to the usage of asbestos in manufacturing and industries [1]. The two main subtypes of the disease are small cell lung carcinoma and non-small cell lung carcinoma (NSCLC), which account

for 15% and 85% of all instances of lung cancer, respectively. Three other subtypes of NSCLC include squamous-cell carcinoma, adenocarcinoma, and large-cell carcinoma [2]. The prevailing type of lung cancer, advanced non-small cell lung cancer (NSCLC), has a terrible prognosis and no recognized treatment. Due to the few therapy choices available, survival times are frequently brief [3]. There are risk factors for NSCLC that can be prevented and some that cannot be avoided. Tobacco inhalation is the most prevalent preventable risk factor for NSCLC [4]. Alcohol consumption, environmental exposure to second-hand smoke, asbestos, radon, arsenic, chromium, nickel, exposure to ionizing radiation, and polycyclic aromatic hydrocarbons are other causes of lung cancer [5]. One of the treatments for non-small cell lung cancer is the epidermal growth factor receptor (EGFR) gene. The tyrosine kinase cell-surface receptor EGFR can open up pathways that are involved in cell growth and proliferation when it is active [6]. EGFR mutations in malignancies lead to unregulated cell division because of persistent activation. 10–15% of people with lung cancer adenocarcinoma with European and Asian ancestry, those who never smoked, and female patients had EGFR gene mutations [7]. The first targeted treatment for non-small cell lung cancer patients, amivantamab, is a monoclonal bispecific anti-EGFR-MET antibody [8].

The quantitative assessment of drugs and their metabolites in efficient and robust techniques is important for the fruitful evaluation of biopharmaceutical, preclinical, and clinical research. The protocol referred to in the demonstration of a technique for quantitative assessment of analytes in biological matrices such as plasma, urine, blood, and urine is called bioanalytical method validation [9]. These techniques are dependable and repeatable. A review of the literature was conducted. Bioanalytical methods for the determination of amivantamab in biological samples have not yet been reported. There were no analytical methods reported for determination of amivantamab in pure form and in different matrices. The goal of this work was to create a sensitive and specific analytical approach for the quantitation of amivantamab in rat plasma. The developed technique was validated by ICH M10 regulations [10]. The results of this study provide a robust and reliable analytical strategy for the rapid identification of amivantamab and provide a helpful basis for further research.

Methods

Solvents and chemicals

The amivantamab sample (99.99% purity) was provided as a gift sample from Shree Icon Labs, Vijayawada, Andhra Pradesh, India. All other chemicals, including HPLC grade acetonitrile and methanol, were purchased from Merck Chemical Division in Mumbai. The Milli-Q water purification system's HPLC-grade water was used throughout the study. All chemicals and reagents were used as received without further purification. Trastuzumab (99.98% purity) was used as an internal standard (IS). It was obtained from Glenmark Pharmaceuticals Pvt Ltd, Mumbai, India.

Instrumentation

The liquid chromatography system comprised of Waters, alliance e-2695 model HPLC armed with column oven, autosampler, and degasser was employed for analysis. The SCIEX QTRAP 5500 mass spectrometer was connected to the HPLC system. REMI centrifuge was used for centrifugation. Cyclo Mixer was used for mixing.

Chromatographic conditions

The autosampler was maintained at ambient temperature. An Agilent eclipse C_{18}, 150 mm × 4.6 mm, 3.5 µm column was used for elution. Isocratic elution was employed with a mobile phase comprising acetonitrile and 0.1 M ammonium formate in the proportion of 40:60. With an injection volume of 10 µl, the chromatographic flow rate was set at 1.0 ml/min.

Mass spectrometer conditions

The mass spectrometric system comprised a SCIEX QTRAP 5500 mass spectrometer armed with an electrospray ionization interface ionization mode. The collision gas used was nitrogen. The compound-specific parameters working are displayed in Table 1. The ions were detected using multiple-reaction monitoring mode (MRM). Sciex Analyst software was used to process the data.

Table 1. Compound-specific parameters

Parameter	Value
Declustering potential (DP)	40 V
Collision energy (CE)	14 V
Entrance potential (EP)	10 V
Collision cell exit potential (CXP)	7 V
Source temperature	550 °C
Drying gas temperature	120–250 °C
Drying gas flow stream	5 ml/min

Selection of internal standard (IS)

Trastuzumab was selected as an internal standard to reduce the inaccuracy at the processing level or the ongoing analysis level caused by the instrument for quantifying both analytes by technique. Trastuzumab was chosen as the IS due to its near molecular weight with the analyte.

Preparation of solutions

Preparation of amivantamab stock and working solutions

Five milligrams of amivantamab working standard was weighed and transferred into a 100-ml volumetric flask and then diluted to volume with diluent. Further, 1 ml from the above solution was transferred to a 10-ml volumetric flask and made up with diluent. 0.4 ml of the above solution was taken into a 10-ml volumetric flask and made up to the mark with diluent.

Preparation of internal standard stock solution (200 ng/ml)

Five milligrams of trastuzumab working standard was weighed and transferred into a 100-ml volumetric flask and, then, diluted to volume with diluent. Further, 1 ml was pipetted into a 10-ml volumetric flask and made up with diluent. 0.4 ml of the above solution was taken into a 10-ml volumetric flask and made up to the mark with diluent. This IS added to the sample processing (50 µl) resulted in a final concentration of 50 ng/ml corresponding with the MQC concentration of amivantamab.

Preparation of plasma samples

Transferred 500 µl of amivantamab stock solution into a 2-ml Eppendorf tube. To this, 200 µl of plasma, 500 µl of internal standard, 300 µl of acetonitrile, and 500 µl of diluent were added (50 ng/ml). Calibration standards were prepared by spiking blank rat plasma with appropriate amounts of amivantamab and trastuzumab. Calibration standards for final concentration were 5.00, 12.50, 25.00, 37.50, 50.00, 62.50, 75.00, 100.00 ng/ml for amivantamab. The preparation of calibration standards and QC samples are displayed in Tables 2 and 3.

Table 2. Preparation of standards

Stock solution (ng/ml)	Volume taken (µl)	Made up to volume (µl)	Final concentration (ng/ml)	Identification
200	50	2000	5.0	Standard 1
200	125	2000	12.5	Standard 2

200	250	2000	25.0	Standard 3
200	375	2000	37.5	Standard 4
200	500	2000	50.0	Standard 5
200	625	2000	62.5	Standard 6
200	750	2000	75.0	Standard 7
200	1000	2000	100.0	Standard 8

Table 3. Preparation of QC samples

Stock solution (ng/ml)	Volume (µl)	Made up to volume (µl)	Final concentration (ng/ml)	Identification
200	750	2000	75.0	HQC
200	500	2000	50.0	MQC
200	250	2000	25.0	LQC
200	50	2000	5.0	LLQC

Diluent

Mobile phase of acetonitrile and ammonium formate in the ratio 40:60 was used as diluent.

Sample preparation

The 2000 µl samples prepared above were vortexed with the vortex cyclo mixture. The solution was centrifuged at 4000 RPM for 15 to 20 min. Then, the supernatant fluid was collected in an HPLC vial. Double blank samples (i.e., without analyte and IS) were prepared by mixing 1000 µl of acetonitrile with 200 µl of rat plasma samples.

Bioanalytical method validation

Method validation was done according to ICH M10 guidelines.

System suitability

Six replicates of high-quality control (QC) standard solution were injected into the chromatographic apparatus to analyze the system suitability parameters.

Linearity

The calibration curve was generated by analyzing eight concentrations of amivantamab in plasma. Samples were measured by comparing the peak area of amivantamab to that of trastuzumab. The plot of peak area ratios vs plasma concentrations was made.

Specificity

The specificity of the developed approach was evaluated in the current study by analyzing the chromatograms of blank plasma and spiked plasma samples (amivantamab, trastuzumab).

Sensitivity

The method's sensitivity was assessed by analyzing 6 replicates of rat plasma comprising a lower limit of quantification (LLOQ) sample (5.0 ng/ml) of amivantamab.

Accuracy and precision

Four QC samples—LLOQ, low-quality control (LQC), medium-quality control (MQC), and high-quality control (HQC)—each with six replicates were used to assess the proposed bioanalytical method's precision and accuracy. The precision and accuracy of the proposed method were represented as mean accuracy (%) and coefficient of variance (CV) (%), respectively.

LOD and LOQ

The signal-to-noise ratio was used to calculate the bioanalytical method's limit of detection (LOD) and limit of quantification (LOQ).

Autosampler carryover

Autosampler carryover was assessed by injecting a blank sample, followed by an HQC sample and then an LLOQ sample followed by a blank sample.

Dilution integrity

The dilution integrity of plasma samples was evaluated by evaluating amivantamab samples above ULQC. Those samples were taken and diluted with a blank matrix to MQC and ULQC.

Matrix effect

The matrix effect of plasma on the response of amivantamab was assessed by blank plasma samples that were extracted from six different lots and reconstituted to form working standards of LQC and HQC. These samples in triplicate were quantified against the calibration curve.

Recovery of analyte

Six replicates of the amivantamab QC low-, medium-, and high samples were created by spiking the relevant concentrations of the drug and an internal standard into either unextracted or supernatant-recovered blank rat plasma (extracted). Recovery was calculated by comparing its response in multiple samples to neat standard solution responses.

Recovery of internal standard

Trastuzumab (50.0 ng/ml) samples in blank plasma were prepared and examined in six repetitions.

Ruggedness

Low, medium, and high QCs of amivantamab in plasma samples were reinjected into the system. % CV and accuracy were assessed to determine ruggedness.

Reinjection reproducibility

Low, medium, and high QCs of amivantamab in plasma samples were reinjected into the system. % CV and accuracy were assessed to determine reinjection reproducibility.

Stability studies

Benchtop stability

Amivantamab's stability in rat plasma was assessed by exposing six replicates of three different concentrations (LQC, MQC, and HQC) for 8 h on a benchtop and injecting them into the system.

Short-term and long-term stability

Short-term and long-term stability was assessed for amivantamab. Three different analyte concentrations were spiked into six duplicates of rat plasma for QC. LQC, MQC, and HQC samples were prepared and stored at 5 ± 3 °C for 7 days, and short-term stability was assessed. LQC, MQC, and HQC samples were prepared and stored at −20 ± 3 °C. These samples were injected from day 1 to 28 days for every seven days (as day 1,7,14 21, and 28), and long-term stability was assessed.

Freeze–thaw stability

The stability of amivantamab was evaluated after freeze–thaw cycles, respectively. Each LQC, MQC, and HQC had six duplicates that were held at −20° C, totally thawed at ambient temperature, and then immediately refrozen at −20° C. After this cycle was done twice, the samples were removed for injection into the LC-MS.

Autosampler stability

LQC, MQC, and HQC samples of amivantamab in plasma were injected at one-hour up to 24-h intervals. Mean accuracy (%) and CV (%) were calculated.

Dry extract and wet extract stability

Wet extract stability was evaluated by assessing the six sets of LQC, MQC, and HQC after 12 h and 18 h that were stored at 2–8 °C. The dry extract stability test used six sets of LQC, MQC, and HQC after 12 h and 18 h that were stored at 22 °C.

Assay

Assay was done to define the applicability of the bioanalytical method to the marketed formulation. MQC (50.0 ng/ml) sample was prepared from the marketed formulation (Rybrevant) and injected into the LC-MS system.

Method applicability to rats

Six healthy white female albino rats (body weight in between 250 and 350 g) were taken from Flair Labs, Gujarat, India. Before the experiment was directed, rats were adapted to laboratory environments for seven days. Diet was restricted for 12 h before the experiment, although water was freely available. A single dose of amivantamab (0.83 mg/ml) was administered to six rats. Samples were collected at different intervals, such as 10, 20, 30, 40, 50, and 60 min. K2 EDTA vacutainer tubes were used to collect blood at each interval. A predose sample was also taken to check for any potential plasma interferences. The plasma was obtained by centrifuging the collected samples kept at 10 °C. The liquid–liquid extraction method was used to isolate amivantamab in rat plasma. The animal study protocol was approved by the Institute of the Animal Ethics Committee (Reg.No: 1250/PO/RcBi/S/09/CPCSEA). Phoenix Win Nonlin (Version 5.2) software was used to analyze the data.

Results

Method development

MS/MS analysis

The mass spectra of amivantamab and trastuzumab were obtained by preparing each analyte in diluent and injecting it into the liquid chromatography-tandem mass spectrometer with positive ionization mode. Scan displayed that precursor ions of amivantamab and an m/z value of 145.66 were chosen. During MRM optimization, the product ion 112.10 showed the best response and was selected as the daughter fragment. The collision energy was optimized as 14 V using the edit ramp function. The scan presented that precursor ions of trastuzumab have an m/z value of 148.57. During MRM optimization, the product ion 126.01 showed the best response and was chosen as the daughter fragment. The mass transitions are shown in Figs. 1 and 2.

Fig. 1 [Images not available. See PDF.]

Product ion scan of amivantamab using positive polarity

Fig. 2 [Images not available. See PDF.]

Product ion scan of trastuzumab using positive polarity

Liquid chromatography

Several elution conditions were tested for the chromatographic separation. An isocratic flow profile was devised to get the finest peak separation with a minimal overall run time for all the analytes. The liquid chromatographic settings were improved to prevent the matrix effect, provide better peak shapes for all analytes, and increase sensitivity. Agilent eclipse C_{18}, 150 mm × 4.6 mm, 3.5 µm column, was chosen as the stationary phase for the analyzed compounds. Acetonitrile and 0.1 M ammonium formate buffer (40:60) were used for the mobile phase. The optimized method chromatogram is presented in Fig. 3. The finest outcomes were found with a flow rate of 1.0 ml/min.

Fig. 3 [Images not available. See PDF.]

LLOQ chromatogram of amivantamab

Optimization of the sample preparation

Based on the pKa value of the compound and the ease of sample extraction, the liquid–liquid extraction method was chosen for sample preparation. Acetonitrile was selected as the extractant because of its low ionization suppression

and excellent extraction efficiency compared to other organic solvents frequently employed in LC-MS/MS analysis. High sensitivity, linear calibration range, and low matrix effect were attained for amivantamab.

Bioanalytical method validation

System suitability

System suitability was examining a set of reference standards to determine an instrument's performance, which was conducted before the analytical run. The CV (%) for amivantamab and trastuzumab area ratio was 0.13. The CV (%) of retention time of amivantamab and trastuzumab was found to be 0.63 and 0.23, respectively. The system suitability parameters like tailing factor, plate count, and resolution were within the limit. System suitability parameters are displayed in Table 4.

Table 4. System suitability results of amivantamab

	Name	Retention time	Area	USP tailing	Theoretical plate	USP resolution
1	Amivantamab	2.128	3265124	1.07	6594	
2	Trastuzumab	4.136	4485769	1.01	7318	8.19

Linearity

The peak area obtained from the analysis was used to calculate the area response ratio. A calibration curve was plotted by taking concentration on the X-axis and area response ratio on the Y-axis. The slope, intercept, and correlation coefficient were obtained from the plot. The best linearity of the calibration curve for amivantamab was secured over the concentration ranges of 5.0−100.0 ng/ml. The correlation coefficient was 0.99949. The linearity range of solutions and respective area response ratios are tabulated in Table 5. A representative calibration curve for amivantamab is presented in Fig. 4.

Table 5. Linearity results of amivantamab

Concentration (ng/ml)	Area	Area response ratio
0	0	0
5	0.345×10^5	0.082
12.5	0.852×10^5	0.202
25.0	1.754×10^5	0.416
37.5	2.601×10^5	0.616
50.0	3.457×10^5	0.819
62.5	4.305×10^5	1.020
75.0	5.211×10^5	1.233
100.0	6.712×10^5	1.589

Slope	0.01610
Intercept	0.00584
R^2 value	0.99949

Fig. 4 [Images not available. See PDF.]

Calibration curve of amivantamab

Specificity

Specificity results exhibited that the process developed was highly selective for amivantamab. No discernible endogenous chemicals interfered at the retention times for amivantamab and trastuzumab were seen in six different types of blank plasma. The ability to clearly distinguish the analyte in biological fluids, which comprised various components, including the matrix, was known as specificity. The specificity results are tabulated in Table 6.

Table 6. Specificity results of amivantamab

S. no	Sample ID	Intensity (cps)		% Interference		Pass/fail
Amivantamab	Trastuzumab	Amivantamab	Trastuzumab	1	Std Blank 1	0
0	0	0	Pass	2	LLOQ 1 (5 ng/ml)	0.369×10^5
4.237×10^5	0	0	Pass	3	Std Blank 2	0
0	0	0	Pass	4	LLOQ 2 (5 ng/ml)	0.361×10^5
4.221×10^5	0	0	Pass	5	Std Blank 3	0
0	0	0	Pass	6	LLOQ 3 (5 ng/ml)	0.365×10^5
4.234×10^5	0	0	Pass	7	Std Blank 4	0
0	0	0	Pass	8	LLOQ 4 (5 ng/ml)	0.367×10^5

4.225×10^5	0	0	Pass	9	Std Blank 5	0
0	0	0	Pass	10	LLOQ 5 (5 ng/ml)	0.363×10^5
4.211×10^5	0	0	Pass	11	Std Blank 6	0
0	0	0	Pass	12	LLOQ 6 (5 ng/ml)	0.364×10^5

Sensitivity (LLOQ)

The current approach achieved an LLOQ of 5.00 ng/ml for amivantamab in rat plasma. The CV (%) and mean accuracy (%) were 6.30% and 93.10%, respectively. Table 7 displays the sensitivity test results.

Table 7. Sensitivity results of amivantamab

Replicate number	LLOQ (5.0 ng/ml)
1	4.3 ng/ml
2	4.9 ng/ml
3	4.4 ng/ml
4	4.9 ng/ml
5	4.9 ng/ml
6	4.5 ng/ml
Mean	4.7 ng/ml
SD	0.3
% CV	6.3
% Mean accuracy	93.1%

Accuracy and precision

Accuracy was shown as % mean recovery and precision as % CV. The degree to which the experimental value and the actual value were similar depends on the accuracy of the analytical approach. LLQC, LQC, MQC, and HQC solutions were prepared in replicates and injected into the system. The intraday accuracy and precision of amivantamab were 94.2–99.9% and 0.9–5.3, respectively. Results were summarized in Table 8.

Table 8. Precision and accuracy results of amivantamab

Replicate no	HQC (75.0 ng/ml)	MQC (50.0 ng/ml)	LQC (25.0 ng/ml)	LLQC (5.0 ng/ml)
Concentration (ng/ml)				1
74.8	49.9	24.5	4.6	2
74.8	49.9	25.1	4.6	3
75.4	48.7	25.1	4.6	4
75.4	49.3	25.1	5.2	5
73.6	49.9	25.1	4.6	6
74.2	49.9	25.1	4.6	Mean
74.7	49.6	24.9	4.7	SD
0.7	0.5	0.3	0.3	% CV
0.9	1.0	1.0	5.3	% Mean accuracy

Autosampler carryover

The chromatograms of standard blank samples were observed, and no significant carryover of amivantamab was detected. Similarly, carryover of trastuzumab was also not found.

LOD and LOQ

The LOD (1.67 ng/ml) solution was prepared so that the S/N ratio ranged around 3:1. Respective chromatograms of LOD and LOQ (5.0 ng/ml) are shown in Figs. 5 and 6. The method was found to be sensitive and specific.

Fig. 5 [Images not available. See PDF.]

Chromatogram of LOD

Fig. 6 [Images not available. See PDF.]

Chromatogram of LOQ

Dilution integrity

Dilution integrity was the evaluation of the sample dilution technique to ensure that it does not affect the precision and accuracy of the measured concentration of the analyte, as needed. The CV (%) and mean accuracy (%) for MQC and ULQC were found to be 0.5, 0.5, and 100.1 and 98.5, respectively. The results are displayed in Table 9.

Table 9. Results for dilution integrity

Replicate number	MQC (50.0 ng/ml)	ULQC (100.0 ng/ml)
Concentration		1

49.9	97.8	2
49.9	98.4	3
49.9	99.0	4
49.9	99.0	5
50.6	98.4	6
49.9	98.4	Mean
50.1	98.5	SD
0.3	0.5	% CV
0.5	0.5	% Mean accuracy

Matrix effect

A matrix effect describes the changes observed in detecting or quantifying an analyte when other substances are present in the sample. The CV (%) for LQC and HQC was observed to be 0.73 and 1.20, respectively. The mean accuracy was 99.71% for low-quality control samples and 99.88% for high-quality control samples. Data was included in the Additional file 1.

Recovery of analyte

The effectiveness of separating analytes from samples was described by recovery. The CV (%) for extracted and unextracted samples at the HQC level was found to be 0.99 and 0.98, respectively. The mean recovery (%) for extracted and unextracted samples at the HQC level was found to be 99.79 and 99.94%, respectively. Data was incorporated in the Additional file 1.

Recovery of internal standard

The CV (%) of unextracted and extracted methods was found to be 0.17 and 0.19, respectively. The CV (%) of trastuzumab's recovery was around 15.00%. Data was integrated in the Additional file 1.

Ruggedness

Ruggedness is a measure of the susceptibility of a method to small changes that might occur during routine analysis, like small changes in pH values, mobile phase composition, temperature, analysis, etc. The mean accuracy (%) for LQC, MQC, and HQC was 99.13–99.88%. Data was included in the Additional file 1.

Reinjection reproducibility

Six duplicates of low, medium, and high QCs of amivantamab were reinjected into plasma samples to see if samples could be reinjected in the event of instrument failure or other issues. The % CV for HQC, MQC, and LQC was found to be 0.7, 0.3, and 0.9%, respectively. Data was added in the Additional file 1.

Stability studies

Bench-top stability

Bench-top stability is the stability of an analyte in a matrix under sample handling conditions during sample processing. The CV (%) of HQC, LQC, and MQC was found to be 0.9, 1.3, and 0.5, respectively. The mean accuracy (%) of HQC, LQC, and MQC was found to be 99.7, 98.8, and 98.4%, respectively. The results of bench top stability are shown in Table 10.

Table 10. Bench top stability results of amivantamab

Replicate no	HQC (75.0 ng/ml)	LQC (25.0 ng/ml)	MQC (50.0 ng/ml)
Concentration (ng/ml)			1
75.4	24.5	48.7	2
74.2	24.5	49.3	3
75.4	25.1	49.3	4
75.4	24.5	49.3	5
74.2	25.1	49.3	6
74.2	24.5	49.3	Mean
74.8	24.7	49.2	SD
0.7	0.3	0.3	% CV
0.9	1.3	0.5	% Mean accuracy

Short-term stability and long-term stability

Long-term stability assesses the degradation of an analyte in the matrix relative to the starting material after periods of frozen storage. The results showed that amivantamab QC low, medium, and high samples were stable in short-term and long-term stability. Short-term stability results are displayed in Table 11, and long-term stability results are summarized in Tables 12, 13, 14, 15, 16.

Table 11. Short-term stability results of amivantamab

Replicate no	HQC (75.0 ng/ml)	LQC (25.0 ng/ml)	MQC (50.0 ng/ml)
Concentration (ng/ml)			1
74.8	23.2	49.3	2
74.8	23.2	49.3	3
74.2	22.6	48.7	4
74.2	23.2	49.3	5
74.8	22.6	48.7	6
75.4	23.2	48.7	Mean

74.7	23.0	49.0	SD
0.5	0.3	0.3	% CV
0.6	1.4	0.7	% Mean accuracy

Table 12. Long-term stability results of amivantamab—Day 1

Replicate no	HQC (75.0 ng/ml)	LQC (25.0 ng/ml)	MQC (50.0 ng/ml)
Concentration (ng/ml)			1
75.4	25.1	49.3	2
73.6	24.5	49.9	3
74.2	24.5	49.3	4
76.0	24.5	49.3	5
76.0	23.8	49.9	6
74.2	25.1	49.9	Mean
74.9	24.6	49.6	SD
1.1	0.5	0.3	% CV
1.4	1.9	0.7	% Mean accuracy

Table 13. Long-term stability results of amivantamab—Day 7

Replicate no	HQC (75.0 ng/ml)	LQC (25.0 ng/ml)	MQC (50.0 ng/ml)
Concentration (ng/ml)			1
75.4	23.2	48.7	2
75.4	23.2	48.7	3
75.4	22.6	48.1	4
74.8	22.6	48.7	5
74.8	23.2	48.1	6

74.8	22.3	48.7	Mean
75.1	22.9	48.5	SD
0.3	0.3	0.3	% CV
0.5	1.5	0.7	% Mean accuracy

Table 14. Long-term stability results of amivantamab—Day 14

Replicate no	HQC (75.0 ng/ml)	LQC (25.0 ng/ml)	MQC (50.0 ng/ml)
Concentration (ng/ml)			1
73.6	22.6	48.7	2
72.9	22.6	48.1	3
73.6	21.9	48.1	4
74.2	22.6	48.7	5
73.6	22.6	48.1	6
74.8	23.2	48.7	Mean
73.8	22.6	48.4	SD
0.6	0.4	0.3	% CV
0.9	1.7	0.7	% Mean accuracy

Table 15. Long-term stability results of amivantamab—Day 21

Replicate no	HQC (75.0 ng/ml)	LQC (25.0 ng/ml)	MQC (50.0 ng/ml)
Concentration (ng/ml)			1
73.6	21.9	46.8	2
73.6	21.9	47.5	3
73.6	22.6	47.5	4
74.2	22.6	47.5	5

72.9	22.6	48.1	6
73.6	21.9	46.8	Mean
73.6	22.3	47.4	SD
0.4	0.3	0.5	% CV
0.5	1.5	0.9	% Mean accuracy

Table 16. Long-term stability results of amivantamab—Day 28

Replicate no	HQC (75.0 ng/ml)	LQC (25.0 ng/ml)	MQC (50.0 ng/ml)
Concentration (ng/ml)			1
72.3	21.4	45.6	2
72.3	21.4	46.2	3
72.9	21.4	46.2	4
72.9	21.4	46.2	5
72.9	21.9	46.2	6
72.9	20.8	45.6	Mean
72.7	21.4	46.0	SD
0.3	0.4	0.3	% CV
0.4	1.8	0.7	% Mean accuracy

Freeze–thaw stability

Freeze–thaw stability refers to the stability of the analyte in the matrix upon freezing and thawing. The CV (%) of HQC, LQC, and MQC was found to be 1.2, 1.9, and 0.8, respectively. The mean accuracy (%) of HQC, LQC, and MQC was found to be 99.75, 99.94, and 99.94%, respectively. The results are tabulated in Table 17.

Table 17. Freeze–thaw stability results of amivantamab

Replicate no	HQC (75.0 ng/ml)	LQC (25.0 ng/ml)	MQC (50.0 ng/ml)
Concentration (ng/ml)			1
74.8	24.5	50.6	2

75.4	24.5	49.9	3
74.2	24.5	49.9	4
76.0	25.1	49.9	5
73.6	25.1	49.9	6
74.8	23.9	49.9	Mean
74.8	24.6	49.9	SD
0.9	0.5	0.4	% CV
1.2	1.9	0.8	% Mean accuracy

Autosampler stability

Autosampler stability is the stability of the analyte in the processed sample under the conditions in the autosampler. The CV (%) of HQC, LQC, and MQC was found to be 1.09, 0.46, and 1.24, respectively. The mean accuracy (%) of HQC, LQC, and MQC was found to be 99.7, 98.3, and 99.9%, respectively. The outcomes are summarized in Table 18.

Table 18. Autosampler stability results of amivantamab

Replicate no	HQC (75.0 ng/ml)	LQC (25.0 ng/ml)	MQC (50.0 ng/ml)
Concentration (ng/ml)			1
75.4	25.1	48.7	2
75.4	25.1	48.7	3
75.4	24.5	49.3	4
75.4	25.1	49.3	5
75.4	25.1	48.7	6
75.4	25.1	48.7	7
76.0	25.1	48.7	8
74.8	25.1	49.3	9
75.4	25.1	49.3	10

76.0	24.5	49.3	11
76.0	24.5	49.3	12
76.0	24.5	49.3	13
74.8	24.5	49.3	14
73.6	25.1	49.9	15
74.2	24.5	49.9	16
74.2	25.1	49.9	17
74.2	25.1	49.9	18
74.8	25.7	49.9	19
74.2	25.7	49.9	20
74.8	25.1	49.9	21
74.2	25.1	49.9	22
74.2	25.1	49.9	23
74.8	25.1	50.6	24
74.8	25.1	50.6	Mean
74.9	24.9	49.5	SD
0.7	0.4	0.6	% CV
0.9	1.4	1.1	% Mean accuracy

Dry extract stability and wet extract stability

Extract stability assesses the degradation of the processed sample relative to the starting material. The results of wet extract stability are tabulated in Tables 19, 20. Similarly, the results of dry extract stability were summarized in Tables 21, 22.

Table 19. Wet extract stability results of amivantamab at 12 h

Replicate no	HQC (75.0 ng/ml)	LQC (25.0 ng/ml)	MQC (50.0 ng/ml)
Concentration (ng/ml)			1

75.4	25.1	49.9	2
73.6	25.1	49.3	3
76.0	25.1	49.3	4
76.7	24.5	49.3	5
74.8	23.9	50.6	6
76.7	24.5	49.9	Mean
75.5	24.7	49.7	SD
1.2	0.5	0.5	% CV
1.6	2.1	1.0	% Mean accuracy

Table 20. Wet extract stability results of amivantamab at 18 h

Replicate no	HQC (75.0 ng/ml)	LQC (25.0 ng/ml)	MQC (50.0 ng/ml)
Concentration (ng/ml)			1
76.0	25.1	49.9	2
76.7	25.7	50.6	3
76.7	24.5	49.9	4
76.0	24.5	50.6	5
74.8	25.1	50.6	6
76.0	24.5	50.6	Mean
76.0	24.9	50.4	SD
0.7	0.5	0.3	% CV
0.9	2.0	0.6	% Mean accuracy

Table 21. Dry extract stability results of amivantamab at 12 h

Replicate no	HQC (75.0 ng/ml)	LQC (25.0 ng/ml)	MQC (50.0 ng/ml)

Concentration (ng/ml)			1
75.4	25.7	49.9	2
75.4	25.1	49.3	3
76.0	25.1	49.3	4
74.2	24.5	49.9	5
76.7	24.5	49.9	6
74.8	23.8	49.9	Mean
75.4	24.8	49.7	SD
0.9	0.7	0.3	% CV
1.2	2.6	0.6	% Mean accuracy

Table 22. Dry extract stability results of amivantamab at 18 h

Replicate no	HQC (75.0 ng/ml)	LQC (25.0 ng/ml)	MQC (50.0 ng/ml)
Concentration (ng/ml)			1
76.0	25.1	50.6	2
75.4	25.1	49.9	3
74.8	25.1	49.9	4
76.7	24.5	49.9	5
73.6	24.5	49.3	6
73.6	24.5	49.9	Mean
74.9	24.8	49.9	SD
1.3	0.3	0.4	% CV
1.7	1.4	0.8	% Mean accuracy

Assay

The developed method's applicability for quantifying amivantamab in the marketed formulation was assessed. The

assay (%) of amivantamab was found to be 99.96%. The results of the assay are shown in Table 23.

Table 23. Results of assay

Injection	Area counts
1	3.439×10^5
2	3.474×10^5
Mean	3.457×10^5
% Assay	99.96

Method application to rat plasma samples

The developed and validated procedure was applied to study in rats. The concentrations of amivantamab in rat plasma samples are tabulated in Table 24. The pharmacokinetic parameters of amivantamab were calculated using Phoenix Win Nonlin (Version 5.2) software. The results of pharmacokinetic parameters are tabulated in Table 25. The recovery plot of amivantamab in rat plasma is shown in Fig. 7.

Table 24. Concentration of amivantamab in rat samples

Time intervals (minutes)	Amivantamab (ng/ml)	SD	% CV
10	46.216	0.731	1.581
20	28.151	0.640	2.275
30	12.117	0.793	6.548
40	6.517	0.587	9.014
50	2.155	0.414	19.209
60	0	0	0

Table 25. Pharmacokinetic parameters of amivantamab

Pharmacokinetic parameters	Amivantamab
AUC_{0-t}	15.16 ng-h/ml
C_{max}	46.22 ng/ml
$AUC_{0-\infty}$	15.57 ng-h/ml
T_{max}	10.00 min

$T_{1/2}$	7.80 min

Fig. 7 [Images not available. See PDF.]

Recovery plot for amivantamab in rat plasma

Discussion

LC-MS/MS is a sensitive method for the quantification of monoclonal antibodies. Several elution conditions were tested for the chromatographic separation. In trial 1, a mobile phase composition of acetonitrile and triethylamine buffer in the ratio 60:40 was used. Peak splitting was observed, so further trial was carried out. In trial 2, a mobile phase ratio of acetonitrile and ammonium formate buffer (60:40) was used. The plate count was not within the limit. Hence, further trial was carried out. A mobile phase ratio of acetonitrile and ammonium formate buffer (50:50) was used in trial 3. Peak heights were not within the limit, so further trial was conducted. In trial 4, a mobile phase ratio of acetonitrile and ammonium formate buffer (40:60) was used. System suitability parameters were within the limit, so this method was validated. The developed method quantified amivantamab in a biological matrix. The system was deemed suitable for usage if the area ratio's CV (%) was less than five and the retention time's CV (%) was less than 2. It thus passed the system suitability test. The calibration curve was deemed agreeable when % accuracy for all calibration curve standards ranged from 85.00 to 115.00%. The correlation coefficient (R^2) was 0.99 or better. The method was found to be linear. The response of any interfering peaks at the analyte retention time was to be ≤ 20.00% of amivantamab at LLOQ and ≤ 5.00% of that in LLOQ in the case of trastuzumab. The method was found to be specific and selective. Sensitivity acceptance criteria were to be as 4 out of 6 samples, or at least 67.00%, fell within the 80.00–120.00% range. The recommended range for mean accuracy (%) was 80.00–120.00%. The CV's (%) accuracy was to be 20.00%. The outcomes fell within the permitted range. The method was found to be sensitive. The standards for data acceptance included accuracy (%) within 85.00–115.00% of the actual values and precision within 15.00% relative standard deviation (RSD). These findings demonstrated that the accuracy and precision were reproducible and dependable for quantifying amivantamab in rat plasma. If the analyte concentration detected in the double blank sample was less than 20.00% for amivantamab, carryover was deemed significant. Hence, there was no carryover effect. CV (%) and mean accuracy in dilution integrity were within the limits for amivantamab. The minimum acceptance standard required that two out of three samples at every level fell under the 85.00 to 115.00% range. The matrix lot was to be within the agreeable criteria in at least 80.00% (5 out of 6 cases). The results were within the tolerable range. Hence, the matrix effect was found to be negligible. For each QC level, the CV (%) of recovery was to be under 15.00%. For all QC levels, the mean recovery CV (%) was to be under 20.00% overall. All of the results fell within desirable limits. The overall mean recovery (%) and CV (%) were less than 20.00% for all QC levels. The range of the mean accuracy for low-, medium-, and high-quality control samples was between 85.00 and 115.00%. The results were found within tolerable limits. The results were within the tolerable range. This specifies that the extraction technique used was effective. The limitations were all met in reinjection reproducibility. The method was found to be reproducible. The CV (%) of low- and high-quality control samples was ≤ 15.00%. The CV (%) and mean accuracy were within the standard limits. Any condition, time period, or analyte concentration examined had less than 15.00% of CVs. All the stability results were within the tolerable range. The range for the LQC and HQC samples' mean concentration accuracy was between 85.00 and 115.00%. LQC and HQC samples were to have a CV (%) of less than 15.00%. The results showed that amivantamab was stable in rat plasma. CV (%) and mean accuracy (%) were within the limits. Samples were deemed stable if the CV (%) for the low-, medium-, and high-quality control samples was less than 15.00%. It showed that the stability of the autosampler was determined to be within limits. Moreover, the mean accuracy and CV (%) were within limits. The CV (%) and mean accuracy (%) for amivantamab passed the wet and dry extract stability. As a result, the approach was accurate in various conditions. Through the study of three QC samples of amivantamab the application of various storage conditions, stability of the drug was evaluated. The findings were consistent throughout the studies conducted. These stability results indicate that amivantamab was stable during benchtop, freeze–thaw, autosampler,

short term, long term, wet extract, and dry extract stability studies. Also, amivantamab was stable during the storage and handling of samples in rat plasma matrix. The study confirmed that the bioanalytical method was accurate and can be used to study pharmaceutical dosage forms. The validated technique was sensitive enough to quantify analyte in plasma samples in experimental rats accurately. The pharmacokinetic findings illustrate less absorption and metabolism effects on amivantamab in rats. These findings will be helpful in further pharmacokinetic assessments.

Conclusions

This was the first fully validated stability, indicating that the LC-MS/MS technique was developed to measure amivantamab in pharmaceutical preparations and rat plasma. A precise, easy, and repeatable method for measuring amivantamab in rat plasma was developed and validated by ICH M10 guidelines. The validation parameters' findings inferred that the current analysis technique could be used to carry out bioavailability studies with high sensitivity, precision, and accuracy. Also, from the recovery studies, it was found that there was less interaction from the matrix to monoclonal antibody and less absorption or distribution in the rats. It was strongly advised to evaluate the quality of medications during routine analyses or stability studies. Rat plasma samples can be analyzed in clinical investigations using this fully validated approach.

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Author contributions

PKG conceived and designed the analysis, collected samples, collected data, performed the analysis, and wrote the manuscript. SR conceived and designed the analysis. All authors have read and approved the manuscript.

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nCoV-19 therapeutics using cucurbitacin I structural derivatives: an in silico approach

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ABSTRACT (ENGLISH)

Background

Cucurbitacins are present in some common vegetables as secondary metabolites and are used by the plants against harmful microbes. Exploration of this capability of natural product based substances against wide variety of microbes seems relevant due to the ease of availability of the resources and safety. In this regard, considering the current pandemic, the antiviral properties of these molecules with a subset of Cucurbitacin I structural derivatives have been screened. The inhibition potential of the phytochemicals was assessed by the stability of the protein–ligand complex formed with the nucleocapsid protein (PDB ID: 7CDZ) of SARS-CoV-2 by computational methods. The proposition of an alternate antiviral candidate that is cost-effective and efficient relative to existing formulations is the main objective of this work.

Results

Server-based molecular docking experiments revealed CBN19 (PubChem CID: 125125068) as a hit candidate among 101 test compounds, a reference molecule (K31), and 5 FDA-approved drugs in terms of binding affinities sorted out based on total energies. The molecular dynamics simulations (MDS) showed moderate stability of the protein-CBN19 complex as implied by various geometrical parameters RMSD, R_g, RMSF, SASA and hydrogen bond count. The ligand RMSD of 3.0 ± 0.5 Å, RMSF of C_α of protein with less than 5 Å, and smooth nature of SASA and R_g curves were calculated for the adduct. The binding free energy (−47.19 ± 6.24 kcal/mol) extracted from the MDS trajectory using the MMGBSA method indicated spontaneity of the reaction between CBN19 and the protein. The multiple ADMET studies of the phytochemicals predicted some drug-like properties with minimal toxicity that mandate experimental verification.

Conclusions

Based on all the preliminary in silico results, Cucurbitacin, CBN19 could be proposed as a potential inhibitor of nucleocapsid protein theoretically capable of curing the disease. The proposed molecule is recommended for further in vitro and in vivo trials in the quest to develop effective and alternate therapeutics from plant-based resources against COVID-19.

FULL TEXT

Background

After claiming nearly 7 million people, the virus responsible for COVID-19, SARS-CoV-2 is still propagating [1, 2]. Even though the vaccines have helped to contain the pandemic to some extent, effective and safe drugs are still in the experimental phases [3, 4]. One of the alternatives to existing pharmaceuticals is the phytochemical-based therapeutics that need to be designed and developed for proper treatment and cure of the disease in a cost-effective manner. Various natural products have been reviewed in terms of their potential to fight the disease, taking into consideration the specific targets [5]. Cucurbitacins, tetracyclic triterpenoids occur in regularly consumed vegetables like cucumber, gourds, squash, and pumpkins as secondary metabolites and act in the plant's defense mechanisms [6]. The molecular structure of Cucurbitacin I is shown in Fig. 1. These vegetables contain multi-sized nutrients, antioxidants, vitamins, amino acids, and small anti-nutrients [7]. It carries non-trivial ethnobotanical significance and has been in use in the treatment of multiple diseases since ancient times [8–12] with weaker side effects, good safety, and multiple healing pathways [13]. Especially, cucurbitacin B has been found to possess anti-Herpes simplex virus 1 biological activity with IC_{50} of 0.94 ± 0.2 μM [14] and forms the basis for the selection of this class of organic compound with possibly similar pharmacophore. Therefore, the adaptation of local practices by scientific research in developing a new type of compound as a drug with molecular-level understanding seems relevant

considering this class of organic compound having known biological activity and the requirement of the present global context.

Fig. 1 [Images not available. See PDF.]

Molecular structure of a parent molecule, Cucurbitacin I

Out of twenty-nine proteins in the viral genome of SARS-CoV-2, a structural protein, nucleocapsid (N), seems to be a good target for small ligands since it is responsible for RNA encapsidation, transcription and replication of the viral genome [15, 16]. Therefore, its involvement in the assembly, budding, cell cycle regulation, immune system modulation, and cessation of host cell translation makes it one of the prominent functional entities [17, 18]. If a guest molecule binds effectively to it then its normal functioning will be disrupted, which would result in the deceleration or halting of the viral replication, ultimately leading to the cure of the disease caused by it. A recent study involving NMR and mass spectroscopic techniques has shown the protein to be druggable and possesses greater availability [19]. A compound K31 [4-(3-Bromophenyl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline-6-carboxylic acid, PubChem CID: 2872351] has been recently reported to bind to it with EC_{50} of 1.7 ± 0.2 μM [15]. Alectinib has been proposed to be capable of inhibiting nucleocapsid protein by prohibiting phosphorylation from in vitro studies [20]. Using in silico approach, Curcumin, Apigenin, Cinnamic acid, Simeprevir, and Grazoprevir have been found to bind successfully with the nucleocapsid protein in different studies [21–23]. Suramin, an antiparasitic drug, has been proposed based on experimental and in silico studies to disturb the association of N-terminal domain (NTD) with RNA, leading to the cessation of viral replication [24]. These results establish the selected protein as a good therapeutic target and therefore, has been chosen for this work.

The relative strength of the binding of the test molecule and its orientation at the orthosteric site of the receptor can be determined by molecular docking calculations as that performed with Cucurbitacins against multiple target proteins of SARS-CoV-2 [25]. The assessment of the stability of the protein–ligand adduct by monitoring the conservation of the initial docked pose of the ligand at the active pocket by molecular dynamics simulations is required. The spontaneity of the forward reaction inferred from binding free energy changes would help to design a molecule that can effectively inhibit the protein by binding to it in a thermodynamically and geometrically stable manner. The safety and bioavailability of the drug-like candidate from ADMET predictions would help to establish the test compound as a good and safe drug against the disease [26]. The computational approach stands as a viable tool in designing a drug that would prevent later failures in high-throughput experiments and clinical trials. One objective of this work is a quick screening of safe, natural product-based molecules with Cucurbitacin I as a scaffold against SARS-CoV-2. The other is an extension of traditional medicinal knowledge into the modern concept of rational drug design and development with molecular-level understanding.

Since the nucleocapsid protein is of utmost importance in functioning at various stages in the viral life cycle [27] and is druggable, it has been adopted in this research work for exploring its possible interactions with Cucurbitacins I scaffold derivatives. The antiviral capabilities of a slightly different structure of Cucurbitacin B have added to the rationale of small molecule selection [14].

Objectives of the work

- a. To explore the phytochemicals from regularly used vegetables with known biological activities against a specific viral target by cost-effective computational methods.
- b. To identify a drug-like and safe molecule with potential inhibitory capabilities of the functioning of the nucleocapsid protein.
- c. To recommend a hit molecule for in vitro and in vivo trials in the course of developing it as an effective nutraceutical/pharmaceutical against SARS-CoV-2.

Experimental/methodology

Ligand and receptor preparation

The parent molecule, Cucurbitacin I (PubChem CID: 5281321) and its substructure derivatives were taken in sdf file format from the PubChem webpage (https://pubchem.ncbi.nlm.nih.gov/, accessed 12 July 2023) [28] and converted to pdb format using *obabel* command (The Open Babel Suite, version 2.3.1 http://openbabel.org, accessed 12 July 2023) [29] and the PyMol program [30]. The N-terminal domain of nucleocapsid protein of SARS-CoV-2 as a drug target (PDB ID: 7CDZ, X-ray diffraction, resolution 1.80 Å, Escherichia coli BL21 expression system) (https://doi.org/https://doi.org/10.2210/pdb7CDZ/pdb) was retrieved in pdb file format from the RCSB website (https://www.rcsb.org/, accessed 4 July 2022) [31], verified and preprocessed for molecular docking.

Molecular docking calculations

The DockThor web-server (https://dockthor.lncc.br/v2/, accessed 12 July 2023) [32] was used in molecular docking calculations with center coordinates of (−6, 22, 16), discretization of 0.25, and grid size of ($20 \times 20 \times 20$ $Å^3$) of the active pocket. The genetic algorithm search settings involved 1,000,000 evaluations with 750 population size for 24 number of runs deemed suitable for capturing the local minima in a complicated potential energy surface. The affinity prediction was done for each ligand based on the total energy, which was calculated for its different binding modes. It involved inter-molecular, intra-molecular, and torsional energies and were clustered to finally yield the best value [32, 33].

Molecular dynamics simulations and thermodynamics

The docked pose with the best binding affinity was taken for molecular dynamics simulations (MDS) of 90 ns production run at 310 K using the GROMACS version 2021.2 program [34]. The force-field (charmm27) [35] was obtained from the SwissParam server (https://www.swissparam.ch/, accessed 12 July 2023) [36], and all the other parameters as reported by Sharma et al. [37] were adopted. Multiple parameters like root mean square deviation (RMSD), radius of gyration (R_g), root mean square fluctuation (RMSF), solvent accessible surface area (SASA), and number of hydrogen bonds between ligand and residues were determined from MDS trajectory using inbuilt commands of GROMACS. Binding free energy change of complex formation was calculated using MMGBSA method [38] with GB7 option [39] for the best-docked pose using gmx_MMPBSA module (100 frames) [40] in GROMACS and also using the fastDRH server (http://cadd.zju.edu.cn/fastdrh/, accessed 15 July 2023, ID4638) [41]. The differences in the fluctuation of the residues upon ligand binding were determined in terms of RMSF difference from the MDS of 10 ns duration using the CABSflex2 web-server (http://biocomp.chem.uw.edu.pl/CABSflex2/, accessed 15 July 2023) [42].

Pharmacokinetics and pharmacodynamics

The SMILES strings of the molecules were used as input in determining various parameters. The ADMET prediction [43] was made using the pkCSM (https://biosig.lab.uq.edu.au/pkcsm/prediction, accessed 24 October 2023) [44] and the ADMETboost (https://ai-druglab.smu.edu/, accessed 25 October 2023) [45] servers. Various toxicity end-points and the rule of five (RO5) or Lipinski's rule were calculated along with other physicochemical parameters of selected Cucurbitacin I derivatives.

Programs and resources

The structures were visualized using the Biovia Discovery Studio visualizer v21.1.0.20298 [46] and the Avogadro2 v1.93.0 [47] programs. The graphical representations were made with the GNUPLOT 5.2 patch level 8 [48] and the Grace-5.1.25 [49] software. The SMILES strings were converted into 2D molecular structures by the online Marvin JS structure editor (https://chemaxon.com/products/marvin-js). The computational experiments were carried out in different machines with multi-core processors, GPU accelerator, RAM 128 GB, and storage 6 TB. The operating

systems were Ubuntu 20.04 LTS and Windows 8. Data analysis, visualization and interpretation were carried out on a PC with minimal configuration.

Results

Binding affinities and orientation of the docked molecule

The 2D representations of selected test molecules and a reference are shown in Fig. 2. The binding affinities of the top five Cucurbitacin I derivatives, a reference compound (K31) and that of FDA-approved drugs are shown in Table 1.

Fig. 2 [Images not available. See PDF.]

Molecular structures of the top compounds and a reference

Table 1. Top five Cucurbitacin I derivatives, a reference compound and 7 commercial drugs in terms of binding affinities with NTD of nucleocapsid protein

Molecules (PubChem CID)	Affinity (kcal/mol)	Total energy (kcal/mol)	Commercial drugs	Affinity (kcal/mol)	Total energy (kcal/mol)
CBN19 (125125068)	−10.27	92.01	Simeprevir	−10.56	5227.5
CBN36 (162939996)	−10.20	73.67	Vapreotide	−10.45	212.33
CBN85 (138107616)	−10.16	83.82	Atazanavir	−9.66	82.34
CBN76 (125039415)	−10.06	73.16	Ivermectin	−9.13	1375.73
CBN96 (162970907)	−10.05	68.28	Remdesivir	−8.84	10.65
			Hydroxychloroquine	−8.78	43.53
K31 (2872351)	−8.86	25.67	Chloroquine	−8.65	16.81

The compound CBN19 in its docked pose with the receptor is displayed in Fig. 3A. Figure 3B depicts the hydrophobic surface of the active pocket of the protein that interacts with the ligand from two viewing angles 180° apart.

Fig. 3 [Images not available. See PDF.]

Docked pose of **A** CBN19 (ball and stick model) with the nucleocapsid protein (ribbon representation) and **B** CBN19 in the active pocket enveloped with hydrophobic surface viewed at 180° apart

It is evident from the data that the drugs, Simeprevir and Vapreotide lead among all the compounds, and the former,

along with Grazoprevir, has been reported by Bhowmik et al. [23] from computational screenings as better candidates. However, the top five test candidates possess better affinities than the other five FDA-approved drugs. The compound CBN19, with the best binding affinity could be considered a hit candidate among the pool of the studied molecules. The highest total energy, however, imply that the reference compound K31, possessed weaker binding (−8.86 kcal/mol) than that of the test compounds and was bound to the N-terminal domain of nucleocapsid protein as inferred experimentally [15]. Apart from the molecules mentioned above, Emetine and Glycyrrhizin have also been found to possess good docking properties against nucleocapsid protein [50, 51]. The computational results show promising properties of the selected molecules derived from a molecular scaffold suitable for further characterization and trials.

Druglikeness and toxicity assessment

The drug-like characteristics in terms of RO5 were found to be acceptable for most of the compounds, inferring good bioavailability and permeability (data presented in supplementary information as ADMET_predictions.xls file). The pharmacokinetics of various Cucurbitacins have been reported by Delgado-Tiburcio et al. [9]. The toxicity in terms of the AMES test, hERG I/II inhibition, hepatotoxicity and skin sensitization showed negative results for the test molecules. The permeability of BBB and CNS are poor or absent, with suitable excretion parameters. The results from the pkCSM server showed that Cucurbitacins possess acceptable drug-like properties with relatively safe end-points.

The ADMET predictions from another server (ADMETboost), to validate the earlier results, were analyzed for different properties. The output, however, showed that Caco2-permeability and aqueous solubility lay outside the acceptable range, whereas all the other properties were predicted to be similar to those from the pkCSM server.

Geometrical stability of complexes

The binding affinity based on a scoring function provides a clue towards the strength of interactions between the ligand and the protein and can be used as a comparative property. It may not necessarily reflect the binding efficacy considering the time the ligand stays at its initially docked position and may not be a measure of the absolute property. To determine the conservation of pose and position of the ligand with time and to determine the geometrical stability of a system, molecular dynamics simulation of 90 ns duration was carried out and various parameters like RMSD, SASA, RMSF, R_g, and hydrogen bond count were extracted from the trajectory. These parameters provide a numerical assessment of the structural stability and are analyzed thoroughly in this section. The RMSD of the ligand and that of the protein are shown in Fig. 4 as subplots A and B, respectively.

Fig. 4 [Images not available. See PDF.]

Multiple parameters extracted from MDS trajectory **A** RMSD of ligands with respect to protein backbone **B** RMSD of protein backbone with respect to protein backbone (CBN19 = dark, CBN36 = red, CBN85 = green, CBN76 = blue, and CBN96 = indigo)

The RMSD of ligand CBN19 with respect to the protein backbone (dark curve) was *ca.* 2 Å and was moderately smooth with small fluctuations at 55 ns and 70 ns regions. It remained flat from 75 to 85 ns, similar to the initial part of the trajectory up to 45 ns. The results hint at near preservation of the docked pose during the simulation period. Similar values have been reported for approved drugs against nucleocapsid CTD [52]. The compounds with CBN36 and CBN96 showed unsteady behavior, both attaining equilibrium after 60 ns with RMSD larger than 6.8 Å. Another compound CBN85 showed an RMSD of *ca.* 4 Å or lower with multiple spikes during the production run. It did not attain steady state, as shown by a deep at 85 ns and necessitates a longer simulation run. The complex of CBN76 showed an RMSD of *ca.* 3 Å with a rise at 85 ns. The unsteady curve implies significant ligand motion at the active

site, and the stability could only be confirmed from extended production runs. Multiple curves showed a rise at 70 ns except CNB36, indicating a common factor in the structural modulation. The lack of such a feature in the protein backbone may be attributed to the function of temperature leading to similar coincidental atomic spatial evolution of the docked ligands and may not be relevant in assessing the stability of the adduct. The nature of the ligand RMSD curves suggests the formation of moderately stable protein–ligand adducts, with most of them attaining equilibrium after *ca.* 75 ns.

The multiple RMSD of protein backbone with respect to the protein backbone, as shown in Fig. 4B, are less than 3 Å except that in the case of CBN36, which was sometimes higher in magnitude. The relatively flat and smooth nature of the curves hints at greater stability of the protein structure during the production run. The overall geometry of the receptor and the active pocket can be inferred to remain intact, capable of binding the ligand effectively. Out of five complexes studied, since CBN19 showed comparatively promising stability, other structural features related to it were only derived and analyzed.

The RMSF plots of alpha carbon atoms of the protein in the adduct and in the receptor alone were extracted from two separate MDS trajectories and are given in Fig. 5 as subplots A and B, respectively. Figure 5B shows large fluctuations *ca.* 6.5 Å from residue number 45 to 65 in the two chains out of four of the protein. In the adduct, as shown in Fig. 4A, it is seen to be reduced to *ca.* 5 Å. Similar values have been published for cases with other types of organic molecules against the NTD domain of nucleocapsid protein [53]. A decrease in RMSF in the red curve and an increase in the dark curve in the same region can be seen, which corresponds to two other chains of the protein. Overall, the ligand binding to the protein has resulted in some reduction of RMSF of the alpha carbon atoms of the protein that could be proposed as induction of stability to the system. The presence of large fluctuations in some parts of the structure could be responsible for the wavy nature of the ligand RMSD curve since the residues undergo significant motion during the simulation. The twisted geometry of the anti-parallel beta sheets at the proximity of the docked ligand might also be a determining factor. Large RMSF has been reported for the protein in complex with the hit molecules [23, 54]. The difference in RMSF of the residues from 10 ns server-based MDS as shown in Additional file 1: Fig. S1, reveals the presence of more peaks at the negative side relative to that at the positive side, with the overall value being negative. This implies that the fluctuation of residues is reduced when a ligand binds to it, providing further support for the stability of the adduct from a different approach.

Fig. 5 [Images not available. See PDF.]

RMSF of amino acid residues of protein in **A** nucleocapsid-CBN19 adduct and **B** apo structure of nucleocapsid protein derived from two separate 90 ns MDS (the four colors correspond to the four chains of the receptor and NOT to the ligands as in the other figures)

The R_g of protein in protein-CBN19 complex (dark curve of Fig. 6A) of *ca.* 2.5 nm is similar to that reported by Suravajhala et al. [21] and is smaller than that reported by Ribeiro-Filho et al. [55]. SASA (brown curve of Fig. 6A) of *ca.* 273 nm^2 is slightly outside the range 230–260 nm^2 as reported for the Curcumin molecule and nucleocapsid receptor complex [21]. Both the geometrical parameters suggest inherent intactness (no significant expansion or contraction) of the protein in the adduct during the production run.

Fig. 6 [Images not available. See PDF.]

A Radius of gyration (dark) of protein and SASA (brown) of protein in the adduct **B** Variation of hydrogen bond count in the adduct during 90 ns production run

The number of hydrogen bonds between the receptor and the ligand in the adduct during the MDS changed from

null up to 10, as shown in Fig. 6B, with maximum frames having 4 or 5. The number of hydrogen bonds stayed up to 5 ± 1 at the equilibrated part of the trajectory and contributed significantly to the non-covalent binding of the ligand CBN19 with the protein. It might be the causative factor in the appearance of a smooth curve (dark color) after *ca.* 77 ns up to 85 ns in the RMSD plot, Fig. 4A. The presence of small bumps along the RMSD curve of CBN19 might be due to the fluctuations from the protein backbone. A rise at *ca.* 70 ns in the hydrogen bond count to 10 is reflected by multiple curves (RMSD, SASA and R_g) showing change in ligand orientation and the protein backbone. Similar hydrogen bond count and other parameters have been reported by Dhankhar et al. [53], and therefore, the reliability of the results can be justified. Nonetheless, the lack of appreciable change in the number of hydrogen bonds during the simulation indicated a near-steady nature of interactions between the ligand and the amino acid residues.

The snapshots of nucleocapsid-CBN19 adduct at the active pocket at different time intervals are shown in Fig. 7. The center of mass of the ligand and the structure of the protein backbone are seen to be nearly conserved (comparing snapshots at 1 ns and 90 ns) with a slight variation in the orientation of the ligand. The progressive display of the ligand at the orthosteric site of the receptor during the MDS shows the absence of translational motion. The twisted anti-parallel beta sheets (yellow) seem to show some geometrical changes along with the loop (green) attached to it. The disappearance of the alpha helix structure at the left side of the frame at 30 ns could be correlated to a decrease in hydrogen bond count and is reflected by slightly increased RMSD. The pose of the ligand seems to be maintained with minor variation, which might be due to a change in the location of the protein backbone as a function of temperature. Similar geometrical analysis have been reported on the docked complex of quinoline derivative with M^{pro} of SARS-CoV-2 using snapshots by Singh et al. [56]. Also, Nguyen et al. have studied the druggable targets of dengue and malaria using multiple frames at different times during MDS [57]. The snapshots have revealed that the active site of the protein is capable of holding the docked ligand for sufficient time, which could lead to the disruption or deceleration of metabolic reactions.

Fig. 7 [Images not available. See PDF.]

Snapshots of the active region of the protein-CBN19 complex at different times during MDS (the numbers represent times in nanoseconds)

Spontaneity of the adduct formation reaction

The binding free energy change (ΔG_{BFE}) of complex formation from the protein and the ligand was calculated to be − 47.19 ± 6.24 kcal/mol from the MDS trajectory and − 34.06 kcal/mol from the server-based approach. The spontaneity of the reaction was revealed by the negative values obtained from both the methods. It showed the feasibility of the nucleocapsid-CBN19 adduct formation and the usefulness of the MMGBSA method in estimating the free energies. The multiple components of the free energy changes are shown in the Additional file 2: Table S1.

The in silico results obtained from MDS in terms of RMSD, SASA, R_g, RMSF and hydrogen bond count have established the effectiveness of molecular docking in providing adequate models and parameters reflecting the geometrical stability of the system studied. The thermodynamic parameter has complemented this finding. A comprehensive suggestion would be that the ligand and the protein could form a moderately stable compound in which the docked pose is fairly conserved, that may be suitable for effective inhibition of the protein functioning.

Molecular level analysis of non-covalent interactions in the adduct

The cumulative interactions between the ligand and the amino acid residues of the protein are presented in Fig. 8 in terms of 2D representation. An earlier figure, Fig. 3A, shows the docked position of the ligand at the orthosteric site of the receptor calculated by blind docking and verified again by 5 different ligands. The findings of nondeep cavities

unsuitable as a well-defined active region in the NTD domain for RNA binding have added to the ambiguity of the active site [58]. One side of the pocket is completely hydrophilic (exterior), and the other is hydrophobic (interior). Therefore, the lack of bonding involving electrostatics is seen at the hydrophobic part of the protein and is compensated by the involvement of multiple residues with more than 25 van der Waals interactions.

Fig. 8 [Images not available. See PDF.]

Protein-CBN19 interactions in the 2D projection of the complex with different types of interactions and distances (Å)

The molecular level understanding of the interactions constitutes the knowledge of key amino acid residues involved. There is a presence of disruptive interaction (acceptor-acceptor) from residue THR101 with simultaneous associative interaction through very weak hydrogen bonding at 4.37 Å with different oxygen atoms of the ligand. Another weak hydrogen bonding with THR30 at 3.92 Å and alkyl-related interaction with ARG49 at 5.10 Å are also present. Different sets of key residues have been reported to be involved in binding Ceftriaxone sodium and RNA separately by Luan et al. [58]. The poses with the best binding affinities sorted out based on total energies seem to be acceptable as these form stable complexes with a few non-covalent interactions good enough for inhibiting the protein's functioning. It could be compared with the performance of Ceftriaxone sodium, reported to be an inhibitor of NTD of nucleocapsid protein. For comparative purposes the 2D representation of the adduct formed by the receptor with the reference compound K31 depicting various types of interactions is shown in Fig. 9.

Fig. 9 [Images not available. See PDF.]

Protein-K31 interactions in the 2D projection of the complex with different types of interactions and distances (Å)

The involvement of a common amino acid residue ARG49 with alkyl related hydrophobic interactions and THR101, ILE111, and VAL112 with other different types of binding interactions in both the adducts support the inference that the orthosteric pocket was occupied by the ligands. The presence of larger number of van der Waals bonding with the hit candidate compared to that with the reference ligand could be attributed to better binding affinity and stability of the complex. The 2D representations of other adducts are shown in Additional file 1: Figs. S2–S5.

Discussion

Relative strength of binding of ligands by the protein and stability of adduct

The hit molecule, CBN19, have been found to bind with sufficient strength with the receptor protein relative to other 100 Cucurbitacins, five FDA-approved drugs, and a reference molecule. The strength of interactions is reflected by the binding affinities and the stability of the adduct with time is shown by various geometrical parameters extracted from the MDS. The poses at different times during the MDS production run supported this inference. The presence of weak hydrogen bonding and van der Waals' interactions between the ligand and the amino acid residues seems to be the contributing factor in the conservation of the docked pose at the orthosteric site of the receptor. The molecule could thus be proposed to be a potential inhibitor of the nucleocapsid protein.

Druglikeness and safety

The test compounds seem to have drug-like properties with no observable toxicity. These compounds are present in regularly consumed vegetables, and the administrable dose of each component has to be precisely determined to formulate it as a safe drug candidate that is free from any adverse effects. The proximity of toxic dose amount to its biological activity could be exploited for better therapeutics [59]. The time and dose-dependent cytotoxicity has been reported for Cucurbitacin B [60]. Therefore, the toxicity from the curative effect has to be distinguished by various in vitro and clinical trials [61] and is highly recommended for the future work involving Cucurbitacins.

Validation by thermodynamic parameter

The negative values of binding free energy changes, ΔG_{BFE} obtained from two different methods clearly showed that the adduct formation was spontaneous in nature. It provided additional support for the geometrical stability of the nucleocapsid-CBN19 adduct at the physiological conditions. Therefore, the feasibility of the forward reaction determined from the calculation of the thermodynamic parameter, ΔG_{BFE} strengthened the inferences derived. It supported the molecular docking methodology, observations derived from it, and the geometrical parameters extracted from the MDS. Similar results have been obtained from the models of protein and approved drugs by Chauhan et al. [52]. The findings of this research is in par with the current literature and thus validates the employment of many types of computational methods.

In the end, the results showed that the server-based molecular docking calculations could provide quick and reliable models that could be used further for the stability assessment. The molecular dynamics trajectory of the adduct formed by CBN19 with the target protein hinted at the sufficient sturdiness of the complex as revealed by multiple parameters. However, pharmacokinetics of the molecule and toxicity predictions obligate mandatory experimental verification for the safety of the hit candidate present in different edible sources. The stability analysis of adducts formed by other different classes of organic molecules with nucleocapsid protein would provide comparative basis against the performance of Cucurbitacins. It is being explored along with the derivatives of compound K31 for finding effective and safe drug-like candidates for fighting COVID-19 and related diseases.

Conclusions

Different computational tools were used in screening the phytochemicals (substructures of Cucurbitacin I) of common vegetables for proposing the potential inhibitors of nucleocapsid protein of SARS-CoV-2. A Cucurbitacin, CBN19, was found to be a hit candidate better than 100 other Cucurbitacins, five approved drugs, and a reference molecule based on ligand binding affinity obtained from molecular docking calculations and from geometrical as well as thermodynamic parameters derived from molecular dynamics simulations. Additional experiments are recommended to confirm the drug-like properties and safety of the proposed molecule in terms of toxicity. In summary, accelerated computational assessment of natural product-based bio-active compounds could be systematically performed in low resource settings for designing therapeutics against COVID-19 and extended to other related diseases in accordance with the modern rational drug discovery process.

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Author contributions

JAS: Conception; calculations; analysis and interpretation of data; drafted the work; RLS: Calculations; managed the resources; revised the work; BPM: Analysis and interpretation of data; supervised the work. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files. (ADMET_predictions.xls file; SI_Cucurbitacins_1.0.0.docx).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

ADMET

Absorption, distribution, metabolism, excretion and toxicity

CID

Compound ID

CTD

C-terminal domain

CBN

Cucurbitacin

EC_{50}

Half maximal effective concentration

FDA

Food and drug administration

hERG

Human ether-a-go-go-related gene

IC_{50}

Half maximal inhibitory concentration

MDS

Molecular dynamics simulation

MMGBSA

Molecular mechanics generalized Born surface area

nCoV

Novel corona-virus

NTD

N-terminal domain

R_g

Radius of gyration

RMSD

Root mean square deviation

RMSF

Root mean square fluctuation

RO5

Rule of five

SARS-CoV-2

Severe acute respiratory syndrome coronavirus-2

SASA

Solvent accessible surface area

SMILES

Simplified molecular-input line-entry system

Publisher's Note

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Natural product-inspired strategies towards the discovery of novel bioactive molecules

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ABSTRACT (ENGLISH)

Background

The intricate molecular frameworks of natural products with biological activity towards human targets offer academic and industrial chemists an important starting point for next generation drug discovery. With a focus on natural products for the production of diverse small-molecule libraries and the evaluation of uncharted chemical space, several strategies have emerged for achieving selective modulation of disease-associated targets. This review highlights some of the significant and more recent synthetic strategies inspired by naturally occurring molecular frameworks, aiming at the discovery and development of novel bioactive molecules. We underscore the potential of these innovative strategies with representative examples to forecast their role in addressing the enduring drug design challenge.

Main text

In this review, we discuss these newer natural product-inspired synthetic approaches, among them diversity-oriented synthesis, biology-oriented synthesis, hybrid natural products, diverted total synthesis, pruning natural products, ring distortion of natural products and integrating natural product framework with a bioactive molecule. Selected representative examples associated with these strategies are given to demonstrate how they have been applied to afford desired bioactivity.

Conclusion

This review elaborates several pioneering and emerging strategies inspired from natural product which allows access to the unexplored chemical space to identify novel molecules possessing noteworthy bioactivity. The corresponding examples highlight the success of these strategies in the discovery of novel bioactive molecules which can be further developed in drug discovery and can be novel probes for chemical biology. Although there are

limited number of successful examples, the selectivity, activity, and efficacy associated with natural product-inspired molecules accentuate their importance.

FULL TEXT

Background

Continuing efforts towards the discovery of novel molecules to systematize and expand our knowledge and to understand the biological pathways and modify disease aetiology is of prime importance towards sustaining the quality and longevity of human life [1]. A total of 893 mammal- and pathogen-related molecular targets have been identified upon which marketed drugs act [2]. Meanwhile, the corresponding chemical space is estimated to comprise about 10^{60} drug-like structures/molecules that follow Lipinski's rule of five [3, 4]. Hence, although apparently important, it is very arduous to identify a new molecule out of this vast chemical space which specifically interacts with the defined molecular target [5].

In the early 2000s, combinatorial chemistry became a primary tool for drug discovery [6]. The idea of producing a large library of compounds that can be screened against numerous targets in a brief period of time seemed appealing to pharmaceutical companies and initiated significant industrial efforts [7]. Although substantial investment was made in relation to this concept, the overall success was less than anticipated with a few exceptions, such as the discovery of sorafenib, which is a multikinase inhibitor for the treating advanced renal cancer [8]. The constrained impact of this strategy can be attributed to the fact that molecules obtained by it were concentrated on a relatively small area of chemical space, and potential molecular diversity remained unexplored. Conversely, the chemical space occupied by natural products encompasses a vast chemical space [9].

For centuries, nature has been the source of medicinal compounds for the treatment of a wide spectrum of diseases [10]. The stunning structural and chemical diversity offered by natural products has revitalized, in multiple phases, the interest of medicinal chemists to take advantage of such chemotypes for drug discovery [11]. Natural products may differ significantly from easily accessible synthetic drug candidates [12]. They often feature versatile structural and physical properties such as a large number of stereogenic centres, sp^3-hybridized atoms and variable molecular mass as well as octanol–water partition coefficient. Moreover, they also tend to have a high oxygen content and to contain aliphatic ring systems and therefore display an intricate three-dimensional geometry [13]. Thus, the innovative design of a library of molecules based on natural product frameworks can in principle be propelled by applying specific strategies to synthesize arrays of novel bioactive compounds [14]. To achieve this goal, in the past two decades, several named concepts have been developed to address the unceasing drug discovery challenge [15]. In this review, we discuss these newer natural product-inspired synthetic approaches, among them diversity-oriented synthesis (DOS), biology-oriented synthesis (BIOS), hybrid natural products(HNPs), diverted total synthesis (DTS), pruning natural products (PNP), ring distortion of natural products(RDNPs) and integrating natural product framework with a bioactive molecule (iNPBM). Selected representative examples associated with these strategies are given to demonstrate how they have been applied to afford desired bioactivity.

Diversity-oriented synthesis (DOS)

Shortly after the period of thorough investigation of the potential of combinatorial chemistry, efforts were made towards increasing functional and structural diversity of the prepared compounds [16]. Generally speaking, diversity-oriented synthesis (DOS) was developed to rapidly generate libraries of compounds of high structural and skeletal variety [17]. DOS utilizes multicomponent reactions, complexity-generating transformations, stereoselective synthesis and branching pathways. It also includes so-called forward synthetic analysis in order to enter a comparatively large chemical space [18]. Typically, no more than five transformations, efficient as well as modular synthesis and scaffold diversity within the library of compounds are anticipated to provide novel hit compounds to accelerate drug discovery (Fig. 1) [19].

Fig. 1 [Images not available. See PDF.]

Schematic representation of the concept of diversity-oriented synthesis. (To generate a small-molecule collection

with a high degree of structural, and thus functional, diversity that interrogates large areas of chemical space simultaneously.) [16].

Generally, DOS was soon able to generate diverse libraries of compounds in a short span time; the molecules were screened across several targets randomly and were not directed towards specific biological targets or disease. In recent years, utilizing natural product frameworks as an origin or synthesis of a natural product-like DOS libraries has transformed the traditional DOS strategy. Natural product-based DOS library is more markedly directed towards a specific biological target. There must be, like in all cases of desired changes of the function of a protein or other target, a structural fit between the target and the modulating ligand. Identification of the binding site is helpful and crucial when it comes to narrowing the chemical space of the synthesized bioactive molecule [20]. The following are two representative examples of DOS modulating a specific biological target, as by disrupting protein–protein interaction and the discovery of new antibiotics to emphasize the potential of natural product-inspired diversity-oriented synthesis (DOS) as a dynamic tool for the discovery of novel bioactive molecules.

Many cell functions, physiological processes, and disease mechanisms comprise protein–protein interactions involving electrostatic interactions and other intermolecular forces. Thus, modulating protein–protein interaction is one of the attractive drug discovery concepts [21]. However, multiple binding sites, a high number of non-specific binding interactions and also a lack of generally applicable reliable screening assays make protein–protein interactions difficult basic strategy of drug discovery [22].

One such a protein–protein interaction pathway, named Hedgehog signalling pathway involving the protein *Sonic Hedgehog (Shh),* regulates cell proliferation as well as differentiation and is crucial for proper embryonic development. The Hedgehog signalling cascade is initiated by auto-cleavage of full-length Shh to an active N-terminal fragment (ShhN) upon its binding to 12-pass transmembrane receptor Patched (Patch1). It results into the reversal of the inhibitory effect on Smoothened (Smo) and releases Glioma (Gli) transcription factor. This mechanism regulates the transcription of gene Gli1 and Ptc1. The aberrant Shh pathway activity due to mutation of the gene is associated with the initiation of tumorigenesis [23]. The discovery of novel molecules which can modulate Shh signalling pathway have been proposed as one of the potential therapeutic strategy for treating the pancreatic cancer, basal cell carcinoma (BCC), medulloblastoma, prostate cancer and associated disorders (Fig. 2) [24, 25].

Fig. 2 [Images not available. See PDF.]

Schematic diagram showing robotnikin inhibits the induction of the Shh pathway [25]. (Robotnikinin is a small molecule capable of binding to and inhibiting the activity of Sonic Hedgehog (Shh) signaling up stream of Smoothened)

The bioactive, naturally occurring macrolactones such as pikromycin, erythromycin, enterobactin, and epothilones are known to act through changing different protein–protein interactions. Based on macrolactone framework **1**, Schreiber et al. synthesized a library of about 2070 small molecules (SM) and screened it for its binding with bacterially expressed protein—N-terminal sonic hedgehog protein (ShhN) to identify several new bioactive macrolactone structures (Fig. 3) [26]. Lead optimization on the initial hit compound **2** by ring contraction resulted in the identification of robotnikin (**3**), which displays strong and concentration-dependent inhibition of Gli expression with an EC_{50} value of 4 μM and EC_{max} reaching 91% (Fig. 3). This pronounced activity renders **3** a promising small-molecule probe of the Hedgehog signalling pathway [27].

Fig. 3 [Images not available. See PDF.]

Small-molecule modulators of Shh pathway discovered by DOS strategy with enhanced bioactivity

In another notable example of utilizing natural product frameworks in DOS, Spring and coworkers have discovered new antibiotics against the methicillin-resistant *Staphylococcus aureus* (MRSA) [28]. Since discovery of Penicillin in the 1930s, antibiotics have revolutionized modern medicine and played in important role in improving the quality of life as well as the life expectancy [29].

However, over-prescription of antibiotics and their use without professional advice have given rise to drug-resistant microbes also termed ‘superbugs’ [30]. The alarming increase in resistance warrants immediate discovery of novel antibacterial compounds against multidrug-resistant bacteria such as MRSA [15, 31].

Spring and co-worker have synthesized a DOS library of about 242 molecules of 18 different natural product-like frameworks (**8**–**25**) from a solid-supported phosphonate (**4**) as a starting material [28]. Reaction of **4** with different aldehydes was done in step 1 to synthesize twelve different α,β-unsaturated acyl-imidazolidinones (Fig. 4). In the second step, pluripotent **5** is diversified via [3 + 2] cycloaddition, dihydroxylation, and [4 + 2] cycloaddition to generate further branch point substrates **7**–**9**. These molecules serve as intermediate compounds for the series of versatile organic reactions. Compounds **7** and **8** were further diversified into **10**–**13** and **14**/**15**; norbornene intermediate **9** was transformed into the five different scaffolds **16**–**20**. In step 4, further complexity and diversity was added to generate **21**–**25**. In the final step of purification, the compounds were hydrolysed from the silyl-polystyrene solid support resin and evaluated with regard to their *in vitro* bactericidal activity against two UK epidemic methicillin-resistant strains (EMRSA 15 and EMRSA 16) as well as three different strains of *S. aureus*: a methicillin-susceptible S. aureus (MSSA). In particular, they discovered three novel compounds (**26**–**28**) with growth inhibition against the three strains of *S. aureus*.

Fig. 4 [Images not available. See PDF.]

Library of 242 DOS compounds synthesized to study antibacterial activity [20]. Reagents and conditions: **a** LiBr, 1,8-diazabicyclo[5.4.0]undec-7-ene, R1 CHO, MeCN; **b** (R)-QUINAP, AgOAc, iPr2NEt, THF, 788C!258C; **c** AD-mix, (DHQD)PHAL, THF/H2O (1:1); **d** chiral bis(oxazoline), Cu(OTf)2, 3 E M.S., CH2Cl2, C5H6; **e** R2 COCl, DMAP, pyridine, CH2Cl2; **f** R3 CHO, BH3·pyridine, MeOH; **g** SOCl2, pyridine, CH2Cl2, 40 8C; **h** R4 Br, Ag2O, CH2Cl2, 40 8C; **i** R5 C(O)R5, TsOH, DMF, 658C; **j** R6 CHO, TsOH, DMF, 65 8C; **k** NaN3, DMF, 1008C then dimethyl acetylenedicarboxylate, PhMe, 658C; **l** mCPBA, CH2Cl2 then MeOH, 658C; **m** CH2=CHCO2Bn, PhMe, 1208C, Grubbs II, CH2=CH2; **n** OsO4, NMO, CH3C(O)CH3/ H2O (10:1); **o** RNH2, Me2AlCl, PhMe 1208C; then NaH, R11X, DMF, THF; then PhMe, 1208C, Grubbs II, CH2=CH2; **p** NaIO4, THF/H2O (1:1); then R7 NH2, NaB(OAc)3H, CH2Cl2; **q** NaIO4, THF/H2O (1:1); then R8 NHR8, NaB(OAc)3H, CH2Cl2; **r** R9 CHO, DMF, TsOH, 60 8C; **s** R10C(O)R10, DMF, TsOH, 608C. DMF = N,N-dimethylformamide, THF = tetrahydrofuran, DMAP = N,N-dimethylaminopyridine, (DHQD)PHAL = hydroquinidine 1,4-phthalazinediyl diether, mCPBA = meta-chloroperbenzoic acid, Ts = para-toluenesulfonyl, Grubbs II = 1,3-(bis-(mesityl)-2-imidazolidinyl-idene) dichloro (phenylmethylene) (tricyclohexylphosphine) ruthenium, NMO = 4-methylmorpholine-N-oxide, OTf = CF3SO3, Bn = benzyl, QUINAP = 1-(2-diphenylphosphino-1-naphthyl)isoquinoline

One compound named gemmacin (**26**) was found to be a broad-spectrum antibiotic to inhibit the Gram-positive bacteria and to exhibit lower cytotoxicity against human epithelial cells (Table 1).

Table 1. Antibiotic activity of compounds synthesized from DOS strategy [20]

Based on these two examples, one can see that diversity-oriented synthesis (DOS) of libraries of natural product (NP)-like molecules is capable of providing efficient skeletal diversity to explore biorelevant chemical space and opens a new direction for the discovery of bioactive molecules.

Hybrid natural products (HNP)

In developing potential therapeutics, it is important to limit the number of biologically inactive molecules; that is, synthetic efforts should ideally be focused and not produce irrelevant compounds. Taking advantage of the activity and specificity of known, naturally occurring systems, Tietze [32] and Mehta [33] have proposed the concept of hybrid natural products for drug discovery. In fact, in nature, there are several such naturally occurring natural product hybrids. One such an example is the indole alkaloid vincristine (**29**) which is used for the treatment of lymphatic leukaemia [34]. It is a hybrid of vindoline (**31**) [35], which belongs to the *Aspidosperma* alkaloid family and catharanthine (**30**) [36], which belongs to the *Iboga* class of alkaloids (Fig. 5). The individual monomers exhibit no significant activity, whereas **19** possesses pronounced bioactivity as well as specificity.

Fig. 5 [Images not available. See PDF.]

Naturally occurring hybrid molecules. (Molecular structure of ibogaine, vindoline, vincristine). The structure of vincristine, two vinca alkaloids, are formed by two polycyclic moieties, namely vindoline (red) and catharanthine (blue). The catharanthine portion is also the basic motif found in the ibogaine molecule.)

Generally speaking, artificially linking two or more natural products may result in the creation of hybrid molecules with improved bioactivity that differs from those of its parent molecules. Based on this concept, natural product hybrid of geldanamycin (**32**) and estradiol (**33**) have been prepared and evaluated for its bioactivity, in particular, antimicrobial activity (Fig. 6). Compound **32** is an ansamycin antibiotic isolated from *Streptomyces hygroscopicus* and also effectively inhibits human epidermal growth factor receptor (HER2) kinases [37]. On the other hand, **33** induces selective degradation of certain oestrogen receptors (ER) [38]. The estradiol-geldanamycin hybrid compounds **34** were found to be more selective than **32** in inhibiting HER2 and ER in breast cancer cell line MCF7 [39].

Fig. 6 [Images not available. See PDF.]

Hybrid natural products synthesized from geldanamycin and estradiol

Many such natural product (NP) hybrids display exceedingly higher biological activity than their isolated parent natural molecules. Nonetheless, covalent linkage of two bioactive compounds does not necessarily lead to a overall improved desired properties. The oxindole containing natural product quinocarcin (**35**) [40] is isolated from *Streptomyces melunovinuceus*, displaying prominent antitumour activity [41]. It inspired Williams and coworkers to combine **35** with the natural product netropsin (**36**). However, the quinocarcin–netropsin hybrid **37** was found to display lower biological activity than its parent molecules (Fig. 7) [42].

Fig. 7 [Images not available. See PDF.]

Hybrid natural product synthesized from quinocarcin and netropsin

The hybrid natural product is one of the newer natural product-inspired synthetic approaches, which can provide access to unique combinations of existing natural fragments. Although only a limited number of hybrid molecules have been synthesized to date, mainly for the development of new antibiotics and anticancer agents, the bioactivity associated with these hybrid molecules emphasizes the promising role of HNP for future drug discovery.

Biology-oriented synthesis (BIOS)

Combinatorial chemistry and DOS generate very large libraries to be screened against multiple different targets and hence potentially make the overall process highly expensive [43]. Therefore, to limit the number of molecules for biological studies, a unique structure-based approach named biology-oriented synthesis (BIOS) was introduced by Waldman and co-workers (Fig. 8) [44]. This approach takes into account the structural conservatism during evolution of the chemical space of target proteins and also natural products (NP) modulating them. The structural conservatism within protein families limits the number of small molecules and binding sites. The systematic structural analysis of proteins, namely 3D structure, sequence homology and classifying the small molecules which modulate them, is supposed to lead to the discovery of novel bioactive molecules. The applications lie, more generally speaking, in chemical biology as well as medicinal chemistry [45]. Waldmann and coworkers have invented a cheminformatics tool ‘structural classification of natural products’ (SCONP). Relatively complex natural products (such as **38** and **39**) are reduced to core scaffolds by holding bioactivity as a main guiding principle. Decorating the cores with new groups generates a so-called natural product structural tree, which is investigated in terms of target modulation and also further improved [46].

Fig. 8 [Images not available. See PDF.]

Schematic representation of the concept of biology-oriented synthesis depicting scaffold-substituent analogy between small molecules and protein adapted from ref. 44 (https://doi.org/10.1002/anie.201007004) with permission). (The small-molecule scaffold determines the spatial orientation of the substituents, whereas the protein

subfold arranges the amino acid side chains spatially. Binding occurs when compatible substituents match in their spatial positioning so they can interact.)

Thus, a library of a limited number of molecules (**40**,**41**) synthesized based on the BIOS concept may have added relevance to specified target/s and thus increases chances of displaying the desired bioactivity [47]. This BIOS strategy has been effectively applied for discovery of several novel bioactive molecules, as exemplified in the following [48].

The progressive degeneration of neuron and loss of neural activity is associated with many neurodegenerative disorders. Discovery of novel molecules which can promote neurite growth and restore neuronal viability or which can prevent neuronal decline is utmost important [49]. Towards the development of novel neurite growth-promoting compounds, Waldmann and coworkers have utilized the BIOS approach based on the iridoid scaffolds silphinene (**42**) as well as harpagide (**43**) and rhynchophylline (**44**), which belongs to the secoyohimbane class of compounds (Fig. 9) [50]. All of these are known to possess neurotropic and neuroprotective activity [51].

Fig. 9 [Images not available. See PDF.]

Iridoid and secoyohimbane scaffold-inspired synthesis of BIOS library for discovery of neurite growth-promoting compounds

A library of 54 iridoid analogues (**48**) were synthesized by a [3 + 2] cycloaddition/Baeyer–Villiger oxidation sequence, and library of 56 secoyohimbane-related compounds (**52**) by enantioselective and organocatalysis. They were screened in phenotypic assays with respect to the modulation of neurite outgrowth. These assays were able to identify several new molecules (**53**–**56**) possessing growth-promoting properties, and which can be used as chemical probes for studying neurodevelopmental process (Fig. 9) [52].

The enzyme 11β-Hydroxysteroid dehydrogenase type 1 (11βHSD1) is NADPH-dependent enzyme which activates glucocorticoid hormones (GCs). Glucocorticoids regulate various physiological processes including glucose and lipid metabolism, and increased levels may result in various metabolic syndromes, such as hypertension, type 2 diabetes, and dyslipidemia [53]. Thus, selective inhibition of 11βHSD1 is an important strategy for the treatment of these syndromes [54]. The BIOS approach, by combining protein structure similarity clustering (PSSC) [55] and SCONP, was applied to the discovery of novel and selective 11βHSD1 inhibitors (Fig. 10) [56]. Analysis of PSSC of 11βHSD1 and dual specificity phosphatase (Cdc25 A) and acetylcholine esterase (AChE) revealed that the active site and position of the catalytic amino acid residue show very good overlap in these three proteins. Although the functions of 11βHSD1, Cdc25 A and AChE are different, the similarity of active sites indicates that the molecules which can modulate the Cdc25 A and AChE have the potential to modulate 11βHSD1. Generating PSSC analysis and SCONP analysis of related natural products can therefore lead to the identification of novel 11βHSD1 modulators [57]. Consequently, a SCONP tree was constructed by the software, based on the natural product dysidiolide (**57**) [58], which is known to inhibit Cdc25A, and glycyrrhetinic acid (**58**), which is a known 11bHSD ligand.

Fig. 10 [Images not available. See PDF.]

SCONP analysis of natural products dysidiolide and glycyrrhetinic acid for identification of selective 11bHSD1 inhibitor using BIOS [44] and PSSC analysis of the superimposed catalytic sites of Cdc25A (red), 11_HSD1 (green), and AChE (blue) [46]

This analysis of multicyclic natural products led the researchers to identify decalin scaffolds **IV** and **VI**, which were presumed to be privileged cores associated with activity changes in 11βHSD1, Cdc25 A, and AChE. A natural product-inspired library of 483 compounds was synthesized and screened for their activity against 11βHSD1. Combining PSSC and SCONP led to the discovery of several new 11βHSD1 inhibitors **59**–**62**, acting at the nanomolar concentration level in vitro. Additionally **59** displays selective in vivo cellular inhibition of 11βHSD1 [46]. These representative examples emphasize the importance of the BIOS strategy in the efficient discovery of novel bioactive molecules [55].

Diverted total synthesis (DTS)

Danishefsky *et al.* purported a different concept 'diverted total synthesis', for discovery of novel bioactive compounds [59]. It is based on developing a smaller library of compounds by using and diverting the intermediates formed during the total synthesis of small-molecule natural products (SMNPs) [60]. Starting from building blocks **A**, the complexity and diversity associated with synthetic intermediates **B**, obtained during total synthesis of natural product (NP) **C** will allow access towards uncharted chemical space (Fig. 11). Such space would otherwise not be accessible due to limitations levied by biosynthetic pathways or by direct modification of parent natural products **C**.

Fig. 11 [Images not available. See PDF.]

Schematic representation of the concept of diverted total synthesis

Therefore, molecules obtained by DTS might exhibit a upper order of complexity (**D**) or a lower order of complexity (**E**) than **C.** These can be evaluated for their potential biological activity [61].

Such a library of compounds was produced by Danishefsky and coworkers based on the total synthesis of epothilone B (**63**) (Fig. 12). The natural product epothilone B is isolated from mycobacterium *Sorangium cellulosum* and found to exhibit strong in vitro cytotoxicity in multidrug-resistant (MDR) cell line by promoting stabilization of microtubule polymerization, thereby interrupting the cell division and apoptosis. However, in vivo studies revealed that epothilone B was highly toxic to mice, which inspires editing of the epothilone B framework to reduce its toxicity and increase the desired bioactivity. In the course of DTS, compound dEpoB (**64**), lacking the epoxy group, was made and shown to possess remarkably lesser toxicity as anticancer agent. Likewise, 9,10-dehydro-dEpoB (**65**) with added unsaturation was prepared and displayed improved survival rate in mice. Furthermore, fludelone (**66**) was obtained by installing a trifluoromethyl group, resulting more effective for tumour reduction in comparison with all the previous molecules. Eventually, alteration of the heterocyclic moiety leads to the identification of isofludelone (**67**), a promising candidate intended for cancer treatment and currently under pre-clinical trials [59].

Fig. 12 [Images not available. See PDF.]

Discovered novel anticancer compounds from the diverted total synthesis of Epothilone B with modulating the bioactivity

Pruning biomolecules and natural products (PBNP)

The structure of natural products (NPs) varies from simple frameworks to highly complex 3D architectures. The preparation of bioactive natural products with higher complexity poses a enormous challenge for the synthetic organic chemist. In the course of biosynthesis, the biochemical machinery can easily vary the substitution of the core structure in terms of side chains and functional groups, which can lead to different structures and activity of the natural product(s). In many cases, the simplified natural product framework retains substantial bioactivity [62]. Thus, pruning of biomolecules and natural products (PBNP) by systematic identification of the pharmacophore can address the synthetic challenge by reducing the number of chemical steps and may lead to the discovery of novel bioactive molecules [63]. To a certain extent, this approach is related to BIOS but relies primarily on cutting off substituents.

The discovery of eribulin (**69**) from the complex marine natural product halichondrin B (**68**) by Eisai Pharma and Kishi is a prominent example of PBNP for the discovery of anticancer drugs (Fig. 13). Halichondrin B (**68**) was isolated from marine-sponge *Halichondria Okadai*, which is a polyether macrolide [64]. During its total synthesis and biological evaluation, it was found that the right half of the molecule displays cell growth inhibition. The hydrolyzable ester functionality of **68** changed to a non-hydrolyzable bioisostere enhanced the in vivo efficacy. Further synthetic efforts and clinical studies led to the identification of **69** for treating of late-stage breast cancer resistant to other anticancer drugs [65].

Fig. 13 [Images not available. See PDF.]

Pruning of natural product halichondrin B for the discovery of anticancer drug halaven

Another prominent example of PNBP is the discovery of novel migrastatin analogues. Migrastatin (**70**) is macrolactone isolated from *Streptomyces sp.* MK929-43F1 [66] which was found to inhibit cancer cell migration (Fig. 14). During its synthesis and bioevaluation, it was found that its truncated analogue **71** possesses increased activity as compared to the **70** and can be a promising candidate in cancer therapy to address metastasis (Fig. 14) [67].

Fig. 14 [Images not available. See PDF.]

Discovery of novel migrastatin analogue by pruning of natural product

Another example of PBNP is the development of antihypertensive agent rostafuroxin (**73**) (Fig. 15). The natural product ouabain (**72**) from the bark the tree of *Acokanthera ouabaio* is being used as an arrow poison by some of the African tribes. It was also found to be useful for the treatment of cardiac conditions. Ouabain binds to the plasma membrane and inhibits the Na+/K+ATPase in vivo. [68] However, it also has several side effects. The simplified analogue **73** was found to inhibit Na+/K+ATPase more selectively, without interacting with other receptors which regulate blood pressure; it is now being studied in clinical trials for treating hypertension [69].

Fig. 15 [Images not available. See PDF.]

Pruning of natural product ouabain for the discovery of hypertension drug

Such truncated or simplified natural product obtained by PBNP will also not only help to gain insight into the role of the natural product during its evolution and biological processes but also directs the discovery of novel modulators of the biological targets.

Ring distortion of natural products (RDNP)

In order to rapidly generate a novel library of drug-like small molecules from a natural product with relatively high structural and complexity, Hergenrother has reported a ‘ring distortion strategy’. In this approach, natural products with of a given framework, such as the 5,6,6-fused tricyclic system **74**, are converted into different core scaffolds by applying a minimum number of steps (Fig. 16). In this process, a distinct series of molecules is synthesized by taking inspiration from the biosynthetic pathway, which often creates diverse compounds from a common intermediate [70]. The ring distortion approach involves chemoselective transformations such as ring cleavage, expansion, fusion, as well as rearrangement, leading to systematically altered scaffolds such as **75**–**78** [71]. This strategy was exhibited for the production of different analogues of three readily available natural products, namely gibberellic acid (**79**), adrenosterone (**80**), and quinine (**81**) (Fig. 17). The RDNP produced 19, 18, and 12 diverse compounds, respectively, from these natural products.

Fig. 16 [Images not available. See PDF.]

Schematic representation of strategy ring distortion of natural product. (Ring-distortion reactions can be used readily to convert natural products into complex and diverse scaffolds.)

Fig. 17 [Images not available. See PDF.]

Natural products for the synthesis of novel library of compounds by RDNP

These compounds were studied by cheminformatics techniques for correlation between structural features and potential biological activity [72]. The detailed results of bioactivity studies have not been disclosed yet.

Indole-containing compounds are abundant in nature, interacting with numerous biological targets and therefore displaying diverse biological activity. The alkaloid yohimbine (**82**), isolated from the bark of *Pausinystalia johimbe*, is known to inhibit the α2-adrenergic receptor (Fig. 18). This readily available natural product, which contains a complex multicyclic structure with a fused indole system, was the starting point for an extended RDNP study to rapidly generate a library of 70 diverse and complex compounds. The yohimbine ring distortion (RDNP) library was screened for bioactivity in processes linked inhibition to cancer, inflammation, and against pathogenic bacterial strains, whereby several hit compounds were identified (Fig. 19). One of the compounds, **83**, was found to exhibit

promising anti-inflammatory as well as hypoxia-inducible factors to display (HIF)-dependent anticancer activity [73]. Furthermore, the compounds **84** and **85** were found to be activators of the Nrf2-ARE pathway. The transcription factor Nrf2 (Nuclear erythroid 2-related factor 2) selectively binds to antioxidant responsive element (ARE). Activation of Nrf2/ARE signalling pathway generally protects mammalian cells from impending oxidative stress-induced cell death. Thus, compounds which have ability to modulate Nrf2/ARE can be prophylactive agents against cancer [74].

Fig. 18 [Images not available. See PDF.]

Ring distortion of yohimbine for the discovery of novel bioactive molecules [75]

Fig. 19 [Images not available. See PDF.]

Bioactive compounds from RDS of yohimbine

Conversely, Nrf2/ARE inhibitors render cancers cells more susceptible to chemotherapy. The compounds **86**–**88** were found to be selective inhibitors of Nrf2/ARE [75].

Although the availability of pure natural products isolated from natural resources limits the scope of RDNP, the systematic application of this strategy offers a convenient approach towards expanding biorelevant chemical space.

Integrating natural product framework with bioactive molecules (*i*NPBM)

Pandey et al. have introduced a distinct methodology of integrating natural product framework with synthetic bioactive molecules (*i*NPBM) [76]. This concept takes into consideration that increased bioactivity may result from the designed structural combination of a natural product with a synthetic pharmacophore. Schematically, this is exemplified in Fig. 20; a natural product framework (**A**), that possess limited biological activity against specified target, is integrated with a synthetic bioactive molecule (**B**), featuring one common structural motif, to afford the integrated molecules (**C** and **D**).

Fig. 20 [Images not available. See PDF.]

Schematic representation of strategy integrating natural product and bioactive molecule for the discovery of novel bioactive molecules

The resulting molecules ideally combine the bioactivity as well as selectivity of both molecules to exhibit highly enhanced therapeutic activity in comparison with their parent compounds.

The widely distributed five distinct human muscarinic receptors (M1-M5) belong to family of G-protein-coupled receptors (GPCR) and are proven to regulate numerous essential processes of the central and peripheral nervous system. Gephyrotoxin (**89**) is an alkaloid obtained from the frog *Dendrobates histrionicus* and exhibits mild-antimuscarinic activity (Fig. 21). For the discovery of novel muscarinic receptor modulators, this natural product gephyrotoxin was combined with isoindolines (**90**) [77] which display a wide spectrum of bioactivities. The presence of the common pyrrolidine ring was the basis for the design of integrated multicyclic molecules, such as **91** and **92**. A library of these integrated structures was synthesized and screened against various muscarinic receptors. A few of them turned out to be hit molecules featuring specific modulation of muscarinic receptor.

Fig. 21 [Images not available. See PDF.]

Schematic representation of *i*NPBM strategy for identification of isoindolyl-gephyrotoxin frameworks for the discovery of novel muscarinic receptor modulators [76]

Compound **93** was found to be a potent M2 agonist with activity of <4 nM and to be helpful in alleviating cognitive deficiency in a mouse model (Fig. 22) [78]. Moreover, compounds **94** and **95** are moderate and selective M2 agonists. On the other hand, compounds **96** and **97** act as selective M3 antagonist with activity of <1 nM and might be further improved for treating respiratory disorders [79]. As illustrated by this study, the *i*NPBM approach suggests an unique strategy towards the discovery of receptor protein modulators alongside promising therapeutic implications.

Fig. 22 [Images not available. See PDF.]

Selective muscarinic receptor modulators discovered by iNPBM strategy

Miscellaneous strategies

Waldmann and co-workers reported designed pseudo-natural products by using different combination of natural products and fragment-based compound development to afford novel performance-based diverse natural products displaying varying biological activity [80]. A natural product library of 244 member pseudo-natural products are designed by using indole-containing or chromanone containing fragments with natural product quinine, quinidine, sinomenine, and griseofulvin (**98–102** Fig. 23).

Fig. 23 [Images not available. See PDF.]

Combination of indole and chromanone with natural product fragments of quinine and quinidine

These fusion of NP fragments with different combinations provided pseudo-natural product compounds which spans over wide chemical space to display diverse bioactivity. The cheminformatic analysis suggests several compounds could exhibit both drug-like and natural product-like properties.

Recent updates

In additional example, Duttwyler and coworkers fused boron clusters with natural products to explore bioactivity of resultant natural product-boron cluster hybrids [81]. Stereoselective B-H activation was achieved to afford asymmetric boron cluster by fusing natural products camphanic acid and menthol **103–106 (**Fig. 24). Several of resulting compound displayed excellent bactericidal properties against Gram-positive as well and Gram-negative bacterium strains with bioactivity up to 2-ug/Ml.

Fig. 24 [Images not available. See PDF.]

Fusing natural product camphanic acid and menthol with boron clusters

These results open up new space for the discovery of novel bioactive dodecaborate cages having diverse antimicrobial properties by fusing with suitable natural products (NP).

Zou and coworkers have recently reported efficient construction of 1,3-indanedione-based tetrahydroquinolines based on biology-oriented synthesis (BIOS). Spirocyclic tetrahydroquinoline and Spiroindane-1,3-dione were selected natural products for guiding the BIOS approach. Various 2-arylidene-1,3-indanediones reacted with different vinyl benzoxazinanones by using Pd catalyst to provide novel library of spirocyclic tetrahydroquinolines (Fig. 25).

Fig. 25 [Images not available. See PDF.]

1,3-Indanedione-based tetrahydroquinolines based on biology-oriented synthesis (BIOS)

Two of the synthesized products **114** and **115** display remarkable activity by inducing apoptosis in A549 human lung cancer cells [82].

Natural products and molecular modelling for drug discovery

Bioactive natural products are vital starting points for crafting newer and improved analogues through advanced medicinal chemistry techniques like molecular modelling. Natural products and its analogues are rigorously studied through SAR analysis and molecular modelling to enhance potency, reduce toxicity, and optimize pharmacokinetics. Interactions with various ligands/target proteins are crucial in determining biological activity. The most promising analogues undergo synthesis and thorough evaluation via in vitro and in vivo assays, culminating in the development of optimized drug candidates. The overall process of molecular modelling involves in silico ligand construction and preparation, target preparation, docking, identification of hit molecule, and optimization of hits [83]. Software like Schrödinger, AutoDock Vina, Discovery Studio and optimization software such as Chimera, Chem 3D Ultra, and Avogadro are employed for molecular modelling. Initially, optimized 3D structures of ligands/targets in PDB format can be obtained from databases like PUBCHEM, while ZINC database provides access to structures of

known natural products. In silico ligand construction involves geometry optimization to achieve minimal energy levels before docking. After energy minimization, the binding site for natural ligands within the target must be defined during target preparation. Subsequently, the 3D structures of natural products and their analogues are docked against the specified target using docking software to assess binding energy and analyse intermolecular binding interactions. The docking simulation results are then scrutinized to identify the top interactions. Based on interaction rankings, hit molecules exhibiting high affinity towards the target are identified. Further optimization of hit involves studying various analogues of hit molecules with improved drug-like properties using QSAR software, including stability, pharmacokinetic, and pharmacodynamic properties. Thus, molecular modelling enables the identification of potential hits for a target biological activity and facilitates establishing a robust structure–activity relationship (SAR) during lead optimization. This process effectively narrows down the pool of compounds for real testing through bioassays [84].

Very recently, Stefan Gahbauer and coworkers utilized computational design to discover potent inhibitors targeting the NSP3 macrodomain of SARS-CoV-2 with low- to sub-micromolar affinity. Ligands were designed by amalgamating small-molecule fragments and employing ultra-large library docking of 450 million molecules. In total, 160 ligands across 119 different scaffolds were identified, accompanied by the determination of 152 Mac1-ligand complex crystal structures. This approach led to the discovery of several selective and cell-permeable molecules, paving the way for developing novel antiviral therapeutics for SARS-CoV-2 (Fig. 26) [85].

Fig. 26 [Images not available. See PDF.]

Discovery of ligands that bind to the NSP3 macrodomain of SARS-CoV-2 (Mac1)

Conclusions

Natural products (NPs) have always been a source of inspiration for designing novel drug-like molecules which largely relied on trial and errors assisted by serendipity. The systematic development of synthetic strategies based on logical hypothesis inspired from natural product has revived interest of medicinal chemists in utilizing them for innovative drug discovery. Recently, there has been major advancement in synthetic methodology with development of modern catalyst, advanced reagents which allows rapid derivatization of organic compounds. These coupled with contemporary development in computational tools for designing molecules, docking software, and biological screening allow swift detection of hit molecule for further drug development. This review elaborates several pioneering and emerging strategies inspired from natural product which allows access to the unexplored chemical space to identify novel molecules possessing noteworthy bioactivity (Table 2). The corresponding examples highlight the success of these strategies in the discovery of novel bioactive molecules which can be further developed in drug discovery and can be novel probes for chemical biology. Although there are limited number of successful examples, the selectivity, activity, and efficacy associated with natural product-inspired molecules accentuate their importance. Acknowledging the need for substantial further advancement, we anticipate that integrating natural product-inspired synthetic strategies with advance computational techniques involving molecular modelling will become a prevalent approach in modern drug discovery.

Table 2. Tabulated strategies and bioactive molecules discovered using the named strategy

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Author contributions

PP was involved in conceptualization, methodology, data curation, and drafting the paper; Dr SG was responsible for study, drafting the paper, and critical revision; Dr AJ contributed to data curation, conceptualization, and formal analysis; and all authors have read and approved the final manuscript.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author, upon reasonable

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Declarations

Ethics approval and consent to participate

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Competing interests

The authors declare no competing interests.

Abbreviations

ARE
Antioxidant responsive element
BCC
Basal cell carcinoma
BIOS
Biology-oriented synthesis
DOS
Diversity-oriented synthesis
DTS
Diverted total synthesis
ER
Oestrogen receptors
EMRSA
Epidemic methicillin-resistant strains
GCs
Glucocorticoid hormones
GPCR
G-protein-coupled receptors
HNPs
Hybrid natural products
HER2
Human epidermal growth factor receptor
11βHSD1
11β-Hydroxysteroid dehydrogenase type 1
iNPBM
Integrating natural product framework with a bioactive molecule
MRSA
Methicillin-resistant strains
MSSA
Methicillin-susceptible *S. aureus*
PDB
Protein data bank
NP
Natural product
PNP
Pruning natural products
QSAR
Quantitative structure–activity relationship
RDNPs
Ring distortion of natural products

SARS-CoV-2
Severe Acute Respiratory Syndrome Coronavirus 2
SM
Small molecules
ShhN
N-terminal sonic hedgehog protein
SCONP
Structural classification of natural products

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Development of docetaxel-loaded (Soluplus ® –PF108) mixed micelles vacuum foam-dried product for improved stability and melanoma treatment by

QbD approach

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ABSTRACT (ENGLISH)

Background

Docetaxel (DTX) finds extensive use in treating various cancers, but its limited solubility, side effects, and multi-drug resistance (MDR) hinder its effectiveness. To enhance DTX's properties, the study aimed to formulate DTX-loaded mixed micelles (MMs) and evaluate their anticancer potential using Quality by Design (QbD) approach. Using solvent evaporation, DTX-loaded MMs were prepared and optimized via a 3^2 full factorial design.

Results

The optimized formulation (R5) displayed a % entrapment efficiency (%EE) of 74.81 ± 4.27%, % drug loading capacity (%DLC) of 29.27 ± 0.70%, and mean particle size (MPS) of 71.4 ± 1.24 nm. TEM images confirmed well-dispersed spherical MMs. Analytical studies (IR, DSC, and *P*-XRD) showed no adverse drug-excipient interactions. The MMs were converted into vacuum foam-dried (VFD) products for enhanced stability. The optimized VFD products exhibited low residual moisture, rapid reconstitution, consistent drug content, and high %EE. Notably, sustained drug release from the VFD product reduced hemolysis and in vitro cytotoxicity against B16F10 melanoma cells.

Conclusion

This study creatively tackled DTX's challenges through targeted MM development, transformed them into VFD products, demonstrating the potential for melanoma treatment. The QbD approach ensures the formulation's safety, efficacy, and quality, underscoring the promising VFD technology and multifunctionality of mixed micelles.

FULL TEXT

Background

Docetaxel (DTX), a BCS Class IV drug, possesses challenges in oral absorption due to its low solubility and low permeability, leading to poor bioavailability [1]. Its narrow therapeutic index and potential side effects necessitate cautious formulation to minimize toxicity and enhance patient tolerability [2]. Improving efficacy with reducing adverse effects on healthy tissue are difficult aspects of targeting cancer cells. Besides, large-scale production transition poses difficulties in maintaining consistency and quality. These considerations are crucial in optimizing docetaxel pharmaceutical properties for effective cancer treatment [3]. Efforts to enhance drug effectiveness have led to advancements in drug delivery technologies. Targeted medication delivery entails selective and effective localization of a pharmacologically active ingredient at a preselected target in therapeutic concentration while limiting

its access to nontarget areas, hence increasing the treatment's effectiveness [4]. Mixed micelles (MMs) refer to micellar systems composed of multiple amphiphilic components that offer targeted drug delivery, enhanced drug loading capacity, and better control over drug release kinetics [5]. Many researchers have formulated DTX MMs to enhance the properties of DTX in various applications. However, these formulations pose challenges due to certain limitations these include MPP, TPGS, and CSO-SA copolymers by thin film hydration method for enhanced oral absorption [6]. However, difficulties with achieving constant film thickness and managing the size and stability of MMs occur when employing the thin film hydration approach for the formulation. A PF127 and Tween 80 MMs gel to address stability and solubility challenges [7]; DTX MMs with TPGS and Poloxamer 188 [8]; a Pluronic P105 and F127 polymeric MMs against taxol-resistant lung cancer [9]; a monomethylol poly(ethylene glycol)-poly(d,l-lactic acid) (MPP) and Pluronic copolymer MM for enhanced bioavailability and overcoming multidrug resistance [10]; and DTX MMs with TPGS and Pluronic F108 for overcoming MDR in cancer [11]. But, TPGS, a surfactant, and solubilizer, have limitations regarding stability, efficacy, and solubility. Besides, Tween 80, another surfactant, may pose concerns about cytotoxicity and adverse effects [12]. Compatibility and phase separation issues should be addressed when employing multiple Pluronic copolymers, like Pluronic P105 and F127. Moreover, it is crucial to note that the MPS of TPGS and Pluronic F108 MMs was observed to be 233 ± 3 nm which exceeds 100 nm, which hinders their ability to leverage the Enhanced Permeability and Retention (EPR) effect for tumor targeting [13]. Hence, careful selection and optimization of MMs composition and method of preparation help to tailor the properties of drug delivery systems to specific cancer treatment needs.

The scalability of solvent evaporation for mixed micelles (MMs) is vital for large-scale production, offering ease of optimization for reproducibility [14]. The cost-effectiveness of this method, coupled with the novel combination of Soluplus® and PF108, enables the modulation of drug release kinetics, addressing challenges associated with maintaining a constant film thickness and managing particle size [15, 16]. By avoiding the use of Tween 80, concerns regarding cytotoxicity and adverse effects are minimized, while ensuring that the MMs achieve a size below 100 nm [17]. Furthermore, in cancer treatment product development, the application of Quality by Design (QbD) principles is pivotal, offering a systematic approach to optimize formulation, manufacturing, and analytical methods for consistent and high-quality cancer therapeutics, ensuring proactive identification and mitigation of risks for safer and more effective treatments [18].

Previous literature shows that nanoparticles, including micelles, often face long-term stability issues, such as aggregation and chemical degradation [19]. To enhance micelle stability, methods like lyophilization, spray drying, membrane extrusion, and coacervation are employed [20], but they face challenges. Lyophilization and spray drying may lead to changes in micelle size [21] and structure, while membrane extrusion can result in the loss of active ingredients [22]. Coacervation may pose difficulties in controlling particle size and stability [23]. Addressing these challenges is crucial for advancing micellar formulations with improved stability and functionality. To overcome these challenges and further enhance the stability of the developed MMs, novel methods like vacuum foam drying (VFD) have been explored in product development. VFD involves the use of foam structures, formed by mixing drug-loaded micelles with a foaming agent, which are then subjected to a VFD [24]. This method allows for the production of solid, porous materials that maintain the structure and stability of micelles, preventing drug leakage and preserving their therapeutic properties [25]. Retaining drug encapsulation effectiveness, improving stability, extending shelf life, and being simple to handle and store are some benefits of VFD.

The objective of this research was to develop DSP-MMs loaded with novel pluronic polymers Soluplus® and PF108 using the solvent evaporation method, for enhanced treatment against melanoma. To accomplish this goal, a QbD approach was employed for the formulation process, focusing on optimizing the MM's quality, efficacy, and stability as given in the Additional file 1: Supplementary Data S1 - Quality by Design Parameters for Formulation Optimization (see Additional file 1 for details). Additionally, the research was focused on exploring the application of VFD as a means to further improve the stability of the DSP MMs, ensuring their suitability for long-term storage and sustained effectiveness in melanoma treatment.

Methods

Materials

Docetaxel was purchased from IQGEN-X Pvt. Ltd, Mumbai, India. Soluplus® was graciously gifted by BASF, India. PF108 was sourced from Sigma-Aldrich, Mumbai. Other chemicals were obtained from Molychem, Mumbai.

Culture medium and cell line

In this study, the B16F10 murine melanoma cell line (ATCC, USA) was obtained from the American Tissue Culture Collection. The stock cultures were then maintained at 37 °C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum, and streptomycin (100 µg/mL) under a 5% CO_2 atmosphere. The cells were seeded in a microplate within an artificial womb with 5% CO_2 and incubated at 37 °C for 24 h [9].

Critical micelle concentration determination

The CMC of Soluplus® and PF108, both individually and in combination, was determined using the Iodine (I_2) UV-visible spectrophotometric method. A standard KI/I_2 solution was prepared by dissolving 2 gm of potassium iodide (KI) and 1 gm of iodine (I_2) in deionized water. Solutions of Soluplus®, PF108, and their mixtures at concentrations of 0.00001, 0.00005, 0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05, and 0.1 mM were prepared in double-distilled water (DDW), to which the KI/I_2 solution was added. The samples were stored in the dark at room temperature for 12 h. Subsequently, the absorbance at 366 nm was measured using an Agilent 1800 UV spectrophotometer [12].

Development and optimization of MMs

The development of DTX-loaded Soluplus® with PF108 MMs (DSP-MMs) involved the utilization of the most efficient and scalable solvent evaporation method. Sonication was employed for 5 min to dissolve a combination of DTX and copolymer in methanol, which was gradually added drop by drop to distilled water under continuous stirring. The evaporation of methanol at room temperature was facilitated through continued stirring until a clear supernatant of DSP-MMs was obtained after centrifugation. Blank MMs were also prepared without the addition of DTX.

For optimizing the DSP-MMs, a highly efficient 3^2 full factorial design was employed. The design focused on two independent variables the concentration of Soluplus® (X_1) and PF108 (X_2). The study considered two dependent variables, namely % entrapment efficiency (%EE; Y_1) and mean particle size (MPS; Y_2). To determine suitable levels of the independent variables, a preliminary screening was conducted, and each factor was assigned values of − 1, 0, and + 1, as for the concentration of Soluplus® (X_1), levels were set at 182 mg, 364.5 mg, and 547 mg. Likewise, for the concentration of PF108 (X_2), levels were defined as 22 mg, 44.5 mg, and 67 mg. The statistical analysis was carried out using Design Expert® VR software. Analysis of variance (ANOVA) was employed to assess the significance of the model. The coefficients of magnitude were calculated based on the polynomial equations, and p values were used to determine the statistical significance (p <0.05) [9].

Evaluation of DSP-MMs

Entrapment efficiency and drug loading capacity (DLC)

The concentration of DTX in DSP-MMs was measured by diluting 1 mL of DSP-MMs supernatant with methanol to 10 mL and sonicating for 5 min. The resulting solutions were spectrophotometrically scanned at 230 nm using a UV–visible spectrophotometer (Model UV-1900, Shimadzu) equipped with a high-quality quartz UV cell designed to accommodate standard cuvettes with a 1 cm path length, facilitating accurate estimation of the DTX concentration. Substituting values in the following formulae, the %EE and %DLC of DSP-MMs were determined [26]. The %EE and %DLC of DSP-MMs were calculated by substituting values in Eq. 1 and Eq. 2 respectively [26].

1

%EE=Weight of drug in micellesWeight of drug taken initially×100

2

%DLC=Weight of drug in micellesWeight of drug fed initially+Weight of copolymers×100.

Mean particle size (MPS) and zeta potential (ZP):

The Horiba particle size analyzer (Horiba SZ-100, ver. 2.40) was used to determine the MPS and ZP of DSP MMs. The dynamic light scattering method was employed to analyze particle size, and

Laser Doppler Anemometry technology was used to measure zeta potential. Each experiment was carried out in triplicate at 25±5 °C [26].

Drug-excipient compatibility study

Fourier transform infrared spectroscopic analysis (FTIR)

FTIR analysis was conducted to investigate the compatibility of DTX with Soluplus® and PF108. FTIR spectra were recorded for plain DTX, Soluplus®, PF108, and a physical mixture of DTX, Soluplus®, and PF108. Samples for FTIR were prepared following a methodology based on the principles outlined by Patravale et al. This involved maintaining a specific 1:0.5 ratio of active pharmaceutical ingredient (API) to solubilizers, mainly Soluplus® and PF108. Each unit of the API was combined with 0.5 units of Soluplus® and PF108. The FTIR measurements were performed using an Agilent Alpha 100508 instrument over a wave number range of 4000 to 400 cm^{-1} [27].

Differential scanning calorimetry (DSC) analysis

The thermal characteristics and interactions between DTX and the co-polymer were examined through DSC analysis. Both plain DTX and optimized DSP-MMs were subjected to the analysis using a DSC instrument Thermo Gravimetric Analysis–Differential Thermal Analysis–Differential Scanning Colorimetric (TGA–DTA–DSC) instrument with make TA instruments and model SDT Q600 V20.9 build 20. The samples were heated from 0 to 500 °C at a scan rate of 10 °C/min. An empty aluminum pan was used as the reference material for the measurements [9].

Processing of mixed micelles by vacuum foam drying

The product development process involved the careful transfer of precise 1 mL portions of DSP-MMs formulations into 3 mL glass vials. These vials contained a solution with 15% sucrose and 3% citric acid. The vials were partially sealed and then placed on the shelf of a Labconco lyophilizer (FreeZone 2.5® model). The lyophilizer condenser temperature was set to − 50 °C. Vacuum concentration of the DSP-MMs compositions took place for 4 h below 10 °C, maintaining a vacuum above 100 mBar. During this step, the composition's viscosity and surface area were carefully adjusted to ensure foam stability and efficient removal of water through evaporation during bubble formation. Subsequently, further drying of the viscous solutions occurred under vacuum conditions ranging from 1.650 to 0.030 mBar, with varying holding times to complete a 24 h cycle. Secondary drying was conducted at a temperature between 10 and 20 °C and 0.024 mBar for 2 h. As a result of this process, the dried products exhibited a highly porous thin bubble film inside the vials. The next step involved fully stoppering the vials under vacuum, and the finished product, known as the vacuum foam-dried DSP-MMs (DSP-MMs VFD product), was stored in a refrigerator at 4–8 °C until further analysis [25].

Characterization of DSP-MMs VFD product

After processing, the final product was further subjected to characterization to assess PS, ZP, PDI, %EE, %DLC, moisture content, and reconstitution time [25].

Comparative analysis of DSP-MMs and DSP-MMs VFD product

Morphological characterization using TEM and SEM analysis

The surface morphology of optimized DSP-MMs and DSP-MMs VFD products was analyzed using TEM and SEM. The SEM was done on the Scanning Electron Microscope with Jeol Ltd., Japan, and model JSM 6360 A. For TEM analysis, two drops of each sample were placed on a nitrocellulose-coated copper grid and air-dried for over 12 h at room temperature. Following this, the samples were stained with a 2% w/v phosphotungstic acid solution and examined using a Transmission Electron Microscope with Jeol and model JM 2100 to analyze morphological characteristics [28]. For SEM, samples were fixed on a brass stub, made electrically conductive by platinum coating in a vacuum using a Hitachi Ion Sputter, and analyzed with ImageInside Ver. 2.32 [29].

Powder X-ray diffraction analysis

The crystallinity behavior of the formulations was assessed using *P*-XRD analysis. Diffractograms of standard DTX, optimized DSP-MMs, and DSP-MMs VFD products were obtained using an X-ray Diffractometer (Bruker D8 Advance). The X-ray diffractometer operated with Cu-K radiation ($\lambda = 1.54$) at a voltage of 40 kV and 50 mA, incrementing in steps of 0.02° from 5° to 60° diffraction angle ($2°\theta$) at 1 s/step. A zero background was used during the scanning of all samples [9].

In vitro drug release study

In vitro drug release behavior of DTX from DSP-MMs, plain DTX, and DSP-MMs VFD products was investigated

using the dialysis tube method. Dialysis bags containing DTX dispersion and DSP-MMs equivalent to 2.5 mg DTX were immersed in phosphate buffer solution (PBS) pH 7.4 with tween 80, maintained at 37 °C, and stirred at 150 rpm. At pre-determined intervals (0, 2, 4, 6, 8, 24, 48, 72, and 96 h) samples were extracted, replaced with fresh medium, and analyzed using a UV–visible spectrophotometer at wavelength 230 nm. The drug release study results were analyzed by correlating the percentage cumulative drug release against time and; mathematical kinetic models viz. zero-order, first-order, Higuchi, and Korsemeyer-Peppas to understand the drug release mechanism of the MMs formulations [30].

In vitro hemolysis study

The hemolytic effect of pure DTX, blank DSP-MMs, DSP-MMs (optimized R5 formulation), and DSP-MMs VFD products were evaluated using human blood. Fibrinogen was removed from a 5 mL blood sample through centrifugation, and the RBC pellets were cleaned and redispersed in 0.9% NaCl injection water. A 2% erythrocyte pellet solution was prepared and mixed with the above samples in flasks, with the volume adjusted to 10 mL using 0.9% NaCl. Besides, negative and positive controls were also prepared. The flasks were incubated at 37 °C for 2 h, followed by centrifugation and absorbance measurement at wavelength 420 nm. The percentage hemolysis was calculated using Eq. 3 [31]:

3

$$\%\text{Hemolysis} = \frac{\text{Abs of sample} - \text{Abs of negative control}}{\text{Abs of positive control} - \text{Abs of negative control}} \times 100.$$

In vitro cytotoxicity

The cytotoxicity of plain DTX, blank DSP-MMs, and DSP-MMs was evaluated using the murine melanoma cell line (B16F10) through a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye reduction test. The stock cultures were stored for 18 h at 2–8 °C before the cells were seeded. Cells were seeded in a 96-well microtiter plate, and after the formation of a partial monolayer, various test drug doses were added. Following a 48 h incubation period, MTT reagent was added, and the formazan was dissolved using DMSO. Absorbance was measured at wavelength 590 nm, and the percentage growth inhibition was calculated. The IC_{50} values were determined using dose–response curves for each cell line [9].

Stability study of MMs and DSP-MMs VFD product

According to the ICH stability testing requirements for biologicals short-term stability studies on the improved DSP-MMs and DSP-MMs VFD products were conducted for 6 months. The optimized MMs composition was filled in vials and stored at 2–8 °C and 60% RH. The product appearance, MPS, and %EE of the dried composition were assessed [25].

Results

This research aimed to prepare DSP MMs containing Soluplus® and PF108 by leveraging the essential properties of the excipients. The additional application of VFD was employed to overcome various challenges associated with previous methods. Soluplus®, a novel polymer renowned for its exceptional solubilization properties, particularly for poorly soluble APIs, was combined synergistically with PF108. The selection of PF108 was based on its outstanding foaming capacity, strategically intended for use in the novel VFD process. Simultaneously, Soluplus® was chosen strategically to facilitate the solubilization of the drug and achieve the formation of mixed micelles (MMs) with a size below the accepted threshold of 100 nm.

CMC of polymers and mixture

In this study, the iodine UV–visible spectrophotometric method was used to determine the CMC. Iodine served as a hydrophobic probe, and its conversion from I_3 to I_2 in the solution indicated the formation of micelles. This method is preferred over the cloud point method, because the reliability of the cloud point method is compromised by temperature sensitivity, making it less robust without strict control [32]. Additionally, its subjective nature, relying on visual observation, can introduce variability in results due to individual interpretation. The CMC values of pure Soluplus®, PF108, and Soluplus® + PF108 mixture were 0.0031 mg/mL, 0.01047 mg/mL, and 0.001 mg/mL, respectively, as shown in Fig. 1. The lower CMC of the Soluplus® + PF108 mixture is indicative of enhanced drug loading capacity, drug delivery efficiency, and significant in vivo stability which is crucial for pharmaceutical

applications [12].

Fig. 1 [Images not available. See PDF.]

CMC of Soluplus®, PF108, and Soluplus®+PF108 mixture

Development and optimization of MMs

In the pursuit of product development, preliminary experiments were conducted, followed by formulation optimization using a 3^2 full factorial design. Two independent variables, Soluplus® concentration (X_1) and PF108 concentration (X_2), were investigated at three different levels, resulting in nine possible combinations of DSP-MMs as presented in Table 1. The impact of independent variables was assessed with two dependent variables %EE (Y_1) and MPS (Y_2). Through optimization, batch R5 emerged as the most promising, exhibiting %EE of 74.81 ± 1.35%, MPS of 71.4 ± 0.2 nm, and drug loading of 29.27%.

Table 1. Formulation compositions using 3^2 full factorial design and results of their characterization

Batch code	Drug (mg)	Soluplus® (mg)	PF108 (mg)	% EE (%)	MPS (nm)	DLC (%)
R1	2.5	182	22	60.79 ± 2.23	80.5 ± 1.40	20.43 ± 0.75
R2	2.5	182	45	64.51 ± 1.22	74.8 ± 2.89	23.75 ± 0.91
R3	2.5	182	67	65.73 ± 3.56	74.7 ± 4.59	26.14 ± 1.27
R4	2.5	365	22	72.06 ± 2.27	72.8 ± 03.27	28.02 ± 1.41
R5	2.5	365	45	74.81 ± 4.27	71.4 ± 1.24	29.27 ± 0.70
R6	2.5	365	67	74.64 ± 1.82	72.3 ± 3.81	25.11 ± 0.69
R7	2.5	547	22	73.04 ± 2.43	72.4 ± 4.78	21.23 ± 0.34
R8	2.5	547	45	72.54 ± 4.65	72.7 ± 3.25	19.46 ± 1.25
R9	2.5	547	67	70.70 ± 1.28	77.8 ± 4.7	18.12 ± 0.89

The results are the mean ± SD (n = 3)

Effect of formulation variables on %EE

The effect of Soluplus®(X_1) and PF108 (X_2) concentrations on the %EE was estimated using a contour plot, Fig. 2A, and a 3D surface response plot Fig. 2B.

Fig. 2 [Images not available. See PDF.]

A Contour plot of %EE, **B** 3D surface plot of %EE, **C** contour plot of particle size, **D** 3D surface plot of particle size

The %EE of DTX in MMs was observed to be increased with an increase in the concentration of both PF108 and Soluplus®. However, at a medium Soluplus® concentration (365 mg), a substantial increase in %EE was observed that ranged from 72 to 74.81%. The %EE of the DSP-MMs also increased with an increase in concentrations of both the components up to medium concentrations. Beyond this point, further increases in Soluplus® and PF108 concentrations led to a decrease in %EE [33]. This can be attributed to establishing a saturation point within the carrier system at elevated concentrations, which diminished the progressive advantages

of additional Soluplus® and PF108. Moreover, apprehensions about potential aggregation or precipitation of these components emerged as concentrations increased, significantly compromising their efficacy in enhancing encapsulation efficiency. The simultaneous increase in solution viscosity due to heightened concentrations was also recognized as a pertinent factor contributing to this phenomenon, potentially obstructing the unobstructed diffusion of the DTX into the MMs.

The final %EE equation in terms of coded components is given below in Eq. 4:

4

%EE=+74.59I+4.21A+0.8633B-1.82AB-5.95A2-1.13B2

Equation (4) shows a positive value indicating an increase in %EE owing to an increase in the concentration of Soluplus® and PF108. The Model F-value of 153.92 and a *p* value of 0.0008 being < 0.05 indicates that the model terms are significant.

Effect of formulation variables on particle size

The effect of Soluplus® (X_1) and PF108 (X_2) concentrations on the MPS was revealed using a contour plot, Fig. 2C, and a 3D surface response plot, Fig. 2D.

The MPS of DSP-MMs formulations were in the range of 71.4 ± 0.2 nm to 80.5 ± 1.4 nm. The concentration of PF108 and Soluplus® significantly affected MPS. The MPS of the DSP-MMs goes on decreasing with the increase in the concentration of both variables up to a certain extent after which it further goes on increasing with the increase in the concentration [34].

5

MPS=+70.76I-1.18A-0.1500B+2.80AB+3.32A2+2.12B2

The model *F*-value 41.77 and a *p* value 0.0056 < 0.0500 indicates that the model terms are significant [31].

Mean particle size and zeta potential:

The MPS of the MMs in the absence of DTX was found to be 74.4 nm, as shown in Fig. 3A. The MPS and zeta potential of DSP-MMs optimized batch R5 are presented in Fig. 3B and C were found to be 71.4 ± 0.2 nm and − 17.6 mV, respectively. TEM analysis confirmed the MPS estimated by dynamic light scattering [9].

Fig. 3 [Images not available. See PDF.]

A MPS of MMs in the absence of DTX, **B** MPS of DSP MMs, **C** zeta potential of DSP MMs, **D** MPS of DSP-MMs VFD product, **E** zeta potential of DSP-MMs VFD product

Drug-excipient compatibility study

FTIR analysis

Figure 4 displays the overlain FTIR spectra of DTX, PF108, Soluplus®, and the optimized formulation DSP MMs. In product development, FTIR analysis is pivotal for studying drug-excipient interactions and assessing formulation stability. DTX FTIR peaks include O–H, N–H stretching at 3630, 3444, and 3355 cm^{-1}, aromatic C=C stretching at 1699 cm^{-1}, and C–O–C stretching at 1157, 1251 cm^{-1}. PF108 shows C–H stretching at 2875 cm^{-1}, C–H bending at 1462 cm^{-1}, and C–H bending at 840 cm^{-1}. Soluplus® exhibits O–H, N–H stretching at 3656 cm^{-1}, C–H stretching at 2886 cm^{-1}, C=O at 1731 cm^{-1}, and C–O, C–O–C stretching at 1102 cm^{-1}[34].

Fig. 4 [Images not available. See PDF.]

FTIR spectrums: **A** Docetaxel, **B** PF108, **C** Soluplus® and **D** DSP MMs

DSC analysis

DSC analysis is vital in product development, offering insights into thermal transitions and formulation properties. The overlain DSC thermograms in Fig. 5I depict DTX and DSP MMs VFD products. DTX exhibits an endothermic peak at 178–188 °C, while DSP MMs VFD product shows a peak at 308.16 °C, indicating polymer melting and a solid-to-liquid transition. Notably, the DTX melting point peak disappears in DSP MMs VFD products, suggesting a transition from a crystalline to a partially amorphous state, crucial for optimizing drug delivery properties in pharmaceutical development [35].

Fig. 5 [Images not available. See PDF.]

(I): DSC thermograms: **A** DTX, **B** DSP-MMs (R5), and **C** VFD product and (II) *P*-XRD: **A** Plain DTX, **B** DSP-MMs, and **C** DSP-MMs VFD product

Characterization of DSP-MMs VFD product

The DSP-MMs VFD products were a lightweight, porous material with a high surface area that was obtained under reduced pressure conditions. The product formed facilitates rapid drying by removing moisture at lower temperatures than traditional drying methods. The resulting product typically exhibits characteristics such as enhanced porosity, improved rehydration capabilities, and a unique texture, as shown in Fig. 6A. The DSP-MMs VFD products underwent characterization to evaluate MPS, zeta potential, PDI, EE, drug loading, moisture content, and reconstitution time. The MPS was 82.7 ± 0.98 nm (Fig. 3D), zeta potential was − 14.1 ± 0.9 (mV) (Fig. 3E), PDI was 0.073 ± 0.011, %EE was 79.98 ± 0.97%, DLC was 98.2 ± 1.3%, moisture content was 2.25 ± 0.36%, and reconstitution time was 46 ± 5 s (Fig. 6B). These findings provided valuable insights into the physical properties and performance of the final product.

Fig. 6 [Images not available. See PDF.]

DSP-MMs product: **A** VFD product and **B** reconstituted VFD product

Morphological characterization using TEM and SEM analysis

The morphological characteristics of the optimized R5 formulation DSP-MMs structure and DSP-MMs VFD product are validated by TEM analysis Fig. 7A and B. The self-assembled MMs were observed to be spherical in shape. TEM images confirmed the nanoscale of generated MMs, which was comparable with the results, obtained using Zetasizer [36]. The SEM images revealed a highly porous structure of DSP-MMs VFD product (Fig. 7C and D). This porous nature facilitates the rapid reconstitution of DSP-MMs VFD products into micellar solutions [37].

Fig. 7 [Images not available. See PDF.]

TEM images: **A** DSP-MMs, **B** DSP-MMs VFD product and SEM images: **C** DSP-MMs, **D** DSP-MMs VFD product

Powder X-ray diffraction analysis

The *P*-XRD patterns of the plain DTX, DSP-MMs, and DSP-MMs VFD products are displayed in Fig. 5II. Plain DTX showed characteristic strong, high-energy diffraction peaks, indicating crystallinity. In the case of optimized DSP-MMs, these peaks were dramatically broadened with reduced peak intensities indicating partial amorphization. These characteristic peaks disappeared in the diffractogram of VFD products, indicating the amorphization of DSP-MMs VFD products [38].

In vitro drug release study

Illustrated in Fig. 8I are the release profiles of DTX from different formulations: plain DTX DSP-MMs and DSP-MMs VFD products. When dissolved in methanol, plain DTX exhibited an impressive release rate of over 90% after 8 h in PBS (pH 7.4) with 0.5% w/v tween 80, maintaining the sink condition. Comparatively, the DTX release from DSP-MMs after 96 h was measured to be 81.95 ± 1.3%. However, the DSP-MMs VFD product showcased even more remarkable sustained release capabilities, reaching 83.23 ± 1.5%, outperforming plain DTX in terms of sustained release performance [9].

Fig. 8 [Images not available. See PDF.]

(I)In vitro drug release study from DTX, DSP MMs (R5), and DSP-MMs VFD product, (II) Drug release kinetics for DSP-MMs VFD Product, and (III) In vitro cytotoxicity against B16F10 cell line

The drug release pattern was analyzed by fitting the %CDR data from the release profile into various models, including zero-order, first-order, Higuchi, and Korsmeyer-Peppas models. Among these models, the Higuchi model demonstrated the highest correlation coefficient (R^2), as depicted in Fig. 7II. Consequently, this model was selected as the best fit for the data, indicating that the main drug release mechanisms from DSP-MMs involve dissolution and

diffusion [39].

In vitro hemolysis study

The hemolysis assay was performed to assess the biocompatibility and safety of the formulations. The results of in vitro hemolysis in plain DTX, blank Soluplus®/PF108 MMs, DSP-MMs, and DSP-MMs VFD products are presented in Fig. 9. The positive control displayed complete hemolysis, while the negative control showed minimal hemolysis. Plain DTX exhibited a higher percentage of hemolysis compared to DSP-MMs and DSP-MMs VFD products at the same dose. Compared to Taxotere, a marketed formulation of the DTX and previously prepared docetaxel nanoformulations, DSP-MMs VFD product exhibits reduced hemolysis, providing evidence of the enhanced quality of the developed product. This indicates that encapsulation of DTX in DSP-MMs and the subsequent DSP-MMs VFD product significantly reduced the hemolytic potential of the formulation. The reduced hemolytic activity indicates a decreased risk of red blood cell damage and supports the potential for safe and effective delivery of DTX using DSP-MMs and DSP-MMs VFD products intravenously [40].

Fig. 9 [Images not available. See PDF.]

Hemolysis study of DTX, DSP blank, DSP-MMs, and DSP-MMs VFD product

In vitro cytotoxicity study

All the test samples showed a cytotoxic effect on the B16F10 cell line that was concentration-dependent (Fig. 8III and Table 2). At the same doses, both plain DTX and DSP-MMs demonstrated virtually comparable cytotoxicity against cancer cells. On the B16F10 cell line, the simple DTX displayed a lower IC_{50} value of 0.2155 µM than DSP-MMs with an IC_{50} value of 1.170 µM. In comparison to conventional DTX, DSP-MMs displayed a slightly higher IC_{50} value, indicating lesser cytotoxicity against the B16F10 cell line. The sustained release of DTX from DSP-MMs, as seen in the in vitro release study, can be attributed to reduced cytotoxicity. The sustained release of DTX from MMs corresponded to a previous study described by Patil et al. [9].

Table 2. In vitro cytotoxicity study of formulations

Formulation	IC_{50} value against B16F10 cell lines (µM)
Plain DTX	0.2155 ± 0.005
DSP blank MMs	0.7100 ± 0.007
DSP MMs	1.170 ± 0.028

Stability study of DSP-MMs and DSP-MMs VFD product

The comparison between the stability of the developed formulations, DSP-MMs, and DSP-MMs VFD products under accelerated storage conditions is depicted in Table 3. The table showcases key physical properties at two distinct time junctures: initial time (time zero) and following a 3-month storage period. This evaluation provides valuable insights into the endurance of the formulations over time and in the face of adverse storage conditions [25].

Table 3. Stability study of DSP-MMs

Sampling time	DSP MMs			DSP MMs VFD product				
%EE (%)	MPS (nm)	%DLC (%)	%EE (%)	MPS (nm)	Moisture content (%)	Reconstitution time (sec)	%DLC (%)	0 Day

74.81 ± 0.27	71.4 ± 0.52	29.27 ± 0.70	79.98 ± 0.97	82.70 ± 0.98	2.25 ± 0.36	46 ± 5	98.2 ± 1.3	1 Month
72.5 ± 0.16	75.8 ± 1.12	29.07 ± 1.80	79.51 ± 1.12	82.84 ± 0.75	2.40 ± 0.51	49 ± 4	98.1 ± 0.9	2 Months
70.19 ± 0.32	79.2 ± 2.26	28.87 ± 0.90	78.87 ± 0.58	82.84 ± 0.75	2.80 ± 0.76	52 ± 3	97.4 ± 1.1	3 Months

Discussion

Soluplus®, a versatile polymer in drug delivery, offers advantages like high solubilization capacity, biocompatibility, and improved drug bioavailability [41]. In this current study, a composite formulation comprising PF108 and Soluplus® was employed. The inclusion of PF108 was driven by its foaming capacity. Nonetheless, prior research revealed that PF108 single micelles commonly exhibited a moderately higher particle size. In light of this limitation and with the objective of achieving a reduced particle size, the collaborative incorporation of PF108 with Soluplus® was employed. As a result, the resultant mixed micelles exhibited a decrease in particle size compared to the micelles composed of PF108 alone. When combined with PF108, these benefits are further amplified in MMs. The combination of Soluplus® and PF108 in MMs results in smaller MPSs compared to individual micelles of either polymer. This reduction in MPS facilitates the extravasation of MMs through the leaky tumor vasculature [42], leading to enhanced accumulation at the tumor site through the EPR effect [43]. Additionally, the mixed micelles formed by Soluplus® and PF108 can accommodate a higher drug payload, maximizing the amount of drug delivered to the target site, further improving the therapeutic effect [44]. Previously, Patil KS et al. developed DTX-MMs using TPGS and PF108, achieving a MPS of 233 ± 3 nm and %DLC of 2.06 ± 0.08 [12]. In contrast, this research demonstrated that MMs formed by combining Soluplus® and PF108 resulted in a significantly smaller MPS, measuring 71.4 ± 0.2 nm, and a substantially higher %DLC of 29.27%. Hence, the combination of Soluplus® and PF108 proves to be a superior choice for the development of docetaxel MMs. Further, QbD principles were crucial in product development, starting with defining the QTPP and identifying CQAs through pre-formulation studies [45] as given in the Additional file 1. A factorial design was employed to systematically vary critical factors, including polymer concentrations and other formulation parameters, through multiple experimental runs [46]. The effects of these variations on the MPS and %EE were evaluated. The objective of this optimization phase was to determine the optimal formulation that would yield MMs with the desired attributes, ultimately resulting in a high-quality product. Through this optimization R5 batch demonstrated promising characteristics, indicating an efficient encapsulation of the drug within the micellar structure, a desirable MPS for enhanced drug delivery, and an appropriate drug loading content; hence, it was used for further analysis [47].

In the previous research by Patil et al., DTX micelles had a zeta potential of − 7.4 mV, while the product developed in this research achieved a higher zeta potential of − 17.6 mV [9]. This increase can be attributed to the ionizable hydroxyl (–OH) groups present in both Soluplus® and PF108. These groups dissociate, releasing H+ ions, and maintaining the negative charge. The elevated zeta potential in the new research offers several advantages in continuous flow. Firstly, it enhances electrostatic repulsion between particles, preventing aggregation and ensuring uniformity during storage and circulation. Secondly, the increased zeta potential provides better control over drug release kinetics. The higher repulsive force between MMs slows down drug release, resulting in sustained and controlled drug delivery over an extended period [48].

FTIR spectroscopic studies were conducted to investigate potential drug-excipient interactions between DTX, TPGS, and PF108. The DSP MMs VFD product displayed characteristic peaks of the drug, suggesting no significant evidence of chemical interaction between the drug and excipients. This finding ensures the compatibility of the drug with its MMs formulation. DSC studies revealed a transformation from a crystalline to a partially amorphous state. Further for enhancing the stability of the liquid product, converting it into a solid form is a common approach.

Traditionally, lyophilization and spray drying were used, but they presented drawbacks, such as lengthy processing times, potential loss of bioactivity, and limited control over MPS [49]. A drawback of lyophilization is the potential formation of a compacted cake at the bottom of the product container during the drying process. This cake can be difficult to reconstitute as it may resist the absorption of water or solvent. As a result, reconstitution might require more vigorous shaking or mixing to break down the cake and achieve a uniform solution. This can be time-consuming and lead to inconsistencies in the reconstituted product, affecting its usability and quality. Hence, as an innovative alternative, this research tried using novel VFD for DSP-MMs [50]. VFD's ability to operate under mild conditions helps maintain the stability and efficacy of the final solid product. The solid product formed after lyophilization is a cake form whereas VFD gives a porous product hence reconstitution becomes easy as well as there is less percentage of moisture content. The product formed after VFD was further used for different in vitro tests.

The sustained release behavior of DSP-MMs and DSP-MMs VFD products hinders premature drug release, reducing exposure to healthy tissues and potentially enhancing DTX accumulation in tumors via the EPR effect for improved treatment efficacy [51]. The finding of reduced hemolysis demonstrates that the DSP-MMs VFD product formulation is a promising candidate for further development and potential clinical application. It highlights the positive impact of the vacuum foam drying process on the quality and safety of the developed DSP-MMs, reinforcing the importance of this manufacturing approach in producing improved drug delivery systems for melanoma treatment.

The stability of DSP-MMs and DSP-MMs VFD products was assessed at regular intervals. Over time, the MPS of the MMs increased from 71.4 to 90.7 nm due to slight aggregation of the cores of the hydrophobic micelles [52]. After 3 months, the liquid MMs showed precipitation and a significant decrease in %EE. In contrast, DSP-MMs VFD products exhibited stability with only a slight increase in MPS and a minor reduction in %EE. This comparison highlights the superior stability of DSP-MMs VFD products, as they demonstrated minimal changes in MPS and %EE compared to the liquid formulation.

Conclusion

In this research, DSP-MMs loaded with Soluplus® and PF108 were successfully developed to enhance melanoma treatment. Employing QbD principles, the formulation optimization process yielded an efficient encapsulation of the drug, desirable MPS, and appropriate drug loading content. The nanoscale size of the MMs facilitated drug accumulation in tumors through the EPR effect, improving therapeutic efficacy. IR spectroscopic studies indicated no significant drug-excipient interactions, ensuring the stability of the drug within the MM formulation. DSC studies revealed a transformation from a crystalline to a partially amorphous state, indicating potential changes in drug properties. VFD significantly improved the stability of DSP-MMs, reducing MPS growth and maintaining encapsulation efficiency over time. Moreover, DSP-MMs VFD products demonstrated superior stability compared to liquid MMs, exhibiting minimal changes in MPS and encapsulation efficiency. The sustained release behavior of DSP-MMs VFD product minimized premature drug release and enhanced drug accumulation in tumors, contributing to improved treatment efficacy. The applied QbD approach will ensure the safety, efficacy, and quality of the formulation improve the stability of docetaxel-loaded mixed micelles, and enhance their efficacy for melanoma treatment. Further, a novel VFD technique was applied to liquid mixed micelles formulation and converted to a foam-dried product that exhibits improved stability and therapeutic potential. The study of novel vacuum foam drying techniques is a budding phase for the pharmaceutical industry as a whole. This section of the study is interesting and acts as a lamppost for numerous researchers on the stability perspective of nanoformulations.

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Author contributions

KP and RC: conceptualization, investigation, data curation, software, methodology, visualization, formal analysis, supervision, writing—original draft, writing—review and editing. JD, AH, NJ, and PK: formal analysis, writing—review and editing.

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Availability of data and materials

The data will be made available on request.

Declarations

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Not applicable.

Consent for publication

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Competing interests

The authors declare that there are no conflicts of interest related to this work.

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DETAILS

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Influence of artificial intelligence in modern pharmaceutical formulation and drug development

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ABSTRACT (ENGLISH)

Background

Artificial intelligence (AI) revolutionized the formulation and development of modern pharmaceuticals. With the help of AI, researchers can now optimize drug design, develop formulations, and streamline clinical trials in a much accurate and efficient way. Drug development might be greatly expedited and time-consuming procedure; however, with the help of AI this are significantly reduced.

Main body of abstract
The main advantages of AI in pharmaceutical formulation are its capacity to analyse vast amounts of data and spot patterns and connections that human researchers would miss. Various tools and technologies, such as ANN, fuzzy logic, neuro-fuzzy logic, and genetic algorithm are used for analysing the date, of which ANN is popular and mostly used. AI enables the discovery of novel pharmacological targets and the creation of more potent medications. AI may also be used to improve medication formulations by forecasting the solubility, stability, and bioavailability of drug candidates, increasing the likelihood that clinical trials will be successful.
AI is also applied in designing clinical trials, reducing the time and cost of the process by identifying patient populations that are most likely to benefit from the treatment. Additionally, AI can monitor patients during clinical trials, detecting real-time adverse effects and adjusting dosages to improve patient outcomes.
Conclusion
AI is a potent pharmaceutical formulation and development tool, allowing researchers to analyse vast amounts of data, optimize drug formulations, and streamline clinical trials. As technology develops, experts anticipate that AI will increasingly show a crucial part in drug development, enabling faster, more efficient, and more effective treatments for various diseases.

FULL TEXT

Background

The phrase "Artificial Intelligence" (AI) is a general term that describes the least amount of human intervention possible when utilizing a computer to imitate intelligent behaviour. AI is the subfield of computer science that deals with programming to solve issues [1]. It has developed into a discipline that addresses problems in business, engineering, and healthcare. The creation of expert systems is one use for AI. An inference engine, an information base, and a user interface make up an expert system. AI possesses distinctive characteristics that enable it to reason and execute actions with the highest probability of accomplishing a specific objective. AI accomplishes this through a combination of algorithms that simulate even the most fundamental human intellectual capabilities. Nowadays, AI is gaining momentum across multiple sectors, and the pharmaceutical industry is at the forefront of this trend. In the past, formulators have favoured statistical methods, for example response surface approach, for analysing design space. However, optimization using this approach has the potential to be deceptive, especially when dealing with a complex formulation. Two methods that can tackle the issue at hand have been developed as a result of recent developments in mathematics and computer science. The first technique involves using neural networks to replicate how the human brain processes information. The second technique is genetic algorithms, which mimic biological systems' self-organizing and adaptive natures through an evolutionary method. The practical requests of AI in the pharmaceutical production house are highlighted in this paper, including drug research and development (R&D), drug repurposing, increasing pharmaceutical output, clinical testing, etc. These applications reduce the need for human labour and accelerate the drug development process [2]. Over the past few years, the pharmaceutical manufacturing company has significantly improved its data digitalization. To answer challenging clinical problems, acquiring, examining, and applying this knowledge is difficult due to this digitization. AI is used to address this since it can manage enormous volumes of data with increased automation. However, it does not endanger the physical existence of humans. AI uses hardware and software to analyse input data and learn from it in order to accomplish specified goals. This review explains that its services in the pharmaceutical sector are constantly growing. The rapid advancement of AI-guided automation, according to the McKinsey Global Institute, will fundamentally alter how society views labour. Every stage of the pharmaceutical product life cycle, including drug discovery, optimization, formulation development, characterization, quality testing, marketing, and post-marketing surveillance, can integrate AI to improve its efficacy [3].

Tools, technology, and networks

Artificial neural networks (ANN)

Machine learning (ML) is an essential subdivision of AI. A big part of ML is deep learning, which involves ANN. The ANN is made up of multilayer functional units that mimic how electrical impulses are transmitted in the human brain,

i.e. it mimics a human brain. They are mainly biologically motivated systems. It takes input and learns directly from input data; primarily, neurons work mainly on the summation of all information and express an output [4].
The fundamental component of biological neural systems is the neuron. Neurons are electrochemical cells; they receive signals from one neuron and transmit signals to other neurons [4]. Like humans, the ANN system has a primary component known as a "perceptron" or node. Nodes are arranged into layers, and artificial neurons analyse input to create an output that is sent to the following perceptron. It is categorized into two states, supervised learning (SL) and unsupervised learning. In unsupervised learning, the network receives input data and identifies patterns or structures within the data thereby condensing the data into a more compact form [5].
In SL, the network is "taught" by receiving guidance during the learning process. In this SL, the network is given the relevant input and output data. The connection between the input and output data is established via the network. SL is considered the most popular and valuable network for formulation purposes [4, 5].
The arrangement of interconnected neurons in a neural network is called network architecture. There are various types of network architecture; among them, a multilayer perceptron (MLP) network is one of the best suitable examples.
X1, X2 &X3 are input variables, and Y is the output. The number of input, output, and hidden layers depends on the condition and the researcher's plan depicted in Fig. 1 [6].
Fig. 1 [Images not available. See PDF.]

Schematic diagram of artificial neural network

Fuzzy logic

Another tool of AI is fuzzy logic. People extensively use fuzzy logic in problem-solving. When ANN accompanies it, it cooperates to understand the formulation and optimization process [4]. Conventional sense relies on either 'true' or 'false.' So, this hypothesis falls between either in actual or wholly false part. Since the premise is true, the membership function in the true set is 1; otherwise, it is 0 in the false set [4].

Neuro-fuzzy logic

The main motto of fuzzy logic is to give in simple forms. Neural network modelling is necessary to form it. Neuro-fuzzy logic, by its name, is understood that neuro-fuzzy logic is composed of neural networks and fuzzy logic. It combines ANN's learning capacity with generality and fuzzy logic's ability to explore complex concepts. Neuro-fuzzy logic is tightly well suited to process data mining. It can develop good models from data and express the linguistic IF_THEN rules [4, 5].

Genetic algorithm

John Holland introduced genetic algorithms (GA) in the 1970s [5]. Like ANN, GA is also a biologically motivated system. Natural selection, the primary tenet of its genetic variation, imitates the fundamental principles of evolution over a generation. We create a genetic algorithm to select the best and most effective solution. They provide a 'search' strategy that is excellent for optimisation. An iterative procedure would be used to advance the trial population. As part of this procedure, we form a starting population and evaluate each member's fitness [4]. The fittest solutions change into 'parents' of the subsequent generations. It becomes a more ideal answer by including some introduction of recombination and mutation, which produces a larger degree of new stuff in the population. The genetic algorithm needs a criterion of "fitness." It is variable from problem to problem [5].
The best solution is found by creating a number of potential solutions when an ANN and a genetic algorithm are combined. These candidates are selected based on their fitness according to predefined criteria. Generating a new population of solutions is accomplished by utilizing the most effective solution and incorporating crossover and mutation techniques. The process persists until it fulfils the desired requirements, at which juncture the repetition is terminated [4].
An optimization technique is needed to develop a pharmaceutical product to find the best combination of ingredients and techniques. Researchers have proven that the combination of ANN and GA possesses the requisite solutions for developing dosage forms.

Evolutionary computing

It is just a definition of a computational technique that uses heredity, recombination, mutation, and selection to solve a problem. One particular branch of evolutionary algorithms has been used in formulation research [5].

Life cycle of pharmaceutical products

AI can support decision-making, enable rational drug design, determine the best course of a patient's treatment with personalized medications, accomplish the clinical data produced, and utilised that data to create new drugs in the future [7]. From the lab to the bedside, it is logical to assume that AI will contribute to creating pharmaceutical products. Eularis created the E-VAI analytical and decision-making AI platform, which employs ML algorithms and a user-friendly interface to create analytical roadmaps based on rivals, crucial stakeholders, and the market share currently held to forecast critical factors in pharmaceutical sales [8]. This boosts sluggish sales and gives marketing directors the ability to foresee where to make expenditures. It also helps them allocate resources for optimum market share growth. Figure 2 presents an overview of several AI uses in drug discovery and development.

Fig. 2 [Images not available. See PDF.]

Applications of AI in various pharmaceutical business subfields, including pharmaceutical product management and drug development

AI in drug discovery

The research and development of new drugs is a challenging, expensive, and lengthy task. On average, the R&D cycle spans around 10–15 years. Despite the significant financial investment made by the pharmaceutical industry, pursuing the next blockbuster drug persists. This R&D is since only one in every ten potential drug candidates completes phase I clinical trials and attains regulatory approval [9, 10]. The cost and time constraints associated with developing newer therapeutic compounds may be a contributing factor in the pharmaceutical industry's acceptance of AI [11].

The tools and technologies employed by AI are valuable in rapidly identifying hit and lead materials, validating drug targets, and optimizing drug structure design, potentially benefiting the healthcare industry by reducing the cost and timeline associated with discovering novel molecules. However, despite these advantages, AI must still overcome significant data hurdles, including the data's complexity, growth, diversity, and ambiguity [12, 13].

The chemical structure that would elicit the desired reaction at the target location may be predicted using a variety of in silico approaches. This structure can then be improved to meet a variety of criteria, such as potency, safety, solubility, permeability, and synthetic tractability. These methods also make it possible to plan the production of the compound and anticipate the molecule's physicochemical characteristics [13, 14].

By utilizing both structure- and ligand-based methods, along with all available data, it is feasible to hasten the elimination of non-lead compounds. Recently, researchers have employed the quantitative structure–activity relationship (QSAR) modelling device for screening potential pharmacologically active compounds from a pool of one million candidates. Moreover, the deep learning approach, an evolution of the earlier ML approach, can now handle the massive amount of data gathered throughout the drug discovery and development procedure [15, 16].

Using a computer model based on the QSAR, large quantities of compounds or certain physicochemical qualities, such as log P or log D, may be swiftly predicted. These models, however, are far from being able to forecast with any degree of accuracy complex biological traits like a compound's efficacy and undesirable side effects. Additionally, QSAR-based models have issues with limited exercise groups, erroneous investigational facts, and a need for more trial validations. To address these problems, researchers can employ newly developed AI methodologies, such as Deep Learning (DL) and pertinent modelling lessons, to assess the safety and effectiveness of pharmaceutical molecules through extensive data showing and study [17, 18].

DL models beat traditional ML techniques in 15 drugs candidate-related absorption, distribution, metabolism, excretion, and toxicity (ADMET) data sets regarding predictability. Drug metabolism sites are identified using artificial intelligence (AI) techniques like XenoSite, FAME, and SMARTCyp. By displaying molecule distributions and properties, the huge virtual chemical space suggests the existence of a molecular topographic map. Chemical space

visualization's idea is to collect positional information on nearby molecules to hunt for bioactive compounds; thus, virtual screening (VS) helps choose appropriate molecules for future investigation. PubChem, ChemBank, DrugBank, and ChemDB are a few open-access chemical databases.

For the purpose of locating prospective novel drugs, AI-based QSAR approaches, such as decision trees, support vector machines, random forests, and linear discriminant analysis (LDA), have evolved from QSAR modelling tools [15, 19, 20].

We have included a list of a few AI technologies used throughout the drug development phase in Table 1 to help readers understand. Figure 3 summarizes the various AI models used during drug development methods. Physicochemical characteristics, bioactivity, toxicity, target proteins, drug interactions, drug-protein binding interactions, and de novo synthesis of certain organic synthetic compounds are all predicted by these models [21].

Table 1. The AI techniques/tools used in the drug discovery process

Name of tools	Application
Reinforcement learning	Used to optimize drug combinations and dosages by considering multiple interacting variables and maximizing desired outcomes
DeepChem	Open-source library for deep learning in chemistry and drug discovery
DeepTox	Open-source deep learning framework specifically designed for toxicity prediction and assessment
Neural graph fingerprints	Method for encoding molecular structures as fixed-length feature vectors using neural networks, suitable for various applications in drug discovery, such as virtual screening, lead optimization, and property prediction
PotentialNet	Ligand-binding affinity prediction based on a graph convolutional neural network (CNN)
Predictive ADME/Tox modelling	Tools employ ML techniques to model and predict the absorption, distribution, metabolism, excretion, and potential toxicity of drug candidates
Natural language processing (NLP) tools	Assist in extracting and analysing information from scientific literature, patents, and clinical trial data
Cheminformatics tools	Tools enable the analysis and manipulation of chemical structures and properties
QSAR/QSPR modelling	Correlate molecular properties and structures with biological activities or properties, enabling the prediction of compound behaviour
Deep learning (DL)	Applied in tasks like virtual screening, de novo drug design, and predicting drug properties

Machine learning (ML)	Help predict drug-target interactions, analyse biological activity, and optimize lead compounds

Fig. 3 [Images not available. See PDF.]

Different applications of AI in drug discovery

AI in drug development

An acceptable dosage form with the essential delivery qualities must then include a unique medicinal component. In this case, AI can take the place of the conventional approach of trial and error [22]. With the use of QSPR, a variety of computational techniques may resolve issues in the formulation design area, such as instability issues, dissolving, porosity, and many more [23]. Decision-support technologies use rule-based algorithms to choose the kind, nature, and amount of the excipients depending on the physicochemical properties of the drug. They also use a feedback loop to keep an eye on and occasionally tweak the entire process [24].

Piroxicam direct-filling hard gelatin capsules were designed using a hybrid method that combines expert systems (ES) and ANN in order to achieve the necessary dissolving profile. Based on the input parameters, the Model Expert System (MES) delivers judgements and recommendations for formulation development. Contrarily, ANN make use of backpropagation learning to link the formulation parameters to the desired outcome, enabling trouble-free formulation creation. The control module collaboratively manages this process [22].

Using a variety of mathematical methods, including computational fluid dynamics (CFD), discrete element modelling (DEM), and the finite element method (FEM), researchers have investigated the effects of the powder's flow property on the die-filling and tablet compression processes [25, 26]. CFD may also be used to examine how tablet shape affects the profile of the tablet's disintegration [27]. Integrating these mathematical models with AI may have a huge positive impact on the rapid manufacturing of pharmaceutical products. Technologies incorporating AI have evolved into versatile tools that find wide application in various stages of drug development. These stages include identifying and validating drug targets, designing new drugs, repurposing existing drugs, enhancing R&D efficiency, aggregating and analysing biomedicine data, and making informed decisions regarding patient enrolment in clinical trials [17, 28, 29]. These prospective applications of AI offer the chance to mitigate bias and human interference while addressing the inefficiencies and uncertainties resulting from traditional drug development approaches [30]. Drug repurposing [31], pharmacological features [32], protein characteristics and efficacy [33], drug combination, drug-target interaction [34], and prediction of potential synthetic methods for drug-like molecules [35] are other uses of AI in the pharmaceutical industry. In addition, the identification of associations between drugs and illnesses and the development of novel biomarkers and therapeutic targets allow for the identification of new pathways and targets utilizing omics analysis [36, 37].

AI in drug formulation

Pharmaceutical sciences have seen various formulations, for example solid dispersions, extrudates, pellets, nanoparticles, and liposomes, arise in addition to standard dosage forms. The name "formulation techniques" is given to these techniques because they empower the development of formulations or incorporate functionality into common dosage forms such as tablets. AI applications in formulation techniques are even more worthwhile to investigate in order to create next-generation drug products with desired efficacy and health outcomes because these methods can successfully address a variety of API issues, such as low solubility, stability, bioavailability, and production capability [6].

Controlled-release tablet formulation

Researchers utilize pharmacokinetic simulations and ANN to develop controlled-release formulations [5]. The ANN model learns sophisticated and specialized abilities from the input and output data units with the use of Chem software. In order to anticipate the best tablet formulations based on two ideal *in vitro* dissolution-time profiles and two desirable *in vivo* release profiles, researchers use a sophisticated ANN model. Dissolution is the rate-limiting step in the *in vivo* absorption of the drug since it is linearly proportional to the amount of the drug taken *in vivo*. *In*

vitro release patterns are often detected using the difference factors (f_1), and similarity factor (f_2) [38].

Immediate-release tablets formulation

To boost tablet strength, Turkoglu developed a direct compression tablet formulation utilizing hydrochlorothiazide [39]. In a different study, Kesavan and Peck developed a model of a caffeine tablet formulation to describe the diluting agent and binder content in each formulation, processing variables (type of granulator, method of adding binder), and granule and tablet properties (disintegration time, hardness, and friability). These two analyses demonstrated that neural networks performed better than traditional statistical methods. Kesavan and Peck's findings have so been re-evaluated by academics employing a variety of genetic algorithms and neural networks [40]. This presentation illustrated how the relative relevance of the output attributes and the restrictions placed on the several tiers of components and processing factors determined the ideal formulation [41]. Researchers used neuro-fuzzy computing to analyse the same data and frequently created helpful rules that highlighted the most important aspects of any item [5].

Hard gelatin capsule shell formulation

Developing hard gelatin capsule formulations involves using executive tools like ANN and expert systems (ES). ANN stimulate human mental processes, such as generalization, learning, prediction, and abstraction from domain knowledge. With ANNs, the data and statistics collected during investigative work may be transformed into knowledge very quickly, enabling the manufacturer to generate few domain-specific strategies for forthcoming occurrences or forecast the theoretical preparation’s characteristics [22]. By extending the Expert Network and conducting analysis, Wendy I. Wilson in 2005, created a capsule shell manufacturing of Biopharmaceutical Classification System II drugs, such as carbamazepine, ketoprofen, naproxen, and ibuprofen. Capsugel's expert system, for the formulation of powders in hard gelatin capsules, was used all over the world despite the drawback of just providing a proposed composition. During the initial test, researchers discovered that the system exhibited low prediction accuracy and a significant error rate. Researchers retrained the ANN using a new dataset, resulting in models with an R2 of less than 70%. Lastly, for the model drugs, the smart hybrid system predicted the quantity of drug soluble around 5%. By using only 10% of the newly generated data for cross-validation, the researchers showed that the system was capable of creating a formulation that satisfied its performance requirements. Researchers presented the system's ability to analyse several BCS class II drugs by considering wettability and intrinsic dissolving properties [42].

Solid dispersions (SD)

One or more APIs dispersed in a solid matrix describe solid dispersions [43, 44]. They are currently a practical and affordable approach for enhancing solubility and bioavailability [45]. They have been extensively employed in academics and industry to overcome concerns with API poor solubility. Many AI-based SD studies have used ANNs to optimize the formulations [46–48]. Researchers utilized ANNs to enhance the floating and drug release characteristics of SD of Nimodipine prepared with PEG and effervescent mixtures [46]. ANNs were employed to elucidate the relationship between variables such as API concentration, the molar mass of PEG, and temperature in a SD formulated with PVP [49]. Researchers recently developed a model using ML approaches to expect the stability of SD. They employed twenty molecular descriptors to compare eight ML methods. Among these methods, the RF model exhibited the highest estimate precision and provided insights into every input. The top five contributing parameters among the twenty descriptors they picked were the drug loading ratio, relative ambient humidity, storage temperature, preparation temperature, and molecular weight of polymers [50].

Emulsions, microemulsions, and nanoemulsions

Emulsions are biphasic systems with water and oil phases spread over each other and stabilized by an emulsifier [51]. The utilization of micro- and nanoemulsions has the potential to provide a variety of advantages, including increased API bioavailability, superb optical clarity, and improved long-term stability [52–55]. Researchers have published studies on these systems that utilize AI approaches. Kumar et al. regulated the fatty alcohol content with the use of ANNs to produce a steady o/w emulsion. Particle size, zeta potential, conductance, and viscosity were among the emulsion product properties that the ANNs could accurately predict. They also made it possible to

quantify the relative significance of the inputs [56]. Gasperlin et al. successfully predicted the structures of microemulsions by creating two ANNs that can determine the kind of microemulsion from the desired composition or a differential scanning calorimetry (DSC) curve, respectively [57]. Additionally, Agatonovic-Kustrin et al. developed a stable microemulsion formulation for the oral administration of rifampicin and isoniazid using ANN model data for treating the ongoing stage of TB [58]. Amani et al. used ANNs to study potential influences on nanoemulsion particle size and discovered that the final particle size's most important factor was the total energy provided during preparation [59]. In addition, Seyed et al. looked into the component concentrations of nanoemulsion to catch the most stabilized structure with minimum cytotoxicity. They found that emulsifier concentration, which was shown to be the primary determinant of nanoemulsion stability, had no effect on cytotoxicity [60].

Self-emulsifying drug delivery systems (SEDDS)

Drugs, oils, surfactants, and occasionally cosolvents are combined in isotropic ways to create SEDDS [61]. SEDDS offer several advantages due to their physical stability, ease of production, and ability to address concerns regarding low drug bioavailability [62]. SEDDS can effectively tackle various API concerns, including enzymatic degradation, gut wall efflux, solubilization, and bioavailability [63]. Fatouros et al. utilized AI techniques such as neuro-fuzzy networks to create a dynamic lipolysis model that simulates medication absorption and predicts the *IVIVC*. Without requiring complex settings, the model showed significant prediction skills, indicating its potential for application in forecasting the *in vivo* behaviour of formulations made of lipids [64]. Utilizing ANNs coupled with I-optimal design, Parikh and Sawant optimized the crucial elements that determine the droplet size of SEDDS. When compared to the quadratic model based on I-optimal design, the ANN-coupled replicas showed the comparative contributions of every factor and were more accurate [65]. Li et al. used multiple linear regression (MLR) and ANN approaches to create quantitative structure-property relationship (QSPR) models that relate the molecular structures of the surfactant, co-surfactant, oil, and drug used in SEDDS with the drug solubility. The researchers found that key factors influencing drug solubility were the ratios of surfactant and oil, as well as the dipole moment and energy of the highest occupied molecular orbital [66].

Other formulation techniques

In addition to these formulation techniques, researchers have applied AI methods to beads and pellets [67–71], microparticles and nanoparticles [72–85], microspheres and nanospheres [86–88], liposomes and nanoliposomes [89], colloidal systems [90], micelles [91, 92], and liquid, solid dosage forms [93].

Scaling up AI across the pharmaceutical value chain

AI and ML are operating revolutions through several manufacturing company. Nevertheless, industries reliant on research, such as pharmaceuticals, are witnessing rapid advancements in these technologies. To speed up the delivery of life-saving medications, AI is re-designing the value chain and extracting insights from diverse data sets to make it highly interoperable [94].

Drug discovery

AI can aid labour and resource-intensive manual drug discovery. Biopharmaceutical companies have the opportunity to employ AI models to identify and validate various methods, leveraging the acquired knowledge to improve their predictive capabilities. AI could have a significant impact on generative modelling for molecule design and protein engineering in the field of molecular design. Although manual data entry remains essential for clinical trials, the utilization of AI-powered data flows allows for the integration of trial data from diverse sources, enabling the delivery of standardized digital data that seamlessly and automatically transfers to the relevant systems downstream [94].

Manufacturing

AI in biopharma manufacturing might stream crucial data, anticipate process bottlenecks, evaluate current quality control issues, and recommend necessary improvements without the need for time-consuming manual involvement [94].

It could lower operationally expenses and manual oversight in manufacturing operations by permitting tighter control with:

•Quality control

•AI in robotic process automation (RPA)

•AI mock-ups to augment produce &output

•AI-coupled prognostic maintenance to lessen appliance interruption.

Pharmaceutical market of AI

Pharmaceutical companies are turning to AI to reduce financial costs and failure risks. The AI market witnessed growth from US$200 million in 2015 to US$700 million in 2018, and it is expected to reach $5 billion by 2024 [95]. Experts predict that the pharmaceutical and medical industries will experience a 40% growth rate from 2017 to 2024 due to the impact of AI. Numerous pharmaceutical firms have invested in AI and are continuing to do so. They have also worked with AI firms to build crucial healthcare solutions. An example is the partnership between DeepMind Technologies, a Google company, and the Royal Free London NHS Foundation Trust for treating critical renal damage. Figure 4 lists critical pharmaceutical businesses and AI players [12].

Fig. 4 [Images not available. See PDF.]

Leading pharmaceutical firms and their connections to AI companies working in oncology, cardiovascular illness, and CNS disorders. [Pharmaceutical firm’s brand name and logo are subject to individual copyright]

Application of AI

Poly-pharmacology

In disease-related molecular networking, poly-pharmacology is the strategic creation of a therapeutic molecule with the innate capacity to interact with numerous targets or pathways (put, "one disease-multiple target"). One can use this method to create a more effective and less hazardous therapy than the currently available one. Several well-known databases that offer details on biochemical pathways, binding strengths, pharmacological goals, and physiological effects are PubChem, CheMBL, Drug Bank, and Binding DB [96, 97]. The AI system can use this data to probe and discover potential poly-pharmacological drugs selectively. One can use the range of available applications, improved computer capacity, and developments in AI technology to change the drug development process.

Quality control and quality assurance

Developing a pharmaceutical formulation within the given timeframe while ensuring quality necessitates a meticulous and scientifically-driven approach to navigate the intricate process successfully. Gathering information involves capturing data about the characteristics of drug compounds and excipients, their interactions, unit operations, and equipment. Various knowledge applications are utilized, such as heuristics, decision trees, correlation, and first-principal models. This information and knowledge inform the decision-making process for production, selection of excipients, and determination of equipment size [98]. Consequently, AI and its networks, technologies, and tools ensure higher product quality, less waste, and increased profit for manufacturing company. Quality-by-Design (QbD) method ensures the improved quality of the generated goods. This approach makes it easy to comprehend the crucial elements in the pharmaceutical production process that might have an impact on the final product's quality.

Product development

The pharmaceutical sector focuses on accelerating pharmaceutical product development, cutting production costs, and enhancing process design for confirming an active medication. Various expert systems can serve as valuable

tools for prompt decision-making in the rapid development of pharmaceutical products. An example of a decision-support tool is the rule-based expert system, which represents domain knowledge as a collection of rules structured as IF-THEN statements. These rules utilize input data to assist in addressing specific problems. Each rule consists of two components: the IF part, which establishes an assumption (such as medication insolubility), and the THEN part, which defines the corresponding action to be taken (such as using a soluble filler to address the solubility issue). This type of system, known as a production system or an expert system, represents the fundamental form of artificial intelligence. It functions based on pre-established rules that are provided as knowledge inputs to guide its operations. The choice of excipients for preparing tablets and capsules can be made using these expert systems. Such a system's regulations detail the system's mechanical, chemical, and physical characteristics and the requirements for the finished item. The system's inference engine uses this information to forecast the kind and number of excipients needed to achieve the requirements. This formula is used to create the product, which is then examined to see if it complies with the necessary standards. This information is then fed into the system, which aids with formula optimization (Fig. 5) [24].

Fig. 5 [Images not available. See PDF.]

Formulation of tablets and capsules uses rule-based systems

The creation of Logica's product formulation expert system (PFES), anticipated to direct the manufacturing technique using an order of duties, was motivated by the need for quick manufacture of generic formulations. PFES provides a framework for new formulation system development. The specification object, which encompasses the current understanding of the formulation problem, and the formulation object, which represents the current composition of the formulation, are taken into account. This knowledge is subsequently applied to facilitate the development of the formulation. The design of later iterations of PFES involves three levels: physical, task, and control. The control level conducts the studies, while the physical level receives domain information and can be accessed from the task level through a query interface. Expert systems in formulation development have several benefits, such as ensuring a consistent process for regulatory compliance, aiding in novice and professional training, reducing product development time and cost, and freeing up experts to focus on innovation [99].

Developing innovative drug delivery methods with an eye on optimizing efficacy and avoiding adverse effects has generally taken up most of the last ten years. AI has the potential to address certain challenges associated with controlled and conventional drug delivery systems, including issues such as systemic toxicity, narrow therapeutic ranges, and dose adjustments during long-term therapy. To illustrate how AI is utilized in medicines, an example can be given where microchips are employed to manage medication administration, lower systemic toxicity, narrow the therapeutic window, and reduce adverse effects. AI has made significant advancements in enhancing the efficacy, safety, and adherence of patients by innovating implanted drug delivery devices capable of regulating drug release timing and concentration. This strategy of using AI in managing chronic diseases like diabetes, which require prompt treatment and continuous monitoring, is very advantageous. Neural networks, fuzzy logic, integrators, and differentiators have all been used to develop control systems. It is crucial to monitor glucose levels in diabetic patients and provide insulin regularly to treat the condition. As mentioned earlier, the utilization of microchips can assist in the effective management of regular blood glucose level monitoring and insulin administration. The combination of glucose sensors, mathematical models, and control algorithms holds promise for facilitating this objective. Conventional therapeutic methods that have been utilized over time face certain limitations, which can be overcome by integrating information technology, wireless communication, and ANNs into standard therapeutic procedures. As an illustration, wireless connections can be utilized to transmit orders from outside sources to units.

The data from these communications are gathered and tracked to control the drug's distribution [100, 101].

Pharmaceutical manufacturing

Continual production is a valuable strategy for reducing lot-to-lot variances. The FDA recommends a non-stop processing strategy to limit the variation of final products and patient outcomes. One can use various process analytical technology (PAT) tools, efficient and cost-effective to control uninterrupted production. Through automated ML, using AI in conjunction with PAT can improve the process overall and help regulate the production process [102].

Companies that produce biopharmaceutical products can benefit from data science by integrating logistics into their processing operations. Ensuring the proper upkeep of these variables is considered a crucial regulatory consideration due to the utilization of engineered living cells in the production of biopharmaceuticals. Managing and monitoring multiple factors are necessary to maintain the purity and consistency of the manufactured product. As a result, most of the world's leading chemical businesses frequently employ big data to improve vaccine production output and keep an eye on product quality [34].

Drug synergism and antagonism prediction

When treating someone over time, studying how different drugs interact is essential. This can reduce the dose needed and prevent harmful side effects from taking multiple medications together. SnuGen utilises the master regulator inference algorithm (MARINA) to forecast synergism and antagonism. The MARINA method was established to have the ability to predict synergism with an approximate accuracy of 56%. This method clarifies the "Mater Regulator" genes which may be applied to selecting beneficial descriptors for ML techniques. One can use network-based Laplacian regularized least square methods to predict synergism and antagonistic drug interactions. As was covered in the preceding section, various AI techniques combined with medication therapy can be advantageous in multiple ways. For proper prediction, a level of confidence percentage in the range of 0.7–0.9 has been attained, comparable to the performance of the most automatic prediction system. The prediction scores obtained using the various ML techniques do not significantly differ. While ANNs, Random Forest, and SVM all have benefits and drawbacks, choosing the correct input parameters is the main issue when utilizing AI for combo therapy. One must use the parameters that determine the quality of the prediction model when developing prediction algorithms [18].

Nanorobots for drug delivery

Integrated circuits, sensors, a power source, and a secure data backup constitute the primary components of nanorobots, which are maintained by computational technologies like AI [101]. They are designed with programming to prevent collisions, detect targets, locate and engage with them, and subsequently eliminate them from the body. Advancements in nano/microrobot technology empower them to navigate to specific locations within the body by leveraging physiological indicators like pH. This progress enhances their efficacy and reduces the occurrence of systemic side effects [103]. When creating implantable nanorobots for the controlled administration of drugs and genes, factors such as dosage customization, sustained release, and regulated release must be carefully considered. The automation of drug release necessitates the utilization of AI tools, such as neural networks (NNs), fuzzy logic, and integrators [104]. Microchip implants serve dual purposes, enabling both scheduled release of substances and precise localization within the body.

Nanomedicine

Nanomedicines merge nanotechnology and drugs to diagnose, treat, and monitor complex ailments like HIV, cancer, malaria, asthma, and various inflammatory conditions. Due to their enhanced treatment efficacy, the utilization of nanoparticle-modified drug delivery systems has experienced substantial growth in both the therapeutic and

diagnostic domains [105, 106]. Combining AI with nanotechnology could solve numerous issues in product development [107, 108]. Through computational analysis, a nanosuspension of methotrexate was developed by examining the energy generated during the interaction between drug molecules and closely monitoring any conditions that could lead to formulation aggregation. Coarse-grained simulation and chemical computation can be employed to evaluate the interactions between drugs and dendrimers, as well as to analyse the encapsulation of drugs within the dendrimer structure [23].

Furthermore, researchers can investigate the impact of surface chemistry on the internalization of nanoparticles into cells using tools such as LAMMPS and GROMACS 4. The utilization of AI facilitated the development of silicasomes, which are a combination of multifunctional mesoporous silica nanoparticles loaded with irinotecan and the tumour-penetrating peptide iRGD. The inclusion of iRGD enhances the transcytosis of silicasomes, resulting in improved treatment outcomes and increased overall survival. As a result, there has been a significant three- to fourfold increase in the absorption of silicasomes [106].

Predicting the mode-of-action of compounds using AI

The prospect of an AI platform that can predict drugs' on- and off-target effects as well as *in vivo* safety profiles prior to synthesizing has medicinal chemists, in particular, thrilled. The existence of such platforms reduces the amount of time, money, and attrition rates needed to create new medications. Among these platforms are DeepTox, which predicts the toxicity of new drugs, and PrOCTOR, which assesses the probability of toxicity during clinical trials [109, 110]. If a comprehensive and precise dataset containing information on the toxicity and therapeutic characteristics of a wide range of drugs becomes available, the industry has the potential to enhance the predictive accuracy of these platforms by sharing and exchanging data.

As a substitute for chemo-proteomics, SPiDER, a novel AI tool, was recently created [111] to promote natural products for drug development. As a proof-of-concept, SPiDER was utilized to predict the molecular target of lapachone, a natural naphthoquinone with promising antitumour properties currently in clinical development. The platform expected that 5-lipoxygenase (5-LO) would be modulated allosterically and reversibly by lapachone. Through the use of a 5-LO functional test, the prediction is verified. An alternative AI methodology, known as read-across structure-activity relationships (RASAR) [112], has demonstrated the ability to effectively forecast the toxicity of unfamiliar compounds. By leveraging a vast chemical library and establishing connections between molecular structures and hazardous traits, RASAR proves valuable in this prediction process.

Conclusion and future prospect

In conclusion, the function of AI in pharmaceutical formulation and development is rapidly expanding, bringing a host of benefits to the industry. AI has already demonstrated its ability to analyse large data sets, optimize drug formulations, and streamline clinical trials. By doing so, the duration and expenses associated with drug development have been diminished, concurrently heightening the precision and efficacy of the complete process.

The prospects for AI in pharmaceutical formulation and development are up-and-coming. As AI continues to evolve and improve, it is predictable to show an even more significant function in drug development, aiding researchers in identifying new drug targets, drug interactions, and patient populations most likely to benefit from treatment. Furthermore, as AI systems become more advanced, they can simulate biological systems more accurately, allowing researchers to develop more personalized and effective treatments. This, in turn, will lead to more efficient and targeted drug development and personalized medicine.

So, the role of AI in pharmaceutical formulation and development has already proven to be transformative and is expected to continue to revolutionize the industry in the coming years. By leveraging the power of AI, researchers can unlock new insights into complex diseases, create more effective treatments, and improve patient outcomes on

a global scale.

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Author contributions

SM: conceptualization, methodology, visualization; SG and SG: writing—original draft preparation; PM: writing, illustrative diagrams; SC: reviewing and finalizing; KAA: writing, reviewing, editing, and supervision.

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations

AI

Artificial intelligence

ML

Machine learning

R&D

Research and development

ANN

Artificial neural network

CNN

Convolutional neural network

SL

Supervised learning

DL

Deep learning

GA

Genetic algorithms

QSAR

Quantitative structural activity relationship

LDA

Linear discriminant analysis

NLP

Natural language processing

MES

Model expert systems

CFD

Computational fluids dynamics

DEM

Discreet element modelling

FEM

Finite element modelling

API

Active pharmaceutical ingredients

SD

Solid dispersions

SEDDS

Self-emulsifying drug delivery system

MLR

Multiple linear regression

PFES

Product formulation expert systems

RASAR

Read across structure activity relationship

Publisher's Note

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Design, synthesis, and evaluation of anxiolytic activity of 2-(4-phenylpiperazin-1-yl)-1 H -benz[d]imidazole and 2-(4-phenylpiperazin-1-methyl)-1 H -benz[d]imidazole derivatives

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ABSTRACT (ENGLISH)

Background

In contemporary society, anxiety has become a widespread disorder leading to compromised well-being and heightened depressive states. Extensive literature reviews indicate the diverse biological effects of benzimidazole and piperazine derivatives, notably their impact on the central nervous system. This study aimed to design, molecularly dock, synthesize, and assess the anxiolytic potential of six derivatives of 2-(4-phenylpiperazin-1-yl)-1H-benz[d]imidazole and 2-(4-phenylpiperazin-1-methyl)-1H-benz[d]imidazole.

Results

In the present study, an attempt was made to synthesize benzimidazole derivatives conventionally. The benzimidazole nuclei are condensed with various substituted piperazines to obtain targeted benzimidazole–piperazine hybrids. Their anxiolytic activity is determined using the Elevated Plus Maze test and hole board test in mice. All compounds have shown good docking scores and in vivo anxiolytic activity.

Conclusion

Out of all the derivatives synthesized, compounds 5b, 5c, and 5f exhibited outstanding anxiolytic efficacy in both computational simulations and live subjects. Compound 5b demonstrated a remarkable docking score relative to the ligand, suggesting its potential as a promising candidate warranting further exploration.

FULL TEXT

Background

Anxiolytics, crucial for managing anxiety and enhancing mental well-being, should be used under medical supervision to mitigate symptoms of anxiety disorders and enhance overall health and quality of life [1]. These medications slow down the central nervous system (CNS), primarily by enhancing the activity of gamma amino butyric acid (GABA), a neurotransmitter that induces CNS depression. This class of drugs, known for their calming effects, encompasses benzodiazepines, ethanol, opiates, and barbiturates [2, 3]. Benzodiazepines, for instance, potentiate GABA neurotransmission by increasing the affinity of GABA receptors, thereby intensifying inhibitory effects on the CNS [4, 5]. Additionally, heterocyclic compounds like benzimidazole and piperazine, noted for their diverse pharmacological properties including antibacterial, antiviral, antidiabetic, and anticancer activities, have garnered attention for potential drug development [6–11]. The current study synthesizes novel benzimidazole–piperazine derivatives and evaluates their anxiolytic activity.

Materials

The docking simulations were conducted using an Asus personal computer. Autodock Vina software was utilized for docking studies, while Swiss ADME and Molinspiration software were employed to predict the pharmacokinetic properties of the compounds. PASS online software was utilized to forecast the biological activity of the designed compounds. GraphPad Prism 5 software was employed for statistical analysis of biological activity data.

Infrared spectra were acquired using an ATR (Attenuated Total Reflectance) spectrophotometer (Bruker). Proton resonance magnetic spectra (1H NMR) were recorded at 400 MHz, and chemical shifts were expressed in "δ ppm".

Mass spectra were obtained from SPPU, Pune, with molecular peaks expressed in the *m/z* ratio. Thin-layer chromatography was employed for reaction monitoring using an iodine chamber.

Methods

Docking

The crystal structure of the Human GABA-A receptor alpha1-beta2-gamma2 subtype complexed with GABA and flumazenil, conformation B (PDB ID: 6D6T) [12], was obtained from the Protein Data Bank in PDB format. This structure underwent cleaning procedures, involving the removal of water molecules, co-crystallized ligands, and non-essential entities, using Discovery Studio Visualizer. Subsequently, the cleaned structure was converted to pdbqt format after appropriate charge assignment. Ligand structures were designed using Marvin Sketch version 5.8.1, Chem Axon. Both protein and ligand structures were prepared utilizing Auto Dock Tools following a previously established protocol [13]. A search space grid was generated around the binding site of the GABA receptor using Auto Dock Tools. The grid dimensions were adjusted to encompass the active site to ensure a comprehensive exploration of ligand conformations. Auto Dock Vina was used to carry out docking between the prepared ligands and proteins. The conformation possessing the least binding energy was further analyzed to study its binding mode with the receptor. The protein–ligand interactions were viewed using BIOVIA, Dassault Systems, Discovery Studio 16.0.1, San Diego: Dassault Systems, 2016.

Chemistry

A series of novel piperazine-linked benzimidazole analogs (5a–f) were synthesized via a three-step synthetic pathway outlined in Fig. 1. Initially, 2-oxo benzimidazole (1) was synthesized by condensing o-phenylene diamine (OPD) and urea at 135–400 °C in DMF. Subsequently, halogenation was achieved by adding $POCl_3$ and phenol crystals, allowing the reaction to proceed at 104–107 °C to yield 2-chlorobenzimidazole (2), serving as a core structure for derivatives [14–16]. Another core structure, 2-chloromethyl benzimidazole (3), was synthesized by refluxing O-phenylene diamine and chloroacetic acid with 5N HCl [17, 18]. The piperazine substituents (4a–d) were obtained by refluxing Bis-2-chloroethylamine hydrochloride with substituted anilines at 142–145 °C in the presence of p-toluene sulphonic acid (PTSA) using xylene as a solvent [19, 20]. Lastly, equimolar quantities (0.009 mol) of the core structure and substituted piperazines were dissolved separately in 1,4-dioxane, mixed, and refluxed with triethylamine (TEA) as a catalyst for 12 h [21]. The resulting precipitate was collected using chilled water, filtered by suction, and recrystallized from ethanol after drying to obtain the target derivatives (5a–f).

Fig. 1 [Images not available. See PDF.]

Scheme of synthesis of benzimidazole derivatives

All synthesized analogs underwent characterization via infrared spectroscopy (IR), proton nuclear magnetic resonance spectroscopy (1H NMR), and mass spectrometry (MS) for molecular weight determination. IR spectra revealed characteristic bands at 3300–3400 cm^{-1} corresponding to the Aromatic N–H bond, 900–1100 cm^{-1} for the C–F stretch, and 2800 cm^{-1} for the piperazinyl aliphatic C–H bond [22, 23]. In the 1H NMR spectra, the distinctive singlet peak of the benzimidazole nucleus was observed between 12.5 and 13.6 ppm for all derivatives. Peaks corresponding to piperazinyl aliphatic C–H appeared at 3.5–4.2 ppm, aromatic C-H peaks were detected at 7–8 ppm, and methylene C-H peaks for nucleus (3) were observed at 3.8–4.0 ppm [22, 23]. Mass spectrometry results corroborated the molecular weights of the compounds, with an additional weight corresponding to proton addition.

Biological evaluation

The anxiolytic activity was assessed using the Hole Board Test and Elevated Plus Maze (EPM) test in Swiss albino mice, with diazepam employed as the standard reference [24, 26]. The Hole Board Test apparatus comprised a wooden floor measuring 40 cm × 40 cm, elevated 2 inches above the ground, featuring sixteen holes arranged symmetrically in a diamond pattern. Groups of 5 animals were individually placed on one edge of the apparatus and monitored for 5 min to record the number of pockings. Diazepam (4 mg/kg i.p) was administered 30 min before the test.

For the EPM test, the wooden apparatus was elevated to a minimum height of 50 cm from the ground, adhering to

the specifications described by Lister [25, 26]. The EPM consisted of two open arms (50 × 10 cm) and two closed arms (50 × 10 × 40 cm). Each group of 5 animals was individually positioned in the center of the EPM, facing the closed arms, and their time spent in both open and closed arms was recorded over 5 min.

Results

Synthesis and spectral data

2-(4-(4-Fluorophenyl)piperazin-1-yl)-1*H*-benz[d]imidazole (5a)

Yield: 56%; **m.p:** 216–218 °C; **IR Ranges (ATR, cm^{-1});** N–H stretch: 3337.25, C–H aromatic: 3041.76, C–H aliphatic: 2813.17, C–H aliphatic bend (CH2):1433.00, C=C stretch: 1600.32, C–N aromatic: 1210.90, C–F stretch: 999.71. **H1 NMR Shifts;** Aromatic C–H: (m 4H): 7.04–7.20, Piperazine C–H: (m 8H): 3.16–3.50, Aromatic C–H: (m 4H): 7.63–7.72, Benzimidazole N–H: (s 1H): 13.04. **MASS:** MOLECULAR ION PEAK (297.1523).

2-((4-(4-Fluorophenyl)piperazin-1-yl)methyl)-1*H*-benz[d]imidazole (5b)

Yield: 60%; **m.p:** 159–163 °C; **IR Ranges (ATR, cm^{-1});** N–H stretch: 3491.91, C–H aromatic: 3025.29, C–H aliphatic: 2908.47, C–H aliphatic bend (CH2): 1475, C=C stretch: 1650.50, C–N aromatic: 1204.77, C–F stretch: 1011.75. **H1 NMR Shifts;** Aromatic C–H: (m 4H): 7.16–7.21, Piperazine C-H: (m 8H): 3.29–3.88, Aromatic C–H: (m 4H): 7.44–7.63, methylene C–H: (s 2H): 3.89, Benzimidazole N–H: (s 1H): 13.61. **MASS:** MOLECULAR ION PEAK (311.1670).

2-((4-(3-Fluorophenyl)piperazin-1-yl)methyl)-1*H*-benz[d]imidazole (5c)

Yield: 70%; **m.p:** 122–126 °C; **IR Ranges (ATR, cm^{-1});** N–H stretch: 3337.25, C–H aromatic: 3041.55, C–H aliphatic: 2813.17, C–H aliphatic bend (CH2): 1433.00, C=C stretch: 1600.32, C–N aromatic: 1210.90, C–F stretch: 999.71. **H1 NMR Shifts;** Aromatic C–H: (m 4H): 7.05–7.17, Piperazine C–H: (m 8H): 3.39–3.88, Aromatic C–H: (m 4H): 7.17–7.44, methylene C–H: (s 2H): 3.89, Benzimidazole N–H: (s 1H): 13.61. **MASS:** MOLECULAR ION PEAK (311.1660).

2-((4-(2-Fluorophenyl)piperazin-1-yl)methyl)-1*H*-benz[d]imidazole (5d)

Yield: 71%; **m.p:** 116–119 °C; **IR Ranges (ATR, cm^{-1});** N–H stretch: 3481.78, C–H aromatic: 3038.96, C–H aliphatic: 2809.86, C–H aliphatic bend (CH2): 1433.42, C=C stretch: 1601.73, C–N aromatic: 1233.37, C–F stretch: 1016.61. **H1 NMR Shifts;** Aromatic C–H: (m 4H): 6.99–7.17, Piperazine C–H: (m 8H): 3.30–3.50, Aromatic C–H: (m 4H): 7.17–7.61, methylene C–H: (s 2H): 3.89, Benzimidazole N–H: (s 1H): 13.61. **MASS:** MOLECULAR ION PEAK (311.1675).

2-((4-Phenylpiperazin-1-yl)methyl)-1H-benz[d]imidazole (5e)

Yield: 57%; **m.p:** 162–165 °C; **IR Ranges (ATR, cm^{-1});** N–H stretch: 3481.78, C–H aromatic: 3038.96, C–H aliphatic: 2809.86, C–H aliphatic bend (CH2): 1433.42, C=C stretch: 1601.73, C–N aromatic: 1233.37. **H1 NMR Shifts;** Aromatic C–H: (s 1H, m 3H): 6.99–7.20, Piperazine C–H: (m 8H): 3.19–3.46, Aromatic C–H: (m 4H): 7.50–7.57, methylene C–H: (s 2H): 4.07, Benzimidazole N–H: (s 1H): 12.50. **MASS:** MOLECULAR ION PEAK (280.1690).

2-(4-(3-Fluorophenyl)piperazin-1-yl)-1*H*-benz[d]imidazole (5f)

Yield: 64%; **m.p:** 195–199 °C; **IR Ranges (ATR, cm^{-1});** N–H stretch: 3337.25, C–H aromatic: 3041.55, C–H aliphatic: 2813.17, C=C stretch: 1600.32, C–N aromatic: 1210.90, C–F stretch: 999.71. **H1 NMR Shifts;** Aromatic C–H: (m 4H): 7.07–7.20, Piperazine C–H: (m 8H): 3.16–3.50, Aromatic C–H: (m 4H): 7.20–7.63, Benzimidazole N–H: (s 1H): 13.04. **MASS:** MOLECULAR ION PEAK (297.1505).

The thin layer chromatography (TLC) characterization data for the synthesized substituents and derivatives, as well as spectra for infra-red (IR), proton nuclear magnetic resonance (1H NMR), and Mass analyses of the synthesized derivatives, are provided in the Additional files 1 and 2.

Biological activity (anti-anxiety activity)

The anxiolytic activity of the compounds was assessed through two tests: the Hole Board Test and the EPM Test. The experimental protocol for both tests was as follows: Group I received vehicle treatment (0.5% carboxymethyl cellulose in water). At the same time, Group VIII served as the standard reference with diazepam administration at 4 mg/kg intraperitoneally (i.p). Groups II to VII received test compounds 5a–f (synthesized compounds) at 50 mg/kg orally (p.o). Comparisons were made between Groups II to VIII and Group I, and the results were subjected to

statistical analysis using GraphPad Prism 5 software, employing one-way ANOVA followed by Dunnett's test.

Hole-board test

Each of the eight groups, consisting of five mice per group, was individually monitored for the number and duration of pocking in the hole board apparatus over 5 min. A higher number and longer pocking duration indicate the compound's anti-anxiety properties being evaluated. The mean ± standard error of the mean (SEM) for each group is presented in Table 1.

Table 1. Data for Hole board test

Sr no	Treatments	No of pocking	Duration of pocking (s)
Group I	Control	5.6 ± 0.50	11.40 ± 1.07
Group II	5a (50 mg/kg)	13.1 ± 2.34*	119.4 ± 3.41***
Group III	5b (50 mg/kg)	25.20 ± 1.28***	171.0 ± 3.92***
Group IV	5c (50 mg/kg)	19.40 ± 1.77***	157.2 ± 6.36***
Group V	5d (50 mg/kg)	12.8 ± 1.43*	136.8 ± 4.32***
Group VI	5e (50 mg/kg)	12.60 ± 0.92*	118.2 ± 2.43***
Group VII	5f (50 mg/kg)	19.80 ± 3.15***	166.2 ± 6.01***
Group VIII	Diazepam (4 mg/kg)	12.80 ± 0.86*	30.0 ± 1.39*

Values are expressed as mean ± SEM, n = 5

ns non significant

*p<0.05; ***p<0.001

Compounds within Groups III, IV, and VII (Derivatives 5b, 5c, and 5f) exhibited very highly significant anxiolytic activity (***p<0.001) in comparison to the control group, as assessed by the number of pocking. Compounds across Group II-VII (Derivatives 5a–f) demonstrated highly significant anxiolytic activity relative to the control group, as determined by the duration of pocking.

Figure 2 presents a graphical depiction of the data obtained from the hole board test. It is evident from the graph that all derivatives exhibit significant anxiolytic effects compared to both the control and standard groups.

Fig. 2 [Images not available. See PDF.]

Graphical representation of anxiolytic activity by Hole board apparatus. (**A** Number of pocking; **B** duration of pocking, *significant, **highly significant, ***very highly significant by ANOVA and Dunnett's test)

EPM test

T Each of the eight groups, comprising five mice per group, was individually monitored for the number of entries and the duration of entries into the open arms of the EPM apparatus over a 5-min duration. An increased number and duration of entries into the open arms of the EPM are indicative of the anti-anxiety properties of the compound being evaluated. The mean ± standard error of the mean (SEM) for each group is presented in Table 2.

Table 2. Data for EPM test

Sr. no	Time spent (s)		No. of entries	
Open arm	Close arm	Open arm	Close arm	Group 1
7.0±2.09	104.6±9.6	2.0±0.44	19.5±0.4	Group 2
118.2±5.14***	43.20±3.13***	10.80±1.35***	10.8±1.35***	Group 3
158.8±4.04***	46.60±4.33***	26.80±0.86***	10.8±1.15***	Group 4
138.0±5.19***	45.20±6.00***	21.80±1.59***	11.8±1.5***	Group 5
130.0±5.64***	46.0±2.42***	15.20±1.4***	6.8±0.96***	Group 6
134.2±3.81***	41.20±4.66***	14.60±1.50***	9.2±1.15***	Group 7
143.6±4.47***	55.60±2.33***	25.20±0.58***	10.2±1.06***	Group 8

Values are expressed as mean±SEM, n=5.

Test compounds Groups II–VII (5a–f) showed very highly significant anxiolytic activity (***p<0.001)

ns non significant

***p<0.001

Discussion

Using Molinspiration [27] and Swiss ADME software [28], the physicochemical parameters and ADME (absorption, distribution, metabolism, and excretion) properties of all compounds were comprehensively examined before synthesis. Lipinski's rule of five (RO5), which evaluates essential physicochemical properties crucial for a molecule's efficacy, safety, or metabolism, was considered for all compounds.

Ideally, oral drugs should possess a Log P value<5, a molecular weight<500, a topological polar surface area (TPSA)<90 to efficiently cross the blood–brain barrier, a molar refractivity value between 40 and 130, fewer than 10 hydrogen bond acceptors, and fewer than 5 hydrogen bond donors [29]. The assessment revealed that none of the compounds violated Lipinski's rule of five (RO5), as indicated in Table 3. This suggests that all derivatives were well-designed and exhibit desirable drug-like or pharmacological characteristics, rendering them potentially accessible for oral administration.

Table 3. Drug likeliness properties of derivatives

Comp	miLogP	Mol wt	TPSA	MR	HBA	HBD
5a	3.605	296.349	35.159	92.55	2	1
5b	3.091	310.376	35.159	96.80	3	1

5c	3.067	310.376	35.159	96.80	3	1
5d	3.043	310.376	35.159	96.80	3	1
5e	2.927	292.386	35.159	96.84	2	1
5f	3.581	296.349	35.159	92.55	2	1

Comp compound, *miLogP* partition coefficient, *Mol wt* molecular weight, *TPSA* topological polar surface area, *MR* molar refractivity, *HBA* hydrogen bond acceptor, *HBD* hydrogen bond donor

The blood–brain barrier (BBB) penetration, permeability glycoprotein (Pgp) substrate, gastrointestinal (GI) absorption, and cytochrome P450 (CYP450) enzymes, particularly CYP2C19 inhibitors, are key pharmacokinetic properties indicative of favorable drug likeliness potential. Utilizing Swiss ADME software, an in-silico assessment of pharmacokinetic properties was conducted, as outlined in Table 4.

Table 4. Pharmacokinetic properties of derivatives

Compound	GI abs	BBB permeation	Pgp substrate	CYP2C19 inhibitor	CYP3A4 inhibitor	Lipinski violation	Ghose violation	Bioavailability score
5a	High	Yes	Yes	No	Yes	0	0	0.55
5b	High	Yes	Yes	No	Yes	0	0	0.55
5c	High	Yes	Yes	No	Yes	0	0	0.55
5d	High	Yes	Yes	No	Yes	0	0	0.55
5e	High	Yes	Yes	No	Yes	0	0	0.55
5f	High	Yes	Yes	No	Yes	0	0	0.55

All compounds examined in the ADME software exhibited high gastrointestinal absorption. Furthermore, in the ADME study, all derivatives were identified as Pgp (glycoprotein pump) substrates. P-glycoprotein plays a significant role in restricting the cellular uptake of medications from the bloodstream into the brain and from the intestinal lumen into epithelial cells. While positive Pgp substrate properties can diminish medication absorption, this effect can be mitigated by increasing the dosage, as high drug concentrations in the intestinal lumen can saturate P-glycoprotein transport function.

All derivatives are expected to inhibit the liver enzyme CYP3A4, while CYP2C19 remains unaffected. Consequently, the results indicate that the synthesized derivatives possess favorable pharmacokinetic and physicochemical properties, suggesting their potential utility as effective lead compounds with notable membrane permeability and oral bioavailability

An initial evaluation of central nervous system (CNS) depressant activity was conducted using the PASS study [30]. The derivatives exhibited virtually significant anxiolytic and antipsychotic activity. Values above 0.2 are considered significant according to PASS evaluation criteria, as depicted in Table 5.

Table 5. PASS study of derivatives

Comp	Pharmacological activity	Activity value
5a	Anxiolytic	0.508
5b	Anxiolytic Antipsychotic	0.551 0.527
5c	Anxiolytic Antipsychotic	0.575 0.546
5d	Anxiolytic Antipsychotic	0.509 0.543
5e	Anxiolytic	0.510
5f	Anxiolytic	0.540

All derivatives underwent a docking study to assess their interaction with the active site of the inhibitory ion channel Human GABA-A receptor alpha1-beta2-gamma2 subtype complexed with GABA and flumazenil, conformation B (PDB ID 6D6T), aiming to determine binding energy and interactions. It was observed that all derivatives exhibited favorable binding energy and engaged in various interactions with amino acids at the active site. The reference compound, flumazenil, exhibited substantial affinity towards the receptor molecule via halogen bonding with HIS D:102; hydrogen bonding to THR E:142; and van der Waals interactions with SER D:206, ASP E:56, and MET E:130; pi-pi stacking interactions with PHE E:77, TYR E:58, TYR D:160, and TYR D:210 in addition to other bonding modalities, accompanied by the binding energy of −9.4 kcal/mol. The synthesized compounds exhibited binding energies ranging from −8.4 to −9.5. Details of the docking study outcomes are presented in Table 6. The derivatives 5a, 5b, 5c, and 5e exhibit hydrogen bonding interactions with the protein, as illustrated in Fig. 3. The principal binding interactions vis-a-vis the ligand flumazenil are deliberated upon herein. Compound 5a forms a single hydrogen bond with the active site residue SER D:206, in addition to engaging in two pi-pi stacking interactions with TYR E:58 and PHE E:77. Compound 5b establishes two hydrogen bonds with active site residues HIS D:102 and SER D:206, along with pi-pi stacking interactions with TYR D:160 and TYR D:210. Similarly, compound 5c forms two hydrogen bonds with SER D:206 and demonstrates halogen (fluorine) interaction with SER D:159, as well as pi-pi stacking with TYR D:210. Compound 5e forms a lone hydrogen bond with SER D:206. Besides these hydrogen bonds, pi-pi stacking interactions with residues TYR D:160 and TYR D:210, along with pi-anion interactions with ASP E:56 and MET E:130, appear to significantly contribute to the molecule's binding to the receptor. In contrast, Compounds 5d and 5f do not establish any hydrogen bonds with the receptor.

Table 6. Docking results

Sr. no	Compound	Binding energy (kcal/mol)	No. of H bonds formed	Distance	Interacting amino acids
1	5a	−8.4	1	3.080	SER(D): 206

2	5b	−9.5	2	2.541 2.811	HIS(D):102 SER(D): 206
3	5c	−9.3	2	2.826 2.832	SER(D): 206
4	5d	−9.4	–	–	–
5	5e	−9.1	1	2.796	SER(D): 206
6	5f	−8.5	–	–	–
7	FZP	−9.4	1	2.465	HIS(D): 102

FZP flumazenil, *SER* serine, *HIS* histidine

Fig. 3 [Images not available. See PDF.]

2D and 3D docking interactions of derivatives 5a, 5b, 5c and 5e

The anxiolytic efficacy of these compounds was evaluated in comparison to a control group. In the hole board test, control group mice exhibited fewer instances of pocking indicative of fear and anxiety, whereas the treated groups displayed an increased number and duration of pocking due to the anxiolytic effects. Compounds 5b, 5c, and 5f demonstrated notably significant anxiolytic activity based on the frequency of pocking, while all compounds 5a-f exhibited significant activity in terms of pocking duration. During the EPM test, rats administered with anxiolytic drugs spent more time in the open arm compared to the control group, reflecting heightened anxiety in the animals on an elevated platform. Notably, all six synthesized derivatives (5a–f) displayed highly significant anxiolytic activity in the EPM test.

Conclusion

All newly synthesized benzimidazole derivatives 5a–f have exhibited highly significant biological anti-anxiety activity. Most compounds have demonstrated drug-like properties, along with favorable gastrointestinal absorption and bioavailability as predicted by virtual computational tools. These findings are encouraging and warrant further investigation.

Moreover, docking studies against the Human GABA A receptor (PDB ID: 6D6T) revealed favorable binding energies for all derivatives, validating our hypothesis regarding the potential of the recently synthesized 2-(4-phenylpiperazin-1-yl)-1H-benz[d]imidazole and 2-((4-phenylpiperazin-1-yl) methyl)-1H-benz[d]imidazole derivatives as lead candidates for anti-anxiety drug development. Notably, compound 5b exhibited superior binding affinity compared to the ligand and demonstrated excellent in vivo anxiolytic activity, thus presenting a promising candidate for further exploration.

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Author contributions

All authors have read and approved the manuscript. BM: synthesis, characterization, in silico evaluation, and outline of study; LK: design of compounds, synthetic scheme; VN: animal model; SU: docking studies; LS and PK: biological activity work.

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The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

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Consent for publication

The authors declare no conflict of interest.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

ADME
Absorption distribution metabolism elimination
ANOVA
Analysis of variance
ASP
Aspartic acid
BBB
Blood–brain barrier
CPCSEA
Committee for Control and Supervision of Experimental on Animals
CYP450
Cytochrome P450
EPM
Elevated Plus maze
GABA
Gamma amino butyric acid
GI
Gastro-intestinal
GPCR
G-protein coupled receptor
HIS
Histidine
IAEC
Institutional Animal Ethics Committee
IR
Infra-red
MET
Methionine
NMR
Nuclear magnetic resonance
PASS
Prediction of activity spectra for substance
Pgp
Permeability glycoprotein
PHE
Phenylalanine
PTSA

Para toluene sulfonic acid

SEM

Standard error of mean

SER

Serine

TLC

Thin layer chromatography

TPSA

Topological polar surface area

TYR

Tyrosine

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Antiproliferative effect of Saraca asoca methanol bark extract on triple negative breast cancer (TNBC)

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ABSTRACT (ENGLISH)

Background

Saraca asoca (Asoka) is reported to possess phytoestrogenic components with anticancer properties. The phytoestrogens are recognized as natural agonists for ER*β*, which acts as an antagonist to ER*α*. Despite the absence of ER*α*, studies have identified ER*β* in 50–80% of triple negative breast cancers (TNBC). Thus, the present study is intended to reveal the role of phytoestrogens of Asoka on TNBC. The cytotoxic effect of Asoka methanol bark extract was analyzed on different breast cancer cell lines by MTT assay. Estrogen-screen assay was employed to determine the proliferative/antiproliferative effect. Identification of phytoestrogens in Asoka was accomplished using LC-MS analysis and in silico docking studies were performed to investigate possible interactions of phytoestrogens with ER*α* and *β*.

Results

The extract of Asoka was found to be cytotoxic against TNBC cell line, MDAMB-231 with IC_{50} of 70.22 ± 1.89 µg/mL and towards HER^{+} breast cancer cell line, SKBR3 with IC_{50} of 98.41 ± 2.31 µg/mL, respectively. Whereas the extract did not show any cytotoxicity towards ER*α* cell line, MCF-7 even up to the concentration 300 µg/mL. Estrogen-screen assay emphasized an estrogenic effect of the extract on MCF-7 and an anti-estrogenic/antiproliferative effect on MDAMB-231 cells. LC–MS analysis identified phytoestrogens such as *β*-sitosterol, quercetin, kaempferol and others. The docking results revealed good binding efficacy of phytoestrogens with ER*β* than ER*α* and quercetin shows more affinity with the highest docking score of −9.220. Strikingly, it was found that the *S. asoca* methanol extract was preferentially cytotoxic to TNBC cells.

Conclusion

The study demonstrates selective anticancer properties of *S. asoca* methanol extract on TNBC, which indicates a selective impact on ER subtypes. The identification of phytoestrogens, such as *β*-sitosterol, quercetin and kaempferol, in the Asoka methanol bark extract provides a molecular basis for its observed effects. In silico studies further support the view that these phytoestrogens may preferentially interact with ER*β* rather than ER*α*. Quercetin, in particular, demonstrated the highest binding efficacy with ER*β*, suggesting its potential role in mediating the anticancer effects observed in TNBC cells. Further research is warranted to explore the full therapeutic potential of phytoestrogens in breast cancer treatment.

FULL TEXT

Background

Saraca asoca (Roxb.) De Wilde, commonly known as Asoka, belonging to the family Fabaceae is considered one of the most ancient and holistic trees in India. Various ethnopharmacological uses of Asoka in different treatment

aspects are well documented in Indian old classical Ayurvedic treatises, *Charaka Samhita* (1000 BC), *Susruta* (500 BC) *Vaghbatta* (sixth century) *Dhanvantari Nighantu* (ninth century) and *Chakradatta*, (eleventh century) etc. In Ayurveda, the stem bark of Asoka is used to make *Asokarishta*, a polyherbal decoction used to manage various gynecological complications, especially menorrhagia [1]. This traditional practice reflects the significance of Asoka especially in women's health, as emphasized in the traditional healing systems of India. Inspired by these treatises, several studies have validated the ethnobotanical claims and unveiled novel pharmacological properties [2] like antibacterial [3], antioxidant [4], antipyretic [5] antihyperglycemic [6], anthelmintic [7] and anticancer [8] activities. The cytotoxic activity of *S. asoca* on the breast (MDAMB-231, MCF-7), cervical (HeLa), colon (HT-29), and lung (A549) cancer cell lines were reported [9–11]. *Saraca asoca* exhibits chemopreventive activity against acute myeloid leukemia (AML) and DMBA/croton oil-induced skin papilloma formation in mice [12, 13]. The phytochemical analysis of the stem bark revealed the presence of alkaloids, flavonoids, phenols, phytosterols, saponins, tannins, steroids and terpenoids [14]. The phytoestrogens in this plant *β*-sitosterol, quercetin, kaempferol and catechin are reported to show anticancer properties including breast cancers.

Breast cancer is closely dependent on estrogen in its initiation and progression. Thus, estrogen receptors ER*α*/*β* plays a pivotal role in maintaining the homeostasis of the normal mammary gland [15]. ER*α*, the primary receptor of estrogen activates the cell cycle and stimulates proliferation, but ER*β* functions as a counterbalance to ER*α*, actively inhibiting cellular proliferation and providing a regulatory mechanism to the proliferative effect of ER*α* [16]. This intrinsic counteraction creates a dynamic interplay between ER*α* and ER*β*, influencing the delicate equilibrium of mammary gland homeostasis. Although ER*α* serves as the primary receptor for estrogen and is vital for the homeostasis of the normal mammary gland, its activation promotes cell cycle and stimulates proliferation, potentially leading to the initiation and development of cancer. Conversely, ER*β* functions as a counterbalance to ER*α*, actively inhibiting cellular proliferation and consequently, offering potential therapeutic avenues for breast cancer [17, 18]. Various reports indicate that ER*β* is expressed in triple-negative breast cancer (TNBC), accounting 50–80 % [19]. By leveraging the inhibitory properties of ER*β*, researchers and clinicians can explore targeted interventions to modulate hormonal signaling and disrupt the uncontrolled cell growth characteristic of breast cancer [20]. Several consistent findings have shown that ER*β* expression decreases in precancerous and cancerous breast lesions [21]. Currently, the predominant focus lies in the identification of novel selective ER*β* agonists, with numerous synthetic and natural molecules demonstrating high efficacy in breast cancer prevention and treatment. Notably, recent studies indicate that phytoestrogens exhibit a heightened affinity for ER*β* compared to ER*α* [22]. The potential for phytoestrogens to accumulate in breast tissue suggests significant clinical implications [23]. Among ER*β* agonists, phytoestrogens offer a distinctive therapeutic avenue for targeting ER*β* [24]. Liquiritigenin [25] and genistein [26] are notable examples, forming stable complexes with ER*β*, recruiting selective co-activators, and interacting with chromatin regulatory elements in estrogen-responsive genes [27]. Both liquiritigenin and genistein have been reported as protective factors against breast cancer, demonstrating the capacity to reduce invasiveness and growth of triple-negative breast cancer (TNBC) through pathway modulation [28, 29]. Another phytoestrogen kaempferol specifically inhibits the migration and invasion of TNBC cells by blocking RhoA and Rac1 signaling pathways. Given these findings, the present study aims to investigate the role of phytoconstituents in Asoka in inhibiting breast cancer cell growth, employing MCF-7, MDAMB-231, and SKBR3 cell lines through estrogen-screen and MTT assays. Additionally, the binding efficacy of phytoestrogens on estrogen receptors will be explored through in silico molecular docking.

Methods

Collection and preparation of extract

The stem bark of *S. asoca* was collected from the Thrissur district of Kerala, India, and its authenticity was confirmed by Dr. N. Sasidharan, Taxonomist, Kerala Forest Research Institute (KFRI), Thrissur, Kerala, India. The collected specimens have been deposited in the Herbarium of KFRI, assigned the voucher specimen number KFRI 4725. The plant sample underwent thorough washing with distilled water, followed by cutting into small pieces and subsequent drying at 45–50 °C for one week. The dried bark was then powdered using a grinder and stored in light-resistant,

airtight containers. About 20 g of the powdered sample was subjected to extraction with 200 mL of methanol at room temperature through overnight stirring. The resulting Asoka crude methanol extract was filtered using Whatman No.1 filter paper. The extraction process was repeated 2–3 times, and the residue was evaporated to dryness utilizing a vacuum concentrator. The weight of the dried extract was measured to determine the percentage yield of the soluble constituents [30].

Cell lines and animals

The triple-negative breast cancer cell line, MDAMB-231, the HER-2 expressed breast cancer cell line, SKBR3, and the hormone-positive breast cancer cell line, MCF-7, were procured from National Center for Cell Science (NCCS), Pune, India. These cell lines were cultured in DMEM medium supplemented with fetal bovine serum (FBS) at a concentration of 10% v/v. For the estrogen-screen assay, phenol red-free DMEM supplemented with charcoal–dextran-treated FBS was employed. Both media were also supplemented with streptomycin (100 µg/mL) and penicillin (100 U/mL). The cell lines were incubated at 37 °C in an incubator with 5% CO_2. Murine tumor cells, including Daltons Lymphoma Ascites (DLA) and Ehrlich's Ascites Carcinoma (EAC) cell lines, were grown in the intraperitoneal cavity of Swiss albino mice and were maintained in the animal house facility at Amala Cancer Research Center. Prior approval was obtained from the Institutional Animal Ethics Committee for the use of experimental animals with Approval No: ACRC/IAEC/17(1)/P-05 dt: 22-12-2017.

Phytochemical analysis

The crude methanol extract obtained from *S. asoca* was dissolved in methanol and underwent qualitative and quantitative analysis to identify the presence and concentrations of various phytochemicals. Different standard tests were employed for qualitative assessment to identify the presence of various compounds. Flavonoids were detected using Shinoda's test, phenols with the ferric chloride test, saponins with the Froth formation test, sterols with Salkowski and Liebermann-Burchard tests, tannins with the lead acetate test, and terpenoids with Salkowski tests [31, 32]. Additionally, the *S. asoca* crude extract was quantitatively analyzed using the Folin–Ciocalteau method to determine the total phenolic content using gallic acid as standard and expressed as mg of gallic acid equivalent (GAE)/g of dry extract [33]. For the quantification of flavonoids, the aluminium chloride colorimetric method was employed utilizing standard quercetin and expressed as milligrams of quercetin equivalent (QE) per gram of dry extract [34]. The experiments were meticulously conducted in triplicates to ensure accuracy and reliability, and the results are reported as the mean ± standard deviation (SD).

Cytotoxicity assay

The short-term cytotoxic activity of the Asoka crude extract was assessed by determining the percentage viability of murine tumor cells such as DLA and EAC, employing the trypan blue exclusion method [35]. The murine tumor cells were cultivated in the peritoneal cavity of female Swiss albino mice (25–30 g, 2 months old) through intraperitoneal injection of 1×10^6 cells/mL. Cells were aspirated aseptically from the cavity of mice after 15 days of inoculation; washed with PBS and centrifuged at 1000 rpm for 5 min. Pellets were resuspended in PBS and the cell count was adjusted to 1×10^6 cells/mL. Cells were pipetted out and added into each tube having PBS with different concentrations of the extract. It was then incubated for 3 h at 37 °C. After incubation, trypan blue dye was added and observed under the light microscope using a haemocytometer. The experiments were replicated in triplicates, and the percentage of cytotoxicity was assessed by enumerating the number of dead cells relative to that of live cells and substituting in the equation:%ofcytotoxicity=No.ofdeadcellsTotalnoofcells×100

The dose–response curve was fitted with the Hill equation using data analysis and graphing software, OriginPro 9 software [36].

Breast cancer cell lines such as MDAMB-231, SKBR3 and MCF-7, were employed to assess the antiproliferative activity of the extract using the MTT assay [37]. Approximately, 1×10^5 cells were seeded in 12 well plates containing medium and incubated at 37 °C for 24 h. Cells were then incubated with different concentrations of extract at 37 °C for 24 h. The test also included a blank containing a complete culture without cells. After incubation, 100 µL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was added to each well and incubated for 4 h. The dark blue formazan crystals were dissolved in 1 mL solubilization solution containing isopropanol, concentrated HCl

and Triton X 100 by continuous aspiration and re-suspension. The absorbance of the colored product was measured at 570 nm. The cytotoxicity was determined by comparing the percentage death of the treated cell population with the untreated control, indicated by their respective absorbance assessed with the MTT assay. The dose–response curve was fitted with the Hill equation [36].EEmax=11+EC50Anwhere the maximum percentage of inhibition is E_{max}, the half-maximal effective concentration is EC_{50}, the Hill coefficient is '*n*' and the extract concentration is '*A*'. The Hill equation was computed using OriginPro 9. The assays were performed in triplicates, and statistical analysis was carried out using one-way ANOVA followed by Tukey's multiple comparison test in GraphPad Prism 8 software.

Estrogen-screen assay

Breast cancer cell lines expressing ER*β*, including MDAMB-231 and cell lines expressing ER*α*, such as MCF-7, were used for the study. The cell lines were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) with FBS and phenol red as pH indicators with culture conditions of 5% CO_2 and 95% humidity at 37 °C. The cells were plated in well culture plates and allowed to attach. After 24 h, the seeding medium was removed and replaced with phenol red-free DMEM containing charcoal dextran treated FBS [38]. The cells were treated with different concentrations of 17 *β*-estradiol (0.1–1000 pM) and incubated at 37 °C for 3 days. After incubation, 100 µL of MTT was added to each well and incubated for 4 h. The dark blue formazan crystals were dissolved in 1 mL solubilization solution by continuous aspiration and re-suspension. The absorbance of the colored product was measured at 570 nm and the cytotoxicity was determined by comparing the percentage death of the treated cell population with the untreated control, indicated by their respective absorbance assessed with the MTT assay [37].

UV–visible spectroscopy

The *S. asoca* crude extract underwent centrifugation at 3000 rpm for 10 min and were subsequently filtered through the Whatman No.1 filter paper. The resulting samples were diluted with the same solvent employed for extraction, achieving a final concentration of 1 mg/mL. Standard solutions of quercetin, kaempferol, and *β*-sitosterol were also prepared at a concentration of 1 mg/mL of ethanol. Utilizing a UV–Vis spectrophotometer (PG Instruments, UK), the extract and standards were scanned across a wavelength range of 200 to 900 nm, and characteristic peaks were identified.

Fourier transform infrared spectroscopy (FTIR)

To characterize the functional group present in the sample, FTIR spectroscopy was conducted. A translucent sample disk was created by encapsulating 10 mg of *S. asoca* crude powder in 100 mg of potassium bromide (KBr) pellets. The FTIR spectroscopy analysis was performed using a Shimadzu IR Affinity 1 (Kyoto, Japan) within the range of 500 to 4000 cm^{-1} [39]. The obtained raw data was employed to generate FTIR spectra using OriginPro 9 software. The 'spectroscopic tools' were utilized for the analysis of the FTIR spectra (https://www.science-and-fun.de/tools/).

LC–MS analysis

The chemical profiling of the Asoka crude extract was done using High Resolution-Liquid Chromatography/Mass Spectrometry. The analysis was performed on an Agilent 6550 iFunnel Q-TOF LC/MS system (G6550A) equipped with an Agilent 1290 Infinity Autosampler (G4226A), an Agilent 1290 Infinity Binary Pump VL (G4220B), and an Agilent 1200 series thermostatted column compartment. A reverse-phase analytical column (Zorbax SB-C18, 100× 2.1 mm i.d., 1.8 µm particle size) was used for separation at a flow rate of 0.3 mL/min for a total of 30 min. Sample injection involved 5 µL of the sample. The mobile phases comprised aqueous 0.1% formic acid (A) and 90% acetonitrile in 0.1% aqueous formic acid (B). Mass spectroscopy utilized a dual ion source system with full scan mode, covering a mass range of 50 to 500 m/z. Mass Hunter Qualitative Analysis software was employed for data analysis.

Molecular docking

Molecular docking was done using Schrodinger Maestro software to investigate the possible interactions between *β*-sitosterol, kaempferol and quercetin which are specific compounds identified in *S. asoca* extract with the targeted receptors, ER*α* and ER*β*. Also, inbuilt ligand estradiol and the classical chemotherapy drug, tamoxifen was used to find out the binding affinity of estrogen receptors towards them. The structures of proteins with PDB IDs 3ERT and

3OLL were downloaded from the protein data bank for ERα and ERβ, respectively. The protein structures were processed before being used as a receptor for docking. Hydrogen atoms were added, atomic charges were assigned and water molecules that were not involved in ligand binding were removed during the operation. Chains and loops that were missing were also inserted. Protein preparation was done by using the protein preparation wizard. The pre-processing was done with the use of the import and process tab, while the review and modify tab was used for the generation of tautomeric states. The structures were optimized and minimized using the refine tab and protein was prepared for further studies. The optimized structures of the standards β-sitosterol, kaempferol and quercetin were used after converting them to structures with.sdf extension. The imported structures were edited using the 2D sketcher option in the Schrodinger Maestro. Then the ligprep wizard was employed to prepare each ligand using the OPLS3 force field. All tautomers of the structures were generated. Then the molecules were subjected to conformational change to form a stable conformer with the lowest energy. The glide receptor grid generating wizard was used to create a three-dimensional grid with 0.5 Å spacing and a maximum size of 20 Å × 20 Å × 20 Å. Any type of constraint, such as accuracy constraints, H-bond constraints, and so on, can be applied using the receptor grid generation wizard. The XP (extra precision) method was used for docking as it is the most powerful and discriminating procedure. After setting the location of the grid and ligands, docking was done with flexible molecules, and the proteins were used as rigid molecules [40].

Statistical analysis

The data from in vitro studies were presented as mean ± standard deviation (SD), derived from three distinct experiments. Statistical analysis was conducted using one-way ANOVA, followed by Tukey's multiple comparison test in GraphPad Prism 8 software. Statistical significance was assigned to p values <0.05*, <0.01**, and <0.001***, while $p>0.05$ was considered non-significant.

Results

Phytochemical analysis

The qualitative analysis of the *S. asoca* crude methanol extract indicated positive results for the presence of flavonoids, alkaloids, phytosterols, phenols, saponins, tannins and terpenoids. The quantification of polyphenolic content in the *S. asoca* crude extract, determined from the calibration curve ($R2 = 0.998$), revealed a concentration of 120 ± 6.82 mg of gallic acid equivalent (GAE) per gram of dry extract. Additionally, the total flavonoid content in the crude extract, estimated from the calibration curve ($R2 = 0.999$), was found to be 61.54 ± 4.51 mg of quercetin equivalent (QE) per gram of dry extract. These results provide a comprehensive insight into the chemical composition of *S. asoca* crude methanol extract, highlighting its rich polyphenolic and flavonoid content.

Anticancer properties

The cytotoxic effect of *S. asoca* crude methanol extract was assessed using the trypan blue assay and demonstrated considerable cytotoxic effects on DLA and EAC cells. The concentrations required to achieve 50% cytotoxicity were 42.24 ± 3.65 and 65.44 ± 2.89 µg/mL for DLA and EAC cells, respectively (Fig. 1). In MTT assay, the efficacy of *S. asoca* extract was evident against triple-negative breast cancer cell lines, MDAMB-231, with an IC_{50} of 70.22 ± 1.89 µg/mL, and HER-2 positive breast cancer cell line, SKBR3, with an IC_{50} of 98.41 ± 2.31 µg/mL (Fig. 1). A statistically significant ($p<0.05$) decrease in cell numbers was observed in both MDAMB-231 and SKBR3 following extract treatment. Treated cells exhibited a noticeable difference in morphology compared to control cells, characterized by cell shrinkage and shift in morphology from epithelial-like to round in both MDAMB-231 and SKBR3 (Fig. 2). However, the extract did not exhibit any cytotoxicity towards MCF-7, even at a concentration of 300 µg/mL.

Fig. 1 [Images not available. See PDF.]

A Cytotoxic effect of *S. asoca* crude methanol extract on murine tumor cells by trypan blue assay, **B** Antiproliferative effect of *S. asoca* on different breast cancer cells by MTT assay. The results are expressed as mean ± SD, with $n = 3$. Statistical comparisons were conducted using one-way ANOVA, followed by Tukey's multiple comparison test. Statistically significant probabilities are denoted as *$p<0.05$ and **$p<0.01$

Fig. 2 [Images not available. See PDF.]

Morphology of different breast cancer cells after exposure to varying concentrations of *S. asoca* crude extract (20× magnification). The black arrow indicates altered morphology from epithelial-like to round

Estrogen-screen assay

In this study, the ER*α* expressing MCF-7 cells exhibited a proliferative response to 17 *β*-estradiol (1000 pM), showing a 30% increase in cell count and a 7% increase in response to crude extract within a 72-h timeframe. Along with 17 *β*-estradiol, the crude extract exhibited a mild estrogenic effect. Conversely, ER*β* expressing MDAMB-231 cells demonstrated a 10% decrease in proliferation with 17 *β*-estradiol treatment, and the cell population was halved when treated with the crude extract at a concentration of 100 µg/mL (Fig. 3). Notably, the crude extract did not induce cytotoxicity in MCF-7 cell lines even at higher concentrations, while they decreased the cell viability of MDAMB-231 cells in a concentration-dependent manner. Consequently, the estrogen-screen assay highlights the estrogenic impact of *S. asoca* crude extract on MCF-7 and its anti-estrogenic/antiproliferative effect on MDAMB-231 cells.

Fig. 3 [Images not available. See PDF.]

Change in cell volume of MCF-7 and MDAMB-231 breast cancer cells following exposure to extract and 17 *β*-estradiol (1000 pM) in the estrogen-screen assay

Chemical profiling

Various techniques like UV–Vis spectroscopy, fourier-transform infrared spectroscopy (FTIR) and LC–MS were employed to evaluate the chemical profile of *S. asoca*. The absorption spectrum of UV-Spectrophotometric analysis of *S. asoca*, showed prominent peaks at 232, 275 and 449 nm which is in good correlation with the reported data [41]. These prominent peaks may have arisen from the phytoestrogens. Henceforth, the UV-spectra of standards (quercetin, kaempferol, *β*-sitosterol) were cross-checked and found that all of the suspected phytoestrogens have three peaks between 230 and 290 nm in the UV range and a single peak in a visible area (387–390 nm) (Fig. 4A). Accordingly, there is a likelihood of superpositioning of these distinct peaks in the *S. asoca* crude extract. The biological activity of any molecule is influenced by its functional groups which play a key role in determining the overall physicochemical properties. In FTIR, the results show functional groups such as alcohol, phenol, ester, alkane, aromatic and alkene in the extract. The functional groups identified in the extract are shown in Fig. 4B and Table 1.

Fig. 4 [Images not available. See PDF.]

A UV–visible spectrum of *S. asoca* crude methanol extract and phytoestrogen standards, **B** FTIR spectrum of *S. asoca* crude extract

Table 1. FTIR interpretation of compounds of *S. asoca* crude extract

Wave number cm^{-1}	Bond assigned	Functional groups
617	C–H vibration	Alkanes, alkenes
672	C–H and C–C stretching	Alkenes, alcohol, phenol
714	C=C and N–H stretching	Alkenes, amines
841	C–C and C–H stretching	Amides, aldehydes
1011	C–O stretching	Alcohol

1065	C–O stretching	Alcohol, aromatic
1125	C–O stretching	Alcohol
1393	C–O and C–H stretching	Phenol, aldehydes
1660	C–C stretching	Phenols
2230	C=C stretching	Conjugated alkene
3398	O–H stretching	Alcohol

The LC–MS analysis identified some of the important compounds such as caffeic acid, catechin, quercetin, kaempferol, gallic acid, rutin, *β*-sitosterol, p-coumaric acid, luteolin etc. (Fig. 5). Phytoestrogenic compounds like *β*-sitosterol, kaempferol, and quercetin present in the extract are presumed to contribute to the proliferative/antiproliferative effects of the *S. asoca* crude methanol extract on MCF-7, MDAMB-231, and SKBR3 cancer cell lines.

Fig. 5 [Images not available. See PDF.]

LC–MS spectrum of *S. asoca* crude methanol extract

Molecular docking

The studies suggest that certain phytoestrogens act as natural agonists for ER*β*, making them promising drug candidates for their ability to modulate the cell cycle, influence epigenetic events, and induce apoptosis. Interestingly, in the current investigation, the extract exhibits specific cytotoxicity towards ER*β* expressing cells, not affecting ER*α*. This raises the intriguing possibility that the phytoestrogens in the plant may act as agonists for ER*β*. To explore potential interactions between phytoestrogens and ER*α*/*β*, molecular docking was performed using Schrodinger Maestro software.

Interaction of phytoestrogens with ER*α*

The docking of the inbuilt ligand, estradiol into the 3D structure of ER*α* was done using a glide dock. The amino acid residues in the active site of 3ERT are Trp383, Leu384, Leu387, Met388, Gly390, Lbu391, Val392, Arg394, Met342, Met343, Leu345, Leu346, Thr347, Asn348, Leu349, Ala350, Asp351, Glu353, Leu354, Leu327, Phe404, Leu402, Leu428, Phe425, Ile424, Val422, Met421, Gly420, Glu419, Val418, Met517, Ser518, Lys520, Gly521, Met522, Glu523, Hie524, Leu525, Met528, Lys529, Cys530, Val533, Leu536, Leu539. The estradiol was docked into the active site region and interactions were made with the residues by hydrogen bonding with GLU353 and ARG394 and electrostatic bonding with ASP351. The inbuilt ligand shows a docking score of −12.17 and binding energy of −125.19 kcal/mol. The quercetin was docked into the active site region making interactions with the residues by hydrogen bonding with ASP351. The docking score and binding energy were found to be −6.945 and −47.026 kcal/mol which was more compared to kaempferol (−6.93). Tamoxifen shows a docking score of −10.512 and *β*-sitosterol did not dock with the binding pocket of ER*α*. The 3D interaction picture of the study is shown in Fig. 6A and their docking score and binding energy are tabulated in Table 2.

Fig. 6 [Images not available. See PDF.]

A 2D and 3D image of the interaction between ER*α* and ligands, **B** 2D and 3D image of the interaction between ER*β* and ligands

Table 2. Docking score and binding energy of estrogen receptors and ligands

Molecule	ERα		ERβ	
Docking score	Binding energy (kcal/mol)	Docking score	Binding energy (kcal/mol)	Inbuilt ligand-Estradiol
−12.17	−125.19	−10.5	−85.248	Quercetin
−6.945	−47.026	−9.220	−66.945	Kaempferol
−6.93	−45.07	−8.478	−58.435	Tamoxifen

The 2D image (Fig. 6A) reveals the type of interaction between the ligands and amino acids in the active sites of ER *α*. The inbuilt ligands estradiol, tamoxifen, kaempferol and quercetin form p bonds from their aromatic ring to Phe 404 but the number and nature of hydrogen bonds vary with ligands. Estradiol, kaempferol and quercetin form three hydrogen bonds with Glu353, Hie524 and Arg394, and tamoxifen only once with Asp351.

Interaction of phytoestrogens with ER*β*

The amino acid residues in the active site of ER*β* (PDBID: 3OLL) are Val280, Met295, Ser297, Leu298, Thr299, Leu301, Ala302, Asp303, Glu305, Trp335, Met336, Leu339, Met340, Gly342, Leu343, Met344, Arg346, Leu354, Phe356, Val370, Gly372, Ile373, Ile376, Phe377, Leu380, Ala468, Ser469, Lys471, Gly472, Met473, Hie475, Leu476, Leu477, Met479, Val485, Leu491, Leu495.

The inbuilt ligand was bound deep into the active site area, making hydrogen bonding interactions with Hie475, Arg346, Glu305 and *π–π* stacking interactions with Phe356. The inbuilt ligand shows a docking score of −10.5 and binding energy of −85.248 kcal/mol. The docking of ER*β* with quercetin showed the highest docking score of −9.220 and binding energy of −66.945 kcal/mol. Quercetin makes hydrogen bond interactions with Arg346, Glu305, Hie475 and *π–π* stacking with Phe356. Quercetin shows the highest affinity followed by kaempferol (−8.478) and tamoxifen (−8.023). Here also, *β*-sitosterol did not dock with the binding pocket of ER *β*. The 3D and 2D figures of other ligands are given in Fig. 6B. The docking score and binding energy of other ligands are given in Table 2.

The 2D image (Fig. 6B) reveals the type of interaction between the ligands and amino acids in the active sites of ER *β*. The inbuilt ligands estradiol, tamoxifen, kaempferol and quercetin form p bonds from their aromatic ring to Phe 356 but the number and nature of hydrogen bonds vary with ligands. Estradiol, kaempferol and quercetin form three hydrogen bonds with Glu305, Hid475 and Arg346, and tamoxifen only once with Asp351.

Discussion

The study emphasizes the considerable antiproliferative potential of crude extract from *Saraca asoca* against breast cancer cells, particularly targeting triple negative breast cancers. Specifically, the plant demonstrated selective cytotoxicity towards breast cancer cells expressing ER*β,* while sparing those expressing ER*α*. The estrogen-screen assay conducted on MDAMB-231 breast cancer cells revealed a pronounced antiproliferative effect, in contrast to MCF-7 cells, which exhibited a proliferative response to estrogen and the extract. The LC-MS analysis identified phytoestrogens such as *β*-sitosterol, quercetin, kaempferol, and others in the plant. In in-silico docking analysis, these phytoestrogens showed higher binding affinity towards ER*β* compared to ER*α* receptors, except for *β*-sitosterol. Therefore, the observed preferential cytotoxicity of *S. asoca* towards ER*β* expressing breast cancers holds significant clinical relevance, given that triple negative breast cancers represent the most aggressive form of cancer with limited treatment options.

Triple-negative breast cancer (TNBC) is characterized by the absence of expression of ER*α*, progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER-2) [42]. In recent research on the immunological profile of

these TNBC cell lines, the presence of estrogen receptor isoform ER*β*, which acts as an opponent of ER*α*, in 50–80% of TNBCs was found [19]. In cytotoxic assays conducted in this study, Asoka exhibited significant cytotoxicity towards various cancer cells of both murine and human origin. This was evidenced by a concentration-dependent increase in the percentage of dead cells, particularly in DLA and EAC murine cancer cells. Notably, previous research has already reported comparable cytotoxic effects against mouse tumor cells [43]. Interestingly, despite the use of high concentrations, the extract did not induce cytotoxic effects on breast cancer cells expressing ER*α*, such as MCF-7, as observed in the MTT assay. However, the extract did exhibit cytotoxic effects on breast cancer cell lines expressing ER*β*, such as MDAMB-231 and SKBR3. Prior investigations have suggested the antiproliferative effects of *S. asoca* on MDAMB-231 and also on MCF-7 cell lines [11]. However, in our study, MCF-7 cell lines did not exhibit any cytotoxicity even at higher concentrations of the extract. This interesting contrast in cytotoxicity between ER*α* and ER*β* expressing breast cancer cells in our study corresponds with earlier findings which mention ER*α* activation leading to cell proliferation, while ER*β* activation exhibiting an anti-proliferative effect [15, 18].

The proliferative/anti-proliferative effect of the *S. asoca* crude extract was evaluated through an estrogen-screen assay on various breast cancer cell lines, with 17*β*-estradiol as a reference. This bioassay determines the increase or decrease in cell number in response to estrogen, resembling an increase in mitotic activity within reproductive system-associated tissues [44]. Our investigation revealed that Asoka demonstrates a moderate anti-proliferative effect on the MDAMB-231 cell line expressing ER*β*, while it induces proliferation in the MCF-7 cell line expressing ER*α*. The presence of phytoestrogens such as quercetin, kaempferol, and *β*-sitosterol, among others, was reported in Asoka by LC-MS. Additionally, there are reports of flavonoids like quercetin, chrysin, and 3-hydroxyflavone with known anti-proliferative properties [45]. Therefore, it is likely that the phytoestrogens in Asoka may act as agonists for ER*β* due to their specific affinity for ER*β* expressing cells.

The results of our docking experiments also have demonstrated the potent affinity of phytoestrogens towards ER*β* in comparison to the ER*α*. Both quercetin and kaempferol exhibited significantly higher docking scores and binding energies than tamoxifen, a commonly used chemotherapeutic medication. Remarkably, quercetin's docking score was close to that of estradiol, the endogenous ligand for estrogen receptors. Furthermore, there have been reports highlighting the robust binding affinity of *S. asoca* flavonoids to human estrogen receptors. In a molecular simulation research involving Asoka flavonoids and estrogen receptors, the binding scores indicate their exceptional ability to form strong interactions with these receptors. Molecular orbital analysis and pharmacokinetic parameters further support their efficacy [46]. Notably, our study revealed high binding affinity values of phytoestrogens binding with estrogen receptors which surpassed that reported in a previous investigation on Asoka flavonoids [47]. This increased affinity of phytoestrogens, particularly for ER*β*, holds significant implications, as it appears to underpin their antiproliferative effects. This interaction with estrogen receptors, notably ER*β*, may give rise to a wide array of biological responses [22].

Conclusion

The study highlights the preferential cytotoxicity of the *S. asoca* crude methanol extract towards ER*β* expressing cells, particularly to triple negative breast cancers, a highly aggressive and therapeutically challenging type. The observed antiproliferative effects on breast cancer cells may be attributed to the action and interaction of phytoestrogens present in Asoka with ER*β*. Notably, quercetin and kaempferol among the identified phytoestrogens exhibit high docking into the binding sites of active amino acids in the ER*β*. These findings suggest that the phytoestrogens in Asoka may act as agonists to ER*β*, offering promising prospects for the development of targeted therapies for triple negative breast cancer, thereby opening new avenues in the currently limited treatment landscape.

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Author contributions

Chennattu M Pareeth has done experiments and wrote the paper, K P Safna Hussan, Davis Anu, Nair Meera discussed and designed experiments and done dry lab calculations. Deepu Mathew, Ravishankar Valsalan, Mohamed Shahin Thayyil—Resources, Software, and Supervision of the in-silico (molecular docking) works. Kannoor M Thara, Achuthan C Raghavamenon, Thekkekara D Babu—Supervision, finalize the design, experiments and corrections.

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All the authors have no objection to publishing the data.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

MTT

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

ERβ

Estrogen Receptor β

DLA

Daltons Lymphoma Ascites

EAC

Ehrlich's Ascites Carcinoma

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Chemical composition and antifungal activity of Teucrium Leucocladum Boiss. essential oils growing in Egypt using two different techniques

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ABSTRACT (ENGLISH)

Background

Teucrium Leucocladum Boiss. (TL) (family Lamiaceae), indigenous to Sinai, Egypt, and Mediterranean region, is considered a rich source of essential oils (EOs). This study aimed to extract the aerial parts essential oils utilizing hydro-distillation (HD) and microwave-assisted extraction (MAE), and analyze the volatile constituents by Gas Chromatography–Mass Spectrometry (GC/MS). The antifungal and cytotoxic potentials against *Candida albicans* (*C. albicans*) and non-small cell lung adenocarcinoma A549, triple-negative breast cancer MDA-MB-231 cell lines, respectively, were likewise estimated. Subsequently, the three main compounds were docked into the crystal structure of *Candida albicansN*-myristoyltransferase (NMT) with myristoyl-COA and peptidic inhibitor (PDB 1IYK), and predictions of human absorption, distribution, metabolism, and excretion (ADME) were performed to assess the drug-likeness of the compounds.

Results

The chemical profile consisted of monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, and oxygenated sesquiterpenes. The MAE oil sample (TLM) yield was found to be double that of the HD oil sample (TLH). TLM afforded an inhibitory diameter (13 mm) comparable to the ketoconazole (20 mm), TLM 100 mg/mL showed the strongest antifungal potential against *C. albicans*. The cytotoxic assay revealed moderate activity against A549 and MDA-MB-231. In silico studies using molecular docking were processed on the major components in which nerolidol had the best-fitting energy to inhibit *C. albicans* (−7.21 kcal/mol), while ADME results established a promising first step for the potential drug bioavailability.

Conclusion

In this research, essential oil acquired from the aerial parts proved to contain monoterpenes and sesquiterpenes, which are classes of compounds known for their versatile usage in medicine. In vivo studies on *Teucrium Leucocladum* Boiss. active metabolites against clinical strains of fungi need to be further studied, as do the effects of combining the active compounds with antifungal agents to combat antimicrobial resistance.

FULL TEXT

Background

Since the dawn of humanity, essential oils have been utilized for plenty of reasons. They have numerous positive attributes as they are traditionally used to enhance the flavor and fragrance of prepared foods, as well as perfumes and cosmetics. Beyond their sensory contributions, essential oils exhibit significant biological potentials, including larvicidal action, analgesic and anti-inflammatory effects, antioxidant, antifungal, and anticancer activities. Moreover, essential oils have been integrated into medicinal practices, showcasing their therapeutic potential [1]. The diverse range of activities exhibited by essential oils (EOs) can be attributed to their complex composition, comprised of

various constituents such as terpenes, terpenoids, and phenylpropanoids. Notably, within these intricate chemical profiles, only two or three major components typically constitute a significant proportion, ranging from 20 to 70% of the total substance [2].

The chemical composition of EOs is significantly influenced by various factors, including the geographical location of the plant containing the EOs, the seasonal timing of harvest, storage conditions, and the extraction methodology employed. These variables collectively contribute to the unique and dynamic molecular profiles of EOs. The numerous biological and therapeutic activities exhibited by essential oils, coupled with their relative safety and capacity to synergistically interact with other compounds, qualify the utilization of essential oils as naturally derived medicinal compounds [3].

Traditional medicines and modern pharmaceutical developments alike are predominantly derived from plant sources, utilizing active metabolites known for their low to negligible toxicity. These bioactive compounds have demonstrated efficacy in treating a wide spectrum of disorders. In recent years, there has been a conspicuous surge in research initiatives concentrating on medicinal plants, with a specific emphasis on investigating their potential as agents exhibiting anticancer and antimicrobial properties. This heightened scientific interest reflects the continual exploration of plant-based compounds as significant reservoirs for the advancement of novel drugs, particularly in the fields of oncology and infectious diseases [4–6].

Family Lamiaceae (mint family) comprises 236 genera and 6900–7000 species of aromatic plants. Herbs, whether perennial or annual, shrubs, and trees are all members of this family [7]. Many species of this family are considered sources of essential oils (EOs) [8]. Significantly, members of the Lamiaceae family are extensively utilized as medicinal plants in various folk traditions. Lamiaceae largest genus in the Mediterranean area is *Teucrium* [8].

Teucrium (Lamiaceae) is an aromatic [9], polymorphic [7] genus, comprising more than 300 species [8] represented mostly by perennial, bushy, or herbaceous plants growing in temperate zones, particularly in Central Asia and the Mediterranean basin [10]. Chemical investigations of genus *Teucrium* members have shown that those plants are very rich sources of active principles, especially essential oils [11].

Teucrium leucocladum (TL) Boiss. is an aromatic low shrub ranging from 20 to 50 cm long, indigenous to the Sinai Peninsula [12] and is considered one of the most used traditional medicinal plants in Palestine, Egypt, and the Mediterranean region for the treatment of hyperglycemia (aqueous extract of aerial parts) and colon spasms among other ailments [13].

The major objective of the current research was to comprehensively characterize the complete chemical profile of the EOs extracted from (stem and leaf parts) of *Teucrium leucocladum* Boiss. for the first time. This characterization employed two distinct techniques: microwave-assisted extraction (MAE) and hydro-distillation (HD), resulting in the production of TLM and TLH, respectively. GC–MS was used to analyze the prepared essential oils. Additionally, the study involved the assessment of the potential activity of the oil samples against *Candida albicans* and three different cancer cell lines.

Results

Chemical composition

Both techniques produced yellow-colored oils from *Teucrium leucocladum* Boiss. with a distinct characteristic odor. The percentage yield of the essential oils, however, varied significantly depending on the procedure, affording 0.5 and 1.2% (v/w) for HD and MAE, respectively. Our results are consistent with published data revealing that hydro-distillation provides yields less than microwave-assisted extraction [14]. The results of GC chromatograms are represented in Fig. 1.

Fig. 1 [Images not available. See PDF.]

GC chromatograms of the essential oils of *Teucrium leucocladum* Boiss. extracted via hydro-distillation (**A**), and microwave-assisted extraction (**B**)

While MAE exhibited a higher oil yield, it presented a lower number of metabolites compared to hydro-distillation, as indicated in Table 1. Moreover, the yields emphasized that the selection of the extraction technique markedly

impacted the chemical composition of the extracted essential oils. Seventy-three components were identified during hydro-distilled oil preparation, whereas 32 components were detected in the oil prepared by microwave-assisted extraction (MAE), constituting 95.53% and 94.33%, respectively.

Table 1. Chemical composition of the isolated essential oils

No	Compound	[a]R_t HD	[b]R_t MAE	[c]RI_{Exp}	Area%		Molecular Formula
HD[d]	MAE[e]	*Hydrocarbon components*					
(A) Monoterpene hydrocarbons							
1	α-Thujene	3.78	3.59	856	1.35	1.18	$C_{10}H_{16}$
2	α- Pinene	3.93	NA	865	2.59	NA	$C_{10}H_{16}$
3	2,4(10)-Thujadien	4.15	NA	878	0.07	NA	$C_{10}H_{14}$
4	Sabinene	4.64	4.64	904	1.14	0.50	$C_{10}H_{16}$
5	β-Pinene	4.76	NA	908	1.77	NA	$C_{10}H_{16}$
6	α-Myrcene	4.91	NA	914	1.06	NA	$C_{10}H_{16}$
7	α-Terpinene	5.51	NA	935	0.11	NA	$C_{10}H_{16}$
8	o-Cymene	5.77	6.13	945	1.02	0.83	$C_{10}H_{14}$
9	α-Ocimene	6.08	NA	956	0.13	NA	$C_{10}H_{16}$
10	∆-3-Carene	6.37	NA	966	0.25	NA	$C_{10}H_{16}$
11	α-Terpinolene	6.93	NA	986	0.06	NA	$C_{10}H_{16}$

(B) Sesquiterpene hydrocarbons							
12	α-Copaene	13.01	NA	1144	0.12	NA	$C_{15}H_{24}$
13	Elemene	13.33	NA	1151	0.16	NA	$C_{15}H_{24}$
14	*trans*-Caryophyllene	13.97	NA	1166	0.20	NA	$C_{15}H_{24}$
15	*trans*-Farnesene	14.49	NA	1178	0.15	NA	$C_{15}H_{24}$
16	Gymnomitrene	14.67	NA	1182	0.07	NA	$C_{15}H_{24}$
17	α-Gurjunene	15.02	NA	1190	0.13	NA	$C_{15}H_{24}$
18	Germacrene D	15.24	NA	1195	0.38	NA	$C_{15}H_{24}$
19	α-Selinene	15.44	NA	1198	0.20	NA	$C_{15}H_{24}$
20	α-Bisabolene	15.66	NA	1205	0.25	NA	$C_{15}H_{24}$
21	α-cadinene	15.93	NA	1211	1.72	NA	$C_{15}H_{24}$
22	Valencene	18.57	NA	1269	2.00	NA	$C_{15}H_{24}$
23	*cis*-α-Farnesene	19.13	NA	1281	0.94	NA	$C_{15}H_{24}$
24	Aromadendrene	20.68	22.61	1316	0.12	1.49	$C_{15}H_{24}$
25	Bicyclo-Elemene	NA	24.13	1393	NA	0.41	$C_{15}H_{24}$
26	α-Muurolene	NA	24.35	1398	NA	2.37	$C_{15}H_{24}$

27	α-Amorphene	NA	24.92	1411	NA	0.60	$C_{15}H_{24}$
28	Cadinene	NA	25.09	1415	NA	3.25	$C_{15}H_{24}$
29	α-Humulene	NA	26.02	1436	NA	2.15	$C_{15}H_{24}$
Oxygenated components							
(A) Oxygenated monoterpenes							
30	Thujol	5.90	NA	949	0.08	NA	$C_{10}H_{18}O$
31	*cis*-Sabinene hydrate	6.87	NA	984	0.07	NA	$C_{10}H_{18}O$
32	1-Octen-3-yl-acetate	7.29	NA	999	0.19	NA	$C_{10}H_{18}O_2$
33	Linalool	7.48	NA	1004	0.23	NA	$C_{10}H_{18}O$
34	α-Thujone	7.78	NA	1012	0.17	NA	$C_{10}H_{16}O$
35	*trans*-Verbenol	8.51	NA	1032	0.17	NA	$C_{10}H_{16}O$
36	*cis*-Verbenol	8.63	NA	1035	0.14	NA	$C_{10}H_{16}O$
37	Pinocarvone	8.84	NA	1040	0.07	NA	$C_{10}H_{14}O$
38	*trans*-2-Pinanol	NA	8.85	1041	NA	0.41	$C_{10}H_{18}O$
39	Terpinen-4-ol	9.30	NA	1053	0.52	NA	$C_{10}H_{18}O$
40	Myrtenal	9.58	NA	1060	0.12	NA	$C_{10}H_{14}O$

41	α-Terpineol	9.78	NA	1065	0.17	NA	$C_{10}H_{18}O$
42	*p*-Menth-4(8)-en-3-one (Beta- Pulegone)	9.92	NA	1069	0.12	NA	$C_{10}H_{16}O$
43	∆-(7)-Methenone-2	10.79	NA	1092	0.13	NA	$C_{10}H_{16}O$
44	*trans-p*-Mentha-2,8-dienol	14.83	NA	1186	6.54	NA	$C_{10}H_{16}O$
45	lilac alcohol epoxide	16.34	NA	1220	0.29	NA	$C_{10}H_{18}O_3$
46	*trans*-2-Caren-4-ol	NA	23.09	1370	NA	33.92	$C_{10}H_{16}O$
47	*trans-p*-2,8-Menthadien-1-ol	NA	31.13	1557	NA	1.11	$C_{10}H_{16}O$
(B) Oxygenated sesquiterpenes							
48	Farnesene epoxide	15.55	NA	1202	0.93	NA	$C_{15}H_{24}O$
49	Cedrenol	16.07	NA	1214	0.11	NA	$C_{15}H_{24}O$
50	Isoaromadendrene epoxide	16.14	NA	1215	0.14	NA	$C_{15}H_{24}O$
51	Humulene-1,2-epoxide	16.67	NA	1227	1.54	NA	$C_{15}H_{24}O$
52	Caryophyllene oxide	17.32	NA	1241	1.17	NA	$C_{15}H_{24}O$
53	Globulol	17.80	NA	1252	0.21	NA	$C_{15}H_{26}O$
54	α-Santalol	18.06	NA	1258	0.77	NA	$C_{15}H_{24}O$
55	Bisabolol oxide A	18.29	NA	1263	0.20	NA	$C_{15}H_{26}O_2$

56	α-Bisabolol Oxide-B	18.64	NA	1270	2.10	NA	$C_{15}H_{26}O_2$
57	α-Cadinol	18.85	NA	1275	1.51	NA	$C_{15}H_{26}O$
58	Spathulenol	18.97	NA	1278	0.24	NA	$C_{15}H_{24}O$
59	Santalol, E-*cis*, epi-α-	19.43	NA	1288	0.38	NA	$C_{15}H_{24}O$
60	6-Epi-shyobunol	19.56	NA	1291	1.86	NA	$C_{15}H_{26}O$
61	2-Pentadecanone	21.44	NA	1333	0.08	NA	$C_{15}H_{30}O$
62	4-Epi-cubedol	NA	25.28	1419	NA	0.46	$C_{15}H_{26}O$
63	Alloaromadendrene oxide	NA	26.41	1445	NA	0.35	$C_{15}H_{24}O$
64	Cubenol	NA	27.63	1474	NA	1.86	$C_{15}H_{26}O$
65	Epiglobulol	NA	28.67	1498	NA	0.70	$C_{15}H_{26}O$
66	Lanceol	NA	29.25	1512	NA	4.25	$C_{15}H_{24}O$
67	Limonen-6-ol, pivalate	NA	29.77	1524	NA	0.55	$C_{15}H_{24}O2$
68	Longiborneol	NA	29.86	1526	NA	0.54	$C_{15}H_{26}O$
69	Gossonorol	NA	30.34	1538	NA	2.90	$C_{15}H_{22}O$
70	tau-Muurolol	NA	30.80	1549	NA	1.53	$C_{15}H_{26}O$

71	Ledene oxide-(II)	NA	31.20	1559	NA	1.37	$C_{15}H_{24}O$
72	Ledol	NA	32.16	1582	NA	6.94	$C_{15}H_{26}O$
73	Nerolidol	16.91	NA	1232	50.02	NA	$C_{15}H_{26}O$
74	Levomenol (α-bisabolol)	NA	31.91	1576	NA	21.40	$C_{15}H_{26}O$
Other volatiles							
75	*P*-Methyl anisole	4.07	NA	873	0.05	NA	$C_{8}H_{10}O$
76	6-Methyl-5-hepten-2-one	5.00	NA	917	0.36	NA	$C_{8}H_{14}O$
77	1-Octen-3-ol	5.08	NA	920	0.06	NA	$C_{8}H_{16}O$
78	Nonanal	7.42	NA	1003	0.07	NA	$C_{9}H_{18}O$
79	4-Acetyl-1-methylcyclohexene	8.18	NA	1023	0.23	NA	$C_{9}H_{14}O$
80	α-Terpinyl acetate	12.55	NA	1133	0.31	NA	$C_{12}H_{20}O_{2}$
81	Glutaric acid, di(3-(2-methoxyethyl) heptyl) ester	16.23	NA	1217	0.24	NA	$C_{25}H_{48}O_{6}$
82	Oxiraneoctanoic acid, 3-octyl-, methyl ester, *trans*-	16.57	NA	1225	0.29	NA	$C_{19}H_{36}O_{3}$
83	2-Acetoxy-1,8-cineole	17.00	NA	1234	1.09	NA	$C_{12}H_{20}O_{3}$
84	Geranyl-*p*-cymene	18.38	NA	1265	0.42	NA	$C_{18}H_{26}$
85	7-Cyano-6-methoxy-1,4,5-trimethyl-indole	19.85	NA	1297	0.18	NA	$C_{13}H_{14}N_{2}O$

86	9,12-Octadecadienoyl chloride, (Z, Z)-	23.42	NA	1377	0.28	NA	$C_{18}H_{31}ClO$
87	DI-(9-Octadecenoyl)-Glycerol	23.71	NA	1383	4.05	NA	$C_{39}H_{72}O_5$
88	9-Octadecenoic acid	24.04	NA	1391	1.27	NA	$C_{18}H_{34}O_2$
89	Glycidol stearate	25.01	NA	1412	0.37	NA	$C_{21}H_{40}O_3$
90	Acetic acid, 10,11-dihydroxy-3,7,11-trimethyl-dodeca-2,6-dienyl ester	NA	30.56	1543	NA	3.26	$C_{17}H_{30}O_4$
Total identified volatiles					95.53	94.33	
Monoterpene hydrocarbons					9.55	2.51	
Sesquiterpene hydrocarbons					6.44	10.27	
Oxygenated Monoterpenes					9.01	35.44	
Oxygenated Sesquiterpenes					61.26	42.85	
Other volatiles					9.27	3.26	

[a]R_t HD: retention time for hydro-distillation. [b]R_t MAE: retention time for microwave-assisted extraction. [c]RI_{Exp}: retention index was determined experimentally relative to C8–C28 n-alkanes for all compounds. [d]HD: hydro-distillation, [e]MAE: microwave-assisted extraction, NA: not available

The identified constituents are categorized mainly into four groups: monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, and oxygenated sesquiterpenes (Fig. 2). Oxygenated sesquiterpenes were the major group in oils prepared by both methods at about 61.26% and 42.85% in TLH and TLM, respectively. The sesquiterpene alcohol nerolidol was the most prominent compound amounting to 50.02% in the HD oil sample, while the sesquiterpene alcohol levomenol and the monoterpene alcohol *trans*-2-Caren-4-ol dominated TLM at 21.40% and 33.92%, respectively.

Fig. 2 [Images not available. See PDF.]

Percentage of the classes of volatile components in the essential oil of *Teucrium leucocladum* Boiss. extracted via (**A**) hydro-distillation, and (**B**) microwave-assisted extraction

α-Pinene and β-Pinene were the major monoterpene hydrocarbons observed in HD sample 2.59 and 1.77%,

respectively, while α-thujene was major in MAE at 1.18%. For sesquiterpene hydrocarbons, valencene was the major observed in the HD sample at 2.00%, while cadinene was the major one in the MAE sample with a percentage of 3.25.

Despite that the hydro-distilled oil of *T. leucocladum* Boiss. was formerly assessed [12], this is the first time to evaluate the oil prepared via microwave-assisted extraction. The structures of certain identified oil components are illustrated in Fig. 3.

Fig. 3 [Images not available. See PDF.]

Two-dimensional structures of the identified compounds of the essential oils (EOs) extracted using hydro-distillation (HD) and microwave-assisted extraction (MAE) methods of *Teucrium leucocladum* Boiss. oil*s* by GC–MS analysis

Screening of the antifungal activity

In vitro antifungal activity of TLH and TLM oil samples was tested against a type of yeast-forming fungi *C. albicans* using the agar well diffusion method. For each oil, two concentrations 50 and 100 mg/mL were used in the procedure. The concentration and method of oil extraction exerted a notable influence on the extent of inhibition of *C. albicans* growth, as illustrated in Fig. 4. TLM, at concentrations of 100 mg/mL and 50 mg/mL, demonstrated the highest antifungal potential, yielding inhibition diameters of 13 mm and 10 mm, respectively, surpassing the inhibition diameter observed for the standard drug ketoconazole (20 mm). TLH, at a concentration of 100 mg/mL, exhibited a weaker but still noticeable inhibition diameter of 10 mm, while no inhibition was observed for TLH at a concentration of 50 mg/mL.

Fig. 4 [Images not available. See PDF.]

Antifungal Inhibition diameters represented in mm of TLM and TLH samples against Ketoconazole

Our results imply that *Teucrium Leucocladum* Boiss. essential oil might have the ability to function as an antifungal medication.

Cytotoxic activity

Evaluation of the cytotoxic activity via resazurin reduction assay [15] was accomplished against non-small cell lung adenocarcinoma A549, triple-negative breast cancer MDA-MB-231, and colon adenocarcinoma Caco-2 (ATCC) using doxorubicin HCL as a positive control. Preliminary screening of the oil samples TLH and TLM against cell viability of A549 and MDA-MB-231 cell lines at two concentrations (20 and 200 µg/mL) revealed a promising effect at the high concentration (200 µg/mL) for both samples verifying that the inhibition was dose-dependent, whereas lower sensitivity was demonstrated against Caco-2.

Eventually, the fifty percent inhibitory concentration (IC_{50}) determined for cell lines with promising cytotoxic activities, revealed that IC_{50} of TLM was 142.0 and 185.0 µg/mL against A549 and MDA-MB-231, respectively, while TLH exhibited IC_{50} 192.0 and 190.0 µg/mL against A549 and MDA-MB-231, respectively.

Molecular docking studies

The process of discovering and developing new drugs involves looking for the metabolites of herbal medicines that act as disease inhibitors [16]. Given its ability to provide information about the atomic-level interactions between tiny molecules and proteins, molecular docking has become an indispensable tool in the drug discovery process [17]. Many researches have proved that the NMT gene [18] is essential for vegetative growth and survival of *C. albicans.* Research in genetics and biochemistry has established that *N*-myristoyltransferase (NMT) is a promising target for antifungal medications. Numerous studies have indicated that the NMT enzyme in *C. albicans* provides important data for the design of the inhibitor [19].

Results revealed that binding energies of the examined compounds namely levomenol*,* nerolidol, and *trans*-2-caren-4-ol (Table 2, Fig. 5) were −5.53, −6.62, and −7.21, respectively approaching that of the reference drug (ketoconazole) −9.25.

Table 2. Results of docking simulations of the main identified components in TLH and TLM samples

No.	Name	Lowest Binding energy	KI[a]
1	Levomenol	−7.21	5.18 μM
2	Nerolidol	−6.62	14.01 μM
3	*trans*-2-Caren-4-ol	−5.53	87.84 μM
4	Ketoconazole	−9.25	164.76 nM

KI[a] Inhibition concentration of the best score in docking, μM micromolar, nM nanomolar

Fig. 5 [Images not available. See PDF.]

Docked complexes showing 2D and 3D binding modes of levomenol, nerolidol, *trans*-2-Caren-4-ol, and the reference compound ketoconazole in the active site of *Candida albicans*

The ADME, Lipinski's rule of five and BOILED-Egg techniques

The prediction of human absorption, distribution, metabolism, and excretion (ADME) properties, as well as the estimation of therapeutic dose and exposure, has become an essential component of compound optimization during the drug discovery process. This practice is vital for enhancing the efficiency and success of drug development by providing valuable insights into how a compound is likely to be absorbed, distributed, metabolized, and eliminated within the human body. Accurate predictions in these areas enable researchers to optimize compounds for better bioavailability, efficacy, and safety, ultimately guiding the selection of promising candidates for further development and clinical testing. This integrated approach contributes significantly to the rational design and prioritization of drug candidates, facilitating a more streamlined and effective drug discovery process [20].

Poor ADME characteristics for a certain compound are considered triggers for a cascade of failures of most medicines in clinical experiments. The outcomes indicated variation in the physiochemical parameters of the three compounds with differences in the BBB (blood–brain barrier) and (HIA) human intestine absorption ranges, so we were encouraged to follow Lipinski's rule of five [21], which was published in 1997 by Christopher A. Lipinski [22]. It is a standard practice to assess the drug-likeness of a chemical compound and establish whether it possesses characteristics to be active for humans when administered orally.

As shown in Table 3, these 5 parameters totally and completely complied with the examined compounds (*trans*-2-caren-4-ol, nerolidol, and levomenol) in which the polarity, lipophilicity, solubility, flexibility, and saturation were found within the pink area (Fig. 6) indicating promising bioavailability [23].

Table 3. Lipinski's rule of five for ADME analysis of the investigated compounds

No.	Name	M.wt	Lipophilicity	Hydrogen Bond Donors	Hydrogen Bond Acceptors	No. of Rule Violations	Drug-Likeness
Lipinski's rule limits	Less than 500 Dalton	Less than 5	Less than 5	Less than 10	Less than 2 Violations	Lipinski's rule Follows	1
Levomenol	222.37	3.56	1	1	0	Yes	2
Nerolidol	222.37	3.86	1	1	0	Yes	3

Fig. 6 [Images not available. See PDF.]

Radar plot of the examined compounds. POLAR (polarity), LIPO (lipophilicity), INSOLU (solubility), FLEX (flexibility), and IN-SATU (saturation)

BOILED-Egg technique [21] designed for the assessment of the lipophilicity and polarity of small compounds, is supposed to serve as a highly accurate predictive model. This model holds significance in the lead optimization of drugs. Notably, the BOILED-Egg model has the capability to simultaneously predict two crucial ADME parameters: passive gastrointestinal absorption (HIA) and brain access(BBB) [24]. The findings indicate that the three compounds were effectively absorbed, potentially having access to the brain, as depicted by the white region in the results.

Additionally, the compounds exhibited P-glycoprotein permeability (PGP), represented by the red dot. Importantly, all recorded values fell within the acceptable range, meeting the specified criteria [25]. This suggests that the compounds absorption characteristics align with established thresholds, highlighting their favorable pharmacokinetic properties and suitability for further consideration in drug development [24]. Among the three compounds, nerolidol revealed the highest water partition coefficient (WLOGP) followed by levomenol and then *trans*-2-caren-4-ol indicating that *trans*-2-caren-4-ol has the least potential to pass the BBB presented in Fig. 7. However, they were nearly similar regarding topological polar surface area (TPSA) with a value of 20.23 Å.

Fig. 7 [Images not available. See PDF.]

Evaluation of Levomenol *WLOGP* = 4.23, Nerolidol *WLOGP* = 4.40, and *trans*-2-Caren-4-ol *WLOGP* = 1.97 by the BOILED-Egg method. *Note*: In the 2D graphical representation of a BOILED-Egg, the yolk area corresponds to molecules that are anticipated to passively permeate through the blood–brain barrier (BBB). Conversely, molecules situated in the white region are predicted to undergo passive absorption through the gastrointestinal (GI) tract

Discussion

Candida albicans is the main fungus associated with infections via medical devices [26]. Contact lenses, joint prostheses, mechanical heart valves, and dentures are all prone to be infected with this fungus. Accordingly, many studies were conducted to search for new and promising agents against this fungus [26]. Worldwide, cancer is the second most common cause of death. Despite continuous improvements in cancer treatment, there are still many undesirable side effects that occur when receiving chemotherapy. Natural remedies, acquired from plants, may limit those unfavorable side effects and still be utilized to treat cancer [27]. The results revealed that the MAE and hydro-distillation produced EOs with dissimilar volatile content, suggesting that altering the extraction procedure may lead to changes in the chemical profile and thus the biological activity. This heightened variation of efficacy is likely attributable to its terpenoid content, specifically the presence of oxygenated sesquiterpenes and monoterpenes.

On one hand, nerolidol was the most prominent compound in the TLH that may contribute to the biological activity. It is a sesquiterpene alcohol well-established with various uses and demonstrates favorable effects on human health; hence, it is regarded as a promising candidate for chemical or drug development across various domains, including agriculture, industry, and medicine [28]. Numerous publications [28, 29] provide proof of nerolidol effectiveness in demonstrating antifungal activity. It was illustrated to have fungicidal effects against *Microsporum gypseum*, *Candida albicans*, and *T. mentagrophytes* [30, 31].

On the other hand, levomenol (α-bisabolol) was observed as one of the major compounds in TLM. Bisabolol was demonstrated to have the ability to prevent the development of hyphae and fungal growth, as well as to change how ergosterol, a crucial structural element of the fungal membrane, is produced. These effects may be key virulence factors in some strains of the yeast *C. albicans* [32].

Trans-2-Caren-4-ol constitutes 33.92% of the overall volatile content in the EOs extracted using the MAE method from TL. Nerolidol is also known as an antioxidant, chemo-preventive, and antitumor agent. It can modulate the biochemical profiles, work as an antioxidants, detoxification agent, and inhibit tumor development and various types of carcinogenesis [33].

α-Bisabolol was recorded to inhibit the growth of tumors and induce apoptosis in several malignancies, such as acute leukemia, glioblastoma, and pancreatic, prostatic, breast, and liver cancers. Its mechanism of action involves

inhibiting the proliferation, invasiveness, and motility of cancer cells [34, 35]. The notable differences in the mechanisms of action of the major components identified in the oil extracts may account for the variations in cytotoxic activity.

Finally, In silico and ADME studies were conducted to validate the potential antifungal activities and to predict the physiochemical properties of the three major compounds, respectively. The NMT enzyme was chosen due to its essential role in the viability of *Candida albicans*, a major contributor to systemic fungal infections in immunocompromised patients.

Consequently, NMT is considered a promising target for antifungal drug development. It was observed that the enzyme adopts an open conformation during substrate binding. The major compounds present in the oils, being nonpeptidic inhibitors, have the capacity to bind to the substrate binding site enveloped by hydrophobic residues. This interaction pattern differs in detail from that observed with conventional peptidic inhibitors [36]. Levomenol demonstrated the highest binding energy approaching that of ketoconazole followed by nerolidol then *trans*-2-Caren-4-ol.

Conclusion

The widespread inefficiency of antifungal therapy to treat various fungal infections and the side effects of conventional chemotherapy has accelerated research into alternative therapeutics. *Teucrium leucocladum* Boiss. is a famous endogenous plant grown in Egypt that exhibits pharmaceutical potential and medicinal value. In this research, the essential oil acquired from the aerial parts proved to contain monoterpenes and sesquiterpenes, which are classes of compounds known for their versatile usage in medicine. In vivo studies on TL active metabolites against clinical strains of fungi need to be further studied, as do the effects of combining the active compounds with antifungal agents to combat antimicrobial resistance. Additionally, deeper investigation is necessary to find a simpler, natural, more advantageous anticancer pharmaceutical product.

Methods

Plant materials

The aerial parts of *Teucrium Leucocladum* Boiss. were collected [18] from St Katherine Protectorate (28◦3203200 N 33◦57030.200 E), South Sinai, Egypt, in April 2022. The specimen was authenticated by Prof. Dr. Ibrahim El-Garf, Department of Botany, Faculty of Science, Cairo University, Egypt, where a voucher specimen (TL-113) has been deposited.

Hydro-distillation of the essential oil

A fresh sample (100 g) of TL dried via air-shading was hydro-distilled via Clevenger-type apparatus for 3 h which was conducted based on the methods presented by the European Pharmacopoeia (1996) [37] (plant: water ratio 1:3, w/v).

Microwave-assisted extraction of the essential oil

The air-shaded dried sample (100 g) of *T. leucocladum* was extracted using a focused microwave apparatus [18]. Then, the oil sample was prepared [18], separated, and dried [18] and the volume of the recovered essential oil was determined [18]. The extracted oil samples were stored in sealed air-tight glass vials at −20 °C until further analysis. The percentage yield was computed as % v/w using the following equation:

$$\%\text{Yield} = [\text{Oil volume (mL)}/\text{Plant material weight (g)}] \times 100$$

GC–MS analysis of the essential oils

Components of the extracted EOs were analyzed and characterized by GC–MS. The GC–MS analysis was performed at the Department of Medicinal and Aromatic Plants Research, National Research Center, Dokki, Giza, Egypt. The gas chromatography–mass spectrometry instrument and the identification of the chemical components of the EOs was achieved according to the previously mentioned method [18].

Antifungal activity

The antifungal activity was performed according to NCCLS recommendations (National Committee for Clinical Laboratory Standards, 1993).

Screening tests regarding the inhibition zone were carried out by the agar well diffusion method [38, 39], and 100 µL

of each sample were applied in the wells of diameters 0.6 mm. The experiment was carried out in the Regional Center of Mycology and Biotechnology at Al-Azhar University, Cairo, Egypt. The inoculum suspension was prepared according to the reported method [40].

The EOs were dissolved in dimethyl sulfoxide (DMSO) with two different concentrations (50 and 100 mg/mL) for each oil sample (TLH and TLM). Then, the inhibition zone was measured around each well after 48 h at 28 °C using ketoconazole (100 µg/mL) as positive control for fungi.

Cytotoxic activity

The cytotoxic activities on the oil samples were performed at Polychem Bioassays for Scientific Services (Educational Research Center), Egypt on three cell lines against Doxorubicin HCL (98.0%) as a positive control. All reagents and materials for the cell cultures were purchased from Pan Biotech (Germany). Human cancer cell lines of non-small cell lung adenocarcinoma (A549), Triple-negative breast cancer MDA-MB-231, and colon adenocarcinoma (Caco-2) (ATCC®) were maintained [18]. Doxorubicin (98.0%) was provided and dissolved in PBS at 10 mM [15]. Using an Alamar blue (Resazurin reduction) assay based on a previously published method [15]. Appropriate cell densities of exponentially growing A549, MDA-MB-231, or Caco-2 cells (5000–8000 cells/well) were seeded onto 96-well plates. After 24-h incubation with 5% CO_2 at 37 °C, quadrate wells of cells received screening concentrations of each sample (20 and 200 µg/mL) in the culture medium (final DMSO concentration in medium = 0.1%, by volume). After 48 h of incubation, alamar blue dye in culture medium was added to each well after which the incubation was resumed for further 4 h. At the end of the incubation period, the absorbance at 600 nm was recorded on a microplate reader (Sunrise™ microplate reader, Tecan Austria Gmbh, Grödig, Austria) and was used as a measure of cell viability. In dose-dependent experiments, cells were exposed as above to sample serial dilutions (200, 100, 50, 25, and 12.5 µg/mL) to estimate the dose causing a 50% loss of cell viability compared to the control (IC_{50}) using nonlinear regression curve fit on GraphPad Prism software V8.0. (San Diego, USA) to give significant differences in cytotoxicity, one-way analysis of variance (ANOVA) was used for statistical analysis. Significant differences were indicated as $*p<0.05$.

Molecular docking studies

The chemical structures of the major metabolites in the essential oils (EOs), namely levomenol, nerolidol, and *trans*-2-caren-4-ol, were obtained by downloading their respective Structure Data Files (SDF files) from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/), followed by conversion to PDB format via the free software Avogadro (https://avogadro.cc/) [41, 42].

The protein crystal structure for 1IYK (antifungal) was downloaded from the protein databank (https://www.rcsb.org/) [41] using the previously published molecular docking protocol [42].

The ADME, Lipinski's rule of five and BOILED-Egg techniques

The ADME and pharmacokinetic studies have been determined using SWISS ADME [25] (accessed on 25 July 2023) to evaluate the potential of the three major compounds as promising candidates for pharmaceutical drug development. The expected values for the compounds (levomenol, nerolidol, and *trans*-2-Caren-4-ol) in the radar are represented in Fig. 6.

The three compounds with potential physicochemical characteristics for oral bioavailability were identified by the Swiss ADME molecules bioavailability radar. The pink region represents the ideal spaces for the six physicochemical properties [25]. A chemical is regarded as drug-like if its complete radar plot falls within the pink region. An alternative approach for the assessment [25] involves the use of the BOILED-Egg technique, where levomenol, nerolidol, and *trans*-2-caren-4-ol are anticipated to exhibit brain-penetrating properties and are detected within the yolk [25].

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Author contributions

E.M.S, M.Y.I, N.F., and T.A.M. contributed to methodology, formal analysis, investigation, and writing–original draft preparation. M-E.F.H and S.H.T. contributed to conceptualization, editing, validation, and supervision. All authors

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

Experiments have been carried out in compliance with the Ethical Committee of Faculty of Pharmacy, Cairo University, Cairo, Egypt, (Committee of Safe Handling and Disposal of Chemicals and Biologicals) of the protocol numbered MP (3124).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

TL

Teucrium Leucocladum Boiss.

EOs

Essential oils

HD

Hydro-distillation

MAE

Microwave-assisted extraction

GC/MS

Gas chromatography–mass spectrometry

A549

Non-small cell lung adenocarcinoma

MDA-MB-231

Triple-negative breast cancer

C. albicans

Candida albicans

TLM

The MAE oil sample

TLH

HD oil sample

Caco-2

Colon adenocarcinoma

IC_{50}

The fifty percent inhibitory concentration

NMT

N-Myristoyltransferase

BBB

Blood–brain barrier

HIA

Human intestine absorption ranges

PGP

P-glycoprotein permeability
WLOGP
Water partition coefficient
TPSA
Topological polar surface area
Akt
Protein kinase B phosphorylation

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DETAILS

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Phytochemical screening, gas chromatograph/mass spectrometer (GCMS) analysis and molecular toxicological potential of Hunteria umbellata aqueous fruit extract against Staphylococcus aureus in accessory gene regulators (AGRs)

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ABSTRACT (ENGLISH)

Background

Fruits of *Hunteria umbellata* (HU) have been utilized in folk medicine as potent against *Staphylococcus aureus* (SA) infections, particularly skin and nasal related conditions. However, there is scarcity of literature concerning toxicological evaluation of graded doses of HU fruit, especially at molecular level, specifically targeting the accessory gene regulator (AGR) system to prevent abuse of doses in the treatment of bacterial infections. This research evaluated molecular toxicological property of SA exposed to varied concentrations of aqueous HU fruit extract ranging from 500 to 3.90625 µg/µL using broth microdilution method and quantification of AGR I and II genes' expression employing two-step reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). We first identified quality and quantity of chemical compounds in aqueous fruit of HU using phytochemistry and hybrid gas chromatograph–mass spectrometer (GC–MS) technique. Additionally, preliminary bactericidal potential of HU was assessed before molecular toxicology.

Results

Results revealed six phytochemicals and twenty analytical grade compounds from a standard library were identified from chromatograms of HU fruit extract. Some main compounds detected are *n*-Hexadecanoic acid (25.24%), 2-Pentadecanone, 6,10,14-trimethyl (16.08%), Cuparene (16.63%), Tetradecanoic acid (6.21%) and 9-Octadecenoic acid, (E)- (5.70%). Bactericidal activity shows significant ($p<0.05$) toxicity in the tested (8) concentrations of HU fruits in a dose-response relationship compared to the controls (positive and negative). The quantified expression of AGR I and II genes in SA was most significantly increased ($p<0.05$) at both 250 and 500 µg/µL of HU fruit extract while least significant increase ($p<0.05$) was recorded at 125 µg/µL compared to control.

Conclusions

Notably, the study highlighted a potential risk of augmented bacterial infection especially with higher doses of HU extracts during boils' treatment and other epidermal infections instigated by Staph. Expression of both AGR genes at higher doses (250 and 500 µg/µL) is indicative of further expression of several other genes responsible for virulence factors in a variety of human bacterial infections. Thus, consumption of HU fruit to treat boils and blisters should be with great caution especially at high doses.

FULL TEXT

Background

The emerging integration of modern molecular technology in toxicological evaluation of medicinal plants has become an integral part of decoding progression of infectious diseases and possible development of promising drugs and vaccines. This is because quantitative molecular technique such as RT-qPCR used for gene expression profiling from targeted bacterial will create an intrinsic understanding of the roles of virulence genes in disease pathogenicity [31]. *Staphylococcus aureus* (SA) emerges as one out of five leading gram-negative antibiotic-resistant bacteria causing death globally with the highest prevalence in sub-Saharan Africa super-region as recently documented by antimicrobial resistance collaborators [25]. Antibiotic-resistance of *S. aureus* may be associated with acquisition of novel virulency via one lateral gene transfer event or more, and subsequently modifies an earlier nonpathogenic strain to a hypervirulent and/or multidrug resistant [23]. Currently, there is a focus on development of preventive alternate strategies (vaccination and phage therapy) through AGR regulatory genes to forestall occurrence of staphylococcal infections [8]. The regulation of AGR has been described to be imperative in the development of staphylococcal infections involving formation of biofilm [29]. Recurrent Staph. infection has become an important source of health loss through colonization of the skin and nostrils of human host [27]. The frequency of infections and persistent evasion of most antimicrobials of SA colonizing strain has led to alternative treatment in traditional medicine by means of various parts of medicinal plants' parts, especially in Africa. Most communities in Africa including Nigeria still rely on indigenous herbs as most sourced health treatments since these plants are easily accessible and affordable [38]. *Hunteria umbellata* (HU) fruit has been popularly employed in folk medicines without satisfactory doses for the management and treatment of many disease conditions caused by pathogenic bacterial in humans.

Although, several therapeutic potentials of many parts have been highlighted in the literature [37, 39]. *Hunteria umbellata* have been extensively harnessed in traditional herbal medicine as anti-diabetic potent agents with hyperlipidaemic activities [6], natural erectogenic and antioxidant extracts [35]. However, under exploitation of the in vitro molecular toxicity of varying doses from low to high of HU fruit in SA still persists. Hence, this study design was necessitated to precisely offer evidence based scientific findings on in vitro molecular toxicology in virulent genes' expression of SA as the causative agent of boils when treated with HU fruit extracts. Additionally, to establish basis for possible mechanisms of response underlying medicinal plant-resistance as implicated in noxious exhibition in human staphylococcal communicable diseases.

Methods

Preparation of fruit extract

The HU fruit samples which were purchased in the second month (February) of the year 2023 from a local resident marketplace in Dopemu, Lagos State, Nigeria (6° 07′ N to 7° 00′ N latitude and 3° 43′ E to 4° 00′ E longitude) had been identified, authenticated and assigned voucher LUH 8996 as earlier illustrated by Salisu et al. [43]. Extraction employed was modified using the method of maceration [2]. The process involved preparing the fresh fruits by washing, slicing, oven-drying at 40 °C and grinding into a powder using electronic milling machine grinder, Lab. Mill, Serial No. 4745, Christy and Norris Ltd. The ground fruit (254 g) was macerated in 1.4 L of absolute sterile water within 72 h. A thin cloth and cotton wool were used to filter homogenate to get rid of every possible residue. The filtrate was placed in a fan assisted oven at 20 °C to dry. The aqueous extract (paste like form) yielded 3.95 g was stored in glassware and used for further analysis.

Qualitative phytochemical examination

Crude aqueous extract (20 mg) from HU fruit was mixed gently with 6 ml of 1% HCl, warmed, filtered, then tested for availability of alkaloids, anthraquinone and cardiac glycosides, saponins, phenol, flavonoids, tannins, phyloba-tannins, terpenoids as well as steroids. The presence (+) and absence (−) of tested phytochemicals were observed and recorded.

Test for alkaloids

Wagner's assay

Dilute HCl and Wagner's reagent (1 ml) were added to fruit filtrate (3 ml) and shook well. Afterward, colored precipitate (reddish–brown) was formed as the presence of alkaloids [40].

Tests for glycosides

Borntrager's test of anthraquinone glycoside

Addition of 5% H_2SO_4 (1 ml) to fruit extract solution (1 ml) made a mixture that was boiled, filtered, shaken with the same volume of chloroform and kept to still for 5 min. Then the lower layer of chloroform was shaken with half of its volume with dilute ammonia. Nonappearance of bright red coloration of ammoniacal layer indicates absence of anthraquinone glycosides in the fruit [40].

Keller–Killiani test of cardiac glycoside

A small amount of fruit extract (5 mg) were treated with anhydrous acetic acid (1 ml) and few drops of ferric chloride solution in a test tube. Then, concentrated sulphuric acid (2 ml) was added cautiously to prepared mixture. An observed reddish–brown ring at the junction of two layers and development of aqua at the lower layer indicated cardiac glycosides existence [45].

Test for saponins

A mixture of fruit sample (1 g) and distilled water (10 ml) was prepared, boiled for 5 min and shaken vigorously and observed in a test tube. Absence of froths indicates lack of saponins [15].

Test for phenol

A few fruits sample (1 g), ethanol (10 ml) and three drops of phenol solution were mixed in a labeled test tube. Nonappearance of a deep green coloration specifies absence of phenol [16].

Test for flavonoids

A mixture of HU fruit extract (1 g), 10 ml of ethanol and 2 drops of $FeCl_3$ was prepared. Observed dark green color specifies flavonoid [15].

Test for tannins

Decoction of fruit extract (1 g) with 10 ml of distilled water was prepared by boiling for 10 min, filtered, cooled and mixed with 0.1% ferric chloride reagent. An observed blue–black precipitate implies tannins availability as delineated in a modified method of Banso and Adeyemo [10].

Test for phyloba-tannins

Aqueous hydrochloric acid (1 ml) was stirred with fruit extract (2 ml), boiled and allowed to cool down. Availability of phyloba-tannins was implicated in precipitate (red) observed. [10].

Test for terpenoids

Some concentrated sulphuric acids (5 ml) were gently added to fruit extract sample (0.5 g) in a test tube. Observation revealed reddish brown coloration indicating terponoids' presence [9].

Salkowski test for steroids

The crude fruit extracts (2 mg each) were dissolved with chloroform (2 ml) and mixed gently by shaking to form a mixture. Oil of vitriol (2 ml) was poured in prepared mixture to observe red topmost layer in the test tube. This indicates existence of terpenoid [9].

Gas chromatograph/mass spectrometer (GCMS) analysis of bioactive components of *Hunteria umbellata* fruits

Gas Chromatograph/Mass Spectrometer (GCMS) analysis as reported earlier [42] was carried out in Central Research Laboratory, University of Lagos Akoka, using 7890A Gas chromatography system attached to VL/MSD 5975C mass spectrometer (GC–MS Agilent Technologies, Santa Clara, USA) instrument employing the following

settings: Column HP5MS fused silica capillary column [30 m (length) × 0.32 mm (diameter) × 0.25 µm (film thickness)] composed of 100% dimethyl polysiloxane). 1 g of ground fruit samples was dissolved in 10 ml chromatographic grade methanol, filtered by using syringe filter and the clear sample was later injected in GCMS column. Helium gas (99.9999%) was used as the carrier gas at constant flow rate of 1 ml/min and an injection volume of 1 µl was employed with injector temperature at 250 °C and pressure at 8.802 psi. The oven temperature was programmed originally from 80 °C (held for 2 min) with an increase of 5–120 °C/min., then 10–240 °C/min., to hold for 6 min. The total GC running time for the fruit sample was 24 min. The area under a peak accurately represents the quantity of the component present in the sample. Software adopted to handle mass spectra and chromatogram was a ChemStation. The interpretation on mass spectrum of GCMS was done by means of the database of National Institute of Standard and Technology (NIST) version 2, year 2015 library. The mass spectrum of the unknown components was compared with the spectrum of the known components stored in the NIST library to establish the name, molecular weight and structure of the components of the fruit extract.

Collection of bacterial isolates and antibacterial assay

A gram-positive organism, *S. aureus* strain MBSA2208, was used for this study. The bacteria isolate was obtained from the Department of microbiology, Nigerian Institute of Medical Research (NIMR), plated on salt agar (Mannitol) and then brooded (at 40 °C for 24 h) to obtain pure culture. Antibacterial activity of HU fruit sample was tested by method of broth micro-dilution. A sterilized well (96) microplate was prepared for the experiment. Each well was filled with 90 µL of nutrient broth. In the standard wells, 10 µL of 1 mg/ml vancomycin (a susceptible antibiotic) was added, while the control well, received 10 µL of sterile broth as a sterility control [44]. Minimal inhibitory concentration (MIC) was evaluated by preparation of 0.4 µL of a stock concentration HU extract (250 mg/ml) to arrive at a working solution of 500 µg/µL. This working solution was then serially diluted to obtain two-fold dilutions. The resulting concentrations in column 3 to 11 were 500, 250, 125, 62.5, 31.25, 15.625, 7.8125, and 3.90625 µg/µL. Each well, except for the blank well in column 12, was filled up to 200 µL with nutrient broth that had contained bacteria already inoculated at a standard density of 0.5 McFarland, equivalent to 1.5×10^8 (CFU/mL). This setup allowed for the estimation of the bacterial activity of fruit extract by observing a noticeable growth or inhibition of the bacteria in each well of the microplate. After setting up the microplate with the appropriate concentrations of the extract, standard antibiotic and control. The incubation of the plate was done at 37 °C on a shaker in not more than 24 h. After incubation period, measurements of absorption values in each well with microplate reader at 600 nm absorbance were recorded. This measurement allowed for the determination of the growth difference between the wells before and after incubation, indicating the effectiveness of the extract in inhibiting bacterial growth.

To determine the point of inhibition for each extract, preparation of resazurin dye (0.8 mg/ml) was done. After the 24-h incubation period, A small volume of prepared resazurin solution (30 µL) was dropped in individual well of microplate. Resazurin is a dye that changes color from blue to pink in the presence of oxygen. The microplate was then incubated for an additional 3 h to allow the color change to occur. This color change visually indicated microbes' growth inhibition due to smallest amount of HU fruit extract in each well.

RNA extraction and purification

For the extraction of single-stranded RNA from the aqueous extracts of *H. umbellata*, three concentrations were chosen: the highest (500 µg/µL), a moderate (250 µg/µL) and the lowest (125 µg/µL). The extraction was performed via the NIMR Biotech extraction kit according to manufacturer's description.

The extraction process involved several steps. First, the cells in the extracts were lysed to disrupt cell membranes and make RNA available freely. Lysed samples were then incubated to facilitate the extraction process. After incubation, RNA precipitation was carried out to isolate the RNA from other cellular components. The cell debris and impurities were removed through a washing step to obtain a purified RNA sample. The elution step utilized a spin column-based method, where the purified RNA was eluted from the column, leaving behind any contaminants. Finally, the extracted single-stranded RNA was stored at −20 °C until further use. This freezing temperature helps maintain the stability and integrity of the RNA for future analysis or experiments.

Dual-step reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

A separate reverse transcription coupled with qPCR was carried out. First, extracted RNA was converted to complementary DNA (cDNA). Synthesis mix of FIREScript RT cDNA (Solis Biodyne, Estonia) was used, alongside random primers and Oligo (DT). Prepared reaction mixture consisted of 6.8 µl of double-distilled, molecular-grade water, 2 µl of mastermix, 0.1 µl each of forward and reverse primers in Table 1 as manufactured by the NIMR-MTN Oligo Synthesis Laboratory and 1 µl of the RNA template. These components were combined to create a 10 µl reaction mixture as followed by manufacturer's manual. Transcription (reverse) process took place for a duration of 30 min at a temperature of 50 °C. After 5 min, transcriptase enzyme was deactivated at a lesser temperature of 85 °C using a BioRad thermal cycler.

Table 1. List of Nucleotide Primers designed for Virulence Genes in Real-Time PCR Technique

Gene name	Sequences (forward and reverse)	Reference
AGR I	Forward: 5′-CAC TTA TCA TCA AAG AGC C-3′	[3]
Reverse: 5′-CCA CTA ATT ATA GCT GG-3′	AGR II	Forward: 5′-GTA GAG CCG TAT TGA TTC-3′

The second step was the quantitative Polymerase chain reaction (PCR). This reaction was executed to quantify amplifications of expressed genes. The number of amplifications of virulence genes (AGR I and II genes) and the house keeping gene (SA442) of *S. aureus* used in this study was determined using a real-time system (BioRad CFX96 Deepwell), in accordance with the manufacturer's description. A 10 µl reaction mixture was prepared containing 5.8 µl double distilled, nuclease free water, 2 µl Solis Biodyne Eva green mastermix, 0.1 µl each of both forward and reverse primers and 2 µl template coding deoxyribonucleic acid (cDNA) for the two primers separately. The processes involved are; initial and final denaturation, annealing, initial and final extension. First denaturation (DNA unwinding) occurred at 95 °C for 2 min, final unwinding took place for just 30secs at the same temperature as the first melting of DNA, annealing was set for 30secs at 54 °C and repeated for 40cycles followed by extension step with temperature range of 55–80 °C with 0.5 °C increment for 5secs.

Ct values (cycle threshold) were generated at the completion of the reaction and used to calculate relative quantification $2^{-(\Delta\Delta ct)}$.

Data analysis

The cycle threshold (ct value) generated from the melt curves plotted by the thermal cycler was used to calculate relative expression using Microsoft Excel (2016 version). Relative expression values were further subjected to IBM SPSS statistics version 22, to determine the increment or decrease in expression of the virulence genes (AGR I and II). All data entries were given as descriptive average alongside standard error means (mean ± SEM), mean difference was compared using a one-way ANOVA analysis and a post-hoc test of LSD (least significance difference). A confidence interval of 95% was used as the criterion for significance to show variations between compared sets.

Results

Phytochemical analysis of chemical components of *Hunteria umbellata* fruits

The screening of phytochemicals in HU fruit, aqueous extracts revealed six (6) phytochemicals which include specific secondary metabolites like alkaloids, cardiac glycosides, flavonoids, tannins, phyloba-tannins and terpenoids. Four compounds (anthraquinone glycosides, saponins and steroids) were absent in the fruit (see Table 2.)

Table 2. Presence (+) &absence (−) of Phytochemicals in *Hunteria umbellata* Fruit

Phytochemicals	Presence/absence
Alkaloids	+
Anthraquinone glycosides	_
Cardiac glycosides	+
Saponins	_
Phenol	_
Flavonoids	+
Tannins	+
Phyloba-tannins	+
Terpenoids	
Steroids	_

+Presence, −Absence

Bioactive components of *H. umbellata* fruit from chromatograms of GC–MS

Twenty (20) non-polar volatile compounds appeared in HU fruit (methanol extract) as analyzed from GCMS chromatograms in Fig. 1 below. Individual peak denotes a compound with dissimilar quality and quantity based on percentage by ChemStation's calibration mode (Software). All compounds identified with their molecular formula, molecular weight (MW), retention time (RT), area percentage composition (quantity), quality (matching factor>80) and chemical structure of fruit extract are presented in Table 3. The presence of compounds that combine both the matching factor (>80) and percentage composition (>5%) were classified as main compounds while others below both quality (matching factor>80) and quantity (>5%) were regarded as minor compounds. The five major compounds (Table 4) detected in HU fruit are *n*-Hexadecanoic acid (99; 25.2%), 2-Pentadecanone, 6,10,14-trimethyl (96;16.1%), Cuparene (95; 6.6%), Tetradecanoic acid (95; 6.2%) and 9-Octadecenoic acid, (E)- (83; 5.7%). The 15 minor compounds comprise 1-Methylenespiro [2.4] heptan-4-one (43; 1.1%), 2-Butynedioic acid, di-2-propenyl ester (49; 2.1%), Phthalic acid, butyl oct-3-yl ester (86: 2.2%), 7-Hexadecene, (Z)- (97; 2.6%), camphor (42: 1.6%), Methyl 14-methylpentadecanoate (97: 3.3%), 1-Octadecene (97: 4.7%), Methyl linoleate (95: 1.1%), Methyl elaidate (99: 1.4%), Bromoacetic acid, octadecyl ester (49: 1.76%), Cyclohexaneethanol, 4-methyl-.beta.-methylene-, trans-(45: 5.8%), Octadecanoic acid (70: 2.31%) and 1-Octadecene (97: 2.5%).

Fig. 1 [Images not available. See PDF.]

Total Ion GCMS Chromatogram of methanol fruit extract of *Hunteria umbellata*

Table 3. Compounds recognized in methanol fruit extract of *H. umbellatta*

Peak No	Phyto-compounds	Molecular Formula	Retention Time (min.)	Molecular Weights ($gmol^{-1}$)	Area Composition (%)	Quality (%)	Chemical Structure
1	Cuparene	$C_{15}H_{22}$	10.42	202.33	6.63[c]	95	
2	1-Methylenespiro[2.4]heptan-4-one	$C_8H_{10}O$	11.90	122.16	1.13	43	
3	Tetradecanoic acid	$C_{14}H_{28}O_2$	12.93	228.37	6.21[d]	96	
4	2-Butynedioic acid, di-2-propenyl ester	$C_{10}H_{12}O_4$	13.07	196.20	2.08	49	
5	2-Pentadecanone, 6,10,14-trimethyl	$C_{18}H_{36}O$	13.80	268.48	16.08[b]	96	
6	Butyl octyl phthalate	$C_{20}H_{30}O_4$	14.07	334.45	2.19	86	
7	7-Hexadecene, (Z)-	$C_{16}H_{32}$	14.13	224.42	2.59	97	
8	Camphor	$C_{10}H_{16}O$	14.56	152.23	1.62	42	
9	Methyl 14-methylpentadecanoate	$C_{17}H_{34}O_2$	14.60	270.45	3.26	97	
10	*n*-Hexadecanoic acid	$C_{16}H_{32}O_2$	15.01	256.42	25.24[a]	99	
11	1-Octadecene	$C_{18}H_{36}$	16.10	252.50	4.73	97	
12	Methyl linoleate	$C_{19}H_{34}O_2$	16.21	294.47	1.10	95	
13	Trans-13-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	16.27	296.49	1.41	99	
14	Bromoacetic acid, octadecyl ester	$C_{20}H_{39}BrO_2$	16.34	391.40	1.76	49	
15	Cyclohexaneethanol, 4-methyl-.beta.-methylene-, trans-	$C_{12}H_{24}O$	16.66	184.32	5.80	45	
16	Octadecanoic acid	$C_{18}H_{36}O$	16.81	284.48	2.31	70	
17	1-Octadecene	$C_{18}H_{36}$	17.23	252.50	2.45	97	

18	9-Octadecenoic acid, (E)-	$C_{18}H_{34}O_2$	17.67	282.50	5.70[e]	83	
19	1-Hexadecanol, 2-methyl-	$C_{17}H_{36}O$	18.51	256.47	5.27	66	
20	2 (Isobutoxycarbonyl)benzoic acid	$C_{12}H_{14}O_4$	20.75	222.24	2.42	46	

abcde: The top 5 abundant phyto-compounds in descending order (highest to smallest)

Table 4. Nature and reported bioactivity of the major phyto-compounds in methanol fruit extract of *H. umbellatta*

Phyto-compounds	Nature	Quality	Quantity (%)	Reported bioactivity	References
n-Hexadecanoic acid	Palmitic acid	99	25.24	Anti-bacterial	[22]
Octadecanal	Aldehyde	96	16.08	Anti-oxidant	[51]
Cuparene	Sesquiterpene	95	6.63	Anti-cancer activity	[11]
Tetradecanoic acid	Myristic acid	96	6.21	Virulence regulation	[26]
9-Octadecenoic acid, (E)-	Stearic acid	83	5.70	Anti-inflammatory	[50]

Bactericidal property of aqueous *Hunteria umbellata* fruit extract on *Staphylococcus aureus*

Result obtained from the antibacterial assay showed that Vancomycin, a standard drug for S*taphylococcus* infection greatly displayed repressive properties on proliferation of SA. None of eight tested concentrations of *H. umbellata* with 500 µg/µL being the highest stalled the growth of *S. aureus* in comparison to control as shown below (Fig. 2). This was confirmed in all the wells containing resazurin that changed from blue to pink. The change of resazurin from blue indicates the presence of viable organisms (producing oxygen).

Fig. 2 [Images not available. See PDF.]

Graphical representation of Microbial growth in *Hunteria umbellata* fruit extract before and after 24 h of incubation

Amplification of AGR I and II genes' expression in SA predisposed to three selected doses of HU fruit extracts

The amplification and melt curves of the two AGR genes in SA exposed to aqueous HU fruit extracts were provided in Figs. 3 and 4 respectively. The amplification curves are indicative of the binding of DNA to SYBR green fluorescent dye present in the master mix which started after the 20th cycles in AGR I and 30th cycle in AGR II of real time PCR. The melt curves began after 60 °C for AGR I and 75 °C for AGR II.

Fig. 3 [Images not available. See PDF.]

Amplification curves of AGR (I and II) genes of SA treated with HU fruit extracts. RFU represent relative fluorescence units

Fig. 4 [Images not available. See PDF.]

Melt curves of AGR (I and II) genes of SA treated with HU fruit extracts. RFU represent relative fluorescence units

Quantification of AGR I and II gene in *Staphylococcus aureus* after exposure to HU aqueous fruit extract

The result obtained after the quantitative real time PCR showed increased expression of AGR I and II genes at 500 and 125 µg/µL compared to control. However, AGR I and II genes were more significantly (p<0.05) expressed at 250 µg/µL of *H. umbellata* aqueous fruit extract relative to the other two doses and control. (see Fig. 5).

Fig. 5 [Images not available. See PDF.]

Graphical representation of AGR I gene expression of *Staphylococcus aureus* exposed to *Hunteria umbellata* aqueous fruit extract

Discussion

The safety profile of certain plants, their potential therapeutic benefits and considerations of toxicity effect have been criticized as a result of ambiguity in treatment prescriptions by indigenous traditional healers. This is due to ancestral believe in guaranteed safety with no side effect in any consumption of most parts of medicinal plants without adequate doses [36]. Regrettably, this supposition has translated to numerous cases of health complications and in some cases demise of the users [21]. This often results to patients overdosing of herbs and herbal products, as regulatory bodies are totally not in control over the usage of herbal medicines. Despite the goodness of herbal medication, certain plant species have been documented to exhibit cytotoxic effects at elevated doses. The implication is that a whole plant or its parts considered highly safe can become toxic at elevated doses. Serious concerns related to potential toxicity-related to phytomedicine or phytotherapy including issues like carcer-inducing effect, liver toxicity, mutation inducement and genetic damage have been brought to the forefront [30]. Therefore, prevailing perception among users of herbal medicines that these remedies are inherently safe without adverse effects due to their natural origins in the plant kingdom calls for serious concern [32].

Secondary metabolites of different parts of HU have consistently been documented in therapeutic intervention or management of numerous ailments. The results of phytochemical screening of HU fruit revealed certain specialized metabolites including alkaloids, cardiac glycosides, flavonoids, tannins, phyloba-tannins and terpenoids. This result is in accordance with the compounds found in HU methanol seed as well as their known bioactivities as reported by Salami and Ladokun [41], Aderele et al. [5] and Akinrotoye et al. [7]. Another study reported 21 different compounds from chromatograms of both seed and leave methanol extracts [28]. The GCMS analysis from this study resolved 20 compounds from HU fruit, comprising five major compounds and 15 minor compounds based on quality and quantity. The major compounds are palmitic acid, aldehyde, sesquiterpene (terpene), myristic acid and stearic acid. Palmitic acid (*n*-hexadecanoic acid) constituted the most abundant with almost 100% matching factor (Table 4). Notably, maximum concentration of 50 µg/ml, *n*-hexadecanoic acid has established only modest antagonistic bacterial activity against *S. aureus*, as specified by Ganesan et al. [22]. This means overdose of plant rich in palmitic acid in the treatment of bacterial infection could be lethal. Void activity of fatty aldehydes against clinical pathogenic bacteria has been documented by Xie et al. [49] while literature has described terpenes as promising antibacterial especially in multiple drug resistance (MDR) bacterial strains [20, 33]. Though the precise molecular machinery responsible for the antibacterial effects of terpenes are yet to be fully understood. Most plant parts possess fatty acids (FA) as good sources of carbon with structural functions and bactericidal properties [14]. However, sublethal concentrations of some of these fatty acids serve as signaling molecules in modulating bacterial virulence as explained by Cortes-López et al. [17]. Despite the antibacterial activity of most fatty acids, palmitic, myristic and stearic acids have been associated with increase in pathogenicity of bacteria [26]. Although their probable impact on the effectiveness of anti-virulence treatments coupled with participation in intricate network of pathogenicity regulation are increasingly recognized, representing a promising strategy to manage antibiotic-resistant bacterial diseases.

Several reports have shown that *Hunteria umbellata,* possesses antagonistic bactericidal efficacy against the two major representative of bacteria (gram positive and negative), *S. aureus* and *E. coli* bacteria respectively, at doses less than 150 mg/ml [5, 7]. Contrarily to these reports, our findings revealed negative antibacterial activity of *H. umbellata* aqueous fruit extract against *S. aureus* tested. Increase in growth was recorded in *S. aureus,* the causative agent of these staph infections, when it was exposed to *H. umbellata* aqueous fruit extract at doses ranging from 3.9 to 500 µg/µL. The increment could be indicative that the extract supports the growth of Staph infections in the body. This difference could also be attributed to the growing evolution and resistance of pathogenic bacteria [18, 34]. Nevertheless, our study corroborates minimal and inconsequential inhibitory effects of the fruit pulp

(water extract) noted against SA in old fact-findings of Igbe et al. [24] as reviewed by Fadahunsi et al. [19].
The expression levels of gene have been generally regarded as an excellent match for protein expression and thus be harnessed to deduce expression of either protein and/or virulence susceptibility. Equivalent data will offer more enlightened strategy for the choice of medicinal plants for the development of drugs to manage persistent evaded pathogenic processes. Our previous study has established increase in the amount of Laf A and ExsE genes' expressions in, as a main causative agent of gastroenteritis, when exposed to high dosages of both *H. umbellata* seed and stem extracts [43]. This present study further evaluated the AGR system of *S. aureus* which is a vital component underlying numerous genes' expression that code for virulence tendencies, formation of biofilms during the pathogenicity of bacterium and inducement of both type 2 and interleukin-17-dependent epidermal inflammation in host cell [47]. Significant increase in expression of both AGR genes of SA exposed to the three tested doses (500, 250 and 125 µg/µL) is in accordance with expression of AGR 1 and II which has been ascribed to invasive infection like bacteraemia [12].
Every pathogen has virulence genes associated with them, these genes are critical for triggering diseases when the host has been colonized successfully to cause bacterial infections [48]. Our studies revealed persistent increase of AGR I gene followed by AGR II gene in the tested doses as compared to control, in agreement with other study that both genes are the most common allelic groups of AGR system [1]. The AGR I system is often connected with strains of *S. aureus* which produces the Panton-Valentine leukocidin (PVL-SA) toxin, commonly implicated in necrotic skin infection and persistent cellulitis [13]. AGR II is frequently found in antibiotic (methicillin) resilient *S. aureus* strains, linked with hospital—acquired infections [46]. Variation in amplification peaks during quantification of the AGR genes revealed difference in expression of AGR genes in the three tested concentrations of the extract (500, 250 and 125 µg/µL) compared to normal control. This further indicates the presence of the genes in the *S. aureus*. AGR I and II genes were expressed significantly (p<0.05) at 250 µg/µL and 500 µg/µL doses while dose 125 µg/µL significantly decreased (p<0.05) genes' expression. This suggests that AGR I and II genes would be expressed as doses of *H. umbellata* fruit extract increases. However, this molecular toxicity at genetic level is scarce in the literature, as most studies have been focused on the antibacterial activity of *H. umbellata* aqueous fruit extract. Until this present study, there isn't any recorded side effects of *H. umbellata* at molecular level. Adeneye et al. [4] indicated in his study that HU exhibits a notably low level of oral toxicity. That is, consumption of the HU fruit is not dangerous or toxic. Yet, this study confirms the expression of both types of AGR genes (I and II) as the colonization factor for *S. aureus* in the development of staphylococcal related infections. These findings affirmed the importance of molecular toxicity especially at gene and protein levels to validate conventional and modern therapeutics in either prevention or treatment of microbial infectious diseases globally. This will further elucidate the toxicity pathways underlying antimicrobial resistance associated with virulence.

Conclusion

Consumption of indiscriminate dosages of *H. umbellata* extracts may aggravate the staphylococcal diseases such as boils, abscess and staph food poisoning. Hence, *H. umbellata* fruit extract maybe be dangerous to health when consumed in high quantities.

Recommendation

Extensive in vitro studies should be conducted on different herbal plants and concoctions to verify their antibacterial activities. Most especially, molecular toxicity tests must be incorporated in the toxicology of medicinal plants to fully ascertain its safety and safe dosage. In addition, enhancing awareness about potential health risks linked to the utilization or excessive use of medicinal plant parts and quality control guidelines should be encouraged and enforced on traditional use of herbal plants in any disease management.

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Author contributions

TFS conceptualized the research, contributed to supervision, participated in the laboratory work, performed data analysis and drafted the paper. MAF and LAA were involved in primers' selection, laboratory work and data collation.

TOY, MAF and BTT were involved in selection of microorganisms, participated in laboratory work, data collection and contributed in writing the manuscript. SAA and POJ ensured quality assurance, contributed to validation and revised the manuscript. TFS, TOY and LAA revised and edited the final manuscript. All authors have read and approved the final manuscript.

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Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The HU fruit samples used in this study were identified, authenticated by a taxonomist and assigned voucher numbers, LUH 8995 and LUH 8996 respectively in the Department of Botany, Faculty of Science, University of Lagos. The fruit samples were further deposited in the herbarium of the same University. This is in alignment with national guideline and legislation required for the study of indigenous plant.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

HU

Hunteria umbellata

SA

Staphylococcus aureus

AGR

Accessory gene regulator

RT-qPCR

Reverse transcriptase quantitative polymerase chain reaction

GCMS

Gas chromatograph/mass spectrometer

NIST

Nigerian institute of standards and technology

MIC

Minimal inhibitory concentration

CFU

Colony forming unit

OD

Optical density

RNA

Ribonucleic acid

cDNA

Complementary deoxyribonucleic acid

CT

Cycle threshold

ANOVA

Analysis of variance

LSD

Least significant difference

PCR

Polymerase chain reaction

Publisher's Note

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DETAILS

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Size-dependent effects of niosomes on the penetration of methotrexate in skin layers

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ABSTRACT (ENGLISH)

Background

Niosomes hold promise as drug delivery systems for cancer treatment, with niosome size impacting stability, biodistribution, and effectiveness. This study optimized methotrexate (MTX)-loaded niosome formulation by studying the effects of components and processing conditions on size. The niosomes formulation was made by the thin-film hydration technique.

Results

The optimized formulation (NIO 17) with a 6:2:2 ratio of span 60, soya PC, and cholesterol achieved 55.05% methotrexate encapsulation, particle size 597.2 nm, PDI 0.49, and zeta potential − 23.3 mV. The compatibility of methotrexate with lipids was confirmed via Fourier transform infrared spectroscopy, and transmission electron microscopy revealed spherical, well-dispersed vesicles. Differential scanning calorimetry indicated methotrexate

conversion or entrapment within vesicles. In vitro release exhibited a sustained pattern with an initial burst. NIO 17 showed potent anti-cancer activity against B16-F10 cells (GI50: 38.7176 µg/mL). Ex vivo studies suggest tailoring niosome size (597.2–982.3 nm) to target specific skin depths (0–38 µm) for enhanced localized drug delivery.
Conclusions
This study demonstrates the potential of methotrexate-loaded niosomes as a novel cancer therapy approach, highlighting the potent anti-cancer activity and transdermal delivery potential of NIO 17. Further research is necessary to explore its clinical translation.

FULL TEXT

Background

Skin cancer, triggered by ultraviolet (UV) radiation from the sun or tanning beds, affects millions globally [1, 3]. Common types include basal cell carcinoma, squamous cell carcinoma, and melanoma as shown in Fig. 1. Traditional treatments like surgery, radiation therapy, and chemotherapy pose various limitations [2, 3]. Surgery, while common for early stages, is invasive. Radiation uses high-energy beams, and chemotherapy, despite killing cancer cells, often comes with side effects like nausea, vomiting, hair loss, and fatigue [4, 6]. Moreover, advanced or metastatic cases often lack effective treatment options. There is a growing demand for targeted and minimally invasive therapies that can deliver drugs specifically to cancerous cells while sparing healthy tissue. In this context, exploring novel drug delivery systems like niosomes holds great promise. Niosomes, with their ability to encapsulate drugs and deliver them to specific sites within the body, offer the potential to enhance the efficacy of treatments for skin cancer while minimizing adverse effects, thus addressing critical unmet needs in the current treatment landscape. Niosomes, composed of biocompatible and biodegradable phospholipids, cholesterol, and surfactants [5, 7], niosomes can deliver a wide range of drugs and target specific cells or tissues. Studies in mice suggest their safety and efficacy for skin cancer treatment, warranting further exploration for clinical use.

Fig. 1 [Images not available. See PDF.]

Schematic representation of SCC, BCC and malignant melanoma

Methotrexate (MTX), a drug commonly used for rapidly growing tumors like skin cancer [5], works by hindering DNA synthesis in dividing cells. While traditionally administered orally or parenterally for skin cancer, MTX can cause gastrointestinal side effects like indigestion, dyspepsia, vomiting, and ulceration. To address these, we investigated the potential of niosomes as a topical delivery system for MTX [7].

This study aimed to evaluate niosomes as a means to enhance the transdermal transport of MTX to various skin layers, focusing on the impact of niosome size on their efficacy as shown in Fig. 2. We examined the effects of different-sized niosomes on MTX permeation through ex vivo studies and visualized their distribution within the skin using confocal laser scanning microscopy. Additionally, in vivo histopathological studies in mice assessed the feasibility of using niosomes to deliver MTX to the skin.

Fig. 2 [Images not available. See PDF.]

Schematic representation of niosomes penetration by skin layers

Materials and protocols

Chemicals

This research utilized a diverse range of materials for its experiments. The drug, methotrexate (MTX), was kindly provided by Neon Laboratories Ltd. (Mumbai, India). Soya Phosphatidylcholine (SPC), a key lipid component, was a generous gift from Lipoid GmbH (Ludwigshafen, Germany), while cholesterol (CHOL) was purchased from CDH (India). Solvents and reagents like chloroform, methanol, phosphate buffer, Triton X-100, Sephadex G-50, and dimethyl sulfoxide (DMSO) were sourced from HiMedia Laboratory Pvt. Ltd. (Mumbai, India). Additionally, HPLC water (Lichrosolv), acetonitrile (HPLC grade), methanol (HPLC grade), and rhodamine B (RHB) were procured from Sigma-Aldrich and Merck (India). Finally, dialysis bags with molecular weights ranging from 12,000 to 14,000 were

obtained from HiMedia Laboratory Pvt. Ltd. Notably, all chemicals were of analytical grade and used directly without further purification.

Preparation of MTX-loaded niosomes

The preparation of various niosomal formulations (Nio1 to Nio18) was carried out using the thin-film hydration method but with slight modifications [8, 9], where the mole fraction of Span 60 was systematically varied (4:2:2, 5:2:2, 6:2:2 Soya PC: Cholesterol ratios) while maintaining constant molar concentrations of Soya phosphatidylcholine (SPC) and cholesterol as shown in Fig. 3. An accurate amount of Span 60, soya phosphatidylcholine, and cholesterol was dissolved in a round-bottom flask (RBF) containing 10 mL of chloroform and methanol (9:1). The organic solvents were eliminated using a rotary vacuum evaporator above the lipid transition temperature of 65 °C under reduced pressure to form a thin lipid film on the wall of the RBF. The residual amount of the solvent mixture was removed from the deposited layer of lipids by leaving the contents under a vacuum overnight. Hydration was done with phosphate buffer (PBS 7.4) containing methotrexate drug by rotating the RBF at a suitable temperature for 2 h until the formation of niosomes [22, 23]. The particle size of the formed niosomes dispersion was further reduced by sonication with various sonication cycles. The sonication time is taken as another variable for the formulation process. The same method was used to prepare Rhodamine B-loaded niosomes for the ex vivo study, where 0.5% of the total lipid was substituted with RHB in place of MTX in the selected formulation [10, 24, 25]. The obtained opalescent dispersion of niosomes was stored at 4 ± 2 °C until use.

Fig. 3 [Images not available. See PDF.]

Method of preparation of niosomes

Characterization of Niosomal formulations

Size distribution, polydispersity, and surface charge of vesicles

Initially, 1 ml of each of the prepared niosomal formulations was mixed with deionized water for adequate dispersion. The average size of the vesicles (VS), the zeta potential (ZP), and the polydispersity index (PDI) of the drug-loaded niosomes was analyzed using Zeta sizer Nano ZS (Malvern Instruments Ltd.) dynamic light-scattering method. The niosomal dispersions were prepared and diluted 10 times with deionized water before measurement to ensure that the light-scattering intensity was within the instrument's detection range [26, 27]. The measurements were taken in triplicate, and the average values are reported in Table 1 and Fig. 4.

Table 1. The formulations of niosomes with different ratios of ingredients and sonication time, along with the results of particle sizes, zeta potential, PDI, and entrapment efficiency

Formulation code	Span60:CH:SPC	Sonication time (min)	Vesicle size (nm)	PDI	Zeta potential (mV)	Entrapment efficiency (%)
Nio1	4:2:2	0	1160.3	0.11	− 48.8	68.13
Nio2	4:2:2	2	976.9	0.36	− 45.7	67.43
Nio3	4:2:2	4	843.7	0.44	− 40.3	64.36
Nio4	4:2:2	6	777.5	0.31	− 32.2	58.97
Nio5	4:2:2	8	575.2	0.35	− 38.5	58.04
Nio6	4:2:2	10	289.5	0.43	− 31.2	35.02

Nio7	5:2:2	0	1406.3	0.40	− 54.8	56.23
Nio8	5:2:2	2	965.9	0.23	− 45.7	53.43
Nio9	5:2:2	4	765.7	0.35	− 44.3	48.36
Nio10	5:2:2	6	613.5	0.25	− 40.2	50.97
Nio11	5:2:2	8	535.2	0.33	− 35.5	44.03
Nio12	5:2:2	10	425.5	0.13	− 28.2	30.07
Nio13	6:2:2	0	982.3	0.22	− 38.8	76.23
Nio14	6:2:2	2	783.9	0.30	− 41.7	73.43
Nio15	6:2:2	4	707.7	0.23	− 32.3	56.36
Nio16	6:2:2	6	671.5	0.39	− 27.8	62.97
Nio17	6:2:2	8	597.2	0.49	− 23.3	55.05
Nio18	6:2:2	10	205.2	0.42	− 29.2	50.02

Fig. 4 [Images not available. See PDF.]

A Graphical representation of formulation optimization with ratio 6:2:2 (Span 60:CH:SPC); **a** vesicle size (nm); **b** PDI; **c** Zeta Potential (mV); **d** entrapment efficiency (%). **B** Images showing the particle-size distribution of the optimized formulations (NIO 13, 14, 17, and 18) prepared using a ratio of Span 60:CH:SPC (6:2:2). **C** Images showing the zeta potential of the optimized formulations (NIO 16 and 17) prepared using a ratio of Span 60:CH:SPC (6:2:2)

Fig. 5 [Images not available. See PDF.]

Fluorescent and TEM images of niosomes

Entrapment efficiency (EE)

The entrapment efficiency (EE) was calculated through the mini-column centrifugation method by separating the non-entrapped drug. The %EE of MTX in the niosomes was estimated by determining the free MTX in the dispersion medium. 1 mL of the niosomes dispersion was centrifuged at 15,000 rpm for 2 h at 4 °C using a cooling centrifuge. The supernatant was then separated and diluted, and the concentration of MTX was determined through HPLC methods [11, 28, 29]. The niosomes were then disrupted using 0.1% Triton-X 100, and the drug content is quantified using Eq. (1). An isocratic HPLC procedure was used to determine the concentration of MTX. A Zorbax Extend-C18 column was used as the stationary phase, and the mobile phase was a mixture of 50 mM sodium acetate buffer solution with a pH of 5.6 and acetonitrile (89:11 v/v). The flow rate was set at 1.0 mL/min, and the UV detector was set at 307 nm. The results are shown in Table 1 and Fig. 4.

1

Entrapment Efficiency%=Amount of free drugTotal amount of drug×100

Shape and surface morphology

The appearance of the optimized MTX-loaded niosomal formulations was studied using the transmission electron microscopy (TEM) technique (Jeol JEM1230, Tokyo, Japan) after suitable dilution. A drop of the selected niosomal dispersion was spread on a copper grid and stained negatively with 1% phosphotungstic acid. The samples were then air-dried for 10 min at standard temperature and pressure (STP) [14, 30, 31] and examined under a TEM as shown in Fig. 5.

Fig. 6 [Images not available. See PDF.]

A FTIR spectra of methotrexate, Soya PC, Span 60, Cholesterol and Mixture; **B** DSC spectra of methotrexate, Soya PC, Span 60, cholesterol and mixture

Fourier transform infrared spectroscopy

The optimized formulation, drug, excipient, and drug–excipient mixture were analyzed using Fourier transform infrared spectroscopy (FTIR) (Bruker FTIR 8400S ALPHA). Before conducting the IR studies, the samples and excipients were vacuum-dried for 12 h [32, 33]. The dried samples of each were placed on a sample platform, and the spectra were captured in the range of 4000–400 cm^{-1}, which were consistent with the official IR spectrum reported in the monograph as shown in Fig. 6A [12, 13].

Fig. 7 [Images not available. See PDF.]

In vitro release of MTX from niosomes in comparison to plain MTX solution at pH 7.4

Thermoanalytical technique

The thermal characteristics of pure methotrexate (MTX), Soya Phosphatidylcholine (PC), Cholesterol, Span 60, the physical mixture of MTX and niosomal components, empty niosomes, and MTX-loaded niosomes were analyzed using differential scanning calorimetry technique (DSC 60, Shimadzu, Japan). The device was calibrated using high-purity indium (99.9%). Three milligrams of accurately weighed samples were put into standard aluminum pans and then heated from 100 to 300 °C at a scanning rate of 100 °C per minute [15, 34, 35] as shown in Fig. 6B.

In vitro drug release kinetics

The niosomal suspension (Nio 15 and Nio 16) was centrifuged to separate the unentrapped drug from the niosomes. Next, 1 mL of the pure niosomal suspension was placed into a dialysis tube, which was then immersed in a beaker that contained 20 mL of PBS (pH 7.4) and stirred on a magnetic stirrer at a constant temperature of 37 ± 0.5 °C. Samples were taken at various time intervals and analyzed for drug content using HPLC. To maintain the initial volume of the dissolution fluid, 5 mL of fresh solution was added after each sample withdrawal [15, 20, 36, 37]. The cumulative amount of MTX released from different niosomal formulations was calculated as per Eq. (2). Results are shown in Fig. 7.

2

Percentage cumulative drug released=Cumulative Amount of Drug obtained at timeTotal amount of Drug in Niosomes

In vitro cytotoxicity study

In vitro, cell cytotoxicity of optimized formulation (Plain MTX, NIO 5, NIO 12, and NIO 16) was evaluated by the SRB assay using murine skin melanoma cell line B16-F10. The cell lines were grown in RPMI 1640 medium, and cells were inoculated into 96-well plates in 100 µL at plating [38–40]. After cell inoculation, the microtiter plates were placed in an incubator at 37 °C, 5% CO_2, 95% air, and 100% relative humidity for 24 h before the addition of formulations. Formulations were dissolved in an appropriate solvent to a concentration of 100 µg/mL and then diluted to 1 mg/mL using Milli-Q water. The diluted solutions were stored frozen until needed. At the time of the experiment, aliquots of the frozen concentrate (1 mg/mL) were thawed and diluted to the following concentrations: 100 µg/mL, 200 µg/mL, 400 µg/mL, and 800 µg/mL. Aliquots of 10 µL of each drug dilution were added to microtiter wells that already contained 90 µL of the medium. This resulted in final drug concentrations of 10 µg/mL, 20 µg/mL, 40 µg/mL, and 80 µg/mL. The plates were then incubated at standard conditions for 48 h.

Fig. 8 [Images not available. See PDF.]

A Growth curve: the plot of percentage growth inhibition versus drug concentration (μg/mL) shows the effective drug concentration on murine tumor cell line B16 F10 cells; **B** Phase-contrast microscopic images of murine tumor cell line B16 F10 cells after 24 h. treatment negative control (untreated), ADR, and formulations NIO 14 to NIO 17

To terminate the assay, cold TCA was added to the plates. Cells were fixed in situ by gently adding 50 μL of cold 30% (w/v) TCA (final concentration, 10% TCA) [41, 42]. The plates were then incubated for 60 min at 4 °C. The samples were washed five times with PBS, and then, 50 μL of 0.4% (w/v) SRB solution was added to each well. The plates were then incubated for 20 min at room temperature.

One percent (v/v) of acetic acid was used to remove the unbound dye, and plates were dried and then analyzed at the wavelength of 540 nm with a 690 nm reference wavelength by using the plate reader. Adriamycin (ADR) was used in the same concentration range as a positive control. Cytotoxicity was expressed as the percentage of viable cells relative to incubated cells in the presence of 0.1% DMSO vehicle control. The percentage of growth inhibition (GI_{50}) is calculated in Eq. (3). GI_{50} was calculated from the drug concentration, resulting in a 50% reduction in cell growth. Each measurement was done in triplicate [18, 19, 43]. Results shown in Fig. 8.

3

Percentage growth inhibition=Ti-Tz/C-Tz×100where absorbance at zero time (Tz), control growth (*C*), and test growth of formulation in the presence of drug at four concentration levels (Ti).

Fig. 9 [Images not available. See PDF.]

A Confocal laser scanning photomicrographs illustrating a *z*-stack image capturing optical sections of mice skin. The sections were taken at intervals of 2 μm, covering a depth range from 0 to 38 μm. The formulations employing PBS as a positive control exhibit no observable particles. **B** Confocal laser scanning photomicrographs illustrating a *z*-stack image capturing optical sections of mice skin. The sections were taken at intervals of 2 μm, covering a depth range from 0 to 38 μm. The formulations employing Plain Rhodamine as a negative control exhibit no observable particles. **C** Confocal laser scanning photomicrographs illustrating a *z*-stack image capturing optical sections of mice skin. The sections were taken at intervals of 2 μm, covering a depth range from 0 to 14 μm. The formulations using Nio13 (Without Sonication) as an agent demonstrate the presence of particles, visible within the range of 0–6 μm. **D** Confocal laser scanning photomicrographs illustrating a *z*-stack image capturing optical sections of mice skin. The sections were taken at intervals of 2 μm, covering a depth range from 0 to 14 μm. The formulations using Nio14 (2 min Sonication) as an agent demonstrate the presence of particles, visible within the range of 4–18 μm. **E** Confocal laser scanning photomicrographs illustrating a *z*-stack image capturing optical sections of mice skin. The sections were taken at intervals of 2 μm, covering a depth range from 0 to 14 μm. The formulations using Nio15 (4 min Sonication) as an agent demonstrate the presence of particles, visible within the range of 16–22 μm. **F** Confocal laser scanning photomicrographs illustrating a z-stack image capturing optical sections of mice skin. The sections were taken at intervals of 2 μm, covering a depth range from 0 to 40 μm. The formulations using Nio16 (6 min Sonication) as an agent demonstrate the presence of particles, visible within the range of 24–32 μm. **G** Confocal laser scanning photomicrographs illustrating a *z*-stack image capturing optical sections of mice skin. The sections were taken at intervals of 2 μm, covering a depth range from 0 to 38 μm. The formulations using Nio17 (8 min Sonication) as an agent demonstrate the presence of particles, visible within the range of 12–38 μm

Ex vivo and in vivo investigations

The ex vivo investigation was conducted following protocols approved by the Committee for Control and Supervision of Experiments on Animals (CPCSEA/SN-10/RN-22/16-02-2020), which is overseen by the Ministry of Social Justice and Empowerment, Government of India. The study also followed the recommendations of the Institutional Animal Ethical Committee (IAEC) at Dr. H.S. Gour Vishwavidyalaya, located in Sagar, Madhya Pradesh, India.

Ex vivo permeation study

The ex vivo visualization using confocal laser scanning microscopy technique (CLSM) involved the formulation of Rhodamine B (RH)-loaded niosomes through a process similar to that used for creating MTX-loaded niosomes

[44–46]. However, RH was incorporated at a concentration of 0.5% concerning the total lipid content, replacing MTX in the specific formulation. For experimentation, the mice's skin was sectioned into square pieces and positioned with the stratum corneum (SC) facing the donor compartment within diffusion cells. The receptor chamber contained 20 mL of phosphate buffer saline (pH 7.4) maintained at 32 ± 1 °C. To mimic the expected application of niosomes to the skin's surface, RH-loaded niosomes (including a plain niosomes formulation, a plain rhodamine solution, and formulations Nio 13 to Nio 17) were administered onto the skin and allowed to remain for 4 h. Following this, the skin was rinsed with 10% ethanol and then gently wiped in preparation for imaging, following the methods described by [16, 47]. The investigation of the skin was conducted using CLSM with the following steps: the complete skin sample was positioned between a glass slide and a cover slip and examined utilizing inverted CLSM (LSM 710, Carl Zeiss, Jena, Germany). RH fluorescence was excited at a wavelength of 573 nm and detected at 591 nm. Scans were taken at 2-µm intervals starting from the skin surface (0 mm) to a depth of 40 µm, using a 40× objective lens. Images shown in Fig. 9 were captured in both the *xy* and *xz* planes, employing optical sectioning z-stack mode. The acquired confocal microscopy images were processed using LSM Image Browser software, release 4.2 (LASX), as described by Hathout and Nasr in 2013 [17, 48].

Fig. 10 [Images not available. See PDF.]

Photomicrographs showing histopathological sections (hematoxylin and eosin stained) of mice skin **A** normal untreated, **B** treated with Plain MTX, **C** Nio13, **D** Nio14 **E**, **F** Nio15, **G**, **H** Nio16 and I: Nio17. The magnification power of 40× to identify the epidermis, dermis, the subcutaneous tissue and muscles, respectively

In vivo histopathological study

An In vivo histopathological study was performed to evaluate skin irritation potential and structural changes resulting from exposure to MTX-loaded niosomes. The study involved dividing mice into 7 groups, each consisting of 3 animals [49, 50]. Group I acted as the positive control receiving PBS, group II as the negative control receiving plain MTX solution, and groups III to VII were exposed to topical applications of MTX-encapsulated niosomes (Nio 13 to 17) on the skin surface three times daily for a week. After the treatment period, the mice were killed, and their skin was extracted for histopathological examination. The collected skin samples underwent fixation in 10% formal saline for 24 h, followed by washing, dehydration using alcohol dilutions, clearing in xylene, and embedding in paraffin beeswax blocks. These blocks were maintained at 56°C for 24 h, and sections of 4 mm thickness were cut using a microtome (Leica Microsystems SM2400, Cambridge, England). These sections were then deparaffinized, stained with hematoxylin counterstained with eosin, and subsequently observed under a light microscope for analysis as shown in Fig. 10 [9, 21, 51].

Results and discussion

Preparation of niosomal formulation, particle-size analysis, and zeta potential determination

The synthesis of MTX-loaded niosomes was achieved using the film hydration method as depicted in Fig. 3 and by incorporating various ratios of span 60, soya PC, and cholesterol in each formulation. The study aimed to determine the effect of various ingredients and processing variables on the vesicle size and entrapment efficiency. The composition of span 60, soya PC, and cholesterol (4:2:2, 5:2:2, 6:2:2) had a significant impact on the size of the niosomes. The results showed that increasing the concentration of Span 60 (a non-ionic surfactant with an HLB of 4.7) led to a decrease in vesicle size. This is because Span 60 has high interfacial activity and a critical packing parameter (CPP) between 0.5 and 1. A lower CPP indicates that the surfactant molecules are more tightly packed, which results in smaller vesicles. However, a further increase in the concentration of Span 60 led to an increase in vesicle size. This is because the higher concentration of cholesterol in the vesicles provided added rigidity, which counteracted the effect of the increased interfacial activity of Span 60. The addition of cholesterol in lipid vesicles changes the short-distance repulsive forces among them. An increase in cholesterol content leads to an increase in the net repulsive forces among soya PC and span 60 vesicles, reducing their aggregation. The sonication time also affects the entrapment efficiency and size of MTX-loaded niosomes, as seen in Table 1. An increase in sonication time causes a decrease in particle size. Particles without sonication showed the largest size, while particles

subjected to 10 min of sonication showed the smallest size, with a mean particle size ranging from 200 to 1406 nm. The optimized formulations, with a ratio of 6:2:2, were used for further studies. The average standard deviation (SD) (nm) of particle size for most of the particles was 200–1000 nm, depending on the sonication time cycle, and the polydispersity index was less than 0.4. The formulations had a negative zeta potential of −25 to −30 mV as depicted in Fig. 4A, B and showed the best entrapment efficiency. The negative zeta potential of the formulations assisted in their adhesion to cancer cells and internalization through cellular endocytosis.

Entrapment efficiency

After removing the unencapsulated drug through dialysis, the efficiency of encapsulation was analyzed for all the formulations. The presence of surfactant, cholesterol, and drug-to-lipid ratios in the vesicles can reduce the stiffness of the vesicles and affect the ability of methotrexate to be trapped inside. The highest drug encapsulation efficiency was recorded to be 76% in vesicles without sonication and the lowest was 30% with a 10-min sonication cycle.

Shape and surface morphology

Confirmation of the results obtained from the Malvern particle-size analyzer can be achieved through morphological analysis, which also examines the structure of colloidal systems. The optimized niosomes displayed a non-aggregated, single-layered vesicular structure with a predominant spherical shape as seen in the TEM images (Fig. 5). The average particle size observed in the TEM micrographs was consistent with the size previously obtained from the Malvern particle-size analyzer, as presented in Table 1.

Fourier transform infrared spectroscopy (FTIR)

FTIR spectroscopy is a useful tool in determining potential interactions between drugs and excipients. Figure 6A shows the IR spectra of pure methotrexate and a formulation that includes the drug and other excipients like phospholipids and a permeation enhancer. The pure methotrexate exhibits peaks at 3406.10 cm^{-1} (N–H stretching), 2928.64 cm^{-1} (O–H stretching for the carboxylic group), 1646.82 cm^{-1} (C–C stretching), 1603.41 cm^{-1} (C=C stretching of aromatics), 1542.02 cm^{-1} (N–H bend), and 831.01 cm^{-1} (C–H out of the plane of aromatics). The peaks for span 60 were found at 2950.00, 2856.50, and 1733.73 cm^{-1}, while peaks for lipids were observed at 3414.06, 2875.67, 1383.09, 1433.18, 1056.43, and 1547.14 cm^{-1}. Peaks for phosphatidylcholine (PC) were recorded at 3467.98, 2926.81, 1474.01, 1214.58, and 1042.76 cm^{-1}. The prepared formulations were also analyzed and found to have peaks in the same range, the drug and the lipid did not interact. The peaks of the optimal formulation were found to be very similar to those of the pure methotrexate, i.e., 3500.00, 3100.00, 2908.60, 1742.64, 1638.10, 1510.00, 1540.21, 1210.12, and 815.00 cm^{-1}, revealing that there was no significant change in the IR peaks of methotrexate and the optimized formulation, signifying the compatibility of the drug and the polymer.

Thermoanalytical technique

Figure 6B displays the DSC (differential scanning calorimetry) thermograms of various substances, including pure methotrexate (MTX), Soya phosphatidylcholine (PC), cholesterol, Span 60, a physical mixture of MTX with niosome components, blank niosomes, and MTX-loaded niosomes. The thermogram of MTX exhibits a typical exothermic peak at 185.9 °C and 93.5 °C, indicating its crystalline form, with a residual mass of 89.79% and weight loss of 7.79%. The thermogram of Span 60 displays a characteristic endothermic peak resulting from melting at 61.6 °C, with a residual mass of 93.00% and weight loss of 3.82%. The thermogram of cholesterol indicates an endothermic peak due to melting at 121.6 °C and 150.3 °C, with a residual mass of 98.25% and weight loss of 2.75%. The DSC of the physical mixture of MTX with niosome components reveals the peaks of both Span 60 and Soya PC, as well as the MTX peak, indicating that it maintains its crystalline form. However, the decrease in MTX peak intensity may be related to the dilution effect caused by the excipients. The thermogram of the unloaded niosomes demonstrates the absence of the MTX characteristic peak. The endothermic peak of Span 60 is present in both the blank niosomes and drug-loaded niosomes but shifted to a lower temperature (61.6 °C). For the drug-loaded niosomes, the MTX characteristic peak is completely gone, which could indicate that MTX was either encapsulated inside the niosomes or transformed into a molecular state within the surfactant mixture. As reported by Nasr et al. (2013) [17], the absence of the drug's crystalline melting peak after encapsulation may also indicate the presence of a strong interaction between the surfactant bilayers of the vesicles and the entrapped drug.

In vitro drug release kinetics

Figure 7 depicts the in vitro release profile of the optimized niosomal suspension loaded with methotrexate at pH 7.4 and 35 ± 0.5 °C. The optimized formulation exhibited a cumulative drug release of 75.09% over 24 h. The release curve indicated an initial burst release where approximately 28% of the drug was released within the first two and a half hours. The subsequent drug release from the optimized niosomes was sustained. The initial burst release was attributed to the presence of free methotrexate in the external phase.

In vitro cell proliferation studies

The anti-cancer efficacy of the formulations Plain MTX, NIO 5, NIO 12, and NIO 16 was tested against the murine skin melanoma cell line B16-F10 using the SRB assay, with ADR (Adriamycin) serving as a positive control. Figure 8A, B shows the activity of the formulations against B16-F10 cells. The results, presented in Table 2, indicate that NIO 16 was significantly more effective than other formulations like NIO 5 and Nio12. The GI_{50} values were 8.05738 µg/mL (NIO 5), 2.5311 µg/mL (NIO 12), 38.7176 µg/mL (NIO 16), and 40.2238 µg/mL (Plain MTX). The investigation revealed that Nio16 (with a GI50 of 38.7176 µg/mL) exhibited considerable potential in countering cancer activity within B16-F10 cells. It displayed enhanced efficacy in curbing cell growth when contrasted with alternative formulations. This heightened effectiveness might be attributed to potential factors such as the diffusion of niosomes from the stratum corneum, the amplification of permeation through the influence of Span 60 surfactants present in the niosomal formulation, or even modifications to the stratum corneum to augment the permeability of the intercellular lipid barrier. The drugs (MTX) can cross through the niosomes due to their interactions with aggregation, fusion, and adhesion, leading to a large thermodynamic gradient. Our research found that Nio16 had a potent cell inhibition capacity against B16-F10 cells, suggesting that the modified formulation would be beneficial in the treatment of cancer.

Table 2. Cytotoxic effects of formulations NIO 14 to NIO 17 on murine tumor cell line B16F10 cells

Treatment	% Growth inhibition at concentration (µg/mL)				GI_{50} (µg/mL)
10	20	40	80	NIO 14	9.1
9.9	6.7	6.9	2.5311	NIO 15	4.3
− 0.5	− 2.1	7.5	8.05738	NIO 16	1.7
− 0.8	6.7	14.1	40.2238	NIO 17	5.9
5.8	14.8	32.3	38.7176	ADR	− 70.5

Ex vivo and In vivo investigations

Ex vivo skin penetration study

The ex vivo drug permeation study was conducted to gain insights into the potential in vivo performance of RH-loaded niosomes as a transdermal drug delivery system. Employing CLSM, z-stack images were captured to depict optical sections of mice skin at 2 µm intervals, covering a depth span of 0 to 38 µm. Formulations utilizing PBS as a positive control did not exhibit any detectable particles. Likewise, formulations with Plain Rhodamine as a negative control also displayed no observable particles. The findings from this ex vivo skin permeation study, which identified specific depth ranges for particle presence in various niosome formulations, have significant implications for potential applications in skin cancer treatment. Notably, Nio13 (982.3 nm), with particles observed at a depth of 0–6 µm, suggests a potential for superficial skin cancers. Nio14 (783.9 nm), spanning from 4 to 18 µm, may have relevance in treating lesions at slightly greater depths. The formulation Nio15 (707.7 nm), demonstrating distinct

particles between 16 snf 22 μm, could be well-suited for targeting tumors residing slightly deeper within the skin. For more deeply seated skin cancers, Nio16 (671.5 nm), with particles at depths ranging from 24 to 32 μm, may offer a solution. Additionally, Nio17 (597.2 nm), with particles distributed within a broad range of 12 to 38 μm, could be instrumental in addressing skin cancers with varying depths or infiltrative characteristics. The particles of Nio 18 (205.0 nm) will go into systemic circulation. This study suggests that tailoring niosome size and properties to specific depth ranges has the potential to enhance localized drug delivery for skin cancer treatment, increasing the precision and effectiveness of therapeutic interventions. These findings offer valuable insights into the distribution patterns and potential penetration capabilities of the RH-loaded niosomes across different skin layers. CLSM has emerged as a valuable tool for studying the skin's permeation and nanocarrier distribution due to its real-time imaging, multi-depth capabilities, and noninvasive nature. In this study, Rhodamine B (RH) acted as a model lipid-soluble fluorophore, mimicking a hydrophilic drug in niosomes. Before imaging, untreated skin displayed no fluorescence within the fluorophore's detection range. The visualizations (Fig. 9) showcased the widespread distribution of fluorescence across skin layers following treatment with fluorolabeled niosomes. The interaction of proposed surfactant-containing niosomes with skin surface lipids might have facilitated intracellular drug penetration. This study's findings highlight the potential of MTX-loaded niosomes as effective transdermal drug carriers and underscore the utility of CLSM in comprehending their skin permeation behavior.

In vivo histopathological study

The in vivo histopathological examination provided valuable insights into the potential skin irritation of different treatment groups. The untreated control group (Group I) displayed a healthy and intact skin structure with well-defined layers, as seen in Fig. 10. This serves as a baseline for comparison. Skin treated with only the MTX solution (Group II) also showed an intact epidermis and dermis, despite minor congestion in deeper blood vessels. This suggests that the MTX itself might not be a significant skin irritant in this context. Skin sections from groups receiving niosomal formulations (Groups III–VII) revealed a key finding: no epidermal erosion. However, crucially, this erosion was not accompanied by any signs of inflammation, such as edema or erythema. This strongly suggests that the irritation was mild and manageable, likely influenced by the presence of penetration enhancers like Span 60 and Soya PC in the niosome formulations. Importantly, other skin layers remained intact, and no major disruptions were observed beyond the topmost keratinized stratum corneum. While the qualitative observations are valuable, adding a quantitative scoring system to assess the degree of epidermal erosion and congestion across groups could further strengthen the conclusions about niosomal safety. This would provide more objective and comparable data for future studies. These findings support the idea that MTX-loaded niosomes exhibit a relatively acceptable safety profile. The absence of significant inflammation despite mild erosion suggests that the potential for irritation in clinical settings is likely low. However, future studies implementing quantitative scoring would further bolster these conclusions and provide even more robust evidence for the safety of these niosomes as drug delivery systems.

Discussion

The discussion of the study's findings reveals important insights into the formulation and properties of MTX-loaded niosomes, as well as their potential for cancer treatment.

The study's investigation into the impact of formulation components, particularly the ratios of span 60, soya PC, and cholesterol, on niosome properties revealed a complex relationship between these components and vesicle size. The initial decrease in vesicle size with a higher span 60 concentration highlights the surfactant's interfacial activity, facilitating smaller vesicle formation. However, beyond a certain point, increased span 60 content coupled with higher cholesterol led to larger vesicle sizes due to increased rigidity. This observation underscores the delicate balance required between these components to achieve optimal vesicle properties. A possible solution lies in fine-tuning the component ratios to strike the right balance between interfacial activity and vesicle stability.

The study's findings on the encapsulation efficiency of MTX shed light on the influence of sonication time on drug loading. The variations in encapsulation efficiency can be attributed to the interplay of sonication energy, vesicle size, and drug solubility. To address this, a controlled and standardized sonication process should be established, accounting for both drug entrapment and vesicle size optimization.

The FTIR spectroscopy results confirm the compatibility of MTX with niosome components, supporting the formulation's potential for effective drug delivery. This is a significant assurance of the stability and efficacy of the loaded drug within the vesicles.

The DSC thermograms' insights into the thermal behavior of components, including the disappearance of the drug's crystalline peak after encapsulation, suggest a change in the drug state or its encapsulation within the vesicles. While this finding holds promise for sustained drug release, further investigations are warranted to understand the exact mechanism of drug encapsulation and release from the vesicles.

The study's anti-cancer efficacy evaluation revealed the superior activity of the Nio17 formulation against B16-F10 cells, attributed to the presence of span 60 as a permeation enhancer. This underscores the potential of niosomes as effective carriers for anti-cancer agents, particularly when incorporating permeation enhancers.

The ex vivo drug permeation study provided visual evidence of the penetration of RH-loaded niosomes into different skin layers, showcasing their potential for transdermal drug delivery. This underscores the practical relevance of the formulation's structure and composition in facilitating drug penetration through the skin. The presence of particles was observed in Nio13 (without sonication), with an average size of 982.3 nm (nm) visible within 0 to 6 μm (μm). This suggests that sonication may be beneficial for enhancing skin penetration. The formulations with sonication durations of 2, 4, 6, and 8 min (Nio14, Nio15, Nio16, and Nio17, respectively) produced progressively smaller particles at increasing depths. The particle sizes ranged from 783.9 nm (4–18 μm), 707.7 nm (16–22 μm), 671.5 nm (24–32 μm), to 597.2 nm (12–38 μm), indicating improved permeation. The positive results of Nio17, revealing the presence of particles within skin layers rather than in the bloodstream, underscore its potential as a promising candidate for effective drug delivery.

The in vivo histopathological examination indicated no epidermal erosion or inflammation, supporting the formulation's potential for transdermal delivery in skin cancer therapy. However, the absence of severe inflammation suggests the formulation's acceptability for clinical use. This observation highlights the importance of considering local skin effects in the development of transdermal drug delivery systems.

This study offers valuable insights into the formulation and properties of MTX-loaded niosomes, demonstrating their potential as effective transdermal drug carriers for cancer treatment. The findings underscore the need for careful optimization of formulation components and process parameters to achieve desired vesicle characteristics, encapsulation efficiency, and drug release profiles.

Conclusion

This study comprehensively investigated the formulation, properties, and potential of MTX-loaded niosomes for transdermal cancer therapy. The complex interplay of formulation components, particularly span 60, soya PC, and cholesterol, on vesicle size and encapsulation efficiency necessitates fine-tuning to achieve optimal properties. Compatibility between MTX and niosomes was confirmed by FTIR, while DSC hinted at a possible sustained release mechanism. Notably, Nio16 demonstrated superior anti-cancer activity against B16-F10 cells, highlighting the benefits of permeation enhancers. Ex vivo studies visually confirmed niosome penetration into skin layers, with smaller particles achieving deeper penetration. While mild epidermal erosion was observed in vivo, the absence of severe inflammation suggests acceptable safety. This study establishes the promising potential of MTX-loaded niosomes for transdermal cancer treatment, emphasizing the importance of optimizing formulation parameters and exploring complementary techniques like CLSM for further validation. This research lays the groundwork for developing innovative and effective transdermal drug delivery systems for cancer therapy.

In the future, our study will be advanced by comparing niosomes with market-available formulations for skin diseases and other nanovesicles under research. Physicochemical properties of our niosomes will be compared with liposomes, polymeric nanoparticles, and other nanocarriers using standardized in vitro assays, focusing on size, stability, and drug loading capacity. In vitro drug release profiles will be analyzed to understand potential differences in release kinetics and therapeutic effects. Additionally, comparative in vivo studies in relevant animal models will evaluate biodistribution, pharmacokinetics, and efficacy of our niosomes compared to other nanocarriers, considering factors like targeted delivery, systemic exposure, and potential side effects.

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Author contributions

SS performed the investigation, conducted the formal analysis, wrote the original draft, reviewed and edited the manuscript, conceptualized the study, designed the methodology, validated the results, and curated the data. VS and SKK contributed to the conceptualization of the study, designed the methodology, validated the results, conducted the investigation, performed the formal analysis, wrote the original draft, reviewed and edited the manuscript and supervised the project.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

The Committee for Control and Supervision of Experiments on Animals (CPCSEA/SN-10/RN-22/16-02-2020) under the guidance of the Ministry of Social Justice and Empowerment, Government of India, and based on the recommendations of the Institutional Animal Ethical Committee (IAEC) of Dr. H.S. Gour Vishwavidyalaya located in Sagar, Madhya Pradesh, India.

Consent for publication

All authors are agreeing for the publication of manuscript.

Competing of interests

The authors declare that they have no competing interests.

Abbreviations

MTX
Methotrexate
PC
Phosphatidylcholine
TEM
Transmission electron microscopy
FTIR
Fourier transform infrared spectroscopy
DSC
Differential scanning calorimetry
GI50
Growth inhibition concentration at 50%
CLSM
Confocal laser scanning microscopy

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Captopril pretreatment augments diabetogenic response to streptozotocin administration: experimental in vivo rat model

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ABSTRACT (ENGLISH)

Background

Streptozotocin (STZ) is a glucose analogue commonly used for inducing diabetes in experimental animals. This study is intended to investigate the ability of captopril (Cap) pretreatment to augment STZ-induced diabetogenic effect in an experimental rat model. If this hypothesis were proven, Cap administration to rats could reduce the dosage of STZ by augmenting its effect and resulting in a subsequent reduction in STZ cost. Forty-two adult male Wistar rats were randomly divided into seven groups: a control group that fed a normal diet, whereas the other six experimental groups were fed a high-fat diet (HFD). The six groups were then divided into STZ-30, STZ-30-Cap, STZ-40, STZ-40-Cap, STZ-50, and STZ-50-Cap. All Cap-received groups were supplemented with 50 mg/kg Cap orally one hour just before intraperitoneal (I.P.) injection of STZ. 30-STZ, 40-STZ, and 50-STZ-treated groups were injected once with STZ I.P. at doses of 30, 40, and 50 mg/kg, respectively. An intraperitoneal glucose tolerance test (IPGTT) was done. Pancreatic tissue was obtained to measure Tumor necrosis factor alpha (TNF-α), interleukin one beta (IL-1β), and nitric oxide (NO) by enzyme-linked immunosorbent assay (ELISA) and glucose transporter 2 (GLUT2) gene expression by reverse transcription polymerase chain reaction (RT-PCR). Pancreatic sections were examined by hematoxylin and eosin (H&E) stain, and immunohistochemical staining by anti-insulin and anti-TNF-α antibodies.

Results

Results indicated that administration of Cap before STZ in different doses significantly augmented the hyperglycemic state that was evident by intraperitoneal glucose tolerance test, and markedly increased pancreatic pro-inflammatory markers. Histological analysis of islets of Langerhans indicated degeneration with extensive vacuolations associated with a significant decrease in mean area % of insulin immunoreactivity and an increase in optical density of TNF-α immunoreactivity.

Conclusion

These findings pointed to the ability of captopril pretreatment to augment the hyperglycemic state and the diabetogenic response that was induced secondary to STZ injection in an experimental rat model.

FULL TEXT

Introduction

The animal models played a crucial role in studying how disease pathogenesis develops and evaluating novel therapeutic agents. Diabetes mellitus (DM) was one of the disorders that necessitated ongoing creative therapy approaches [1]. DM refers to a set of metabolic illnesses that result in high blood glucose levels and a state of chronic hyperglycemia. In this context, the two most prevalent types of diabetes mellitus are type-1 diabetes and type-2 [2].

Type-1 diabetes is classified as an autoimmune disease due to the attack and destruction of insulin-producing pancreatic β-cells [3], while type-2 diabetes is accompanied by insulin resistance and insufficient insulin secretion due to a lack of appropriate compensation by the beta-cells. Consequently, the selection of appropriate animal models for diabetes research should be contingent upon the specific aspects of the disease under investigation [4]. To induce DM. in animal models, the traditional toxic chemicals that selectively target pancreatic β-cells have been used by many researchers. For instance, alloxan has been utilized to induce β-cell destruction through the production of free radicals. However, alloxan demonstrated several prominent drawbacks, including high animal mortality, the generation of animal ketosis due to the formation of free fatty acids, reversible diabetic states, species-specific resistance to its diabetogenic effects as in guinea pigs, and renal toxicity within a narrow effective dose range [5]. As a result, streptozotocin (STZ) has emerged as a substitute for alloxan due to its more favorable characteristics for diabetes induction [6]. STZ can be used to induce animal models of both type-1 and type-2 diabetes by applying either high or low doses [7]. High doses of STZ can severely impair insulin release resembling what happened in type-1 diabetes and can also lead to the formation of ketone bodies, a feature of uncontrolled type-1 diabetes. In contrast, low doses of STZ can cause mild impairment in insulin secretion that mimics type-2 diabetes. However, the low-dose STZ-induced animal model does not address insulin resistance which is in general

a feature of type-2 diabetes [8].

A model, that combines a high-fat diet (HFD) followed by a low STZ dose to induce peripheral insulin resistance, would closely resemble human type-2 diabetes [9]. Although there are several experimental models of DM reported to date, the HFD-STZ-type-2 diabetes model is still considered the preferable one [10, 11]. The STZ dose-dependent direct and indirect toxicity is still the main significant drawback. Studies reported that some albino rats developed stomach ulcers, decreased bone volume and muscle mass, reproductive problems, hepatotoxicity, and nephrotoxicity at a dose of 65 mg/kg and 100% mortality in Wistar rats due to the lethal end-point at a dose≥ 70 mg/kg [11].

Even while it appears to be easy to induce diabetes in many different animal species with STZ, it is difficult because of factors like unpredictability, high cost, and high mortality rates. Based on their particular experiences, the investigators employ different techniques when implementing this paradigm. The STZ dosage, the way it is administered, the animal species, the gender, and the body weight are all subject to variation. The investigators have made numerous attempts aiming to create more stable and consistent hyperglycemia [12].

The local renin–angiotensin system (RAS) has been discovered to play a crucial role in pancreatic physiology and is involved in pancreatic function. Angiotensin II receptors include two types 1 and 2 (AT1R and AT2R), they have been demonstrated to be present in pancreatic islets, whereas the function of AT2R is still controversial and not fully understood [13, 14].

Recent evidence revealed that RAS could regulate the endocrine and exocrine function of the pancreas and short-term Angiotensin II (Ag II) infusion changes the intra-islet blood flow while the chronic exposure effects are still unknown [15]. Another study found that Ang II has a significant vasoconstrictive effect on islet vasculature and reduces islet blood flow. In contrast, the angiotensin receptor blockers vasodilate islet arterioles and stimulates insulin secretion in response to high glucose levels [16, 17].

Captopril (Cap), an angiotensin-converting enzyme (ACE) inhibitor medication, is used in controlling hypertension, management of left ventricular dysfunction after myocardial infarction, and diabetic nephropathy. Many researchers discovered its role in increased glucose uptake by pancreatic tissues, the exact mechanism is unclear, but a vasodilation effect may be incorporated [18].

This study aimed to investigate the possibility of developing a modified experimental model of diabetes mellitus by estimating the adding effect of captopril on different low doses of STZ in HFD adult Wistar rats. The authors compared the changes in serum glucose levels in response to the intraperitoneal glucose tolerance test (IPGTT), and local pro-inflammatory markers in pancreatic tissues secondary to injecting different doses of STZ that were given either alone or in combination with captopril for inducing animal model diabetes.

Material and methods

Experimental animal &grouping

Forty-two adult male Wistar rats (8–10 weeks in age and 180–200 g weight) were purchased from and housed in the Animal House of the Faculty of Medicine, Cairo University. Animals were left for seven days to acclimate to ordinary environmental living conditions regarding humidity, temperature, and dark/light cycles. Rats had free access to food and water to ensure normal growth and behavior; they were kept in wire mesh cages (three rats in each) before starting the experimental procedures. All animal procedures were done in accordance with the highest International Criteria for Animal Experimentation of Helsinki. The Faculty of Medicine, Cairo University Ethical Approval No is; CU/III/F/38/22.

Drugs and chemicals

STZ was purchased from Sigma-Aldrich, MO, USA, and was given at doses of (30, 40 &50 mg/kg). STZ was dissolved in freshly prepared 0.1 M citrate buffer (pH 4.5; Talpate, Bhosale, Zambare, &Somani). Captopril purchased from Sigma-Aldrich, MO, USA, as a powder, was dissolved in sterile distilled water and was given at 50 mg/kg orally. All other chemicals and reagents were of high analytical grade.

Investigation protocols

Rats were randomly divided into seven groups (six rats each), **Group I**; control normal rats (fed a normal rat chow

(12% fat, 60% carbohydrate, and 28% protein), and the other six main experimental groups were maintained on a HFD; 41% fat, 41% carbohydrate, and 18% protein) for 2 weeks. Then, the overnight-fasted rats in the experimental groups were injected with STZ. The rats were allocated to: **Group II (STZ-30);** the rats in this group received STZ at a dose of 30 mg/kg (I.P.) [19], **Group III (STZ-30-Cap)**; the rats were subjected to the intake of captopril (50 mg/kg orally) [20, 21] one hour just before injecting STZ at a dose of 30 mg/kg, **group IV (STZ-40)**; the rats in this group were subjected to STZ injection at a dose of 40 mg/kg I.P.), **group V (STZ-40-Cap)**: the rats were subjected to oral administration of captopril at the same of group III one hour just before injecting 40 mg/kg of STZ, **group VI (STZ-50)** ; the rats were subjected to STZ injection at a dose of 50 mg/kg (I.P.), and **group VII (STZ-50-Cap)**; the rats were subjected to oral administration of captopril (at the same of group III, and V one hour just before injecting 50 mg/kg of STZ.

At the end of the study, after 28 days (one week allowed for acclimatization of the experimental rats, followed by a two-week span during which the rats were maintained on a HFD). The final week was dedicated to the implementation of the treatment protocol and served as the end-point of the study, the blood pressure of each rat was measured using the tail-cuff method. The animals were fasted overnight for about 12 h, and then the blood samples were obtained from the femoral vein under ketamine/xylazine (60/7.5 mg/kg, i.p) [22] anesthesia using non-heparinized capillary tubes for separation of sera. Samples were kept immediately in ice-chilled, siliconized disposable glass tubes. The serum samples were obtained by centrifuging blood samples at 4,000 g for 15 min at 4 °C and kept at − 80 °C until further analysis. After that, the rats were sacrificed by cervical dislocation under anesthesia, and the pancreatic tissues were excised and fixed in a 10% buffered formalin solution for histopathologic analysis. More treatment protocol clarification is shown in Fig. 1.

Fig. 1 [Images not available. See PDF.]

Schematic illustration of the timeline of scheduled interventions for all examined groups. *STZ* Streptozotocin, *Cap* Captopril

Noninvasive blood pressure measurement

The noninvasive rat tail systolic arterial blood pressure was recorded at the end of the study using a noninvasive blood pressure meter (LE 5002, Harvard Apparatus). The rat is placed in a restrainer and warmed. A pneumatic pulse sensor is attached to its tail. A cuff is placed around the tail, and slowly inflated above the systolic pressure until it causes pulsations to cease, measured by the piezo-electric pulse sensor. The cuff pressure at which pulsations cease is taken to be the SBP in the tail.

Verification of diabetes

Intraperitoneal glucose tolerance test (IPGTT)

All rats were subjected to IPGTT [23, 24]. Male rats were fasted overnight and injected with glucose at a dose of 2 g/kg (I.P.). Blood glucose levels were measured at times of fasting (time zero), 30, 60, and 120 min with a glucometer (GlucoDrTM, All Medicus Co. Ltd, Gyeonggi, Korea) [25].

Biochemical measurement

TNF-α in pancreatic tissue by ELISA

TNF-α in pancreatic tissue protein was assessed by ELISA technique according to kit instructions; Rat TNF-α ELISA Kit, Catalog No: MBS2507393. MyBioSource, Inc. San Diego, CA 92195–3308, USA.

IL-1β in pancreatic tissue by ELISA

IL-1β in pancreatic tissue protein was assessed by ELISA technique according to kit instructions; Rat IL-1β ELISA Kit. Catalog No: MBS824956.MyBioSource, Inc. San Diego, CA 92195–3308.

NO level in pancreatic tissue

NO level in pancreatic tissue was assessed according to kit instruction; Nitric Oxide Assay Kit (Colorimetric). Catalog No: ab65328, Abcam, Waltham, USA.

GLTU2 gene expression in pancreatic tissue by RT-PCR

The pancreatic tissues from all studied groups were homogenized and total RNA was isolated with Gene JET Kit

(Thermo Fisher Scientific Inc., Germany, #K0732). A one-step qRT-PCR reaction was done; for reverse transcription; about 5 µl from the total RNA from each sample (once for the GLUT2 gene and once for ß actin gene) was used with subsequent amplification with Bioline, A median Life Science company, UK (SensiFASTTM SYBR R Hi-ROX) One-step Kit (catalog number PI-50217 V) in a 48-well plate using the Step-one instrument (Applied Biosystems, USA). Thermal profile was as follows: 45 °C for 15 min in one cycle (for cDNA synthesis), 10 min at 95 °C for reverse transcriptase enzyme inactivation, followed by 40 cycles of PCR amplification. 10 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C were adjusted for each cycle.

RNA quantitation assessment was carried out using a spectrophotometer the Nano Drop® (ND)-1000 (Nano Drop Technologies, Inc.) by measuring the absorbance at 260 nm.

RNA purity was assessed by comparing the absorbance ratio using a spectrophotometer the Nano Drop® (ND)-1000 (Nano Drop Technologies, Inc.) as we measured the absorbance at 280 to exclude contamination with protein and reagent that absorbed near 280, the 260/280 ratio was 2, also the absorbance at 230 was measured to exclude contamination with another compound as carbohydrate and phenol that absorb near 230, the 260/230 ratio was 2.1

From the expression of the studied genes, genes were normalized relative to the mean critical threshold (CT) values of ß actin as the housekeeping gene by the Ct method. Primers' sequences for studied genes are listed in Table 1.

Table 1. Primer sequence for a studied gene

Gene	Primer sequence from 5′- 3′
GLTU2	Forward: GCACACTCTTGGCCCGG Reverse: GCCTGGGAAGAAGAGACT
ß actin	Forward: GCA CCA CAC CTT CTA CAA TG Reverse: TGC TTG CTG ATC CAC ATC TG

Pancreatic tissues histological study

The Pancreas was dissected, fixed in 10% formaldehyde solution, and processed into paraffin blocks. Five-micron sections were cut and mounted on glass slides for hematoxylin and eosin (H&E) staining to examine structural changes in islets of Langerhans in the pancreas.

Immunohistochemical staining of the pancreatic tissues

Pancreatic sections were de-paraffinized, hydrated with descending grades of alcohol, and treated with citrate buffer pH 6 in the microwave for antigen retrieval, then incubated overnight with: 1-anti-insulin antibody (ab63820; rabbit polyclonal antibody, dilution (1:100), Abcam, Cambridge, UK). 2-anti-TNF-α receptor (ab220210; mouse monoclonal antibody, dilution (1:100), Abcam, Cambridge, UK). Two drops of biotinylated secondary antibody were applied for 20 min and then two drops of streptavidin peroxidase for 10 min. The reaction was visualized with diaminobenzidine (DAB) as a chromogen and Mayer's hematoxylin as a counter-stain. The primary antibodies were replaced in negative control serial sections with PBS.

Morphometric assessment

The "Leica Qwin 500 C" image analyzer computer system Ltd. (Cambridge, UK) was used to collect the data. The image analyzer was made up of an Olympus color video camera, a colored monitor, and an IBM hard drive that was attached to a light microscope and ran "Leica Qwin 500 C" software. To automatically translate the measurement units (pixels) generated by the image analyzer program into actual micrometer units, the image analyzer was first calibrated.

The mean diameter of the islet of Langerhans, area % of anti-insulin immunoreactivity, and optical density of TNF-α immunoreactivity were assessed in 10 non-overlapping randomly selected high-power fields (×400).

Statistical analysis

The results were obtained as mean ± standard deviation (SD). Statistical analysis for parametric measures was done via one-way analysis of variance (ANOVA) version 25, followed by the Tukey–Kramer multiple comparisons test as a post hoc test. A comparison of glucose levels in each group with time was done using repeated measure ANOVA. Statistical significance was considered when $P<0.05$. Statistical analysis will be carried out using IBM SPSS Statistics for Windows, version 25 (IBM Corp., Armonk, N.Y., USA).

Results

The intraperitoneal glucose tolerance test (IPGTT) and the systolic and diastolic blood pressure result in all studied groups

The blood pressure was recorded both before and after the administration of STZ and Cap to confirm any changes in blood pressure. The findings showed that there were no significant differences in the reading among all studied groups.

As shown in Table 2, all treated groups showed a significant increase in fasting, and estimated blood glucose was measured after 30, 60, and 120 min following I.P. injection of glucose compared to the control group. Moreover, receiving captopril before injecting STZ at 30 mg/kg and 40 mg/kg was able to significantly increase the levels of blood glucose measured at different times compared to STZ-40 and STZ-50, respectively. Additionally, the maximum results of estimated blood glucose were obtained STZ-50-Cap group at different times compared to other groups.

Table 2. Timeline changes in fasting blood sugar, estimated blood glucose 30 min, 60 min, and 120 min (mg/dl) after IPGTT in all studied groups

	Control	STZ-30	STZ-30-Cap	STZ-40	STZ-40-Cap	STZ-50	STZ-50-Cap
Fasting	88.17 ± 1.17	198.17 ± 7.28*	355.5 ± 6.35*#	222.5 ± 10.39*$	380.5 ± 3.94*#€	345 ± 48.2*#€¥	490 ± 10.49*#$€¥@
30 min	144.5 ± 10.56	239.83 ± 9.26*	436.33 ± 22.43*#	259.17 ± 9.35*$	498.83 ± 15.99*#$€	414 ± 28.48*#€¥	487.67 ± 4.97*#$€¥@
60 min	172 ± 3.79	231.5 ± 6.5*	466.67 ± 4.46*#	246.5 ± 3.21*$	523.83 ± 8.84*#$€	421.33 ± 25.15*#$€¥	481.17 ± 9*#€¥@
120 min	116.83 ± 2.64	214.5 ± 4.14*	387.5 ± 82.13*#	227.17 ± 8.18*$	490 ± 5.37*#$€	385.5 ± 20.27*#€¥	506.5 ± 1.38*#$€@

Data presented by mean ± SD No = 6

Statistical analysis was done using one-way ANOVA test followed by Tukey–Kramer post hoc test

*denotes significant difference compared to control, # denotes significant difference compared to STZ-30, $ denotes significant difference compared to STZ-30-Cap, € denotes significant difference compared to STZ-40, ¥ denotes significant difference compared to STZ-40-Cap, and @ denotes significant difference compared to STZ-50

IPGTT Intraperitoneal glucose tolerance test, *STZ* Streptozotocin, *Cap* Captopril

Captopril administration was able to enhance the expression of GLUT2 and increased nitric oxide levels in pancreatic tissues in the studied groups

The expression levels of GLUT2 were estimated in all studied groups and the pre-intake of captopril before the administration of STZ was able to enhance its expression levels that were documented by PCR. Our data documented enhanced expression levels in the STZ-30-Cap compared to STZ-30, and a significant increase in the same parameter in the STZ-40-Cap compared to its corresponding values in the STZ-40 group, additionally significant increase in GLUT2 expression was recorded in the STZ-50-Cap compared to its corresponding values in

the STZ-50 group, without recording any significant difference between STZ-30-Cap, STZ-40-Cap, STZ-50-Cap groups (Table 3). Additionally, the levels of nitric oxide were significantly increased in all STZ-treated groups compared to the control group, and captopril administration before STZ injection was able to significantly increase the levels of NO in these groups compared to the corresponding values of the STZ-treated groups (Table 3).

Table 3. The levels of NO (nmol/ml) and the expression levels of GLUT2 in all studied groups

	Control	STZ-30	STZ-30-Cap	STZ-40	STZ-40-Cap	STZ-50	STZ-50-Cap
NO nmol/ml	10.4 ± 0.21	29.88 ± 6.28*	59.38 ± 5.73*#	30.45 ± 7.6*$	58.03 ± 4.34*#€	33.33 ± 2.98*$¥	61.62 ± 2.4*#€@
GLUT2 relative expression	1.02 ± 0.18	1.07 ± 0.08	1.73 ± 0.12*#	1.02 ± 0.03$	1.77 ± 0.08*#€	1.01 ± 0.1$¥	1.73 ± 0.12*#€@

Data presented by mean ± SD No = 6

Statistical analysis was done using one-way ANOVA test followed by Tukey–Kramer post hoc test

*denotes significant difference compared to control, # denotes significant difference compared to STZ-30, $ denotes significant difference compared to STZ-30-Cap, € denotes significant difference compared to STZ-40, ¥ denotes significant difference compared to STZ-40-Cap, and @ denotes significant difference compared to STZ-50

NO Nitric oxide, GLUT2 Glucose transporter 2, *STZ* Streptozotocin, *Cap* Captopril

Augmenting pro-inflammatory state secondary to captopril pre-intake with an increased dose of STZ

As shown in Fig. 2**,** the levels of pro-inflammatory cytokines (TNF-α and IL-1β) were significantly increased in all STZ and STZ-Cap-treated groups compared to the corresponding results of the control group that denotes the effectiveness of STZ to produce the pro-inflammatory condition**.** Moreover, the captopril intake before STZ administration was able to augment the production of both TNF-α and IL-1β in STZ-30-Cap compared to STZ-30, and STZ-40-Cap compared to STZ-40 and STZ-50-Cap compared to SZT-50.

Fig. 2 [Images not available. See PDF.]

Changes in tissue levels of TNF-α and IL-1β in all studied groups. Data presented as mean values and SD. No = 6. Statistical analysis was done using one-way ANOVA test followed by Tukey–Kramer post hoc test. * denotes significant difference compared to control, # denotes significant difference compared to STZ-30, $ denotes significant difference compared to STZ-30-Cap, € denotes significant difference compared to STZ-40, ¥ denotes significant difference compared to STZ-40-Cap, and @ denotes significant difference compared to STZ-50. *TNF*-α Tumor necrosis alpha, *IL-1*β Interleukin one beta, *STZ* Streptozotocin, *Cap* Captopril

Effect of STZ and captopril pre-intake on pancreatic tissue degeneration

As shown in Fig. 3, the histological examination using H&E staining of the pancreatic tissues demonstrated that the control group showed normal architecture of the pancreas with the islet of Langerhans surrounded by pancreatic acini. The experimental groups, STZ-30; STZ-30-Cap; and STZ-40, exhibited a degenerated islet of Langerhans. However, STZ-40-Cap; STZ-50; and STZ-50-Cap groups revealed massive degeneration of islets of Langerhans with large areas of vacuolations and numerous depleted β-cells.

Fig. 3 [Images not available. See PDF.]

Representative histological photomicrographs of H&E-stained rat pancreatic Sects. (400x). **A** The control group shows a pale-stained islet of Langerhans surrounded by a darkly stained closely packed pancreatic acini. The acini are formed of pyramidal cells displaying apical acidophilia and basal basophilia with vesicular nuclei and prominent nucleoli. **B** &**C** Groups STZ-30 and STZ-30-Cap illustrate degenerated islets of Langerhans with vacuolations

(arrows) and a few depleted β-cells that display darkly stained nuclei (arrow heads). **D** &**E** Group STZ-40 illustrates a degenerated islet of Langerhans with few vacuolations (arrows) and few depleted β-cells that display darkly stained nuclei (arrow heads). Group STZ-40-Cap exhibits more vacuolations (arrows) in islets of Langerhans and more depleted B-cells with darkly stained nuclei (arrow heads). **F** &**G.** Group STZ-50 displays marked degeneration of islets of Langerhans with vacuolations (arrows) and many depleted β-cells. Group STZ-50-Cap shows massive degeneration with vacuolations (arrows) and most of the cells are depleted (arrow heads). **H.** Histogram representing mean area % Islet of Langerhans size; *denotes significant difference compared to control, # denotes significant difference compared to STZ-30, $ denotes significant difference compared to STZ-30-Cap, € denotes significant difference compared to STZ-40, ¥ denotes significant difference compared to STZ-40-Cap, and @ denotes significant difference compared to STZ-50. *STZ* Streptozotocin, *Cap* Captopril

Immunohistochemical assessment of the pancreas stained with anti-insulin

As shown in Fig. 4; the control group revealed strong widespread insulin immunostaining in the islet of Langerhans. All STZ-treated groups exhibited a significant decrease in the mean area % of insulin immunoreactivity in comparison with the control group. β-cells in experimental groups, STZ-30; STZ-30-Cap; and STZ-40, demonstrated moderate heterogeneous insulin immunostaining. STZ-40-Cap group showed weak insulin immunostaining. Moreover, STZ-50 and STZ-50-Cap groups exhibited faint heterogeneous insulin immunostaining in β-cells, revealing a significant decrease in mean area % of insulin immunoreactivity when compared to groups STZ-30; STZ-30-Cap; and STZ-40. Furthermore, the STZ-50-Cap group demonstrated a significant decrease in mean area % in comparison with all experimental groups. A significant difference was recorded between STZ-30 and STZ-30-Cap groups as well as between STZ-40 and STZ-40-Cap groups in addition between STZ-50 and STZ-50-Cap groups.

Fig. 4 [Images not available. See PDF.]

Representative photomicrographs of anti-insulin immunohistochemistry-stained rat pancreatic Sects. (400x). **A** Control group shows a strong homogeneous distribution of insulin immunostaining in β cells. **B** &**C** Groups STZ-30 and STZ-30-Cap illustrate moderate heterogeneous insulin immunostaining in β cells. **D** STZ-40 group exhibits moderate insulin immunostaining in β cells. **E** Group STZ-40-Cap demonstrates weak insulin immunostaining of β cells. **F** &**G** STZ-50 and STZ-50-Cap exhibit faint heterogeneous insulin immunostaining in β cells. **H** Histogram representing mean area % anti-insulin immunostained sections; * denotes significant difference compared to control, # denotes significant difference compared to STZ-30, $ denotes significant difference compared to STZ-30-Cap, € denotes significant difference compared to STZ-40, ¥ denotes significant difference compared to STZ-40-Cap, and @ denotes significant difference compared to STZ-50. *STZ* Streptozotocin, *Cap* Captopril

Immunohistochemical assessment of the pancreas stained with anti-TNF-α

As shown in Fig. 5, β-cells of the control group exhibited negative TNF-α immunostaining. The optical density of TNF-α immunostaining in all experimental groups revealed a significant increase when compared to the control group. β cells of STZ-30; STZ-30-Cap; and STZ-40 groups revealed moderate TNF-α immunostaining. In addition, groups STZ-40-Cap; STZ-50; and STZ-50-Cap illustrated strong TNF-α immunostaining of β cells. STZ-40 and STZ-40-Cap groups revealed a significant increase in the optical density of TNF-α immunostaining when compared to STZ-30 and STZ-30-Cap groups. Moreover, STZ-50 and STZ-50-Cap groups demonstrated a significant increase of optical density of TNF-α immunostaining in comparison with all experimental groups. A significant difference was recorded between STZ-30 and STZ-30-Cap groups as well as between STZ-40 and STZ-40-Cap groups in addition between STZ-50, and STZ-50-Cap groups.

Fig. 5 [Images not available. See PDF.]

Representative photomicrographs of anti-TNF-α immunohistochemistry-stained rat pancreatic Sects. (400x). **A** The control group shows negative immunostaining of β cells with TNF-α. **B** &**C** The groups STZ-30 and STZ-30-Cap exhibit moderate TNF-α immunostaining in β cells. **D** Group STZ-40 exhibits moderate TNF-α immunostaining. **E** Group STZ-40-Cap demonstrates strong TNF-α immunostaining of β cells. **F** STZ-50 illustrates strong TNF-α

immunostaining of β cells. **G** STZ-50-Cap exhibits intensive immunostaining of β cells with TNF-α. **H** Histogram illustrating optical density of anti-TNF-α immunostained sections. * denotes significant difference compared to control, # denotes significant difference compared to STZ-30, $ denotes significant difference compared to STZ-30-Cap, € denotes significant difference compared to STZ-40, ¥ denotes significant difference compared to STZ-40-Cap, and @ denotes significant difference compared to STZ-50. *TNF*-α Tumor necrosis alpha, *STZ* Streptozotocin, *Cap* Captopril

Mean area % of insulin antibody immunoreactivity result

In addition, STZ-30-Cap, STZ-40-Cap, and STZ-50-Cap revealed a significant decrease when compared to group STZ-30, STZ-40, and STZ-50, respectively (Fig. 4).

Optical density of TNF-α antibody immunoreactivity result

The optical density of TNF-α immunoreactivity in all experimental groups revealed a significant increase when compared to the control group. Groups STZ-30-Cap, STZ-40-Cap, and STZ-50-Cap exhibited a significant increase in comparison with groups STZ-30, STZ-40, and STZ-50, respectively (Fig. 5).

Discussion

To our knowledge, this study is the first to investigate the possibility of developing a modified experimental model of DM by estimating the adding effect of captopril on different low doses of STZ in HFD adult Wistar rats. Our goal was to reduce the high doses of STZ and to reduce the cost of the experimental protocols.

The components of RAS have been proven to be expressed locally in rodent and human pancreatic islets a long time ago. Twenty years ago, researchers [26] discovered the intrinsic angiotensin system in dog exocrine pancreas, and numerous data support the idea of an intrinsic RAS in the rat pancreas indicating its role in pancreatic function regulation and insulin secretion [27].

In this study, we tried to explore a new modified experimental model for the induction of DM in which we investigated the effect of the addition of ACE inhibitor, captopril, to different doses (30, 40, &50 mg/kg) of STZ aiming to augment the diabetic response caused by low doses of STZ by combining it with captopril. This approach assists in achieving a more efficient model of diabetes while reducing the hazards associated with giving high doses of STZ; which can sometimes be lethal to the animals.

Our results were consistent with our working hypothesis as the administration of 50 mg/kg of captopril just an hour before the I.P. injection of 30 mg/kg STZ to the HFD rats resulted in a significant increase in both fasting and postprandial glucose levels more than in groups that were injected with STZ alone and all animals were still alive till the end of the experiment. However, there is some mortality at the other two higher doses (40 &50 mg/kg) in STZ combined with captopril. Captopril is a medication used as an antihypertensive drug that prevents the conversion of angiotensin I to the potent vasoconstrictor angiotensin II [28]. However, its precise mechanism in producing hyperglycemia is not evident but a long time ago, several studies have suggested the relationship between RAS and both pancreatic and islet blood flow.

Studies revealed that islet RAS exhibits a significant role in regulating islet glucose-stimulated insulin release and the locally produced Ang II markedly impair islet blood flow and blocks glucose-induced insulin secretion. RAS antagonists, such as enalaprilat and irbesartan (an AT1 receptor antagonist), can selectively augment pancreatic islet blood flow and improve glucose-stimulated insulin release [29].

A study was done by Olsson et al. [30] to investigate the effect of Ang II on revascularization and local blood flow regulation in transplanted rat pancreatic islets and they concluded that Ang II decreased the vascular conductance of the islet grafts in both nondiabetic and diabetic animals. Carlsson et al. [29] suggested that RAS has marked impacts on the growth, differentiation, secretion, and insulin sensitivity of pancreatic islets, which in turn affect glucose uptake, metabolism, and pathogenesis of DM. Campbell and his colleague [31] 1986 have reported that mRNA encoding angiotensinogen, renin, and Ang II have been discovered in the pancreas of various species. This Ang II can adversely influence pancreatic and islet blood flow through the vasoconstrictive mechanism. A further study was conducted by Lau et al. [32], which used a double immunostaining technique to detect high-affinity binding sites for Ang. Results showed that Ang II was specifically located in islet beta-cells, and it was found to block

glucose-induced insulin release either directly by inhibiting β-cell insulin exocytosis or indirectly through suppressing islet blood perfusion. This effect was fully reversed by losartan indicating a potential role of locally produced pancreatic Ang II in glucose homeostasis. Based on these earlier studies, RAS inhibitors may have a promising role in increasing local pancreatic and islet blood flow which may increase glucose and glucose analog (STZ) uptake through the GLUT2 mechanism. According to these prior studies, the authors of the current study found that the biochemical and both histological and immunohistochemical findings support this theory.

In the present study, the histological examination of pancreatic sections revealed degeneration of islets of Langerhans with β-cell necrosis after STZ administration which is augmented with captopril intake. These results agree with Raza and John [33], stating that administration of STZ at low doses results in β-cell dysfunction and apoptosis while at higher doses leads to β-cell necrosis. Various data explained that STZ induces diabetes in experimental animals through different pathways, The first mechanism involves DNA fragmentation due to the STZ nitrosourea moiety, the second is releasing nitric oxide (NO), the third is creating reactive oxygen species (ROS) and inducing oxidative stress, the fourth mechanism is inhibiting the activity of the glycoside hydrolase O-GlcNAcase enzyme in the β-cell, which is responsible for removing O-GlcNA from proteins. This produces irreversible O-glycosylation of intracellular proteins causing β-cell apoptosis [34].

In addition, STZ contains deoxyglucose molecules that are very reactive and can produce direct cytotoxic damage on pancreatic β-cells. It targets the GLUT2 receptor, which is copious on β-cells plasma membranes making them its primary target [35]. Hence the STZ mechanism begins with GLUT2, a transmembrane carrier protein found in β-cells of the pancreas, the absorptive epithelial cells of the intestinal mucosa, liver, and renal cells [36]. The current results showed that the administration of captopril before STZ exposure resulted in more damage of β-cells, this may be due to enhanced expression of GLUT2 in pancreatic tissues of all groups regardless of the dosage compared to the groups that only received STZ.

Furthermore, this could be attributed to the fact that pancreatic β-cells selectively accumulate STZ via GLUT2 and STZ damage to the β-cells is dependent on GLUT2 expression [37].

The contribution of NO and oxidative stress is a crucial aspect of STZ toxicity [38]. Since β-cells have lower levels of enzymes that scavenge NO and free radicals, they are particularly vulnerable to damage caused by NO and free radicals [39]. In our study, we found that NO levels in pancreatic tissues were increased significantly in all groups that administered STZ. This increase was particularly significant in the STZ-Cap groups, and this may be related to the marked uptake of STZ by captopril. Results were supported by the work done by Eleazu et al. [40] who explained the mechanism of increased NO as the nitrosamine group found in STZ serves as an intracellular donor of NO in pancreatic β-cells, and the amount of NO released locally in pancreatic tissues increased with a high level of STZ. When released in excessive amounts NO hinders the action DNA enzyme, aconitase, which ultimately leads to alkylation and damage of DNA. Aconitase is an enzyme that safeguards mitochondrial DNA from degradation. This enzyme can be inhibited by various ROS as well as reactive nitrogen species [41].

Selective and progressive destruction of insulin-secreting β-cells secondary to the diabetogenic action of STZ can enhance local pancreatic infiltration with inflammatory cells and cytokines, such as TNF-α, IL-1β, and interferon-γ (IFN-γ). Furthermore, IFN-γ and TNF-α, either alone or in combination, produce high levels of NO in pancreatic cells [42, 43].

The current results reported a significant increase in pancreatic TNF-α and IL-1β levels in STZ and STZ-Cap-treated groups which reflect the inflammatory state of the pancreatic tissues. Moreover, the captopril intake before STZ administration was able to augment the production of both TNF-α and IL-1β in STZ-Cap which might be due to increased STZ uptake. Also, histopathological findings of the pancreas showed a significant degeneration of islets of Langerhans and an increase in the optical density of TNF-α immunoreactivity at the doses 30 mg/kg in both STZ and STZ-Cap and the degree of degeneration increased by increase STZ-Cap doses. Also, the mean area % of anti-insulin immunoreactivity had a significant decrease in all groups STZ and STZ-Cap. This decrease ranges from moderate heterogeneous insulin immunostaining in β-cells to faint immunostaining according to STZ doses. Our results are consistent with Akash et al. [44] who denoted that the first pro-inflammatory cytokine that was recognized

due to its involvement in insulin resistance pathogenesis and T2DM was TNF-α and the release of other cytokines like IL-6 and IL-1β is enhanced by the pro-inflammatory TNF-α. During acute inflammation, the elevation in both TNF-α and IL-1β impairs β-cell activity and disrupts the insulin signaling pathway.

In conclusion, our study suggests that captopril may be a crucial regulator of glucose analog uptake (STZ) by β-cell. We found that the prior administration of captopril significantly enhanced the effect of low-dose STZ, helped the induction of diabetes, and decreased the mortality rate that occurred at the other higher doses this could make it a potential novel modified experimental model for the induction of diabetes in experimental animals.

Limitation and future recommendations

Future work is highly recommended to elucidate the long-term effect of coadministration of captopril and STZ regarding the state of hyperglycemia. We combined captopril with different doses of STZ, and we found that the intake of STZ at a dose of 40 mg/kg produced the same results of 50 mg/kg. By introducing the appropriate dose and route of administration for STZ and Alloxan chemical compounds, it could be possible to produce an optimal experimental model for type II DM with a low dose of STZ in rats. However, the long-term effect on plasma glucose and insulin levels, and islet morphology was not investigated.

In addition, it is highly recommended to investigate the effect of the combined treatment protocol versus STZ on the liver and kidney.

Author contributions

The authors declare that all data were generated in-house and that no paper mill was used. All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Ateyya Hayam; Harb Inas; ShamsEldeen Asmaa; Adel Sara; Samir Samaa; Rashed Laila; Mostafa Abeer. HI., AH., and S A. conceived and designed research. AH, HI, and HS conducted experiments. RL, SA, and KS contributed new reagents or analytical tools. AH, HI, AM, and AH analyzed data. AH and SA wrote the manuscript. All authors read and approved the manuscript.

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Availability of data and materials

The data that support the findings of this study are available from the authors upon reasonable request.

Declarations

Ethics approval and consent to participate

The study was done in accordance with the highest International Criteria of Animal Experimentation of Helsinki and was approved by the animal house of the faculty of medicine, Cairo University, approval No; CU/III/F/38/22. The authors confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal.

Consent for publication

Not applicable.

Competing interests

We would like to declare that there were no conflicts of interest in conducting this research.

Abbreviations

STZ

Streptozotocin

Cap

Captopril

IPGTT

Intraperitoneal glucose tolerance test

TNF-α

Tumor necrosis factor alpha

IL-1β

Interleukin one beta
ELISA
Enzyme-linked immunosorbent assay
GLUT2
Glucose transporter 2
RT-PCR
Reverse transcription polymerase chain reaction
H&E
Hematoxylin and eosin
DM
Diabetes mellitus
RAS
Renin–angiotensin system
AT1R
Angiotensin II receptor types 1
AT2R
Angiotensin II receptor types 2
ACE
Angiotensin converting enzyme
ANOVA
Analysis of variance
NO
Nitric oxide
ROS
Reactive oxygen species

Publisher's Note

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Optimizing organically nano-fabricated Ni metal complexes for enhanced antioxidant and anticancer activity using response surface methodology

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ABSTRACT (ENGLISH)

Background

Researchers, prompted by the toxicity and side effects associated with cisplatin, are exploring alternative approaches for developing transition metal-based anticancer agents. Employing a green biochemical approach, we transformed Nickel pyridine dicarboxylic acid compounds into the nanoscale using the aqueous extract of Macrotyloma uniflorum (horse gram).

Results

Characterization of the biosynthesized nanoparticles involved electronic and IR spectroscopy. A scanning electron microscope revealed a predominant spherical shape for most Nickel nanoparticles (Ni-NPs), with XRD patterns

indicating particle sizes ranging from approximately 30–150 nm. The nanoparticles were evaluated for their free radical scavenging efficiency and in vitro anti-malignant properties against HeLa and A549 cancer cell lines. Numerical optimization of the DPPH and MTT assays was conducted using response surface methodology (RSM), focusing on the effects of 3,4-pyridine dicarboxylic acid (ML_1), 2,4-pyridine dicarboxylic acid (ML_2), nickel nanoparticles concentration, and temperature. In this investigation, the incorporation of Horse Gram seed extract (Macrotyloma uniflorum) has unveiled its abundance in phenolic and flavonoid compounds, widely acknowledged for their robust antioxidant activity in the existing literature.

Conclusion

The present study highlights the potential for refining the bio-toxicity and biochemical attributes of Ni-NPs to pave the way for a new generation of versatile anticancer agents with clinically established efficacy. Notably, the anticipated data closely corresponds with experimental outcomes, reinforcing the trustworthiness and validity of the RSM model for examining anticancer and antioxidant properties in this context. ML_2 exhibited heightened antioxidant and anticancer activities in comparison to ML_1 nanoparticles.

FULL TEXT

Background

Nanoparticles derived from metals exhibit a range of advantageous properties, including excellent conductivity, a significant surface-to-volume ratio, and notable nano-plasmonic characteristics [1]. The extensive study of metal nanoparticles is driven by their potential applications in sensor devices, bio-devices, data storage, catalytic processes, and spectrophotometric techniques [2]. Biogenic nano-sized particles obtained from plant-based materials offer a straightforward and time-efficient synthesis method, with plant extracts proving more conducive to size reduction compared to microbiological cultures [3]. Literature supports the efficacy of botanical extracts in treating skin diseases, outperforming results obtained from various microbes [4].

In this research, the focus is on exploring different ligands for the creation of Ni-nanoparticles. Two isomers of pyridine dicarboxylic acid, namely pyridine-2, 4-dicarboxylic acid (Lutidinic acid) and pyridine-3,4-dicarboxylic acid (Cinchomeronic acid), have been selected as ligands for the study in conjunction with Nickel metal. Lutidinic Acid, recognized as a corrosion inhibitor [5], is also noted for its cytotoxic nature [6]. The objective is to examine the outcomes of these ligands in the synthesis of Ni nanoparticles.

The rationale behind selecting the organic moiety pyridine-2, 4-dicarboxylic acid and its derivatives for the study is clear. These compounds have demonstrated significant effects on physiological activity, acting as immuno-suppressants [7] and fibrous-repressive drugs crucial for the initiation and growth of certain plant families [8]. Additionally, they play a protective role by preserving specific enzymes in the cells of Bacillus subtilis species under temperature reduction conditions [9]. Notably, the pyridine compound with 2,4-dicarboxylic acid features structure identical to 2-oxoglutarate, a well-known inhibitor of 2-oxoglutarate-dependent dioxygenases. This inhibition, as observed in the growth of tomato seedlings exposed to various concentrations of pyridine dicarboxylic acid, resulted in diminished root size and smaller epicotyl [10].

Bio-nanotechnology involves the synthesis of nanoparticles through the utilization of organic biomolecules, encompassing living organisms such as fungi, bacteria, herb, yeast, as well as various naturally occurring moieties like proteins, peptides, sugar and vitamins [11, 12].

The integration of physical and chemical methodologies with fundamental standards, such as redox reactions, in the presence of biological adjuvants or natural phytonutrients, results in the production of nanoparticles with specific functions [13]. The biological synthesis of nanoparticles offers an environmentally friendly, uncomplicated, and cost-effective approach for researchers. This method also possesses the advantage of stabilizing nanoparticles through the utilization of plant secondary metabolites, serve equally reducing and capping molecules. Notably, nanoparticles produced using green technology exhibit minimal toxicity compared to chemically prepared counterparts, making them efficient carriers for drug delivery systems in in vivo applications [14].

Nickel nanoparticles, for instance, show promising applications in various fields including magnetism [15], microelectronics, power skills [16], and biomedical applications [17]. Due to their rapid reactivity, ease of operation,

and environmentally friendly properties, nanoparticles play a pivotal role in accelerating various organic reactions. These include oxidative coupling of thiols with multiple reaction pathways [18], reduction of aldehydes and ketones [19], hydrogenation of olefins [20], preparation of stilbenes through alcohol by Wittig-olefination [21], and α-alkylation of ketones [22]. Furthermore, they serve as catalysts for the decomposition of ammonia and its products [23]. A cutting-edge application of nanoparticles involves the fabrication of nanotubes, particularly carbon nanotubes (CNTs) [24].

The literature extensively covers the biological synthesis, characteristics, and applications of both Ni and Ni-oxide nanoparticles, with numerous articles and reviews focusing on environmentally friendly approaches for their preparation [25]. Nasseri et al. presented a method for synthesizing NiO nanoparticles using an aqueous extract of Tamarix serotina, showcasing their catalytic properties and confirming the nanoparticles' size to be in the range of 10–15 nm with a cuboid shape [26].

Presently, various plants segments, including leaves, flowers, seeds, fruits, barks, and peels, are employed for distillation to produce nanoparticles [27]. The plant distillate, rich in phytonutrients, antioxidants, and essential organic compounds, holds potential therapeutic significance [28, 29]. Over the past decade, the preparation and fabrication of nickel oxide nanoparticles have been continuously advancing, exploring their applications in various natural biological systems [30].

A strong statistical technique for experiment design and parameter optimisation is response surface methodology (RSM). Using process parameters, this can be utilized to create an accurate model for the response function in the optimal area [31]. Minimum quantity of research has been documented using RSM to optimize different ligands as parameters.

Our summary encompasses the accomplishments in the eco-friendly biotechnical preparation of Nickel nanoparticles and explores the influence of organic moieties to the physical characteristics of newly synthesized Ni-nanoparticles. Additionally, we present findings on the relative influence of various ligands using RSM, examining their structural effects and the biological properties of organically synthesized Nickel nanoparticles.

Methods

Chemical procurements

Chemicals are sourced from Sigma-Aldrich and employed in chemical reactions, with all solvents subjected to drying and additional purification through standard methods.

Synthesis of Ni complex

The compounds used in this study were obtained from the Department of Chemistry at the University of Rajasthan. The template condensation process was applied to produce Nickel (II) macrocyclic complexes, specifically utilizing benzyl dihydrazone with 3,4-pyridine dicarboxylic acid (ML_1) and 2,4-pyridine dicarboxylic acid (ML_2) in the presence of metal chloride. The synthesis followed established protocols as described in the literature. Subsequently, these complexes were further converted into nano-sized structures within our laboratory for their several applications. The illustrated macrocyclic structures are depicted in Fig. 1.

Fig. 1 [Images not available. See PDF.]

Structures of the macro cyclic complexes

Preparation of plant extract

Macrotyloma uniflorum is acquired from the supermarket, cleaned with deionized H_2O, and drenched in distilled H_2O at 40 °C for 12 h. Subsequently, it undergoes a germination process for 48 h at 30 °C, with regular moistening using deionized H_2O every half day. The sprouts are then crushed to form a dense paste by incorporating phosphate buffer solutions (pH 8). The resultant blend is strained through a twin layer of whatman No.1 filter paper, and the filtrate was centrifuged at 12,000 rotations per minute for 8 min below 40 °C. The weightless floating liquid is collected, and the extract solution is employed for the reduction of compounds into nanoform (Fig. 2).

Fig. 2 [Images not available. See PDF.]

Synthesis of extract of *Macrotyloma uniflorum*

Synthesis of Ni nanoparticles

The synthesis of Ni nanoparticles through a green route involves mixing the seed extract with the specific quantity of aqueous solution of nickel compounds, followed by allowing mixture to remain undisturbed until a noticeable colour change indicates nanoparticle formation. This process occurs either at room temperature or at 60–70 °C, depending on specific requirements.

For the microwave-assisted synthesis, a reduced amount of extraction solvent and a shorter reaction time yield efficient results. The reaction mixture is heated in a microwave at a power of 100–150 W for about 1–2 min. Later the extract undergoes centrifugation to room temperature, and the resulting precipitate is dried to obtain the desired metal nanoparticles. Notably, the percentage yield of nanoparticles from both synthesis methods was found to be nearly identical.

Utilizing an aqueous solution comprising plant material and Ni metal complexes in a 10:1 ratio, nanoparticles were synthesized following literature guidelines [32]. The mixture underwent incubation over a water bath, with careful pH control using 1 Normal H_3PO_4. Within 2–3 h, a distinct colour transformation from yellow to dark brown signified the conversion of the mixture into Nickel nanoparticles (Fig. 3).

Fig. 3 [Images not available. See PDF.]

Synthesis of Ni nanoparticles

The synthesized nanoparticles were subsequently subjected to electronic spectroscopy analysis. The size and crystallinity of Ni-nanoparticles were determined through XRD. The surface morphology of Ni-NPs was then examined using a scanning electron microscope (SEM) to obtain micro/nano-images of the complexes (ML_1 and ML_2). SEM analysis revealed that the Ni-nanoparticles exhibited a round shape within the nano size range. Furthermore, the nanoparticles have been evaluated for their anti-malignant action.

Statistical analysis (factorial design and optimization)

The experimental strategy, data scrutiny, and statistical optimization were carried out using Design-Expert software from Stat-Ease, USA. The objectives were to minimize the absorbance level in the sample, employing three variables: ML_1 with concentrations ranging from 0 to 60 µg/l(ppb), ML_2 the concentrations range from 0 to 60 µg/l, and temperature varying from 20 to 50 °C. These variables were explored at three levels, and the absorbance value was observed as the response.

Design-Expert software generated a 2^3 factorial design consisting of 20 experiments to systematically explore the parameter space. Table 1 presents the range and variables—both in actual and coded forms—that were investigated. Notably, the chosen mid-level values for ML_1 concentration, ML_2 concentration, and temperature were 30 µg/l, 30 µg/l, and 35 °C, respectively.

Table 1. Depiction of independent variable levels in both actual and coded formats

Variables (independent)	Signs	Levels of code		
−1	0	+1	ML_1 concentration (µg/l)	X1
0	30	60	ML_2 concentration (µg/l)	X2

0	30	60	Temperature (°C)	X3

The outcomes of the experiments were displayed using a second-order polynomial equation.

1

$$Y=\beta 0+\sum i=13\beta iXi+\sum i=13\beta iiX12+\sum\sum i\neq j=13\beta ijXiXj$$

In the context of the regression equation, where *Y* denotes the anticipated value of the response variable and *β* denotes the coefficient, the outcomes were analysed using Design Expert. The influence of independent terms on dependent variables was evaluated, and through resolving the regression equation and scrutinizing the graphs, optimal conditions were determined. These optimal conditions were then employed to assess antioxidant and anticancer activities. The experimentally acquired responses were juxtaposed with the numerically predicted counterparts for comparison. To statistically assess the variance between mean values at a 95% confidence interval, analysis of variance (ANOVA) was employed. As per the quadratic model of ANOVA, the significance of model terms is signified by "Prob>*F*" values below 0.0500.

Antioxidant activity

Antioxidant estimation employs the use of 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), a compound that aids in assessing the radical capacities of antioxidants [33]. The assessment relies on the capacity of antioxidants to diminish DPPH, measured through the DPPH radical assay principle. This involves determining the reduction in optical density (Abs) at 517 nm using a spectrophotometer after the interaction with nanoparticles (NPs). The reduction in DPPH colour during the reaction is tracked, and the antioxidant assay is computed through spectrometric analysis. The percentage of DPPH radical scavenging is calculated using the equation provided below [34].

The DPPH scavenging percentage is determined using the following formula:

$$DPPHscavenging\%=100\times Absorbanceofcontrol-AbsorbanceofsampleAbsorbanceofcontrol$$

Here Absorbance of control represents the absorbance before adding test sample to DPPH and Absorbance of sample is the absorbance after reaction has taken place between *DPPH* and test sample.

The formula quantifies the reduction in absorbance due to the scavenging activity of the tested sample against the DPPH radical.

Anticancer activity

Upon procurement of cell lines from NCCS, Pune, the cells were maintained in DMEM augmented with 10% Fetal Bovine Serum (FBS) with antibiotic drugs (0.5 mL^{-1} penicillin/streptomycin) at 38 °C in a 4–5% CO_2/97% air atmosphere. To examine the MTT assay, cells were planted in a 96-well plate at a density of 5.0×10^3 cells/well in 100 µL of culture and allowed to incubate for 8–12 h at 35–40 °C, as previously documented [35]. To assess cell viability, three independent triplicate experiments was conducted three times using six concentrations of test chemicals ranging from 5 to100 µg/mL. After 24 h of incubation, each treatment was removed, and fresh media with varying concentrations of test compounds were added. Subsequently, the test solution was replaced with fresh media containing MTT solution (0.5 mg/mL), and the plates were further incubated at 35–37 °C for three hours. Following the reduction of MTT salt to chromophore formazan crystals, a precipitate was formed at the end of the incubation period. This conversion was facilitated by cells with metabolically active mitochondria. The absorbance of solubilized crystals in DMSO was then measured at 560 nm using a microplate reader.%Inhibition=100×Control-TreatmentControl

Characterization of nanoparticles

The prepared samples underwent characterization using various analytical techniques. UV–visible spectroscopy was carried out using a UV1800S instrument from Shimadzu, Japan. SEM analysis was performed using a JEM-IT 800 SHL version, Japan. Fourier-transform infrared (FTIR) analysis was conducted using an Alpha-T instrument from Bruker. X-ray diffraction (XRD) analysis was accomplished using the X'Pert PRO XRD PW 3040 system.

Results

IR-spectroscopy

IR spectroscopy offers high reliability in characterizing the bulk of nanoparticles. By estimating vibrations of functional groups on their surface, FTIR, in particular, is incredibly versatile for surface analysis of nanoparticles in specific conditions, enabling determination of their surface chemical composition with precision.

The IR spectra analysis of both metal nanoparticle complexes was conducted to elucidate their structural features and bonding interactions. Remarkably, the spectra of the complexes show absence of bands corresponding to ν_{as} ($-NH_2$) at 3360 cm^{-1}, ν_{as} ($-NH_2$) at 3280 cm^{-1}, and ν (C=O) with the range of 1680–1690 cm^{-1}. This absence in the spectra indicates the condensation process and the construction of a macrocyclic structure. Peaks observed in the vicinity of 1648–1580 cm^{-1} were accredited to ν(C=N). The band position for ν(C=N) was found to be lower than the typical values associated with azomethine groups, supporting the inference of coordination of the group with the metal atom and the creation of macrocyclic complexes. Furthermore, the absorption bands corresponding to the phenyl ring were identified in the regions of 1465–1495 cm^{-1} and 1355–1390 cm^{-1}, assigned to $\nu_{asym}C_6H_5$ and $\nu_{sym}C_6H_5$, respectively (Fig. 4).

Fig. 4 [Images not available. See PDF.]

IR-spectra of Ni-NPs of ML_1 and ML_2

Electronic spectra of Ni-NPs

UV–Visible spectroscopy is a powerful method for studying the growth of metal nanoparticles within a polymer network or the formation of a polymer network around a metal nanoparticle core. UV–Visible spectroscopy is commonly employed to investigate the plasmonic resonance of nanoparticles. By analysing absorbance data in the range of 200–700 nm, we have confirmed the formation of Ni nanoparticles and their plasmonic resonance. This enables us to characterize and confirm the presence of reduced Ni metal in the form of nanoparticles.

The observation of electron oscillation points to the occurrence of surface plasmon resonance (SPR), where absorption bands are evident. The phenomenon arises from the absorption of UV light by Ni-NPs, leading to consistent oscillation of conduction electrons. This resonance is achieved when the frequency of surface electrons of metal NPs aligns with that of incident photons. The UV–Visible spectrophotometer is employed to analyse the optical density of the absorbed light [36] (Fig. 5).

Fig. 5 [Images not available. See PDF.]

UV–Vis spectra of Ni-NPs of ML_1 and ML_2

XRD analysis

X-ray diffraction (XRD) is a technique utilized to analyse the crystalline properties of materials, providing insights into their structural characteristics, phase nature, lattice parameters, and grain size. The lattice parameter is determined through the application of the Scherrer equation, which involves assessing the broadening of the most prominent peak observed in an XRD pattern for a given sample.

The XRD spectra of Nickel-nanoparticles, synthesized through the reduction of Ni metal complexes (ML_1 and ML_2) using the seed extract of *Macrotyloma uniflorum*, were examined. For the nano-particles of ML_1 compound, five unique diffraction peaks were identified with corresponding 2θ values of 18.85, 20.56, 22.55, 17.65, and 16.17. Particularly, the peak at 18.85° exhibited the maximum intensity. Similarly, the XRD spectrum of the ML_2 compound displayed six characteristic diffraction peaks at 2θ values of 31.13, 22.38, 19.82, 25.1, 27.3, and 16.5, with the peak at 31.13° demonstrating the highest intensity (Fig. 6).

Fig. 6 [Images not available. See PDF.]

XRD spectra of Ni-NPs of ML_1 and ML_2

SEM (scanning electron microscope) of nickel-NPs

SEM is a powerful technique used for analysing particle characteristics, such as size, shape, and texture with high precision. Operating with only small amounts of material, typically in the milligram range, SEM employs a focused electron beam that scans the prepared sample in a series of parallel tracks. Figures 7 and 8 depict SEM images of

two distinct metal nanoparticles.

Fig. 7 [Images not available. See PDF.]

SEM images of ML_1 NPs

Fig. 8 [Images not available. See PDF.]

SEM images of ML_2 NPs

Statistical analysis using response surface methodology

Response surface methodology (RSM) stands out as a highly efficient technique within the realm of design of experiments (DOE). Its primary goal is to streamline the optimization of crucial parameters to achieve an optimal response while ensuring a robust model fit. By employing RSM, we have effectively optimize key factors such as ML_1 concentration, ML_2 concentration, and temperature, all while conducting a limited number of experiments (20). This approach is particularly advantageous as it avoids the need for an extensive array of experiments (Figs. 9).

Fig. 9 [Images not available. See PDF.]

a 3D plot for absorbance as a function of ML_1 and ML_2 concentration. **b** 3D plot for absorbance as a function of ML_1 concentration and temperature. **c** 3D plot for absorbance as a function of ML_2 concentration and temperature. **d** Predicted versus actual values of absorbance

The design table was obtained (Table 2) and experiments were conducted following the conditions of the table. The absorbance was measured and a quadratic model with 20 runs was built. It was investigated how design process parameters affected absorbance.

Table 2. Central composite design of input variables with responses

S. no	Independent variables			Response	
Factor 1 (ML_1, µg/l)	Factor 2 (ML_2, µg/l)	Factor 3 (Temperature, °C)	Experimental (Absorbance)	Predicted (Absorbance)	1
60.00	30.00	35.00	0.66	0.7	2
30.00	60.00	35.00	0.74	0.75	3
0.00	0.00	20.00	0.92	0.94	4
30.00	30.00	50.00	0.81	0.807	5
30.00	30.00	35.00	0.74	0.75	6
30.00	30.00	35.00	0.74	0.75	7
0.00	60.00	50.00	0.92	0.94	8
60.00	0.00	50.00	0.81	0.84	9
60.00	60.00	50.00	0.83	0.81	10

0.00	60.00	20.00	0.85	0.86	11
30.00	30.00	35.00	0.73	0.75	12
60.00	60.00	20.00	0.67	0.68	13
30.00	30.00	35.00	0.78	0.76	14
30.00	30.00	35.00	0.77	0.75	15
30.00	30.00	20.00	0.80	0.78	16
0.00	30.00	35.00	0.89	0.86	17
0.00	0.00	50.00	0.95	0.94	18
60.00	0.00	20.00	0.78	0.75	19
30.00	0.00	35.00	0.76	0.77	20

In this context, key model terms include ML_1 and ML_2 concentrations, temperature, and the interaction term involving temperature. The model's importance is substantiated by an *F*-value of 15.05. Furthermore, the "Lack of Fit *F*-value" of 3.18 suggests that the lack of fit is not significantly different from pure error, underscoring the adequacy of the model. This signifies that the model fits the data well. This indicates a good fit of the model to the data. The coefficient of determination (R^2) for the response is 93%, affirming the appropriateness of the applied models. The adjusted coefficient of determination (Adj. R^2) for the response is determined to be 86%. As a result, it can be said that the experimental value meets Eq. (2) and the regression coefficients fit into a second-order polynomial equation.

2

Y=1.209-0.00546x1-0.00267x2-0.01855x3+1.388×10-6x1x2+2.5×10-5x1x3+4.722×10-5x2x3+3.232×10-5x12+4.545×10-6x22+2.626×10-4x32

In the given equation, where *Y* represents absorbance, x_1 stands for ML_1 concentration, x_2 for ML_2 concentration, and x_3 for temperature; it is noteworthy that concentration of ML_1 and ML_2 as well as temperature all exhibit a negative impact on absorbance. In order to explore the impact of interactions, 3D plots were created by graphing absorbance versus independent variables while maintaining optimal circumstances for the other variables. These graphs effectively interpreted the interactions between the two factors.

Variables optimization and experimental validation

To guarantee the precision and dependability of the formulated model, experiments were conducted to compare predicted and actual responses, as shown in Fig. 11. Once validated, the model was employed to optimize conditions for maximum antioxidant and anticancer activities. As depicted in Fig. 10a, an increase in ML_1 concentration up to approximately 40 µg/l resulted in a decrease in absorbance. This suggests that higher concentrations of ML_1 may induce cytotoxic effects on the cells, leading to a decline in cell viability, reflected by lower absorbance measurements and further increases in ML_1 concentration did not significantly affect absorbance. Conversely, the increase in ML_2 concentration did not exhibit a notable change in absorbance, possibly indicating that ML_2 concentration had already reached its saturation point (Table 3).

Table 3. Analysis of variance with the second order polynomial fit equation

Source	Sum of squares	DF	Mean square	*F* value	*p* Value
Model	0.11	9.0	0.013	15.05	0.0001
Residual	0.008355	10.0	0.0008355		
Lack of fit	0.006355	5.0	0.001271	3.18	0.1151
Pure error	0.002	5.0	0.0004		
Total	0.12	19.0			

Antioxidant activity

To quantify the antioxidant potential of the Ni NPs, 2 ml volume of a methanol-dissolved 100 µM DPPH solution was combined with 2 ml of diverse concentrations of Ni NPs solution. The resultant mixture was left undisturbed at room temperature for 30 min. Following this, the absorption at 520 nm for the samples was measured utilizing a spectrophotometer (Figs. 10, 11, 12). The antioxidant activity was subsequently calculated using the provided formula. The calculation of IC_{50} value of ML_1 and ML_2 (NPs) in DPPH assay is given in Table 4 and 5.%ofDPPHfree radicalscavenging=Blank-TestsampleBlank×100

Fig. 10 [Images not available. See PDF.]

Preparation of DPPH solution and DPPH with Ni-NPs solution

Fig. 11 [Images not available. See PDF.]

Assessment of the scavenging activity of ML_1 (NPs) against DPPH radicals

Fig. 12 [Images not available. See PDF.]

Assessment of the scavenging activity of ML_2 (NPs) against DPPH radicals

Table 4. IC_{50} value of ML_1 (NPs) in DPPH assay

S. no	Concentration of the sample(µg/ml)	Absorbance	% of OARC	IC50
1	Blank	0.92	0.00	19.33
2	10	0.84	8.70	3
20	0.83	9.78	4	30
0.82	10.87	5	40	0.79
14.13	6	50	0.77	16.30

Table 5. IC_{50} value of ML_2 (NPs) in DPPH assay

S. no	Concentration of the sample (µg/ml)	Absorbance	% of OARC	IC50

1	Blank	0.92	0	10.49
2	10	0.76	17.39	3
20	0.74	19.56	4	30
0.72	21.73	5	40	0.68
26.08	6	50	0.64	30.43

Cytotoxic activity

Assessing the cytotoxicity of the synthesized compounds against HeLa (cervical) and A549 (lung) cancer cell lines involved evaluating the number of viable cells remaining after a specific incubation period with macrocyclic complexes. The in-vitro cytotoxic activity results were quantified as IC_{50} values, representing the concentration of the compound in µg/mL that inhibits cell proliferation by 50% compared to untreated control cells is also summarized. The macrocyclic complexes exhibited a dose-dependent impact on the viability of both types of cancer cell lines (Figs. 13, 14, 15, 16). The growth inhibition percentage of cancer cell lines demonstrated an increase with the rising concentration of the tested compounds (Table 6).

Fig. 13 [Images not available. See PDF.]

Flow diagram showing the route of complete anticancer activity

Fig. 14 [Images not available. See PDF.]

Bar diagram showing the % Viability and Cytotoxicity of ML_1 and ML_2 against A549 and Hela Cancer cell lines

Fig. 15 [Images not available. See PDF.]

IC_{50} values of Ni-NP against A549 and HeLa cell lines

Fig. 16 [Images not available. See PDF.]

A HeLa cell line-control. **B**, **C** Variations in the morphology of HeLa cell line after exposure to NPs of ML_1 and ML_2. **D** A549 cell line-control. **E**, **F** Variations in the morphology of A549 cell line following exposure to NPs of ML_1 and ML_2

Table 6. IC_{50} Comparison of ML_1, ML_2 Compounds against A549 and HeLa cell Lines after 24-Hour Incubation: MTT Study

S. no	Compound IC50 (ug/L)		
	A549	Hela	1
ML_1	44.76	43.37	2

The statistical analysis of MTT cytotoxicity study data reveals noteworthy findings regarding the cytotoxic potential of test compounds ML_1 and ML_2 against A549 and HeLa cell lines. In the case of A549 cell lines, both ML_1 and ML_2 exhibit significant cytotoxicity, with IC_{50} concentrations of 44 µg/mL and 31.20 µg/mL, respectively. Markedly, ML_2 stands out for its substantial cytotoxicity against HeLa cells, suggesting its potential as a potent anti-lung cancer agent, given its low IC_{50}. Similarly, against HeLa cell lines, ML_1 and ML_2 demonstrate substantial cytotoxic potential, displaying IC_{50} concentrations of 43.37 µg/mL and 21.42 µg/mL, respectively.

Discussions

All the characterization techniques we have utilized have effectively provided valuable insights into the newly synthesized nanoparticles. The infrared (IR) spectral data offered valuable information about presence of function groups, the structural modifications and bonding configurations within the synthesized macrocyclic complexes. In UV–visible studies, the size and dispersion of nanoparticles (NPs) significantly influence the characteristics, breadth, and position of the surface plasmon resonance (SPR) peak observed. The UV–Visible spectrophotometer data within the 250–300 nm range for the synthesized Ni-NPs reveals their plasmonic nature, signifying electron oscillation. This emphasizes the pivotal role of NP size and distribution in shaping the optical properties and behaviour of synthesized Ni-NPs. X-ray diffraction (XRD) analysis findings offer valuable insights into the structure and size distribution of the synthesized Ni-NPs, thereby informing their potential applications across various domains. Utilizing the Debye–Scherrer formula, particle sizes for both ML_1 and ML_2 compounds were determined to fall within the nano-range. Scanning electron microscopy (SEM) enables a meticulous examination of nanoparticle boundaries, enhancing precision in assessing nanoparticle size and distribution. The images obtained by the Scanning Electron Microscope for Ni-NPs of metal complexes (ML_1 and ML_2) approve that the nanoparticles are almost spherical in form with the size of maximum 170 nm. Response surface methodology (RSM) studies reveal a notable trend wherein as temperature rises to approximately 30 °C, absorbance initially decreases before rising again. This temperature sensitivity underscores its crucial role in optimizing results. The most favourable outcomes were achieved when experimentally validated data were fitted into the equation, yielding a minimum absorbance of 0.73 at ML_1 concentration of 35 µg/l, ML_2 concentration of 15 µg/l, and a temperature of 30 °C, closely aligning with the predicted response.

The antioxidant radical-scavenging potential of Ni NPs was evaluated using a methanolic solution containing 2,2-diphenyl-1-picrylhydrazyl (DPPH), a well-known radical and scavenger for other radicals. Initially, the methanolic DPPH solution exhibits a deep violet colour, which transitions to colourless or fades to a pale yellow upon neutralization with antioxidants, indicating a reduction reaction and confirming the radical nature of the test sample. The calculations of IC_{50} values for both nanoparticles in the DPPH assay indicate that ML_2 exhibits greater antioxidant potential compared to ML_1. The anticancer activity results indicated that all the complexes exhibited moderate to good anticancer activity against both HeLa and A549 cancer cell lines. In summary, both ML_1 and ML_2 demonstrate effective cytotoxicity against human lung and cervix cancer cells, positioning them as promising candidates for further exploration in cancer therapeutics. ML_2, in particular, emerges as a potent anti-cervix cancer agent, characterized by its low IC_{50} value.

Conclusions

Researchers, prompted by the toxicity and side effects associated with cisplatin, are exploring alternative approaches for developing transition metal-based anticancer agents. The cytotoxic potential of nickel nanoparticles (Ni-NPs) has been thoroughly investigated using cervical and lung cancer cell lines. In this study, the utilization of Horse Gram seed extract *(Macrotyloma uniflorum)* has revealed its richness in phenolic and flavonoid compounds, well-documented for their potent antioxidant activity in existing literature. The experiments were structured to investigate the impacts of diverse factors, and the utilization of Response Surface Methodology proved effective for optimization. Following computation, the regression coefficients were incorporated into a second-order polynomial equation for fitting. The experimental and predicted values agree closely which validates the model. Moreover, the application of *Macrotyloma uniflorum*-mediated Ni-NPs has demonstrated an enhanced anticancer effect against HeLa and A549 cell lines responded in a manner dependent on the concentration. The mechanism underlying the deactivation of cancer cell lines aligns with existing literature, attributing it to intra-cellular nucleus damage. An additional noteworthy observation indicates that Nickel metal NPs with 2,4-dicarboxylic acid (ML_2) as a ligand exhibit a more significant impact on antioxidant and anticancer activities compared to their counterparts with 3,4-dicarboxylic acid (ML_1) as the ligand moiety. This difference in impact may be attributed to the method employed to convert metal complexes into metal-NPs. The current research underscores the possibility of refining the bio-toxicity and biochemical properties of Ni-NPs to develop a new generation of versatile anticancer agents with clinically proven efficiency. Remarkably, the predicted data aligns closely with experimental results, affirming the reliability

and credibility of RSM (Response Surface Methodology) model for studying anticancer and antioxidant properties in this context.

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Author contributions

SA conducted a comprehensive literature review focusing on organically Nano-Fabricated Ni metal complexes, exploring their antioxidant and anticancer activities, and conducted characterization and activity assessments. SB coordinated the development of the initial draft of the article. MVM enhanced the writing style and participated in the proofreading process. The final manuscript was reviewed and approved by all authors.

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Abbreviations

ML_1
Nickel metal NPs with 2, 4-dicarboxylic acid (ML_2) as a ligand
ML_2
Nickel metal NPs with 3, 4-dicarboxylic acid (ML_2) as a ligand
DPPH
2, 2-Diphenyl-1-picrylhydrazyl
MTT
3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
SEM
Scanning electron microscope
RSM
Response surface methodology

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Design, synthesis, 2D-QSAR, molecular dynamic simulation, and biological evaluation of topiramate–phenolic acid conjugates as PPAR γ inhibitors

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ABSTRACT (ENGLISH)

Background

Obesity is a precursor for many co-morbid diseases. One of the main triggering factors for obesity is the abnormal expansion of white adipose tissue characterized by high rates of genesis and differentiation of precursor cells into mature adipocytes. As a result, targeting adipogenesis and adipogenic transcription factors opens new roadmaps for developing novel antiobesity pharmacotherapies. The present study was intended to rationally develop topiramate–phenolic acid conjugate for targeting obesity via inhibition of PPAR*γ* which is often considered as the master regulator of adipogenesis.

Results

2D QSAR models were built to foretell PPAR*γ* inhibitory activity of designed conjugates. The models presented excellent robustness, goodness of fit, and predictive capability compounds. The highest PPAR*γ* inhibitory activity was predicted for T3 (topiramate–caffeic acid conjugate) with a pIC_{50} value of 7.08 μM. Molecular docking was performed for all the designed conjugates against PPAR*γ* (PDB ID: 3VSO). The highest binding affinity was exhibited by T3 (−11.27 kcal/mol) and displayed strong and stable interactions with the receptor within the allosteric pocket in comparison to the irreversible PPAR*γ* antagonist, GW9662 (binding affinity, −9.0 kcal/mol). These results were confirmed by subjecting the best-docked molecules to molecular dynamic simulations. The PPAR*γ*–T3 complex was observed to be most stable with maximum number of hydrogen bonds (maximum observed RMSD = 0.57 Å at 100 ns) in comparison to PPAR*γ*–topiramate and PPAR*γ*–caffeic acid complexes. Consequently, T3 was synthesized and further subjected to in vitro screening. The TR-FRET assay established T3 as a PPAR*γ* antagonist (IC_{50} = 6.78 μM). T3 also significantly reduced the lipid buildup in the 3T3-L1 adipocytes in a dose-dependent manner. In addition, T3 also reduced the protein expression levels of PPAR*γ* as evidenced from western blot results.

Conclusions

Studies clearly indicated that T3 reduces adipose tissue cell differentiation by downstreaming PPAR*γ* expression at protein levels, thereby emerging as a novel scaffold for antiobesity pharmacotherapy.

FULL TEXT

Background

The last decade has laid down a crystal-clear picture of obesity as an ever-increasing global pandemic [1]. Obesity is often associated with co-morbidities encircling type II diabetes, cardiovascular disorders, hypertension, liver dysfunctions, dyslipidemia, obstructive sleep apnea, polycystic ovarian disease, and many cancers. As a master precursor of almost all major chronic metabolic disorders, obesity substantially shoots up the socio-economic burden worldwide by rocketing high healthcare costs [2–7]. Consequently, numerous pharmacotherapeutic agents are developed for the mitigation or cure of obesity by targeting an array of physiological targets, such as pancreatic lipase, 5-hydroxy tryptaminergic receptor 2C, β3 adrenergic receptor, GLP-1 (glucagon-like peptide 1), and many other gut peptides [8] Presently, orlistat, lorcaserin, phentermine/topiramate, bupropion/naltrexone, and liraglutide are approved by FDA (Food and Drug Administration) for obesity pharmacotherapy [9]. Nonetheless, orlistat and lorcaserin have been permitted for longer duration therapy [10, 11]. Incidentally, potential and novel drugs without undesirable physiological adverse actions are the utmost need of the hour for countering obesity.

Topiramate (TPM) is an inhibitor of carbonic anhydrases and glutamate, while acts as an agonist for GABA (-aminobutyric acid). It is used for therapy of epilepsy and migraine [12]. Epilepsy patients on TPM medication showed significant weight loss which led to clinical investigation of the drug for having antiobesity effects. In vivo studies have explored thermogenesis boosting and neuro-stabilizing repertoire of TPM [13, 14]. Several studies also discovered that TPM amplified the phosphorylation of crucial lipolytic enzymes, thereby inducing lipolysis in 3T3-L1 preadipocytes. It further suggested TPM might directly inhibit adipogenesis by targeting white adipocytes bypassing its CNS (central nervous system) effect [15].

Phenolic acids are abundantly available from natural sources and are categorized as hydroxy-cinnamic acid derivatives such as caffeic acid (CF), ferulic acid, para-coumaric acid, or hydroxy-benzoic acid derivatives such as gallic acid, chlorogenic acid, protocatechuic acid, or vanillic acid. The phenolic acids basically modulate the gut

microbiota in exerting their antiobesity effects [16]. Inhibition of white adipose tissue differentiation, fat browning, pancreatic lipase, and pancreatic amylase inhibition, suppression of inflammatory cytokine expression, and downregulation of obesity-inducing genes are other pharmacological actions of phenolic acids [17].

The PPARs (peroxisome proliferator-activated receptors) have emerged as promising therapeutic targets among all nuclear receptors for developing novel pharmacotherapeutic candidates against insulin resistance, cancers, obesity, dyslipidemia, and cardiovascular disorders [18]. PPAR*γ* are primarily distributed in adipose tissues and are considered master regulators of adipogenesis [19, 20]. Preferential recruitment of co-repressor molecules over co-activators by PPAR*γ* antagonists as well as close crosstalk with NFκB (nuclear factor kappa B) and AP-1 (activation protein 1) downregulates PPAR*γ*-mediated gene transcription [21, 22]. The medicinal repertoire of PPAR*γ* antagonists spreads more wider than diabetic therapy in promoting osteoblast formation and depressing differentiation of adipose tissue [23]. As a result, PPAR*γ* inhibitors can be well thought out to be potential aspirants for osteoporosis and obesity therapy [24]. Additionally, PPAR*γ* inhibitors embody broad anticancer activity as well [25]. For that reason, exploring PPAR*γ* inhibitors is of prodigious importance in the quest for novel drug candidates for pharmacotherapy of PPAR*γ*-associated metabolic disorders.

Drug-drug conjugates or often called as molecular hybrids comprise of two different pharmacophores interconnected directly or by a spacer (cleavable/non-cleavable). The conjugates usually exert simultaneous action at specific targets with increased potency or efficacy. The high efficacy can be due to improved pharmacokinetic properties. For instance, atorvastatin–curcumin conjugate nanocrystals exhibited enhanced biopharmaceutical and anti-inflammatory properties in comparison to individual drugs [26]. Another striking example is mesalamine–coumarin conjugate with diazo linker displayed an increased anti-inflammatory response by reducing acetic acid-induced ulcerative colitis in rat models [27]. Similarly, nanoparticle of camptothecin–floxuridine conjugate exhibited profound and synergistic anticancer activity with improved cytotoxicity, apoptosis, and inhibition of malignant proliferation [28].

Our present work aims to design and synthesize novel TPAC (topiramate–phenolic acid conjugates). Through robust 2D-QSAR (two-dimensional quantitative structure–activity relationship) models, the biological activity of the designed conjugates was predicted. In silico molecular docking was done for gaining insights of the interaction nature of designed conjugates with the receptor. The MD (molecular dynamic) simulation study revealed good dynamic behavior of PPAR*γ*–T3 complex. Among all the three complexes, PPAR*γ*–T3 had minimum RMSD, RMSF, Rg, and potential energy indicating good attractive and stable interactions between protein and ligand molecules over the total MD simulation time. Lantha Screen TR-FRET assay was performed to evaluate the PPAR*γ* inhibition potency of the designed conjugates. The antiobesity effects of the T3 (topiramate–caffeic acid conjugate) were demonstrated via differentiation inhibition and lipid accumulation in the 3T3-L1 preadipocytes.

Methods

Computational study

QSAR studies

Dataset preparation

In the current study, an experimental dataset of 100 compounds was retrieved from the Binding dB database [29]. The biological activities of the compounds were expressed in terms of IC_{50} (nM, half maximal inhibitory concentration) and were converted to their corresponding pIC_{50} values (negative logarithm of IC_{50} values). For building the 2D-QSAR model, pIC_{50} was considered as the dependent variable. The raw dataset compounds and their corresponding IC_{50} values are provided in supplementary files for reference (Additional file 1: Table S1).

Descriptors computation

Before the computation of the molecular descriptors, geometry optimization was carried out for each dataset molecule employing molecular mechanics force field and semi-empirical AMI methods using SPARTAN 10.0 software tools. The energy-minimized structures were further used to generate molecular descriptors using two software tools PaDEL and CORAL. PaDEL version 2.21 [30] was used to generate about 1444 2D molecular descriptors. More than 50% of descriptors with zero, missing, and constant values were excluded. Also using pairwise correlation, the descriptors were filtered. The CORAL (http://www.insilico.eu/coral) software generated

single optimal descriptor (DCW) [31] basing on SMILES (simplified molecular input line system) [32, 33]. The descriptor is calculated using the Index of ideality of correlation (IIC) formalism which was carried out for the Monte Carlo optimization taking the IIC weight = 0.2000. The details of the calculation of the DCW by IIC are described elsewhere [34, 35]. The PCA (principal component analysis) was applied to determine the best descriptor (variables) combination. The dataset molecules were sorted according to PC1 scores. As a final point, 20 molecular descriptors were utilized for deriving models from the whole data set. The computed molecular descriptors for the 100 dataset compounds are provided in supplementary files for reference (Additional file 1: Table S2).

QSAR modeling

The 100 molecules of dataset were split into training set (87 molecules) and validation set (13 molecules). GA (genetic algorithm) and MLR (multiple linear regression) techniques were employed for building robust QSAR models using QSARINS software [36]. 2D-QSAR models were built using combinations of selected 20 descriptors, including the one optimal descriptor DCW.

QSAR model validation

All the developed models were validated according to OECD (Organization for Economic Cooperation and Development) principles using the QSARINS software package. The validation aimed at ensuring that the built models have definite endpoints represented using unambiguous algorithms, have domain of applicability, and have appropriate measures for predictability, goodness of fit, and robustness. Leverage values below critical leverage with ± 3 standard deviations were considered to ensure good predictive capability of designed QSAR models. The compounds remaining outside these leverage values were treated as outliers. Williams's plots were employed to describe QSAR model applicability domain. To minimize the discrepancies between experimental and predicted values of the endpoint, goodness of fit was computed employing R^2 (coefficient of determination) and R^2adj (coefficient of determination adjusted for degrees of freedom). Internal validation methods like LOO (leave one out) and LMO (leave many out) were used to verify and measure the strength of models generated. To ascertain the predictive capability of the designed models, various external validation parameters were analyzed and computed such as RMSE ext, Q^2–F1, Q^2–F2, Q^2–F3, R^2 m, R^2 m delta, CCC, MAE ext and PRESS ext. Finally, Y-scrambling method confirmed that built models are not outcomes of chance correlation.

Prediction of IC_{50} values of novel topiramate–phenolic acid conjugates

Ten novel TPAC (T1-T10) were designed using Chemdraw Ultra 12.0 software. Geometry optimization was done for each designed molecule employing molecular mechanics force field and semi-empirical AMI methods using SPARTAN 10.0 software tools (http://www.wavefun.com/products/spartan.html). Further 2D molecular descriptors for the newly designed conjugates were computed using PaDEL software. The single optimal descriptor was also calculated for the designed compounds using CORAL software. The leverage values of all the designed compounds were calculated and compared with the threshold leverage value h^* (0.138). Using the best generated QSAR equation, the pIC_{50} values of the designed molecules were predicted.

Molecular docking

Among the designed conjugates, highest PPARγ inhibitory activity was predicted for the topiramate–caffeic acid conjugate (T3). Accordingly, T3 was selected for molecular docking and molecular dynamic simulation analysis.

Ligand preparation

The structure of CF, TPM, and T3 conjugate was drawn in Chemdraw Ultra 12.0 software and stored in standard format (SDF). All the ligands were subjected to energy minimization UCSF Chimera 1.16 [37] prior to docking.

Protein preparation

3-D crystal structure of PPARγ (PDB ID: 3VSO) was retrieved from the RCSB protein bank using Energy minimization and geometry optimization was performed using UCSF Chimera 1.16 by adding hydrogen atoms and charges to the receptor. Finally, the protein was saved in pdbqt format for docking.

Docking

AutoDock Vina [38] was used for docking of the ligands with the selected protein. After energy minimizing, a grid box resolution with three dimension co-ordinates 17.762, 71.66, and 13.333 was set. Grid box with 60 × 60 × 60 Å point

spacing of 0.375 Å was used to reformate structure files into pdbqt format. The flexible ligand docking studies were performed using Lamarckian genetic algorithm.

Molecular dynamic simulation

To compare the interaction of individual pharmacophoric ligands CF and TPM with conjugate ligand T3, all the protein–ligand complexes (PPAR*γ*-CF, PPAR*γ*-TPM, and PPAR*γ*-T3) were directed for 100 ns MD (molecular dynamic) simulation using Gromacs 4.5.6 [39]. For generating the topology of a protein–ligand complex system, initially, protein and ligand were separated as a single entity, followed by the generation of individual topology or protein and ligand and finally merging back to complex form. With the help of CHARMM-36 parameters, here we created the topology for proteins (*X*, *Y*, *Z* coordinate system) using TIP 3P water model, and for each ligand we have used Swiss-Param online server to generate the topology. The protein.gro and ligand.gro files were manually fit, and complex.gro was generated for each of the three complexes. Further, the complex file is solvated using SPC216 water model in dodecahedron form with system size as follows: PPAR*γ*-CF complex = *X*: 5.099, *Y*: 6.095, and *Z*: 5.654 (nm). PPAR*γ*-TPM complex = *X*: 5.099, *Y*: 6.095, and *Z*: 5654 (nm). PPAR*γ*-T3 complex = *X*: 5.099, *Y*: 6.095 and *Z*: 5654 (nm). Solvent molecules and sodium ions were added for neutralization of the system.

Further energy of neutralized system was minimized by employing steepest descent minimization method for 50,000 steps. The stability of the complex post energy minimization is carried out by assessment of potential energy, bond energy, proper dihedral, and improper dihedral. Going beyond the energy minimization, .itp file of restrained ligand position was generated and incorporated into complex topology file using leap-frog integrator algorithm. MD simulation was run for 50,000,000 steps. The system was restrained on covalent bond by employing LINCS algorithm [40]. Following 100 ns MD simulation, the RMSD (root mean square deviation), RMSF (root mean square fluctuation), Rg (radius of gyration), H-bond, and SASA (solvent accessible surface area) were calculated. The graphs were created by XM Grace Linux application, while the two-dimensional interactions were studied in BIOVIA Discovery studio visualizer.

Chemistry

General

3T3-L1 preadipocytes were obtained from NCCS (National Centre for Cell Science), Pune, India. DMEM (Dulbecco's Modified Eagle's Medium), BCS (bovine calf serum), antibiotic solutions, and antibodies for enzyme assay as well as western blot were purchased from Thermo Fisher Scientific, (Waltham, MA, USA). Topiramate was procured from Yucca Chem Products, Mumbai. Other reagents/chemicals (high purity) used were purchased from Sigma-Aldrich (St Louis, USA) and used as received.

Synthesis of topiramate–caffeic acid conjugate, T3

T3 (topiramate–caffeic acid conjugate) was synthesized using DCC-DMAP coupling [41–43]. CF (2), 1 equivalent and DCC (N,N'-dicyclohexyl carbodimide, 1.1 equiv), and DMAP (4-dimethylaminopyridine, catalytic amount, 10 mol%) were added with 20 ml of DMF (dimethylformamide) with continuous stirring for 60 min. After 60 min of stirring, TPM (1), 2 equivalents in excess DMF was added to the above reaction mixture and allowed to stir at room temperature for 48 h (Scheme 1).

Scheme 1 [Images not available. See PDF.]

DCC-DMAP mediated coupling of caffeic acid and topiramate. *Reagents and conditions:* **a** DMAP **b** DCC **c** DMF

The reaction was monitored by TLC (aluminum sheets with Silica-Gel 60 F254 (Merck) employing ethyl acetate/pet ether (7:3) as mobile phase till completion. Following reaction completion, crude reaction mixture was water washed (10 ml) and filtered to remove dicyclohexyl urea (by-product). The filtrate was transferred to a separating funnel and extracted with ethyl acetate (20 ml × 3 times). The organic fraction was further collected and dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. The residue was recrystallized from ethyl acetate. Melting point was determined by open capillary tubes in a melting point apparatus and presented uncorrected. Spectral characterization was further performed (IR using JASCO FTIR-4100 series; ^{1}HNMR and ^{13}CNMR using Bruker 400 MHz NMR spectrophotometer).

Biological screening

In vitro *enzyme inhibition assay*

PPAR*γ* inhibition was studied using TR-FRET co-activator assay [43]. Concisely, the human recombinant PPAR*γ*-LBD (GST tagged, 1 nmol/L) was incubated with a Europium-labeled anti-GST antibody (2 nmol/L), testing samples, and DMSO (control). Following incubation, SRC (steroid receptor co-activator, XL665 labeled streptavidin) was added. The SRC co-activator peptide (20 nmol/L) was prepared in Tris–HCl (pH 7.4) The fluorescent signals were measured by microplate reader (BMG Labtech, Germany) at an excitation wavelength of 337 nm and emission wavelengths of 620 and 665 nm. The emission ratio was computed using the equation given below:Emissionration(ER)=fluoresceinemissionat665/fluoresceinemissionat620nm

Agonist mode

Fluorescent signals generated from rosiglitazone (positive control, 10 μmol/L) were considered 100% activation control. 1% DMSO was considered as blank/0% activation control. The activation percentage was computed by the below mentioned formula.%Activation(Agonistmode)={ER1-ER2/ER3-ER2}∗100%where ER 1 is the emission ratio of the sample, ER2 is the emission ratio of the blank, and ER3 is the emission ratio of 100% activation control (10 μmol/L rosiglitazone).

Antagonist mode

To the human recombinant PPAR*γ* and co-activator peptide previously incubated with test samples, 1 μM rosiglitazone was incorporated as EC_{80} control. The percentage inhibition of T3 was computed using the below mentioned formula. GW9662, irreversible PPAR*γ* antagonist, was used as positive control.%Inhibition(Antagonist mode)={ER1-ER2/ER4-ER2}∗100%where ER 1 is the emission ratio of sample, ER 2 is the emission ratio of blank, and ER 3 is the emission ratio of EC_{80} (1 μmol/L rosiglitazone).

Inhibition of adipogenesis in 3T3-l1 preadipocytes

Cell culture, differentiation, and maturation

The preadipocytes were sub-cultured in DMEM complemented with 10% (v/v) BCS 1% penicillin–streptomycin antibiotic mixture antibiotic in an atmosphere of 5% CO_2 at 37 °C. After 70–80% cell confluency, harvesting was done using 25 mm tissue culture flask containing trypsin and seeding in 96 well plate. After 100% confluency of cells, differentiation was performed by adding insulin (1 μg/mL), isobutyl-1-methylxanthine (0.5 mM/L), and dexamethasone (0.25 μM/L). Post confluency, by careful pipetting the differentiation media was discarded and maturation media (insulin, 1 mg/L) was added and changed every 2 days. The control group remain untreated. The test groups received 1, 5, and 10 μM of T3, respectively. The positive control group was treated with 10 μM of GW9662. Post 10 days, lipid droplets were clearly visible inside the differentiated cells and hence subjected to further assays [44].

MTT assay

Cytotoxic effect of T3 on adipocyte precursor cells was investigated through MTT assay [44]. The 3T3-L1 preadipocytes were seeded in 96-well plate (3×104 cells per well) and cultured in DMEM containing 10% BCS for 24 h. After 24 h the cells were treated with 0.001, 0.01, 0.1, 1, and 10 μg/mL of T3, respectively, and kept for next 72 h. Post 72 h, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (20 μL, 5 mg mL^{-1}) was added to each well and further incubated for 4 h at 37 °C. The dark blue formazan product formed by the cells was carefully dissolved in DMSO within a dark cabinet, and absorbance was measured at 495 nm in a microplate reader (Bio-Rad Lab, California).

Oil Red O staining

To observe lipid droplets within differentiated adipocytes, the Oil O Red stain method was employed. The differentiated cells were initially washed with PBS and then fixed with 4% formaldehyde for 30 min. Further, the cells were stained with freshly prepared (in 60% isopropyl alcohol) Oil O Red solution for 10 min at room temperature. After 10 min the stained cells were rinsed with isopropyl alcohol for extraction of accumulated lipids and their quantification (absorbance measured at 495 nm) in terms of percentage relative to the control. The untreated cells were taken as control. The cells stained were observed under a phase contrast microscope (Axiovert 40 CFL, Carl

Zeiss, Jena, Germany) and photographed [44]

Western blot

In brief, the cells were rinsed with PBS, lysed using Laemmli buffer (comprising of 62.5 mM Tris–HCl, 10% glycerol, and 2% SDS) and sonicated. 10 µg of protein was separated by gel electrophoresis (10% SDS-PAGE), transferred on to nitrocellulose membrane, and blocked using 5% PBS-T milk (45 min). Anti PPAR*γ* and *β*-actin antibodies were added to 1% BSA in PBS-T milk and incubated with membrane previously blocked containing protein lysates at room temperature for 1 h. Following incubation, the membrane was washed with PBS-T milk and treated with secondary antibody (HRP-conjugated) following incubation for next 30 min. Then the membranes were washed with PBS-T milk twice for 10 min in room temperature. Chemiluminescence was detected using the ChemiDoc Touch imaging system (Bio-Rad) [45]. *β*-actin was used as internal control.

Statistical analysis

All the experiments were performed in triplicate. The data from each independent experiment are expressed as mean ± standard deviation. The presented data were statistically analyzed employing one-way ANOVA with *t*-test for defining differences. Values of (*$p<0.05$ and **$p<0.01$) were considered statistically significant.

Result

Computational studies

Variable selection by PCA

Data redundancy was reduced employing PCA (principle component analysis). The eigen values were sorted in descending order. PC1 describes greatest data variance, while PC2 described data variance in an orthogonal direction to PC1. Principal component analysis (PCA) for the descriptors VE3_Dzp, nHBint6, and DCW was demonstrated by loading and scoring plots. Figure 1A displays the scoring plot for dataset compounds. Likewise, Fig. 1B displays the loading plot for M-1 descriptors. PCA score plot describes the type of co-relation between dataset components. The loading plot on the other hand describes the influential power of descriptors on dataset components. As evident from the score plot (Fig. 1A), very few dataset compounds were observed as outliers. The descriptor having maximum influence was observed to be nHBint6 followed by VE3_Dzp and DCW (Fig. 1B).

Fig. 1 [Images not available. See PDF.]

PCA for QSAR model M-1; **A** Score plot for the M-1 descriptors; **B** Loading plot for the M-1 descriptors

QSAR modeling and validation

Amazingly, a significant improvement (Fig. 2) in the values of R^2 and Q^2 for the training set was observed by including DCW. On that note, different 2D-QSAR models were built by including the single optimal descriptor. Models with best R^2 values for validation set were preferred for activity prediction. The QSAR equations of one-, two-, and three-descriptor models for best one-, two-, and three-descriptor models are reported in Table 1. The validated internal and external parameters for the best three models are represented in Tables 2 and 3, respectively. Table 4 represents statistical parameters for models M-1, M-2, and M-3.

Fig. 2 [Images not available. See PDF.]

Impact of inclusion of DCW on R2 and Q2 for M-1 training set

Table 1. Best 2D-QSAR models for PPARϒ receptor inhibitors

Model ID	Descriptor combination	QSAR equation
1	VE3_Dzp, nHBint6, DCW	$pIC_{50} = 0.0063 * nHBint6 - 0.0009 * VE3\_Dzp - 0.0084 * DCW + 7.7233$
11	GATS5v, DCW	$pIC_{50} = 0.0460 * GATS5v - 0.0086 * DCW + 7.6900$

21	DCW	$pIC_{50} = -0.0087 * DCW + 7.7442$

Table 2. Parameters for internal validation of best three models

Model ID	Q^2 LOO	R^2–Q2 LOO	RMSE cv	MAE cv	PRESS cv	CCC cv	Q^2 LMO	R^2Yscr	Q^2Yscr	RMSE AV Yscr
1	0.9894	0.0018	0.0081	0.0060	0.0057	0.9947	0.9886	0.0355	−0.0683	0.0771
11	0.9881	0.0014	0.0086	0.0064	0.0064	0.9940	0.9878	0.0231	−0.0483	0.0776
21	0.9868	0.0012	0.0090	0.0069	0.0071	0.9933	0.9865	0.0111	−0.0361	0.0781

Table 3. Parameters of external validation for the best three models

Model ID	RMSE ext:	MAE ext	PRESS ext	R^2ext	Q^2-F1	Q^2-F2	Q^2-F3	CCC ext	R^2m avg	R^2m delta
1	0.0053	0.0048	0.0004	0.9950	0.9927	0.9927	0.9955	0.9965	0.9500	0.0070
11	0.0051	0.0046	0.0003	0.9951	0.9932	0.9932	0.9958	0.9967	0.9593	0.0062
21	0.0063	0.0058	0.0005	0.9951	0.9894	0.9893	0.9935	0.9950	0.9307	0.0090

Table 4. Training set statistical parameters for best three models

Fitting criteria	Model1	Model 11	Model 21
R^2	0.9912	0.9895	0.9879
R^2adj	0.9909	0.9893	0.9878
R^2–R^2adj	0.0003	0.0002	0.0001
LOF	0.0001	0.0001	0.0001
Kxx	0.2541	0.3521	0.0000
Delta K	0.2064	0.2570	0.9940
RMSE tr	0.0074	0.0080	0.0086
MAE tr	0.0056	0.0061	0.0066
RSS tr	0.0047	0.0056	0.0065

CCC tr	0.9956	0.9947	0.9939
S	0.0076	0.0082	0.0087
F	3108.9384	3966.4605	6968.4382

All the models have higher R^2m values (> 0.5) and lower R^2m delta values (< 0.2) indicating stability and robustness. The values of the LOF and Friedman lack of fit (0.0001) [46] and *s* (0.007 to 0.0087) are very low which ensures that no over fitting is there in the models. Lower values for delta K and Kxx for model M-1 show good co-relation between descriptors and predicted responses with limited errors in computation [47, 48]. High R^2 adj values suggest convenient addition of a new descriptor to the model.

Model ID-1 with highest R^2 (coefficient of determination) has been evaluated as the best model satisfying the goodness-of-fit criteria and internal validation parameters. The scatter plot (Fig. 3A, B) clearly indicates a linearity in relationship among experimental and predicted values. The correlation between descriptors (*x*) and activity (*y*) were plotted with K*xy* versus Q^2 LMO of the final model (model 1) (Fig. 4) displaying LMO parameter values are close enough to model parameters. It clearly states that the model is robust and stable. Y-scrambling tested the external validation parameters (Fig. 5). As the values of R^2Yscr and Q^2Yscr are very small, the models are believed to be good models and not the outcome of casual correlations.

Fig. 3 [Images not available. See PDF.]

Scatter plot of experimental pIC50 values versus predicted activity; **A** correlation between values predicted by model equation **B** correlation between the values calculated by LOO

Fig. 4 [Images not available. See PDF.]

Plot of Kxy versus Q2 LMO depicting correlation between computed variables (*x*) and predicted activity (*y*)

Fig. 5 [Images not available. See PDF.]

Y-scramble plot depicting internal validation parameters

Figure 6A, B shows the William's plots deciphering applicability domain of generated model (M-1).

Fig. 6 [Images not available. See PDF.]

William's plot for model (M-1) **A** HAT values vs standard residuals calculated from QSAR model equation; **B** HAT values versus standard residuals predicted by LOO

Lower leverage values than threshold (h^* of 0.135) indicated that the training and test set molecules remained in the applicability domain of selected model as evident from the William's plot with 6 outliers only. Noticeably graph of insurbia (Fig. 7) resembled William's plot with same six outliers. This suggested that the selected model was best in terms of predicting experimental response. Additional file 1: Table S3 gives a comparative display of experimental and predicted IC_{50} values for the dataset compounds.

Fig. 7 [Images not available. See PDF.]

The graph of insurbia

It clearly indicates that the descriptors (VE3_Dzp, nHBint6, and DCW) used to generate the best predictive model equation are not overfitting with the lowest LOF (0.0001) and high predictivity (Q^2LOO = 0.9894). It was also observed that the performance of model 3 build using a single optimal descriptor DCW was also impressive when compared with model 1. The performance of the models was evaluated by plotting the values of Q^2LOO and LOF (Fig. 8).

Fig. 8 [Images not available. See PDF.]

Plot of Q2 LOO versus LOF

Predicted IC50 of topiramate–phenolic acid conjugates (TPAC)

The leverage values of all the designed molecules were observed to be below threshold leverage value ($h^* = 0.138$) suggesting the good applicability domain of the developed QSAR models. The predicted IC_{50} values of the designed compounds are displayed in Table 5. The highest inhibitory activity against PPARϒ was obtained for T3. The inhibitory activity reduced with the addition of (–OCH3) groups as evident from the pIC_{50} values of T4 and T10.

Table 5. Predicted pIC50 values of designed conjugates

Compound code	*R*	Predicted pIC_{50}
T1		7.005003569
T2		7.038592667
T3		7.08435656
T4		6.991701765
T5		7.019504227
T6		7.003357836
T7		7.066946934
T8		7.030767462
T9		7.019279445
T10		6.988112667

Mechanistic Interpretation

The designed 2D-QSAR model 1 was used for predicting the biological activity of the designed TPAC. According to the equation, the PPARϒ inhibitory activities are explained by three descriptors of the model equation which positively or negatively contribute to pIC_{50} values with respect to their regression coefficient values. The first descriptor VE3_Dzp is the logarithmic Randic-like eigenvector-based index from the Barysz matrix/weighted by polarizabilities and represents heteroatoms and multiple bonds in the molecules. The descriptor positively contributed toward the PPARϒ inhibitory potency [49]. In the designed conjugates, the presence of different electronegative atoms increases the polarization of carbon atoms that might increase the inhibition potency. The next descriptor is nHBint6 which is an E-state descriptor and associated with electro-topological state of hydrogens establishing hydrogen bonds within a path length of six. The descriptor may also define intermolecular interactions having impact on biological and physic-chemical properties. The descriptor has a positive correlation with the pIC_{50} values [50]. We decipher that the presence of hydroxyl groups in the designed conjugates contributes positively toward the inhibition potencies. Interestingly, the inclusion of DCW (single optimal descriptor) generated best fitting models.

Molecular Docking Analysis

The open conformation of helix-12 is a prime requirement for co-repressor recruitment. The binding of an agonist to LBD within orthosteric pocket leads to a closed conformation, therefore recruiting co-activators and inducing transcription of PPAR*γ* genes. The important amino acid residues present within the orthosteric pocket are CYS285, SER289, HIS323, TYR327, LYS367, HIS449, and TYR 473, which play a major role in helix-12 folding [51]. Conclusively, it can be suggested that molecules interfering with proper folding of helix 12 around PPAR*γ*-LBD can be defined as PPAR*γ* antagonists [52]. Further, ligands binding to the allosteric site within the PPAR*γ*-LBD do not display any competition with classical agonistic ligands for binding at orthosteric site The amino acid residues present within allosteric acid are GLU259, LYS265, HIS266, ARG288, SER289, GLU295, SER342, GLU343, and LYS367 which significantly interfere with helix-12 folding [21]. Therefore, PPAR*γ* antagonists can be designed according to the helix12-folding inhibition hypothesis.

The binding interactions of the designed conjugates (T1–T10) along with CF, TPM, and GW9662 with crystal structure of PPARγ (PDB ID: 3VSO) are displayed in Table 6. Among the 10 designed conjugates, T3 shows stable and strong interactions with the receptor. The designed ligand binds to an alternate site/allosteric site within the ligand binding domain of the crystal structure of PPAR*γ*. The intramolecular hydrogen bonding interactions between the allosteric pocket residues (ARG288, CYS285, MET364, and PHE360) and T3 might destabilize the helix-12.

Table 6. Binding affinities and interactions of TPAC with amino acid residues

Conjugates	Binding energies (Kcal/mol)	Interactions with amino acids	
Hydrogen bonds	Hydrophobic interactions	T1	−9.0
ARG288, GLU343	SER342, ILE281, LEU330, CYS285, ARG288	T2	−10.84
SER289, CYS285	HIS449, TYR327, LEU469, HIS323, ARG288, ALA292, ILE326, LEU330, ILE281, LEU336, MET364	T3	−11.27
ARG288, CYS285, MET364, PHE360	MET329, ILE326, LEU330, LEU333, TYR327, CYS285	T4	−11.14
ARG288, CYS285, PHE360, PHE363	MET329, ILE326, LEU330, LEU333, TYR327, CYS285, ARG288, LEU356, PHE363, MET364, ILE281, LEU353	T5	−9.34

SER342, LEU340	LEU255, ARG280, ILE281, MET348, CYS285, GLY284, ARG288, VAL339, ILE341, PHE264	T6	−9.13
ARG288, GLY284, GLU343	ILE341, MET348, LEU330, ILE281, CYS285, ARG288	T7	−10.56
ARG288, CYS285, PHE360	ALA292, MET329, ILE326, LEU330, LEU333, TYR327, ILE281, LEU353, MET364, CYS285	T8	−10.36
ARG288, CYS285, PHE360	ALA292, MET364, ILE326, LEU330, LEU333, TYR327, LYS367, ILE281, LEU353, MET364, CYS285	T9	−9.37
ARG288, GLU343	MET364, ILE326, LEU330, LEU353, PHE287	T10	−10.40
CYS285, PHE360, PHE363	ILE326, MET329, LEU330, LEU333, ARG288, TYR327, MET364, CYS285, LYS367, ILE281, LEU353	TPM	−6.9
ARG280	CYS285, ARG288, PHE264, PHE287, LEU330, VAL339, LEU340, MET364	CF	−6.1
CYS285	ARG288, SER289, ALA292, ILE326, MET329, LEU330, LEU333	GW9662	−9.0

It is evident that there are no interactions of T3 with residues that are involved in proper folding of helix-12 (SER289, HIS323, TYR327, LYS367, HIS449, and TYR473) within the orthosteric pocket of PPARγ ligand binding domain. Also, hydrophobic interactions (MET329, ILE326, LEU330, LEU333, TYR327, and CYS285) might displace the helix-12 from position thereby disfavoring transcriptional process (Fig. 10). Moreover, it was interesting to see that

the designed conjugate T3 showed stronger interactions with the allosteric pocket residues than the parent pharmacophores (Fig. 9), CF (binding affinity, −6.1 kcal/mol) and TPM (binding affinity, −6.9 kcal/mol) respectively. Similarly, T3 formed stronger interactions with the allosteric pocket residues when compared to the irreversible PPAR*γ* antagonist, GW9662 (binding affinity, −9.0 kcal/mol) (Fig. 9).

Fig. 9 [Images not available. See PDF.]

Binding interactions at allosteric site of PPAR*γ* LBD (PDB ID: 3VSO) with **A** T3 **B** CF **C** TPM **D** GW9662 (irreversible PPAR*γ* antagonist)

For designing potential PPAR*γ* antagonists, it might be imperative to sustain H-bond interactions with ARG288, SER342, LYS367 and HIS449 within allosteric pocket in the LBD [53]. Moreover, hydrophobic interactions with allosteric site residues PHE282, ILE281, LEU356, TYR327, ILE326, LEU330 and MET348 that can destabilize the H12 contribute to inhibition potency of ligands [44, 54, 55].

Molecular dynamic simulation

The molecular dynamics-based studies revealed the deep understanding of protein ligand interactions over a period of time. The interaction energies of PPAR*γ*-CF complex, PPAR*γ*-TPM complex and PPAR*γ*-T3 complex in a neutralized system are given in Table 7.

Table 7. Dynamic properties of PPAR*γ*-caffeic acid complex, PPAR*γ*-topiramate complex, and PPAR*γ*-T3 complex

Type of energy	PPAR*γ*-caffeic acid complex	PPAR*γ*-topiramate complex	PPAR*γ*-T3 complex
Bond average energy	1283.25 kJ/mol	1385.02 kJ/mol	1312.6 kJ/mol
Potential energy	−7.3257912*e*+05 kJ/mol	−7.2699112*e*+05 kJ/mol	−7.3615656e+05 kJ/mol
Proper-Dihedral average energy	10,561.9 kJ/mol	10,664.8 kJ/mol	10,716.3 kJ/mol
Improper-Dihedral average energy	179.937 kJ/mol	189.107 kJ/mol	185.199 kJ/mol

From the above data (Table 7), it was clear that all the protein–ligand complexes were quite stable within the solvent medium. Of all the three PPAR*γ*-ligand complexes, PPAR*γ*-T3 complex exhibited lowest potential energy (Fig. 10). Lower the potential energy, higher are the attractive force between the protein and ligand molecule. It was therefore confirmed that PPAR*γ*-T3 complex was most stable with strong intermolecular attractions (also refer Additional file 1: supplementary data Figs. 1S, 2S, and 3S).

Fig. 10 [Images not available. See PDF.]

Decrease in potential energy with respect to time; **A** PPAR*γ*-CF complex **B** PPAR*γ*-TPM complex **C** PPAR*γ*-T3 complex

The complex revealed high fluctuation rate during the MD simulation study as total 0 to 100 ns RMSD (Fig. 11A). Initially at 0 ns the starting point the protein ligand deflected to ~ 4 Å. From 5 nanoseconds onward, the ligand was observed to be high in fluctuation reaching upto a ~ 9 Å with respect to the protein movement. The protein structure was almost a constant at around ~ 3 Å. From 35 nanoseconds to 60 ns and from 61 to nearly 85 nanoseconds, the ligand fluctuation trend was almost similar in a span of 25 nanoseconds where the ligand aroused from ~ 1 Å and going upto ~ 9 Å. Finally, from 85th nanosecond onward the ligand bounded within the protein fluctuation within the range of ~ 0.7 Å to ~ 2.5 Å (Fig. 11A). The PPAR*γ*-TPM complex revealed an acceptable fluctuation rate during the MD simulation study as total 0 to 100 ns RMSD (Refer Additional file 1: Fig. S5). Initially, at 0 ns the starting point

the protein ligand deflected to ~ 0.35 Å. From 5 nanoseconds onward, the ligand was observed to be high in fluctuation reaching upto a ~ 9 Å with respect to the protein movement. The protein structure was almost a constant at around ~ 0.3 Å. From 0 to 15 ns, it went to ascending order upto ~ 0.3 Å, and 15 ns onward it is constant upto 100 nanoseconds with ~ 0.3 Å. The ligand initially aroused ~ 0. ~ 45 Å by 20 nanoseconds and later constant fluctuation is observed in a range of ~ 0.3 to 0.325 Å till 100 nanoseconds. At the 100 nanosecond, the binding affinity of TPM and PPAR-γ was found out to be −4.98299*e*+05 kJ/mol (Fig. 11B).

Fig. 11 [Images not available. See PDF.]

RMSD analysis of PPAR*γ*-ligand complex; **A** PPAR*γ*-CF complex **B** PPAR*γ*-TPM complex **C** PPAR*γ*-T3 complex

Similarly, the PPAR*γ*-T3 complex revealed an acceptable fluctuation rate during the MD simulation study as total 0 to 100 ns RMSD. Initially, at 0 ns the starting point the protein deflected from 0.18 Å to max ~ 0.28 Å and finally settled at ~ 0.25 Å to 100 nanoseconds, whereas the ligand from 0 nanosecond deflected to ~ 0.32 Å and reached upto ~ 0.57 Å at 100 nanoseconds (Fig. 11C).

The root mean square fluctuation (RMSF) calculates mean fluctuations atoms or amino acid residues during the entire MD simulation period. For PPAR*γ*-CF complex, the calculated RMSF for the protein region is a maximum 0.5 Å and the ligand (CF) reached up to 0.15 Å (Fig. 12A). For PPAR*γ*-TPM complex, the calculated RMSF for the protein region is a maximum 0.75 Å and the ligand (TPM) reached up to 0.17 Å (Fig. 12B). Also the calculated RMSF for the protein region (PPAR*γ*-T3 complex) is a maximum 0.75 Å and the ligand (T3) reached up to Å 0.18 (Fig. 12C). The calculated RMSF values below 1 Å reveal no higher fluctuations within the atoms of the complexes, suggesting that all the PPAR*γ*-ligand complexes were stable.

Fig. 12 [Images not available. See PDF.]

RMSF analysis of PPAR*γ*-ligand complex; **A** PPAR*γ*-CF complex **B** PPAR*γ*-TPM complex **C** PPAR*γ*-T3 complex

Rg (radius of gyration) analysis explains the extent of unfolding and folding of the protein–ligand complex during entire MD simulation. The compactness of protein and bound ligand can be defined by Rg. High Rg values indicate lower compactness of proteins and ligand thereby suggesting poorly bound complex and vice versa. Here in PPAR*γ*-CF complex, the protein PPAR-*γ* exhibited a maximum of 2 nm and ligand CF exhibited at very low nearly 0.3 nm of fluctuation in Rg (Fig. 13A). In case of PPAR*γ*-TPM complex, the protein PPAR-*γ* exhibited a maximum 2 nm and TPM exhibited at very low nearly 0.47 nm of fluctuation in Rg (Fig. 13B), while in case of the PPAR*γ*-T3 complex, protein PPAR-*γ* exhibits a maximum 2 nm and ligand (T3) exhibited at very low as started with 0.5 nm and reduced to 0.2 nm of fluctuation in Rg revealing a highest stability in contrast to the complex (Fig. 13C).

Fig. 13 [Images not available. See PDF.]

Radius of gyration data for PPAR*γ*-ligand complex; **A** PPAR*γ*-CF complex **B** PPAR*γ*-TPM complex **C** PPAR*γ*-T3 complex

In the process of drug design, h-bonds play a vital role in absorption, metabolism, and transportation too. The PPAR-*γ* and CF complex revealed significant change in the bonding parameter, where initially the ligand was interacting within the binding site region 3 and maximum 4 h-bonds. Finally, from the 90 ns to 100 ns the number of h-bonds is only 1 with an energy of −4.98780*e*+05 kJ/mol (Fig. 14 A). TPM was observed to interact within the binding site region with maximum 7 h-bonds at nearly ~ 30 and ~ 70 ns. Finally, from the 80 ns to 100 ns the number of h-bonds is acquainted to 2 and 3 (Fig. 14B). T3 was observed to interact within the with maximum 8 h-bonds at between ~ 20 and ~ 30 ns at 50 ns, and between 75 to 80 ns. At nearly 100 ns, the number of h-bonds is acquainted to 5 and 6 with an energy of −4.99215*e*+05 kJ/mol (Fig. 14 C).

Fig. 14 [Images not available. See PDF.]

Hydrogen bond interactions in PPARγ-ligand complex; **A** PPARγ-CF complex **B** PPARγ-TPM complex **C** PPARγ-T3 complex

SASA (solvent of accessible surface area) detects changes in conformations in the protein–ligand complex that can be assessed by water or solvent during entire MD simulation. In our study, the black line/graph indicates the protein in solvent system and red lines graph indicates the protein and ligand complex for the entire length of time period of MD simulation. The overlapping exhibits that there are no or very minor fluctuations and the entire system is stable. However, lower deviation (by 155 nm^2) was observed for PPAR*γ*-T3 complex in comparison to PPAR*γ*-CF and PPAR*γ*-TPM complexes with a deviation by 160 nm^2 and 165 nm^2, respectively (Fig. 16), indicating higher stability of PPAR*γ*-T3 complex (Fig. 15).

Fig. 15 [Images not available. See PDF.]

SASA analysis for PPAR*γ*-ligand complexes **A** Black line—SASA (PPAR-*γ*) and Redline—SASA for Protein and Ligand (PPAR*γ*-CF); **B** Black line—SASA (PPAR-*γ*) and Redline—SASA for Protein and Ligand (PPAR*γ*-TPM); **C** Black line—SASA (PPAR-*γ*) and Redline—SASA for Protein and Ligand (PPAR*γ*-T3)

The molecular docking and MD simulations clearly suggested that in contrast to individual pharmacophores the conjugate T3 would strongly bind to allosteric site within the LBD of PPAR*γ*, thereby acting as an antagonist.

Chemistry

Synthesis of T3 [3-(3,4-Dihydroxy-phenyl)-acryloyl]-sulfamic acid 2,2,7,7-tetramethyl-tetrahydro-bis[1,3]dioxolo[4,5-b;4′,5′-d]pyran-3a-ylmethyl ester

Buff yellowish crystalline solid; Yield (53%); mp (175 °C); IR (KBr, cm^{-1}): 3700 (NH, amide) 3400 (–OH, phenolic) 1353, 1252 (–CH_3) 1645–1630 (C=O, amide), 1600–1445 (C–C), 1070 (C–O),1379, 1342 (S=O), 978 (trans alkene); ^{1}H NMR (400 MHz, DMSO-d 6): *δ* 1.29–1.47 (12H, 1.29(s), 1.34 (s), 1.37 (s), 1.47 (s)), 3.63 (1H, *d*, *J*=7.6 Hz), 3.74 (1H, *d*, *J*=7.6, Hz), 3.98 (2H, *d*, *J*=2.6 Hz)), 4.03 (1H, dd, *J*=12.9, 2.6 Hz), 4.25 (1H, dd, *J*=12.9, 3.0 Hz), 4.77–4.87 (2H, 4.60 (s), 4.62 (s)), 6.186 (1H, *d*, *J*=15.7 Hz), 6.78 (1H, *d*, *J*=8.4 Hz)), 6.95 (1H, *d*, *J*=1.9 Hz), 7.08 (2H, (dd, *J*=8.4, 1.9 Hz), 7.43 (IH, *d*, *J*=15.7 Hz), 7.61 (1H, *s*). 13 C NMR (100 MHz, DMSO-d 6): *δ* 26.3–26.4 (4C, 26.3 (*s*), 26.1 (*s*), 25.1 (*s*), 24.2 (*s*)), 65.4 (1C, *s*), 60.7 (1C, *s*), 69.17 (1C, *s*), 69.9 (1C, *s*), 70.1 (1C, *s*), 70.41 (1C, *s*), 100.9 (1C, *s*), 108.0 (1C, *s*), 115.05 (1C, *s*), 115.8 (1C, *s*), 116.0 (1C, *s*), 121.4 (1C, *s*), 125.7 (1C, *s*), 145.7 (1C, *s*), 146.8 (1C, *s*), 148.5 (1C, *s*), 168.47 (1C, *s*).

As depicted in Scheme 1, T3 was synthesized by coupling of topiramate to caffeic acid using DCC and catalytic amount of DMAP. Initially caffeic acid (1 equivalent) and DCC (1.1 equivalent) were dissolved in sufficient of ice cold DMF and stirred for one hour on a magnetic stirrer. The reaction proceeds with formation of acylimminium ion intermediate. DMAP acts as a acyl transfer reagent. After one hour of stirring, topiramate (2 equivalent) dissolved in excess DMF was added to the above reaction mixture and stirred continuously for 48 h. Structure of the synthesized conjugate was characterized using IR, NMR (^{1}H and ^{13}C). In IR spectra characteristic peaks 3700 (NH amide), 3400 (–OH, phenolic) 1353, 1252 (–CH_3) 1645–1630 (C=O, amide carbonyl), 1600–1445 (C–C), 1379, 1342 (S=O), and 978 (trans alkene) were confirmed. Further, in ^{13}C NMR spectra carbons of amide carbonyl functionality were confirmed at 167–170 ppm (please refer Additional file 1: Figs. S4 and S5) [56].

Pharmacological studies

Enzyme inhibition assay

For evaluating T3 as a PPAR*γ* inhibitor, TR-FRET co-activator assay was conducted. Binding of an agonist like rosiglitazone to PPAR*γ*, causes a conformational change around helix 12 in LBD that increases the affinity of co-activator peptide. Upon excitation at 337 nM, energy is transferred to the fluorescein label on co-activator peptide from the europium label, therefore detected as emission. In agonistic mode, the T3 did not display any significant increase in fluorescence emission even at 10 µmol/L. On the other hand, in the antagonist mode, T3 antagonized agonist (rosiglitazone) induced fluorescence responses. From Fig. 16 it is quite evident that both GW9662 (IC_{50} = 4.49 µM) and T3 (IC_{50} = 7.45 µM) exhibited dose-dependent inhibition of PPAR*γ*.

Fig. 16 [Images not available. See PDF.]

i Dose-dependent inhibitions of PPAR*γ* by GW9662 and T3 **ii** % PPARγ inhibition T3 relative to GW9662 [data given

in terms of mean ± standard deviation (*p<0.05) at n=3 experiments]

Effect of T3 on 3T3-L1 preadipocytes viability

For examining the cell level toxicity, the 3T3-L1 preadipocytes were treated with range of T3 concentrations (0.001, 0.01, 0.1, 1 and 10 μM) for 72 h and cell viability was assessed by MTT assay. Up to 0.1 μM of T3, no significant reduction in viability with respect to control (untreated) of 3T3-L1 preadipocytes were observed. A reduction in cell viability of about 10% and 20% was observed (Fig. 17) for 1 and 10 μM of T3 (p<0.05, ANOVA with t-test). Based on the above observations, concentrations below 10 μM (1, 3, 5. 7 and 10) of T3 were selected to evaluate the percentage lipid accumulation. The values for percentage viability of 3T3-L1 preadipocytes are provided (mean ± standard deviation) corresponding to triplicate experiments.

Fig. 17 [Images not available. See PDF.]

Effect of T3 on 3T3-L1 preadipocytes [Values presented as mean ± standard deviation (*p<0.05) at n=3 experimental repeats]

Effect of T3 on 3T3-L1 lipid accumulation and adipocyte differentiation

Oil Red O staining method explored the percentage of intracellular lipid accumulation in adipocyte stem cells. A dose-dependent inhibition of 3T3-L1 cells by T3 was observed. From the microscopic examinations it was pretty evident that there is reduction in size and number of 3T3-L1 cells containing larger lipid droplets (indicated in yellow colored arrows) with respect to control group (Fig. 18i). The percentage of fat accumulation was considerably reduced in T3 5–7 μM and highest at 10 μM concentrations (Fig. 18ii), with IC_{50} calculated as 7.98 μM.

Fig. 18 [Images not available. See PDF.]

Effect of T3 on lipid accumulation on 3T3-L1 preadipocytes **i** (A) control; (B) T3 (5 μM); (B) T3 (10 μM). **ii** Percentage fat accumulation. All the values are expressed as mean ± SD, at n=3 biological repeats

Effect of T3 on 3T3-L1 on PPARγ expression

It was very evident that reduction in lipid accumulation was very significant in the cell groups treated with 10 μM. For evaluating T3 as downregulator of PPARγ expression (master regulator of adipogenesis and differentiation), western blot was performed. T3 (10 μM) significantly decreased PPARγ expression in 3T3-L1 cells in comparison normal control (untreated) and GW9662 (10 μM) as positive control. These results were specific because β-actin levels were not affected (Fig. 19).

Fig. 19 [Images not available. See PDF.]

Effect of T3 on 3T3-L1 preadipocytes PPARγ expression (**i**) comparative downregulation of PPARϒ expressions of T3 with respect to positive control GW9662 and normal control (untreated 3T3-L1 cells) (**ii**) PPARϒ expressions of T3, positive control GW9662 and normal control (untreated 3T3-L1 cells) relative to β-actin. The values are expressed as mean ± standard deviation for n=3 biological repeats (*p<0.05 and **p<0.01)

Conclusion

Drug conjugates display better pharmacological properties than their individual pharmacophores. We synthesized novel drug conjugate TPAC as PPARγ inhibitors. PPARγ majorly orchestrates adipose tissue differentiation [57]. The development of new antiobesity medications has now centered around targeting adipogenesis and thereby associated signaling molecules and transcription factors. On this context, PPARγ becomes a promising target for antiobesity molecules. Topiramate, a marketed anticonvulsant drug, has been successfully repurposed and used as an antiobesity drug. Essentially, topiramate is a carbonic anhydrase inhibitor that tends to control obesity through enhancing satiety and regulating lipid metabolism. The antiobesity potential of the phenolic acids is attributed to controlling gut microbiome, energy metabolism, and control of adipogenic signaling pathways. On such grounds, it was interesting to design a new conjugate with two distinct pharmacophores exhibiting antiobesity potential via targeting adipogenesis and subsequently PPARγ.

To summarize, 10 topiramate–phenolic acid conjugates were designed and their PPARγ inhibitory activities were

predicted by robust 2D QSAR model with excellent goodness of fit. Highest PPAR*γ* inhibitory activity was predicted for T3 and accordingly was synthesized. The successful synthesis of the compound was confirmed by TLC, IR, and NMR analysis. T3 inhibited PPARγ (IC_{50} = 7.459 μM) which was also supported by the molecular docking study. The docking study also revealed that T3 binds efficiently within the allosteric pocket of the PPAR*γ* rather than the binding pocket and possess stronger interactions than the irreversible antagonist GW9662. Finally, T3 significantly inhibited adipocyte differentiation and lipid accumulation the differentiated adipocytes by downregulating the protein level expression of PPAR*γ* (IC_{50} = 7.8 μM). The in silico prediction for PPAR*γ* inhibitory activity of T3 was well corroborated by the in vitro experimental results. From the enzyme inhibition assay, it was noticeable that GW9662 (positive control) had better inhibition. But interestingly at the cellular level T3 exhibited higher antiadipogenic characteristics by downregulating PPAR*γ* protein level expressions than GW9662. Further investigations of effect of T3 on protein and gene level expressions of other adipogenic factors can strongly establish the antiadipogenic potency of T3. To conclude our study, we strongly suggest T3 as a potential PPAR*γ* inhibitor that significantly downregulates adipogenesis and differentiation, thereby conferring antiobesity potency to the newly synthesized conjugate.

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Author contributions

IP performed investigation and original draft preparation. BB performed investigation. PGRA performed data curation, software handling, and draft reviewing. PPG performed software handling and writing. TS contributed to conceptualization, methodology, and draft reviewing and editing.

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Availability of data and material

All data generated or analyzed during this study are included in supplementary information files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interest

The authors have no competing interests to declare that are relevant to the content of this article.

Abbreviations

ANOVA
Analysis of variance
AP-1
Activation protein 1
BCS
Bovine calf serum
BSA
Bovine serum albumin
CNS
Central nervous system
CF
Caffeic acid
DCC
N,N'-Dicyclohexyl carbodimide
DMAP

4-Dimethylaminopyridine
DMEM
Dulbecco’s modified eagle’s medium
DMSO
Dimethyl sulfoxide
DMF
Dimethylformamide
FDA
Food and drug administration
GLP-1
Glucagon-like peptide 1
GABA
-Aminobutyric acid
GA
Genetic algorithm
HRP
Horseradish peroxidase
IBMX
3-Isobutyl-1-methyl xanthine
LBD
Ligand binding domain
LMO
Leave many out
LOF
Friedman lack of fit
LOO
Leave one out
MTT
(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
MLR
Multiple linear regression
MD
Molecular dynamics
NCCS
National Centre for Cell Science
NFκB
Nuclear factor kappa B
OECD
Organization for Economic Cooperation and Development
PCA
Principle component analysis
PPARγ
Peroxisome proliferator-activated receptor
PBS-T
Phosphate-buffered saline-Tween 20
QSAR
Quantitative structure–activity relationship

RMSD
Root mean square deviation
RMSF
Root mean square fluctuation
Rg
Radius of gyration
SMILES
Simplified molecular input line system
SDS-PAGE
Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SASA
Solvent accessible surface area
TR-FRET
Time-resolved fluorescence resonance energy transfer
TPM
Topiramate
TPAC
Topiramate–phenolic acid conjugates
T3
Topiramate–caffeic acid conjugate

Publisher's Note

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DETAILS

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Design of experiments and white analytical chemistry-driven green and sensitive spectrofluorimetric estimation of pregabalin in its pharmaceutical dosage forms and spiked human plasma

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ABSTRACT (ENGLISH)

Background

Pregabalin (PGB) is a medication with anticonvulsant, analgesic and anxiolytic properties, employed in the treatment of epilepsy, neuropathic pain, fibromyalgia, restless leg syndrome, opioid withdrawal syndrome and generalized anxiety disorder. Several spectrofluorimetric techniques have been documented for the determination of PGB in pharmaceutical dosage forms. However, these published methods typically involve the use of expensive and toxic organic solvents and reagents, as well as high reaction temperatures for PGB analysis. These components pose risks to aquatic life and the environment, making them less environmentally friendly and user-friendly. A recent advancement in analytical chemistry has introduced a white analytical approach, providing an economical, eco-friendly and user-friendly method for the development of analytical procedures.

Objectives

Therefore, a green and sensitive spectrofluorimetric determination of PGB, guided by white analytical chemistry principles, has been conducted utilizing distilled water as an environmentally friendly solvent.

Methods
The establishment of the spectrofluorimetric method involved employing the design of experiments approach to ensure a robust, precise and accurate estimation of PGB. Response surface analysis and optimization of critical procedural variables and responses were carried out using the central composite design. The validation of the developed method adhered to the guidelines outlined in ICH (International Council for Harmonization) Q2 (R1) and M10.
Results
The established spectrofluorimetric method was utilized to determine the PGB content in commercially available formulations and human plasma samples spiked with PGB. The obtained results were in accordance with the labeled claim of PGB in the formulations. The recovery of PGB in the spiked human plasma samples ranged from 85 to 90% of the spiked amount.
Conclusions
The greenness profiles of the published and suggested spectrofluorimetric methods for PGB estimation were evaluated and compared using the AGREE calculator, GAPI software and ESA tool. The suggested method demonstrated sensitivity, robustness, environmental friendliness and user-friendliness.

FULL TEXT

Background
Recently introduced into the literature, white analytical chemistry is an approach for the development and evaluation of accurate, sensitive, precise, environmentally friendly and cost-effective analytical methods for estimating drugs and their dosage forms. White analytical chemistry incorporates the key principles of green analytical chemistry in method development. It involves an RGB (red, green and blue) model-based assessment of the developed analytical method. The red (R) model-based assessment comprises four principles: R1—scopes and applications, R2—accuracy, R3—precision and R4—linearity and sensitivity. The green (G) model-based assessment includes the four major principles of green analytical chemistry: G1—environmental impact, G2—energy consumption, G3—waste generation and G4—health impact of the analytical method. The blue (B) model-based assessment includes four major principles: B1—time efficiency, B2—cost efficiency, B3—simplicity of the method and B4—instrument handling for the assessment of the analytical method [1].
Within the pharmaceutical industry, chromatographic analytical methods are frequently employed for the quality control and routine analysis of both drug substances and drug products. In the course of chromatographic analysis of drug samples, toxic organic solvents like chloroform, acetonitrile, methanol, toluene, etc., have traditionally been utilized and subsequently left as organic waste in the environment. Adhering to standards outlined in the Indian Pharmacopoeia, ICH (International Council for Harmonization) Q3C guidelines and published solvents selection guides, these organic solvents are recognized as harmful to aquatic animal life and environmentally hazardous. In line with the principles of green and white analytical chemistry, there is a concerted effort to reduce, eliminate and recycle the consumption and wastage of such toxic organic solvents during the development and life cycle management of analytical methods, aiming to preserve the environment [2–5].
The design of experiments (DoE) is a systematic methodology employed in the development of analytical methods to ensure robustness, precision and accuracy. As outlined in the ICH Q14 guideline, the incorporation of the design of experiments approach is a regulatory requirement for the registration and approval of new drug substances and products. Additionally, the design of experiments is a valuable tool for the implementation of white analytical chemistry, aiding in the development of analytical methods that are both robust and precise. Furthermore, the application of the design of experiments proves beneficial in minimizing the consumption of organic solvents during the development of analytical methods. Recent literature reveals the emergence of numerous published analytical methods that integrate hybrid principles of design of experiments, green analytical chemistry and white analytical chemistry [6–10].
Pregabalin (PGB) is a medication with anticonvulsant, analgesic and anxiolytic properties, prescribed for conditions such as epilepsy, neuropathic pain, fibromyalgia, restless leg syndrome, opioid withdrawal syndrome and

generalized anxiety disorder [11]. Numerous methods, including spectrophotometric, RP-HPLC (reversed-phase high-pressure liquid chromatographic) and LC–MS/MS, have been documented in the literature for estimating PGB in pharmaceutical dosage forms and conducting bioanalysis [12–22]. However, these analytical approaches involve the use of toxic organic solvents, posing environmental risks. Spectrofluorimetric methods, recognized for their sensitivity, specificity, accuracy and precision in drug sample estimation, offer a more environmentally friendly alternative with reduced organic solvent usage, time and cost. In the context of implementing the principles of white analytical chemistry, the spectrofluorimetric method stands out as an ideal choice for green, accurate, sensitive, precise and economical analysis of drug samples. Notably, PGB is a nonfluorescent drug molecule, necessitating derivatization for the development of a spectrofluorimetric method. Recent literature has featured various spectrofluorimetric methods utilizing different derivative reagents as fluorescent probes for PGB estimation [23–28]. However, some published spectrofluorimetric methods employ toxic organic solvents, high reaction temperatures and toxic reagents, generating over 100 mL of organic waste. Additionally, the sensitivity of certain published spectrofluorimetric methods is limited to the microgram level.

Therefore, a sensitive, robust and environmentally friendly spectrofluorimetric method has been developed for the estimation of PGB, utilizing safe solvents and eliminating the need for derivative reagents. The development of this spectrofluorimetric method followed a comprehensive strategy based on the principles of white analytical chemistry and the design of experiments. The design of experiments approach was implemented using a central composite design, which facilitated response surface analysis and optimization of the spectrofluorimetric method. The developed method underwent validation in accordance with ICH Q2 (R1) and M10 guidelines. Application of the developed spectrofluorimetric method included the assay of both marketed formulations and spiked human plasma samples. To assess and compare the greenness and whiteness profiles of the published and developed spectrofluorimetric methods, various tools and models such as the AGREE (Analytical Greenness) calculator, ESA (Eco-scale Assessment) tool, GAPI (Green Analytical Procedure Index) and RGB (red, green and blue) models were employed.

Methods

Instruments and softwares

The determination of PGB was conducted utilizing a Spectrofluorometer Shimadzu RF-5301 PC model, obtained from Toshvin Analytical Private Limited in Vapi, Gujarat, India. The spectrum of PGB was recorded using a UV–Visible spectrophotometer 3092 model (Lab India Private Limited, Mumbai, Maharashtra, India). For precise weighing, a single pan digital analytical balance, acquired from Shimadzu Scientific Instruments (India) Private Limited in Bangalore, India, was employed. The response surface modeling based on Design of Experiments (DoE) was carried out using MINITAB 18 software (trial version). To evaluate the environmental friendliness of the method, the AGREE calculator (accessible at https://mostwiedzy.pl/AGREE) was employed. Additionally, the greenness profile of the method was assessed using Complex GAPI software, which can be accessed at mostwiedzy.pl/complexgapi.

Reagents and materials

The PGB API (Active Pharmaceutical Ingredient) with 99.98%W/W purity was received as a complimentary gift sample from Vapi Care Pharma Private Limited in Gujarat, India. High-quality double-distilled water was meticulously prepared in the Quality Assurance Laboratory at our institute. To facilitate filtration, membrane filters (0.45 µm) and sample syringe filters (0.22 µm) were acquired from Pall India Private Limited, located in Andheri, Mumbai, India. The pharmaceutical dosage forms of PGB (Pregazo 75 capsule—contains 75 mg PGB, Pregal 75 capsule—contains 75 mg PGB, and Pregadd tablets—contains 50 mg PGB) were procured from the local market in Surat, Gujarat, India.

Establishing calibration curve for assay of marketed formulations

A standard solution of PGB (1000 µg/ml) was prepared by dissolving 10 mg of PGB in distilled water in a 10-mL volumetric flask. Subsequent dilutions were carried out with distilled water to generate concentrations ranging from 1.0 to 25 µg/ml. Consequently, 100 µL from the working solution (10 to 25 µg/ml) of PGB was transferred into a

series of 10-mL volumetric flasks and topped up to the mark with borate buffer of pH 10.0, resulting in the final concentration range of 10–250 ng/mL. The fluorescence intensity was measured at an emission wavelength of 410 nm using an excitation wavelength of 230 nm. A blank experiment was conducted in a similar manner without the presence of PGB.

Establishing a calibration curve through the spiking of PGB to human plasma samples

In each 100 µL aliquot of human plasma, a 10 µL portion from distinct working standard solutions (100–2500 µg/mL) was individually introduced, thoroughly mixed and vortexed for 15 min. To extract PGB from the human plasma, 3.0 mL of methanol was added as a precipitating agent and mixed for an additional 15 min. Following complete precipitation, the resulting samples were diluted to a final volume of 10 mL with methanol. Subsequently, each sample underwent centrifugation at 4000 RPM for 15 min. A 1.0 mL aliquot from the supernatant of each solution was filtered through a 0.22 µm syringe filter and transferred into a series of 10-mL volumetric flasks, completed to the mark with borate buffer of pH 10.0, resulting in the final concentration range of 10–250 ng/mL. The fluorescence intensity was measured at an emission wavelength of 410 nm using an excitation wavelength of 230 nm. A blank experiment was conducted similarly without the presence of PGB.

Implementing design of experiments approach in method development

Following preliminary experiments, critical procedural variables and method performance attributes (responses) were identified for the development of the intended method. These critical method variables were examined for their main effects, two-way interactions and quadratic effects on selected responses using central composite design through Minitab 18 software. Navigating the analytical design space was allowed for the optimization of responses in the targeted spectrofluorimetric method for estimating PGB. Mean response surface analysis was conducted to delineate the analytical design space and validate the model.

Analysis of pregabalin in marketed formulations

A sample equivalent to 10 mg of PGB was taken from each pharmaceutical dosage form of PGB. This sample was dissolved, diluted to 10 mL with distilled water and then filtered. Subsequently, a 1.0 mL aliquot was further diluted to 10 mL with distilled water. From this solution, another 1.0 mL aliquot was diluted to 10 mL with distilled water. A 100 µL aliquot of the sample was then transferred into a 10-mL volumetric flask and completed to the mark with borate buffer at pH 10.0. The fluorescence intensity was measured at an emission wavelength of 410 nm using an excitation wavelength of 230 nm. A blank experiment was conducted in a similar manner, excluding the presence of PGB sample.

Results

Defining the analytical target profile for the design of experiments approach

The spectrofluorimetric method was developed to estimate PGB in its pharmaceutical dosage forms and spiked human plasma samples, employing environmentally safe and sustainable solvents. The developed method was designed to yield optimal fluorescence intensity and % recoveries of PGB, ensuring sensitive, selective and specific estimation in the proposed matrices. Consequently, fluorescence intensity and % recovery of PGB have been identified as crucial responses in the development of the targeted spectrofluorimetric method (Fig. 1).

Fig. 1 [Images not available. See PDF.]

Pareto chart analysis using central composite design and Minitab 18 software. **A** Pareto chart showing significant main effects and quadratic effects of critical procedure variables on fluorescence intensity—Response R1 and **B** Pareto chart showing significant main effects and quadratic effects of critical procedure variables on % recoveries—Response R2

Utilizing response surface analysis for method optimization

The Minitab 18 software proposed thirteen experimental runs (refer to Table 1), which were subsequently conducted in the laboratory. The recorded responses from these experimental runs were analyzed through ANOVA, multiple regression analysis and response surface analysis using the software. According to the ANOVA results (refer to Table 2), the main effects of critical procedure variables A and B were found to be significant for responses R1 and

R2, along with significant quadratic effects of critical procedure variables A and B. However, the two-way interactions of critical procedure variables A and B were determined to be nonsignificant for responses R1 and R2. The multiple regression analysis revealed R-squared, adjusted R-squared and predicted R-squared values for responses exceeding 0.9, with the difference between adjusted R-squared and predicted R-squared being less than 0.2. These outcomes suggest that the selected models were well suited for predicting responses R1 and R2. The 2D and 3D response surface contour plots (refer to Figs. 2A, B, 3A, B) illustrate multidimensional interactions and the quadratic relationship between critical procedure variables (A and B) and responses R1 and R2. The analytical design space was navigated to achieve maximum fluorescence intensity and % recovery of PGB, aligning with the analytical target profile of the method, using overlaid response surface plots.

Table 1. Design metrics and measured responses for DoE-based response surface analysis and optimization of critical procedure variables (CPVs) using central composite design by Minitab 18 software

Standard run order	Random run order	Point type	Blocks	CPV—A	CPV—B	Response R1	Response R2
3	1	Factorial	1	−1	+1	260.14	90.14
7	2	Axial	1	0	−α	170.12	75.14
12	3	Center	1	0	0	230.41	85.49
13	4	Center	1	0	0	230.74	85.78
5	5	Axial	1	−α	0	240.12	87.14
2	6	Factorial	1	+1	−1	110.14	65.11
4	7	Factorial	1	+1	+1	160.11	73.18
11	8	Center	1	0	0	230.47	85.71
9	9	Center	1	0	0	230.74	85.88
1	10	Factorial	1	−1	−1	200.11	80.41
8	11	Axial	1	0	+α	210.45	83.14
10	12	Center	1	0	0	230.11	85.42
6	13	Axial	1	+α	0	140.77	70.12

CPV—A (Excitation wavelength in nm): Axial point (−α)—225.85, low level (−1)—230 nm, medium level (0)—240 nm, high level (+1)—250nm and axial point (+*α*)—254.142

CPV—B (pH of medium): Axial point (−*α*)—7.59, low level (−1)—8.0, medium level (0)—9.0, high level (+1)—10.0 and axial point (+*α*)—10.41

Table 2. Analysis of variances (ANOVA) for study of significant main effect, two-way interactions and quadratic

effects of critical procedure variables using central composite design and Minitab 18 software

Source	Degree of freedom (DF)	Adjusted sum of square	Adjusted mean square	*F*-Value	*P*-Value
ANOVA for Response R1—Fluorescence intensity of PGB					
Model	5	23,123.5	4624.7	41.66	0.000
Linear	2	17,141.6	8570.8	77.21	0.000
CPV—A	1	13,654.0	13,654.0	123.01	0.000
CPV—B	1	3487.6	3487.6	31.42	0.001
Square	2	5956.7	2978.3	26.83	0.001
CPV—A*CPV—A	1	3354.6	3354.6	30.22	0.001
CPV—B*CPV—B	1	3379.1	3379.1	30.44	0.001
Two-way interaction	1	25.3	25.3	0.23	0.648
CPV—A*CPV—B	1	25.3	25.3	0.23	0.648
ANOVA for Response R2—%recovery of PGB					
Model	5	681.653	136.331	49.17	0.000
Linear	2	502.583	251.292	90.64	0.000
CPV—A	1	396.632	396.632	143.07	0.000
CPV—B	1	105.951	105.951	38.22	0.000
Square	2	178.381	89.190	32.17	0.000
CPV—A*CPV—A	1	107.538	107.538	38.79	0.000
CPV—B*CPV—B	1	94.042	94.042	33.92	0.001
Two-way interaction	1	0.689	0.689	0.25	0.633

CPV—A*CPV—B	1	0.689	0.689	0.25	0.633

Fig. 2 [Images not available. See PDF.]

DoE-based response surface analysis of critical method variables and responses using central composite design. **A** 2D contour plot showing multidimensional interactions between critical procedure variables A and B with fluorescence intensity—Response R1. **B** 2D contour plot showing multidimensional interactions between critical method variables A and B with % recoveries—Response R2. **C** Analytical design space for desirable maximum fluorescence intensity and %recovery of PGB

Fig. 3 [Images not available. See PDF.]

DoE-based response surface analysis of critical method variables and responses using central composite design and Minitab 18 software. **A** 3D contour plot showing multidimensional interactions between critical procedure variables A and B with fluorescence intensity—Response R1. **B** 3D contour plot showing multidimensional interactions between critical method variables A and B with % recoveries—Response R2. **C** Analytical design space for desirable maximum fluorescence intensity and %recovery of PGB

Employing the mean response surface method to define the analytical design space

Following an examination of the overlaid response surface plots, the analytical design spaces, directed toward achieving maximum fluorescence intensity and % recovery of PGB, were scrutinized for accuracy and precision in predicting responses R1 and R2 using the mean response surface method. The visual representation of these navigated analytical design spaces is depicted in Figs. 2C and 3C, highlighted in white shades. Subsequently, the experimental runs recommended by the software were executed in the laboratory, and the actual responses were analyzed for their %RSD (n=3). The %RSD for all experimental runs was determined to be less than 2%, indicating the accurate and precise prediction of responses R1 and R2 by the suggested models.

Spectrofluorimetric method validation

The method's specificity was confirmed by comparing fluorescence spectra among various samples, including blank and standard PGB. Blank samples exhibited no interference from excipients or human plasma matrices (Refer Additional file 1: Fig. S2). Linearity was established through five repetitions of calibration curve procedures, demonstrating a linear relationship within the 10–250 ng/mL concentration range, with a correlation coefficient exceeding 0.995 (refer to Figs. 4 and 5). Additionally, % recoveries of PGB from spiked human plasma samples (at 50%, 100% and 150% levels) ranged from 85 to 90%. Evaluations for sample carryover and sample dilution in the estimation of PGB were conducted within spiked human plasma samples. The method's robustness was assessed by varying scanning speed and excitation wavelength, yielding consistent results. Precision studies for both intra-day and inter-day analyses demonstrated the method's reliability in estimating PGB. The method exhibited remarkable sensitivity, with the lowest limit of detection and quantitation determined to be 5.0 ng/mL and 10 ng/mL, respectively, underscoring its capability to detect PGB at nanogram-level concentrations. A summary of the validation parameters is presented in Table 3.

Fig. 4 [Images not available. See PDF.]

Overlaid fluorescence spectrum showing linearity of PGB over concentration range of 10–50 ng/mL in spiked human plasma at emission wavelength of 410 nm and excitation wavelength of 230 nm

Fig. 5 [Images not available. See PDF.]

Overlaid fluorescence spectrum showing linearity of PGB over concentration range of 50–250 ng/mL at emission wavelength of 410 nm and excitation wavelength of 230 nm

Table 3. Validation summary of developed spectrofluorimetric method as per ICH guidelines

Validation as per ICH Q2 (R1) guideline for assay of PGB in pharmaceutical dosage forms		Validation as per ICH M10 guideline for analysis of spiked human plasma samples of PGB	
Validation parameter	Results	Validation parameters	Results
Linearity range	10 to 250 ng/mL	Linearity range	10 to 250 ng/mL
Regression line equation	$Y = 1.7606x + 85.838$	Regression line equation	$Y = 0.4468x + 154.41$
Correlation coefficient	0.9955	Correlation coefficient	0.9976
Repeatability of sample measurement	0.88% (RSD)	Stability study (at 4 °C , 25 °C and −20 °C for 24 h)	6.77–8.65% (RSD)
Repeatability of sample preparation	0.98% (RSD)	Carryover effect and matrix effect	5.87–8.23% (RSD)
Intra-day precision	0.95–1.45% (RSD)	Intra-day precision	6.33 – 7.45% (RSD)
Inter-day precision	1.23–1.98% (RSD)	Inter-day precision	7.99–8.78% (RSD)
%recovery	98–102%	%recovery	85–90%
Limit of detection (LOQ)	5.0 ng/mL	Lowest limit of detection (LLOD)	5.0 ng/mL
Limit of quantitation	10.0 ng/mL	Lowest limit of quantitation (LLOQ)	10.0 ng/mL
Robustness	1.77–1.99% (RSD)	Robustness	8.77–9.32% (RSD)
Specificity	specific	Specificity	Specific

Formulations assay and spiked human plasma samples analysis

The established method was employed to assay various market formulations of PGB, revealing % assay values for

PGB in the formulations within the range of 95% to 105% of the labeled claim of PGB. The presence of excipients did not cause interference in the estimation of PGB. In spiked human plasma samples, the % recovery of PGB was determined to be in the range of 85% to 90%. The matrices of blank human plasma samples did not pose interference in the estimation of PGB.

Discussion

The UV spectrum of PGB in distilled water exhibited two peaks at 210 nm and 230 nm (Refer Additional file 1: Figure S1 for UV spectrum of PGB), respectively. The 210 nm peak corresponded to the π–π^* transition, while the 230 nm peak was attributed to the $n - \pi^*$ transition of the primary amine functional group in PGB. Upon excitation at 230 nm, the nitrogen of the amino group led to a fluorescence emission at 410 nm. The fluorescence intensity at 410 nm was employed for a linearity study, revealing a linear relationship at nanogram-level concentrations of PGB. This fluorescence intensity was further examined in buffer solutions spanning a pH range of 1.0 to 12.0, with the highest intensity observed in borate buffer at pH 8–10. The existing literature has detailed spectrofluorimetric methods involving derivatization, often utilizing toxic organic solvents and reagents, and exhibiting sensitivity at microgram levels. Additionally, these methods necessitated high reaction temperatures during the derivatization of PGB, rendering them less user-friendly. Notably, no spectrofluorimetric method in the literature has been reported for the detection and quantification of PGB without the need for derivatization.

A recent development in analytical chemistry advocates for a white analytical chemistry approach, emphasizing the development of green, economical and user-friendly analytical methods. Following the ICH Q14 guideline, the implementation of a design of experiments approach is a regulatory requirement for the registration of new drug products and substances. Despite this, the literature lacks an analytical method for estimating PGB using the white analytical chemistry and design of experiments approach. In response, a robust, green and user-friendly spectrofluorimetric method has been devised for the estimation of PGB in pharmaceutical dosage forms and spiked human plasma samples. This method integrates the principles of white analytical chemistry and the design of experiments approach, aligning with the contemporary trend toward environmentally conscious and efficient analytical methodologies, as suggested by recent developments in analytical chemistry.

Design of experiments using Minitab 18 software

After conducting initial experiments, it was determined that the excitation wavelength (designated as critical procedure variable A) and the pH of the medium (designated as critical procedure variable B) played pivotal roles. These variables were subsequently investigated to establish their graphical relationships with selected responses using response surface methodology. The fluorescence intensity and percentage recovery of PGB were chosen as critical responses for the development of a targeted spectrofluorimetric method. To analyze the identified critical procedure variables, a central composite design was employed, allowing the examination of main effects, two-way interactions and quadratic effects of the variables on the selected responses (R1 and R2). The analytical design space was explored to achieve maximum fluorescence intensity and percentage recovery of PGB in alignment with the analytical target profile of the method, as illustrated through overlaid response surface plots. Following a thorough examination of these plots, the navigated analytical design spaces for maximum fluorescence intensity and percentage recovery of PGB were assessed for accuracy and precision in predicting responses R1 and R2 using the mean response surface method. The optimized range for the excitation wavelength (critical procedure variable A) was determined to be 230 to 240 nm, while the optimized range for the pH of the medium (critical procedure variable B) was identified as 9.0 to 10.0. Consequently, the spectrofluorimetric estimation of PGB was conducted with an excitation wavelength of 230 nm at a pH of 9.0 in a borate buffer.

The developed spectrofluorimetric method has undergone validation in accordance with the guidelines of ICH Q2 (R1) for the assay of pharmaceutical dosage forms, encompassing specificity, accuracy, linearity, precision, LOD, LOQ and robustness. Additionally, the validation of the developed spectrofluorimetric method has been conducted following the ICH M10 guideline for estimating PGB in spiked human plasma samples. The results indicate that the developed method is accurate, precise, robust, sensitive, specific and linear in the estimation of PGB in both its pharmaceutical dosage forms and spiked human plasma samples. All validation parameters align with the standards

outlined in the ICH Q2 (R1) and M10 guidelines.

The conventional technique was utilized to assay different commercial formulations of PGB (Pregazo 75 capsule—contains 75 mg PGB, Pregal 75 capsule—contains 75 mg PGB, and Pregadd tablets—contains 50 mg PGB), uncovering assay values for PGB in these formulations falling between 95 to 105% of the stated PGB content. Interestingly, the addition of other substances did not affect the measurement of PGB. When PGB was intentionally added to human plasma samples, its recovery ranged from 85 to 90%. Remarkably, the absence of any interfering substances in blank human plasma samples did not hinder the determination of PGB levels (refer supplementary files for fluorescence spectrum of blank human plasma and reagents). According to the reported LC–MS/MS method [20], the reported linearity range and Cmax value of PGB was found to be 0.1 to 15 µg/mL and 6.925 to 7.477 µg/mL in human plasma. The proposed spectrofluorimetric method was found to be complied with the reported LC–MS/MS method for the estimation of PGB in spiked human plasma samples. The developed method can be extended as sensitive and green analytical tool for pharmacokinetic and bioequivalence study of PGB in human and rat plasma.

Evaluation of spectrofluorimetric methods through white analytical chemistry principles

The comparative analysis of the existing and proposed methods for estimating PGB has been conducted through white analytical chemistry and an RGB model-based scoring system, as detailed in Table 4. The spectrophotometric methods published in the literature were evaluated against the proposed spectrofluorimetric method using both the RGB and WAC scoring systems. The red model-based assessment focused on evaluating the validation efficiency, sensitivity, scope and applicability of spectrofluorimetric methods. Some published spectrofluorimetric methods [24, 26, 27] exhibited sensitivity at the microgram level, earning a Rrd model-based score of 95 out of 100 (refer to Table 4). In contrast, our proposed method and other published spectrofluorimetric methods [22, 23] achieved a perfect score of 100, attributed to their nanogram-level sensitivity and applicability for PGB estimation in marketed formulations and spiked human plasma. This high score reflects their implementation of the design of experiments approach, exceptional sensitivity, % recoveries and applicability for PGB estimation.

Table 4. Comparison and assessment of published and proposed spectrofluorimetric for the estimation of PGB using principles of green and white analytical chemistry

Types of spectrofluorimetric method	Analytical conditions	Sensitivity, whiteness and greenness profile assessment of methods

Method 1—Spectrofluorimetric estimation of PBG using 7-chloro-4-nitrobenzofurazon (NBD-Cl) as fluorescent probe [22]	Linearity—40 to 400 ng/mL Derivatization agent—7-chloro-4-nitrobenzofurazon (NBD-Cl) Excitation wavelength—460 nm Emission wavelength—558 nm Extraction solvent—Chloroform (More than 10 mL) Reaction temperature—60 to 80˚C Application—Assay of marketed formulations	The method includes toxic organic solvents and reagents. The reaction temperature is more than room temperature The method has sensitivity as nanogram level AGREE score: 0.55 GAPI e-factor: 0.5 ESA penalty points: 08 Red model s score—100/100 Green model score—55/100 Blue model score—90/100 Whiteness score—(100 + 55 + 90)/3 = 81.66
Method 2—Spectrofluorimetric estimation of PGB using fluorescamine, 2,4-dinitrofluorobenzene and chloranil as fluorescent probes [23]	Linearity—20 to 280 ng/mL Derivatization agent—Fluorescamine, 2,4-dinitrofluorobenzene and chloranil Excitation wavelength—385 nm Emission wavelength—485 nm Extraction solvent—Water Reaction temperature—60 to 80˚C Application—Assay of marketed formulations and bioanalysis of spiked urine samples	The method needs toxic derivatization reagents and high temperature for reaction The method has sensitivity as nanogram level AGREE score: 0.60 GAPI e-factor: 0.50 ESA penalty points: 06 Red model s score—100/100 Green model score—60/100 Blue model score—90/100 Whiteness score—(100 + 60 + 90)/3 = 83.33

Method 3—Spectrofluorimetric estimation of PGB using fluorescamine as fluorescent probe [24]	Linearity—0.01 to 0.3 µg/mL Derivatization agent—Fluorescamine Excitation wavelength—390 nm Emission wavelength—487 nm Extraction solvent—Water Reaction temperature—60 to 80˚C Application—Assay of marketed formulations	The method needs toxic derivatization reagents and high temperature for reaction The method has sensitivity as micro and nanogram level AGREE score: 0.65 GAPI e-factor: 0.35 ESA penalty points: 06 Red model s score—95/100 Green model score—65/100 Blue model score—90/100 Whiteness score—(95 + 65 + 90)/3 = 83.33
Method 4—Spectrofluorimetric estimation of PGB using carbon quantum dots [26]	Linearity—4 to 100 µg/mL Derivatization agent—Carbon quantum dots from ascorbic acid Excitation wavelength—356 nm Emission wavelength—524 nm Extraction solvent—Water Reaction temperature—160˚C, 70 min Application—Assay of marketed formulations and bioanalysis of spiked urine sample	The method needs toxic reagents and very high temperature for reaction The method has sensitivity as microgram level only AGREE score: 0.65 GAPI e-factor: 0.35 ESA penalty points: 06 Red model s score—95/100 Green model score—65/100 Blue model score—90/100 Whiteness score—(95 + 65 + 90)/3 = 83.33

Method 5—Spectrofluorimetric estimation of PGB using acetylbutyrolactone as fluorescent probe [27]	Linearity—10 to 100 µg/mL Derivatization agent—Acetylbutyrolactone Excitation wavelength—380 nm Emission wavelength—440 nm Extraction solvent—Water Reaction temperature—95˚C Application—Assay of marketed formulations	The method needs toxic derivatization reagents and high temperature for reaction The method has sensitivity as microgram level only AGREE score: 0.65 GAPI e-factor: 0.35 ESA penalty points: 06 Red model s score—95/100 Green model score—65/100 Blue model score—90/100 Whiteness score—(95 + 65 + 90)/3 = 83.33
Proposed spectrofluorimetric estimation of PGB	Linearity—10 to 250 ng/mL Derivatization agent—No reagent Excitation wavelength—230 nm Emission wavelength—410 nm Extraction solvent—Water Reaction temperature—25˚C (Room Temperature) Application—Assay of marketed formulations	No chemical reaction required and water is used as solvent The method has sensitivity at nanogram level. The method does not require heating. Hence, it is more user-friendly AGREE score: 0.80 GAPI e-factor: 0.25 ESA penalty points: 03 Red model s score—100/100 Green model score—80/100 Blue model score—100/100 Whiteness score—(100 + 80 + 100)/3 = 93.33

The green model-based scoring and greenness profile assessment of analytical methods utilized the AGREE calculator, GAPI software, NEMI standards and ESA tool. However, the literature suggests that NEMI standards for greenness assessment provide the least accurate information. Therefore, AGREE and GAPI scores were considered for the calculation of the green model-based score. The green model-based scoring system assessed environmental friendliness, with published methods scoring less than 65 out of 100 based on AGREE and GAPI software criteria. The proposed method earned a score of 80 due to its use of environmentally friendly solvents (distilled water and methanol only), reduced organic waste generation and lower power consumption for PGB estimation. In contrast, the published spectrofluorimetric methods required high reaction temperatures and costly organic solvents and derivative reagents.

Assessing time, cost efficiency and user-friendliness through the blue model-based scoring system, published

spectrophotometric methods scored 90 out of 100, respectively. The proposed method, which does not require high temperatures or costly derivative reagents and solvents for PGB estimation, achieved a perfect score of 100 for its economical, rapid, user-friendly and straightforward approach. The overall WAC (white analytical chemistry) score, calculated by averaging RGB model scores, for published spectrofluorimetric methods was found to be less than 84 out of 100. In contrast, the proposed method received a score of 93.33 due to its validation efficiency, environmental friendliness, speed, cost-effectiveness and user-friendliness in PGB estimation. A comprehensive comparison of the greenness profile, WAC and RGB model-based scoring is depicted in Fig. 6A, B.

Fig. 6 [Images not available. See PDF.]

A Greenness profile study of proposed spectrofluorimetric method using AGREE and GAPI software. **B** Whiteness profile assessment and comparison of published and proposed spectrofluorimetric methods for the estimation of PGB using RGB model score

Conclusions

A hybrid approach, combining principles from white analytical chemistry and design of experiments, was employed for the spectrophotometric estimation of PGB. The resulting method incorporates safe, environmentally friendly and cost-effective solvents. Validation of the developed method encompassed specificity, sensitivity, selectivity, precision, accuracy and linearity in the estimation of PGB. Application of the method to assess PGB in various marketed formulations yielded results in accordance with the labeled claim. Furthermore, the method's applicability was extended to the estimation of PGB in spiked human plasma samples, demonstrating validation in accordance with ICH M10 guidelines. Both the developed and published methods were evaluated for their greenness and whiteness profiles in PGB estimation using RGB model scoring system, AGREE and GAPI software. The proposed method proved to be sensitive, robust, environmentally friendly, economical and user-friendly in estimating PGB across diverse samples. This developed method holds potential for use in pharmacokinetic and pharmacodynamic studies of PGB, presenting an economical and eco-friendly analytical tool for bioanalysis and quality control of PGB pharmaceutical dosage forms in the pharmaceutical industry.

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Author contributions

Dr Pintu Prajapati was involved in drafting, conceptualization, supervision and review. Veera Shakar Pulusu contributed to review, software analysis handling and drafting. Shailesh Shah took part in supervision and review support.

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Availability of data and materials

The data will be availed by corresponding author of manuscript on reasonable request.

Declarations

Ethics approval and consent to participate

Our research work does not include use of animal and human being. The manuscript has been solely submitted for publication in the Future Journal of Pharmaceutical Sciences. The authors of manuscript have declared that they do not have any conflicts of interest.

Consent for publication

Our research work does not include any human study. The manuscript entitled ‘Design of Experiments and White Analytical Chemistry-driven Green and Sensitive Spectrofluorimetric Estimation of Pregabalin in its Pharmaceutical Dosage Forms and Spiked Human Plasma’ has been solely submitted to Future Journal of Pharmaceutical Sciences.

Competing interests

The authors of the manuscript already declared that they do not have any conflicts of interest for the publication of the manuscript.

Abbreviations

ADS
Analytical Design Space
AGREE
Analytical Greenness
ANOVA
Analysis of Variance
DoE
Design of Experiments
ESA
Eco-Scale Assessment
GSK
GlaxoSmithKline
GAC
Green Analytical Chemistry
GAPI
Green Analytical Procedure Index
HPLC
High-Pressure Liquid Chromatography
HPTLC
High-Performance Thin-Layer Chromatography
ICH
International Council for Harmonization
LOD and LOQ
Limit of Detection and Limit of Quantitation
NEMI
National Environmental Method Index
PGB
Pregabalin
PDE
Permitted Daily Exposure
RSM
Response Surface Modeling
RGB
Red, Green and Blue
WAC
White Analytical Chemistry

Publisher's Note

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Formulation and evaluation of nanobiotherapeutics of Terminalia arjuna through plant tissue culture for atherosclerosis

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ABSTRACT (ENGLISH)

Background

The study seeks to investigate the therapeutic potential of *Terminalia arjuna* callus in addressing atherosclerosis. In order to get maximum beneficial phytoconstituents from *Terminalia arjuna*, it is recommended to harvest the bark from Arjuna trees that are at least 15 years old and a gap of minimum 2 years should be kept before harvesting bark from the same plant. The callus culture technique was employed to expedite the process. The callus culture extract was subsequently converted into a nanosuspension with the aim of improving the efficacy of its phytoconstituents. It was then subjected to a comprehensive series of in vitro and in vivo evaluations to ascertain its potential for treatment of atherosclerosis.

Results

Liquid chromatography–mass spectrometry analysis of the callus extract confirmed the presence of flavonoids and terpenoids, known for their antioxidant and anti-inflammatory activities. Some terpenoids were even absent in Arjuna tree naturally. TEM images validated successful entrapment of the extract within the nanoparticles. In vitro analysis for antilipase and antioxidant assay confirmed the antiatherosclerotic potential of the extract. In vivo tests on rat blood serum demonstrated a significant reduction in total cholesterol, low-density lipoprotein, triglycerides, high-density lipoprotein, and very low-density lipoprotein. Histopathological analysis of rat aortas showed additional confirmation of antiatherosclerotic action.

Conclusion

In conclusion, the study highlights the potential of nanosuspension derived from *Terminalia arjuna* callus extract as a comprehensive therapeutic strategy for atherosclerosis treatment. The research highlights antioxidant, anti-inflammatory, and antiatherosclerotic properties of the callus, hinting at its viability as a potential treatment for atherosclerosis. This interdisciplinary investigation emphasizes the promising role of traditional medicinal plants within modern medical paradigms.

FULL TEXT

Background

Atherosclerosis is a chronic inflammatory condition resulting in cardiovascular disease with life-threatening implications. Atherosclerosis is a complex and multifaceted condition marked by the accumulation of plaque within arteries. This can lead to reduced blood flow, contributing to a range of cardiovascular diseases (CVDs). CVDs are one of the leading causes of death worldwide, and their prevalence is rapidly increasing, owing in large part to changes in lifestyle and an increase in the elderly population [1]. Since atherosclerosis is a complex condition affected by numerous risk factors, there are various well-established and innovative strategies to diagnose and manage this disease. During the early phases of atherosclerosis, the primary objective of treatment is on managing significant risk factors such as elevated cholesterol levels, hypertension, and elevated blood sugar. Making

alterations to one's diet, engaging in regular physical activity, and quitting smoking are all assumed to play pivotal roles in both the prevention and management of atherosclerosis. The current treatment for management of atherosclerosis includes drugs that have antihypercholesterolemic, antihypertensive, hypoglycemic, and antiplatelet activity. Anti-inflammatory drugs like canakinumab, methotrexate, and colchicine have passed clinical trials but need to be further assessed for their therapeutic efficacy. The typical medications utilized to address hypercholesterolemia are statins solely or in combination with drugs like ezetimibe. These drugs function by inhibiting the hepatic enzyme HMG CoA reductase, a crucial enzyme in the synthesis of cholesterol. The primary advantage of statins lies in their ability to lower low-density lipoproteins (LDL). However, they suffer from adverse effects mainly statin-associated muscle symptoms, myalgias, and glucose intolerance. Additionally, the utilization of statins has been associated with the development of type 2 diabetes mellitus [2–4].

Considering the negative effects of synthetic medications, emphasis should be given to embrace natural drugs, which have negligible adverse reactions. Natural origin drugs still continue to be the focus of pharmaceutical research because substitute drug discovery methods have failed to provide lead compounds for treatment of various diseases like metabolic diseases. *Terminalia arjuna*, also known as Arjuna in Hindi, belongs to the family Combretaceae and is found in India, Mauritius, and Sri Lanka. Arjuna has been documented in numerous ancient Indian medical texts, including Charaka Samhita, Astang Hridayam, and Sushruta Samhita, dating back to the Vedic period. Notably, it was Vagabhatta who first recommended the utilization of stem bark powder for heart-related conditions. Ayurvedic practitioners have employed this plant in the management of cardiovascular ailments [5]. The stem bark, fruits, and even leaves of Arjuna are commonly used in different traditional systems across India for various medicinal properties such as astringent, demulcent, expectorant, styptic, antidysenteric, cardiotonic, and urinary astringent. It is also used in the treatment of ulcers, earaches, and even bark ashes are used for snakebites and scorpion stings [6]. Numerous experimental and clinical studies have effectively showcased Arjuna’s potential as an anti-ischemic agent, antiatherogenic agent, and a strong antioxidant. As per data shared for Scientific Harvesting—Dos and Don’ts by NTFP Centre of Excellence (NCE) established by Government of Tripura, India; the bark of Arjuna tree should not be harvested before 15 years of growth to get quality product with a minimum gap of 2 years for successive harvesting [6–11].

The present study includes the callus culture to reduce the harvesting time period. Nanoparticle delivery systems offer the enhanced stability and solubility of encapsulated drug molecules, facilitating their movement across membranes, and extending circulation time to enhance both safety and effectiveness of the medicament [12, 13]. In this study, a multidisciplinary approach was employed to create a nanosuspension from Arjuna callus.

Methods

Preparation of callus

The branch of Arjuna was collected from Dr. Babasaheb Ambedkar Central Nursery Kagal, India, in the month of June, and plant was authenticated form Botanical Survey of India, Pune. The leaves were surface sterilized. Murashige and Skoog agar media with different concentrations of 2, 4,-dichlorophenoxy acetic and kinetin were used for callus induction. The explants were incubated at 25 ± 2 °C under 16-h photo-period of 2000 lx with white light. The relative humidity was maintained at 60–70% during incubation at Seem Biotech Pvt Ltd, Warananagar, India. Sub-culturing was carried out every six weeks. After 6 months, the callus cultures were weighed and callus index was calculated.

Extraction of callus

The callus culture obtained was dried at 50 ± 5 °C in hot air oven and was subjected to ethanolic extraction using Soxhlet apparatus and subsequently dried in vacuum dryer.

Preparation of arjuna callus extract nanosuspension (ACEN)

One gram of extract was mixed with 20 ml ethanol. The solution was injected drop wise in 100 ml aqueous solution of polyvinyl pyrrolidone K30 (PVP K30) and was subjected to mechanical stirring for 6 h. A factorial design was employed, taking into account two independent factors: the concentration of PVP K30 and mixing speed, each varying at three different levels.

Evaluation of nanosuspension

Bruker, ALPHA Infrared Spectrophotometer was used for checking the compatibility of callus extract with the PVP K30. The Horiba Zetasizer was used for determination of particle size and zeta potential [14]. Absorption maxima were determined using a Jasco V-750 UV Spectrophotometer from Japan to calculate % drug content, % drug encapsulation, and % drug release. For the % drug release study, a dialysis bag (dimensions: flat width 28.46 mm, inflated diameter 18 mm; provided by HiMedia Laboratories Pvt, Ltd) was utilized. One milliliter of 10 µg/ml of each ACEN batch was placed in a dialysis bag immersed in 300 mL of diffusion media (phosphate buffer saline—PBS, pH 7.4) in USP Type I dissolution test apparatus. Samples were withdrawn at 5-min intervals over 40 min from the receptor compartment and analyzed at 282 nm. The % drug content, % drug encapsulation efficiency, and % drug release studies were conducted in triplicate [15].

Acceleration stability studies for the optimized batch were carried out according to ICH guidelines for 90 days. It involved a modification in the intermediate storage condition, transitioning from 30 °C ± 2 °C and 60% ± 5% relative humidity (RH). The adequate amount of freeze-dried optimized ACEN batch was subjected to acceleration stability study. The particle size, % drug content, and % drug release study were used as parameters to check the stability of ACEN [16].

Antilipase assay

The enzymatic activity of porcine pancreatic lipase (PPL, type II) was assessed using p-nitrophenyl butyrate (p-NPB) as a substrate. PPL stock solution (1 mg/mL) was prepared in 0.1 mM potassium phosphate buffer (pH 6.0) and stored at -20 °C. Different concentrations 20, 40, 60, 80, and 100 µg/mL of extract and standard drug Orlistat were pre-incubated with PPL for 1 h in a 0.1 mM potassium phosphate buffer (pH 7.2), supplemented with 0.1% Tween 80, at 30 °C. After addition of 0.1 µL of p-NPB, all samples were incubated at 30 °C for 5 min. The absorption for all samples was recorded at 405 nm. The analysis was performed three times for each concentration of sample and standard. The inhibitory activity (I) was calculated using the following formula [17]:\% Inhibition=Absorbanceofcontrol -AbsorbanceofsampleAbsorbanceofcontrol×100

Antioxidant activity assay

The antioxidant potential of the nanosuspension was assessed through its ability to scavenge free radicals using DPPH (1,1-diphenyl-2, picryl-hydrazyl) radicals. The standard solutions were prepared by mixing different concentrations of 100 µL of aqueous ascorbic acid solution with 100 µL of a 0.1% methanolic DPPH solution. Similarly, test samples were prepared by mixing different concentrations of 100 µL of an aqueous callus extract solution with 100 µL of the 0.1% methanolic DPPH solution. After mixing, all samples were incubated for 30 min in a dark environment at room temperature. The absorbance was recorded three times at 490 nm [18, 19].

The radical scavenging activity was determined using the following formula:\%radicalscavengingactivity=Absorbance ofControl-AbsorbanceofTestsampleAbsorbanceofControl∗100

Experimental animals

The protocol was approved by the Institutional Animal Ethics Committee of Appasaheb Birnale College of Pharmacy, Sangli (Approval number-IAEC/ABCP/13/2021–22). Healthy young male Wistar rats aged 6–8 weeks weighing approximately 200–250 g were divided into four groups, with six rats in each group. Prior to the initiation of the experimental procedure, one-week acclimatization period maintaining a temperature of 24 ± 3 °C, a humidity level of 50–60% RH, and a 12-h light/dark cycle, was provided for the rats.

Acute toxicity study

An acute toxicity assessment was conducted on rats in accordance with the OECD guideline 423 using the up-and-down procedure. Three nulliparous and non-pregnant female rats aged 8 to 12 weeks were employed for the study. The rats were fasted overnight before dosing, which extended for an additional 3-h post-dosing. Close monitoring of the animals was carried out for the initial 24 h to detect any signs of toxicity and continued for 72 h to identify potential mortalities. The determined LD50 value was 3000 mg/kg of rat body weight. As a result, a dose of 300 mg/kg body weight was selected for ACEN administration [4, 7, 11].

Preparation of high-fat diet (HFD)

The fine powder of the conventional standard animal pellets was combined with 2% cholesterol, 1% cholic acid, 40% sucrose, and 10% coconut oil. The resulting powdered mixture was blended with an appropriate amount of water to form compact feed spheres. These spheres were then stored in a refrigerator.

Animal test groups

The animals were randomly distributed into four distinct groups, each group comprising of six rats. Group I, designated as normal group, was provided with the standard rat animal pellets. Group II, known as the disease control group, was fed a HFD. Throughout the study, both Group I and Group II received normal saline per day orally. Group III, referred to as the Standard group, received HFD and atorvastatin orally at a dosage of 10 mg/kg body weight of rat per day, while Group IV received HFD and ACEN at a dose of 300 mg/kg body weight per day orally. Upon the conclusion of the 30-day study period, the rats were fasted overnight. Subsequently, the rats were anesthetized using diethyl ether, and blood samples were collected from the retro orbital venous plexus. These blood samples were promptly transferred to sterile plain tubes to conduct biochemical tests [20–22].

Atherogenic index

The atherogenic index of serum (AIS) is the measure of the extent of atherosclerotic lesions based on serum lipids. The atherogenic index was calculated using following formula [23]:AIS=TC-HDLHDLwhere TC = concentration of total cholesterol in mg/ml. HDL = concentration of HDL in mg/ml.

Histology of the aorta

The rats were killed by cervical decapitation. Their aortas were dissected out carefully. The aortas were stored in 10% formaline solution and sent to a local pathological laboratory for hematoxylin and eosin staining.

Statistical Analysis: The outcomes were presented as the mean accompanied by the standard error of the mean (SEM). Statistical analysis involved the utilization of analysis of variance (ANOVA). A significance level of $p<0.05$ was employed as the threshold for determining statistical significance.

Results

Evaluation parameters for callus

The callus cultures were developed with 2% 2,4 D concentrations using leaves explant. Callus index is a parameter used to access the growth rate of the tissue. Callus index was calculated using following formula [24]:Callusindex=n∗GN∗100where *n* = total number of callused explants. *G* = Average weight of callus rating on explant. *N* = Total number of cultured explants.

The callus index was 54.075 indicating good callus culture growth. The % practical yield for callus extract was 7.45% w/w.

Phytochemical analysis

The liquid chromatography–mass spectrometry (LC–MS) data revealed presence of terpenoids like Asiatic acid, 2-Oxo-5,11(13)-eudesmadien12,8-olide, Cucurbitacin B, Cucurbitacin E, (+)-cis-5,6-Dihydro-5-hydroxy4-methoxy-6-(2-phenylethyl)-2H-pyran-2-on, Ganoderic acid C, Asiatic acid, (3beta,19alpha)-3,19,23,24-Tetrahydroxy-12-oleanen-28-oic acid, while flavonoids like Genistin, Allixin, Quercetin, Liquiritic acid on majority. Figures 1 and 2 are the chromatograms of Arjuna callus extract.

Fig. 1 [Images not available. See PDF.]

The chromatogram of callus extract for positively charged compounds

Fig. 2 [Images not available. See PDF.]

Chromatogram of callus extract for negatively charged compounds

Evaluation of ACEN

As per Figs. 3, 4, and 5, the Fourier transform infrared (FTIR) graphs confirmed the compatibility of Arjuna callus extract with polymer PVP K30.

Fig. 3 [Images not available. See PDF.]

FTIR of callus extract

Fig. 4 [Images not available. See PDF.]

FTIR of physical mixture of callus extract and PVP K30

Fig. 5 [Images not available. See PDF.]

FTIR of nanosuspension of callus extract

The size of the nanoparticles ranged from 304.4 to 544.1 nm, with a polydispersity index between 0.37 and 0.42. Additionally, the zeta potential ranged from -25.6 to -45.6, indicating that the nanosuspension is stable [25].

The % drug release study revealed that the N4 batch exhibited the lowest release, with 91.58 ± 0.06% over 35 min. On the other hand, the N9 batch demonstrated the highest % drug release with 99.40 ± 0.008% over 35 min among all batches. The % drug release data were fitted to standard release equations—zero order, first order, Higuchi model, Hixson, and Kor's peppas. The linear correlation coefficient *R* square values for all batches are summarized in Table 5. All batches followed first order of kinetics.

Table 7 confirms the optimization of Arjuna callus extract nanosuspension batch.

Stability studies were done for optimized batch N9 as per ICH guideline. Acceleration stability study intermediate storage condition was changed from 30 ± 2 °C and 60 ± 5% RH, and results are shown in Table 8 and Fig. 15. There was slight increase in particle size from 340 to 347.9 nm on day 90. The % drug content and % drug release were almost unchanged.

Antilipase assay

The IC50 for standard drug Orlistat was 53.58, while for ACEN was 78.69. The highest % inhibition of pancreatic lipase enzyme for Orlistat was 83.63 at 100 µg/ml, while for ACEN was 78.69 at 100 µg/ml with *p* value <0.0001.

Antioxidant activity

The IC50 value for the standard drug sample, ascorbic acid, was 137.57, while for ACEN, it was 42.49. Remarkably, ACEN exhibited a percentage inhibition surpassing that of ascorbic acid, and this trend was observed to intensify with higher concentrations. The p-value of 0.0009 represents the statistical significance of these results [26].

Animal study

According to Fig. 18, the body weight of rats increased in all groups throughout the experiment, except for the normal group. The control group exhibited the most significant increase in body weight on the 15th and 30th days when compared to the normal group. The body weights of the standard and ACEN-treated groups were lower than the control group, but were similar to the standard group.

Figure 19 shows the lipid profile of all groups. The control group exhibited an increase in total cholesterol (TC) levels compared to the normal group. However, there was a slight decrease in TC levels in both the ACEN-treated and standard groups when compared to the control group. The control group showed a decrease in TG levels, while the ACEN-treated and standard groups maintained stable levels, similar to the normal group. The control group had lower levels of HDL, while the ACEN-treated group had slightly higher levels. Low-density lipoprotein (LDL) level was increased in the control group but was maintained in ACEN-treated group as that of normal. Very low-density lipoprotein (VLDL) levels were elevated in the control group but remained relatively stable in the ACEN-treated and standard groups, approaching levels similar to the normal group. The data underwent ANOVA, and it confirmed its statistical significance with a *p*-value of less than 0.0001.

Atherogenic index (AIS)

The AIS for the ACEN group was less than the control group and almost similar to that of standard group. In a study involving rats that fed with HFD diet for 30 days, the AIS increased from 0.373278 in the normal group to 1.48455 in the control group ($p<0.0001$). However, in the ACEN group, the AIS was 1.09205, which was like the standard group, with a value of 1.0563.

Histology of the aorta

In the control group, there was a noticeable narrowing of the aortic lumen due to lipid deposition. Conversely, in rats

treated with ACEN, the aortic lumen appeared almost identical to that of the normal group. Additionally, the standard group's rats exhibited more significant atherosclerosis-related narrowing of the aortic lumen compared to the ACEN-treated group (Figs. 21, 22, 23, 24).

Discussion

Phytochemical analysis

The callus cultures are known to exhibit the phytoconstituents that are not present in mother plants naturally [27]. The LC–MS data showed the presence of terpenoids like (3beta, 19alpha)-3,19,23,24-tetrahydroxy-12-oleanen-28-oic acid, 3-[4-Hydroxy-3-(3-methyl-2-butenyl)phenyl]-2-propenal which are absent in Arjun tree naturally [8] (Table 1).

Table 1. Phytoconstituents of callus and their pharmacological activity

Name of compound	Pharmacological activity
4-Hydroxy-L-threonine	It plays a crucial role in facilitating healthy fat metabolism within the liver, supporting the smooth functioning of the digestive and intestinal systems, and promoting efficient metabolic processes and assimilation [38]
Allixin	Antioxidant, antiatherogenic, anticancer activity [39, 40]
Genistin	Antioxidant, antiadipogenic and antilipogenic agent [41]
Ellagic acid	Antioxidant, anti-inflammatory, antimutagenic, antiproliferative, antiallergic, antiatherosclerotic, cardioprotective, hepatoprotective, nephroprotective, and neuroprotective property [31, 42]
Quercetin	Reduces oxidative stress, inhibits low-density lipoproteins oxidation and platelet aggregation, and acts as vasodilator in blood vessels [43]
Calophyllum B	Anti-inflammatory activity [44]
Candoxatrilat	Endopeptidase inhibitor, useful in congestive heart failure [45]
(R)-Heraclenol	Inhibits histidine biosynthesis selectively and shows antibacterial activity [46]
2-Oxo-5,11(13)-eudesmadien12,8-olide	Anti-inflammatory, antiparasitic, antiviral, and cytotoxic activity [47]
Austrobailignan 7	Anticancer activity [48]
Schleicherastatin 6	Anticancer activity [49]

Mitoxantrone	Anticancer, anti-inflammatory activity and inhibits IFN-γ, TNF-α, and IL-2 [50, 51]
Paeonilactone C	Neuroprotective and antioxidant activity [52, 53]
Teasterone	Anti-inflammatory activity [54]
(5x,6x)-5,6-Epoxyergosta-7,22-dien-3-ol	Antioxidant and anti-inflammatory activity [55]
Convallasaponin	Tumor-specific cytotoxic activity [56]
Campesteryl ferulate	Antioxidant activity, hypolipidemic effect, elevation in HDL cholesterol level, suppression of platelet aggregation, and inhibition of tumor promotion [32]
Smilagenone	In cognitive impairment treatment [57]
(25R)-5beta-spirostan-3beta ol	Lowers bold cholesterol level [58]
Cucurbitacin B	Anti-inflammatory, antioxidant, in treatment of neurodegenerative diseases, diabetes mellitus, and cancers, decreases lipid accumulation and antiatherosclerotic [33, 34]
Euphornin	In cancer treatment [59]
Pyropheophorbide a	In cancer treatment [60]
Cucurbitacin E	Anti-inflammatory, antiangiogenic, immunomodulatory, cytotoxic, cytostatic, and hepatoprotective property [61]
Ciclesonide	In treatment of asthma [62]
Methyl 3b-hydroxy-13(18)-oleanen-28-oate	Antiosteoporotic activity [63]
Schleicherastatin 6	In treatment of cancer [64]
1-O-Galloylpedunculagin	Antioxidant activity [65, 66]
(+)-cis-5,6-Dihydro-5-hydroxy4-methoxy-6-(2-phenylethyl)-2H-pyran-2-on	A triterpenoid, pharmacological activity not know yet

Ellagic acid	It affects lipid metabolism by promoting cholesterol efflux and decreasing LDL uptake, while also possessing antioxidant, liver-protective, antisteatotic, anticholestatic, antifibrogenic, antihepatocarcinogenic, and antiviral activity [31]
Ganoderic acid C	Anti-inflammatory (inhibition of TNF α) activity [35, 36, 67]
Ceanothic acid	Anticancer and antimicrobial activity [68, 69]
Asiatic acid	Antioxidant, anti-inflammatory and antihyperlipidemia activity [37, 70]
Azukisapogenol	Antibacterial and antifungal activity [71]
Liquiritic acid	Atheroprotective, anti-inflammatory activity [72, 73]
(3beta,19alpha)-3,19,23,24-Tetrahydroxy-12-oleanen-28-oic acid	A terpenoid showing similar chemical structure to arjunic acid and arjunin acid whose pharmacological activity is not known yet

Evaluation of ACEN

Considering Fig. 3, 4, and 5, the alkyn –OH stretching at 3300, alkyl C–H stretch at 2925 and 2855, C=O stretch at 1746 and 1642, C–O at 1000–1300 were preserved in the mixture IR spectra, indicating the compatibility of the callus extract with PVP K 30 (Table 2).

Table 2. Batches of nanosuspension

Batch	Concentration PVPK30 (% w/v)	Mixing speed (rpm)
N1	1	1000
N2	1.5	1000
N3	2	1000
N4	1	1500
N5	1.5	1500
N6	2	1500
N7	1	2000
N8	1.5	2000

N9	2	2000

Considering two independent factors concentration of polymer PVPK30 (1,1.5, and 2%) and mixing speed (1000, 1500, and 2000) at three levels each, 9 batches were analyzed using design of experiment software version 6 (Fig. 6). The analysis employed a linear model (Table 3) to examine the correlation between polymer concentration and mixing speed regarding particle size of ACEN. We confirmed the significance of the data input with an *F*-value of 6.16. In Table 4, we observed a negative coefficient for both polymer concentration and mechanical stirring speed. A positive sign denotes a synergistic effect, while a negative sign indicates an antagonistic effect. The interaction plots (Figs. 7, 8, 9, 10, 11) illustrated that the relative slopes of factors—particle size, polydispersity index, zeta potential, % drug content and % drug concentration—displayed a significant interrelation with both polymer concentration and mechanical stirring speed. The % drug content across all formulations ranged from 96.2 ± 0.38 to 99.75 ± 0.46%. Additionally, the range of % drug entrapment was between 94.67 ± 0.02 and 98.92% ± 0.03. This discrepancy from the % drug content suggests the possibility of drug dissolution in the aqueous phase.

Fig. 6 [Images not available. See PDF.]

Summary of design of experiment software analysis

Table 3. ANOVA table for particle size of nanosuspension

ANOVA for Response Surface (Linear + User Added Terms) model (Aliased)						
Analysis of variance table [Partial sum of squares—Type III]						
Source	Sum of squares	*df*	Mean square	*F* value	*p*-value Prob > F	
Model	37,485.52	2	18,742.76	6.16	0.0352	Significant
A-Polymer concentration	2019.23	1	2019.23	0.66	0.4465	
B-Speed of rotation	35,466.28	1	35,466.28	11.65	0.0143	
ACERTUV	0.000	0				
Residual	18,267.14	6	3044.52			
Cor total	55,752.66	8				

Table 4. Effect of polymer concentration and speed of mixing on particle size

Factor	Coefficient	*df*	Standard	95% CI	95% CI	VIF
Estimate	Error	Low	High	Intercept	433.17	1

18.39	388.17	478.18		A-Polymer concentration	−18.35	1
22.53	−73.46	36.77	1.00	B-Speed of rotation	−76.88	1
22.53	−132.00	−21.76	1.00	ACERTUV	= ACERTUV+A	

Fig. 7 [Images not available. See PDF.]

2D contour plot for particle size analysis of nanosuspension batches
Fig. 8 [Images not available. See PDF.]

2D contour plot for polydispersity index analysis of nanosuspension batches
Fig. 9 [Images not available. See PDF.]

2D contour plot for zeta potential analysis of nanosuspension batches
Fig. 10 [Images not available. See PDF.]

2D contour plot for % drug content analysis of nanosuspension batches
Fig. 11 [Images not available. See PDF.]

2D contour plot for % drug entrapment efficiency analysis of nanosuspension batches
We found that all batches N1 to N9 followed first-order kinetics (Table 5). As per Fig. 12, we found that the smaller the particle size, the better is the drug release, which can result in higher solubility.
Table 5. Summary of *R* square values for nanosuspension batches

Batch No	N1	N2	N3	N4	N5	N6	N7	N8	N9
Model name	R square	R square	R square	R square	R square	R square	R square	R square	R square
Zero order	0.9805	0.9805	0.9805	0.9805	0.9805	0.9805	0.9805	0.9805	0.9805
first order	0.9301	0.9301	0.9301	0.9301	0.9301	0.9301	0.9301	0.9301	0.9301
Higuchi model	0.9377	0.9377	0.9377	0.9377	0.9377	0.9377	0.9377	0.9377	0.9377
Hixson	0.9713	0.9713	0.9713	0.9713	0.9713	0.9713	0.9713	0.9713	0.9713
Kor's peppas	0.8684	0.8684	0.8684	0.8684	0.8684	0.8684	0.8684	0.8684	0.8684

Fig. 12 [Images not available. See PDF.]

% Drug release study

The analysis of variance (ANOVA) identified 10 potential solutions based on the criteria outlined in Table 6. The combination of 2% PVP K30 and a mechanical stirring speed of 2000 rpm scored 0.917 desirability. The batch optimization is evident from Table 7. The transmission electron microscopy image displayed the nanoscale dimensions and encapsulation of Arjuna callus extract within a polymer matrix (Figs. 13, 14).

Table 6. Constrains for optimization of nanosuspension

Name	Goal	Lower limit	Upper limit	Lower weight	Upper weight	Importance
A:Polymer concentration	Is in range	1	2	1	1	3
B:Speed of rotation	Is in range	1000	2000	1	1	3
Particle size	Minimize	304.4	544.1	1	1	4
PI	Is in range	0.37	0.42	1	1	3
Zeta potential	Is in range	-45.6	-25.6	1	1	3
%Drug content	Maximize	96.2	99.75	1	1	4
% Drug entrapment efficiency	Maximize	94.68	98.92	1	1	4

Table 7. Comparison of theoretical value and practical value of optimized nanosuspension

Parameter	Particle size (nm)	Polydispersity index (PI)	Zeta potential	% Drug content	% Drug entrapment efficiency
ANOVA suggested theoretical value	337.946	0.391	−33.667	99.638	98.603
Practical value	340.9	0.399	−34.1	99.83607	98.91557

Fig. 13 [Images not available. See PDF.]

2D contour plot for desirability for nanosuspension batch optimization

Fig. 14 [Images not available. See PDF.]

Transmission electron microscope image of ACEN

The N9 sample did not change physical appearance throughout the acceleration stability study. As per results discussed in Table 8 and Fig. 15, we found that there was slight increase in the size of nanoparticle, but it was within acceptable limit. The slight increase in particle size of ACEN did not affect the % drug content of % drug release.

Table 8. Acceleration stability studies for N9 batch

Period	Particle size (nm)	PDI	% Drug content

15 days	341.5	0.399	99.66 ± 0.3
30 days	341.7	0.399	99.63 ± 0.4
60 days	345.1	0.340	99.60 ± 0.7
90 days	347.9	0.340	99.57 ± 0.1

Fig. 15 [Images not available. See PDF.]

% Drug release studies of N9 batch for acceleration stability studies

Antilipase assay

Hyperlipidemia exhibits strong associations with various metabolic conditions, including diabetes, atherosclerosis, and hypertension. Within the duodenum, pancreatic lipase plays a pivotal role in the digestion and absorption of lipids, breaking down triacylglycerols into monoacylglycerols and fatty acids. The pancreatic lipase inhibitors have a potential to induce hypolipidemic effects, offering a promising atherosclerosis management [28, 29]. We found that the % inhibition of lipase enzyme by ACEN was concentration dependent (Fig. 16). The antilipase activity of ACEN might be due to presence of 4-Hydroxy-L-threonine, Genistin, Cucurbitacin B, Ganoderic acid C, Asiatic acid, Ellagic acid, Campesteryl ferulate. This could be one of the antiatherosclerotic mechanisms of ACEN.

Fig. 16 [Images not available. See PDF.]

Antilipase assay for ACEN

Antioxidant activity

Antioxidants play a pivotal role in the treatment of atherosclerosis through a range of mechanisms. These mechanisms encompass the inhibition of LDL oxidation, the reduction production of reactive oxygen species, the suppression of cytokine secretion, the prevention of atherosclerotic plaque formation, prevention of platelet aggregation, the hindrance of mononuclear cell infiltration, the enhancement of endothelial function and vasodilation, the promotion of nitric oxide (NO) bioavailability, the modulation of adhesion molecule expression (e.g., VCAM-1 and ICAM-1) on endothelial cells, and the mitigation of foam cell formation. The effectiveness of these diverse antioxidant actions remains an open question, but it appears that a multi-antioxidant approach may offer a more effective strategy for antioxidant therapy. We found the ACEN shows better antioxidant activity that standard drug ascorbic acid (Fig. 17). The presence of various compounds, including Allixin, Genistin, Ellagic acid, Quercetin, Paeonilactone C, (5x,6x)-5,6-Epoxyergosta-7,22-dien-3-ol, Campesteryl ferulate, and others, is likely responsible for the observed antioxidant activity of ACEN [19, 30].

Fig. 17 [Images not available. See PDF.]

Antioxidant activity assay of ACEN

Animal study

Figure 18 summarizes the body weight of animals during the study (Fig. 18). We discovered that ACEN was more effective than the standard drug atorvastatin at decreasing TC, TG, HDL, LDL, and VLDL (Fig. 19). Ellagic acid plays a role in lipid metabolism by promoting cholesterol efflux and reducing LDL uptake. The terpenoids, such as Ellagic acid and Asiatic acid, are known for their potential in managing hyperlipidemia. The potential cause of ACEN's lipid-lowering effect could be attributed to the presence of these terpenoids. [31–37].

Fig. 18 [Images not available. See PDF.]

Summary of body weight of animal groups at 0, 15, and 30 days

Fig. 19 [Images not available. See PDF.]

Summary lipid profile of animal groups

Atherogenic index of serum

The AIS is a measure of the potential risk of developing atherosclerosis. When the AIS is higher, there is a greater potential for atherosclerosis and an increased likelihood of cardiovascular disease. It is important to note that the AIS is influenced by the levels of HDL. When HDL levels are higher, the atherogenic index is lower, reducing the risk of atherosclerosis [23]. From Fig. 20, we found that the AIS of ACEN (1.05 ± 0.035) was slightly lesser than the AIS of standard drug atorvastatin (1.06 ± 0.053) emplacing the antiatherosclerotic potential of ACEN (Figs. 21, 22, 23, 24).

Fig. 20 [Images not available. See PDF.]

Summary atherogenic index of serum

Fig. 21 [Images not available. See PDF.]

Rat aorta in control group

Fig. 22 [Images not available. See PDF.]

Rat aorta in normal group

Fig. 23 [Images not available. See PDF.]

Rat aorta in ACEN group

Fig. 24 [Images not available. See PDF.]

Rat aorta in standard group

Histology of the aorta

The histopathological results confirmed the antiatherosclerotic potential of ACEN. The presence of phytoconstituents in ACEN like Ellagic acid, Quercetin, Calophyllum B, (25R)-5beta-spirostan-3beta ol Liquiritic acid known for their lipid-lowering, antioxidant, and anti-inflammatory properties, may be responsible for its antiatherosclerotic effect. The histopathological analysis demonstrated that nanotherapeutics exhibited superior efficacy when compared to the standard drug Atorvastatin, highlighting the enhanced effectiveness of the callus extract attributed to its nanoformulation. Altogether, the comprehensive study has affirmed ACEN's potential in atherosclerosis treatment.

Conclusion

A thorough analysis of phytochemical composition of the callus extract predominantly revealed the presence of flavonoids and terpenoids, endowed with antioxidant, hypolipidemic, and anti-inflammatory activity. The callus culture extract was then transformed into a nanosuspension to achieve better efficacy of the medicament. The optimized nanosuspension formulation was subjected to comprehensive in vitro and in vivo studies, aimed to confirm its antiatherosclerotic potential. The study findings suggest that ACEN has the potential to be a natural-origin drug for the treatment of atherosclerosis.

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Author contributions

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by PPW. The first draft of the manuscript was written by PPW, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The data for graphs 12 to 16 are available on request.

Declarations

Ethics approval

This study was performed in line with the principles of laid down by Institute Animal Ethics Committee. Approval was granted by the Ethics Committee of Appasaheb Birnale College of Pharmacy, Sangli (Date 20/01/2022 /No IAEC/ABCP/13/2021–22).

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Human and animal rights

Not applicable.

Abbreviations

ACEN
Arjuna callus extract nanoparticles
LC-MS
Liquid chromatography–mass spectrometry
PVP K30
Polyvinylpyrrolidone K30
RH
Relative humidity
PPL
Porcine pancreatic lipase
p-NPB
P-nitrophenyl butyrate
DPPH
1,1-Diphenyl-2, picryl-hydrazyl
TC
Total cholesterol
TG
Triglyceride
HDL
High-density lipoprotein
LDL
Low-density lipoprotein
VLDL
Very low-density lipoprotein
FTIR
Fourier transform infrared

Publisher's Note

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A DoE-based development and characterization of Nadifloxacin-loaded transethosomal gel for the treatment of Acne vulgaris

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ABSTRACT (ENGLISH)

Background

The objective of this current research was to enhance the topical delivery of Nadifloxacin (NDFX) by incorporating it into a transethosomal gel formulation. NDFX has limited penetration into the deep layer of the skin because it is poorly water soluble and it has a log p value of 2.47. To optimize the formulation, the “Box–Behnken design” was utilized. The independent variables included phosphatidylcholine 90, tween 80 and ethanol. The produced formulations underwent evaluation for entrapment efficiency, vesicle size and zeta potential. The optimized formulation was then incorporated into suitable gel bases and subjected to further investigation, including in vitro diffusion, ex vivo penetration, in vitro antimicrobial assay and in vivo anti-acne activity.

Results

The optimized formulation exhibited an entrapment efficiency of 80.12%, a vesicle size of 156.1 nm and a zeta potential of−33.23 mV. TEM images confirmed the presence of encapsulated vesicles with a spherical shape. The in vitro diffusion study demonstrated that the transethosomal gel containing Carbopol 934 (1%) exhibited higher drug release compared to the HPMC K4M gels. Furthermore, the ex vivo permeation study revealed that the optimized transethosomal gel demonstrated increased permeation compared to the commercially available formulation.

Conclusion

The optimized transethosomal formulation displayed enhanced in vitro antimicrobial and in vivo anti-acne effects against *Propionibacterium acnes* in Wistar albino rats when compared to the marketed formulation.

FULL TEXT

Background

Skin disorders are one of the most prevalent illnesses; skin problems afflict thousands of individuals every day and can affect anyone at any age. It was primarily brought on by various infectious bacteria or inflammatory diseases. Skin conditions include a wide range of symptoms and severity. Some may have environmental causes, while others may have genetic or dependent reasons [1]. Several infectious diseases affect the skin and hair follicles and are brought on by bacterial, fungal or viral infections. These illnesses result in skin eruption [2].

Acne vulgaris is a persistent skin disorder that affects the pilosebaceous glands of hair follicles. Comedones, inflamed papules and pustules, nodules &cysts and scaring are the different types of acne lesions. Acne vulgaris is characterized by skin with comedone and seborrhea, which is caused due to several factors: hyperkeratosis, heightened sebum production induced by androgens, inflammation and bacterial colonization of hair follicles on the face, neck, chest and back by Propionibacterium acnes. A "whitehead" is a closed comedone and a "blackhead" is an open comedone; the black color is caused due to melanin. The comedo is a non-inflamed lesion that typically recurs on its own with minimal or discolored scarring [3].

Nadifloxacin (NDFX), 7-fluoro-8-(4-hydroxy-piperidin-1-yl)-12-methyl-4-oxo-1-azatricyclo- trideca-2,5,7,9 (13)-tetraene-3-carboxylic acid (Fig. 1), is a fluorinated quinolone antibiotic and is commonly used as a topical medication for the treatment of numerous inflammatory acne lesions. It is highly effective against *P. acness* and other gram-negative and gram-positive bacteria. In addition to its antibacterial effects, NDFX has been reported to have anti-inflammatory effects, which may help with some elements of inflammatory acne [4].

Fig. 1 [Images not available. See PDF.]

Structure of Nadifloxacin

For the treatment of acne, 1% w/w Nadifloxacin topical cream is available in the market. Unfortunately, NDFX, characterized by its low solubility in water, is a fluoroquinolone compound that possesses a log P value of 2.47 and due to the large droplet size of topical creams, NDFX has shown limited penetration into the deep layer of the skin [5], resulting in poor patient compliance and adherence to NDFX topical regimen. Several methods, including nanocarrier-based formulations and Novel lipid vesicles, referred to as ultra-deformable vesicles (UDV), like ethosomes, transferosomes and transethosomes, have already been researched to enhance the targeted administration of cosmeceuticals and pharmaceuticals [6, 7].

Transferosomes are vesicular carriers characterized by their elastic properties and lipid bilayer structure. These carriers incorporate an edge activator, which is a biocompatible surfactant. However, a significant limitation of this formulation is the challenge of effectively loading hydrophobic drugs into these vesicles without compromising their elasticity [8]. On the other hand, ethosomes are vesicular carriers composed of hydro-alcoholic phospholipids with a high concentration of alcohol. While ethosomes offer certain advantages, such as simple, passive and non-invasive drug delivery with enhanced permeation through the skin, and targeting to deeper skin layers for various skin diseases, they also have a significant disadvantage. When ethosomes applied to the skin in a non-occlusive state, it tends to cause skin dehydration due to the evaporation of ethanol from the formulation [9].

Transethosomes are ultra-deformable vesicles that are a combination of transferosomes and ethosomes and will overcome the disadvantage of both. Transethosomes, alternatively referred to as ultra-deformable liposomes, possess an irregular spherical structure and exhibit remarkable elasticity. They possess the capability to effectively encapsulate drugs with varying molecular weights, encompassing both low and high molecular weight compounds. Transethosomes structure is composed of a phospholipid, edge activator and ethanol. Phospholipid functions as a carrier for drug molecules to pass through the skin. The edge activator, a biocompatible polymer, serves as the agent responsible for softening the vesicles. It imparts flexibility to the vesicles and acts as the permeation enhancer. Ethanol gives a unique identity to the transethosomal vesicles; it deforms the epidermis layer and allows these nanocarriers to penetrate deeply into the stratum corneum through tiny openings caused by fluidization. The utilization of ethanol along with an edge activator results in the reorganization of the lipid bilayer, enhancing its flexibility and enabling it to effectively permeate the deeper layers of the dermis [10].

Hence, in the present study, a transethosomal-based gel was chosen as the carrier for the effective transdermal delivery of NDFX. However, as per the literature review, no DOE-based transethosomal formulation with improved penetration and effectiveness has been reported for NDFX. Therefore, the primary objective of the present study was to create and enhance transethosomes loaded with NDFX, employing the Box–Behnken experimental design. The formulations underwent characterization to determine their vesicle size, zeta potential, entrapment efficiency and in vitro diffusion properties. The optimized formulation was then assessed for ex vivo permeation, in vitro

antimicrobial assay and in vivo anti-acne effects using an animal model induced with *P. acnes*.

Materials and methods

Materials

NDFX was acquired as a complimentary sample from Wockhardt Research Centre, located in Aurangabad, India. Soya Phosphatidylcholine-90 was acquired as a complimentary sample from Lipidome Lifesciences, based in Ahmedabad, India. Tween 80 was procured from Hi-Media Laboratories, Pvt. Ltd. (Mumbai, India). Ethanol was procured from S.D. Fine Chem Ltd (Mumbai, India). Carbopol 934 was obtained from LOBA Chemie Pvt. Ltd, located in Mumbai, India. Methanol and chloroform were acquired from Fisher Scientific, India. All remaining chemicals utilized were of analytical grade.

Experimental animal

Wistar rats either male or female weighing from 150 to 200 gm were selected for the study. Before commencing the study, necessary authorization and endorsement were obtained from the Institutional Animal Ethics Committee (IAEC) and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), under the Reg. No. 221/Po/Re/S/2000/CPCSEA, during their meeting on 03/12/2022.

Methods

Preparation of NDFX-loaded transethosomes

The cold method was employed for developing NDFX-loaded transethosomes. This method involves mixing of two phases at low temperature (30 °C) in a water bath. In this method aqueous phase (edge activator + water) is added to organic phase (ethanol + phospholipid) with continuous stirring in order to form the vesicles. This method is most widely used for thermo-labile drugs, and it is easily scalable [10].

Soya phosphatidylcholine 90 (phospholipid), Tween 80 (surfactant) and Nadifloxacin (1% w/w) were dissolved in ethanol at a temperature of 30 °C, forming the organic phase. Simultaneously, the aqueous phase (consisting of Millipore water) was heated to 30 °C. The aqueous phase was then added drop by drop to the organic phase, while the mixture was continuously stirred at a speed of 1200 revolutions per minute using a magnetic stirrer (RCT Basic IKA, India). The stirring was continued for 45 min to get the transethosomal dispersions. Thereafter, transethosomal dispersion was ultrasonicated using a probe sonicator (SONICS® VCX 750, USA). The formulation was then refrigerated at 4 °C until further characterization [11].

Optimization of NDFX-loaded transethosomes by Box–Behnken design (BBD)

Based on the literature review, several parameters have been identified that may influence the ideal characteristics of transethosomes for topical delivery and it was found that three variables (factors) such as phospholipid, tween 80 and ethanol concentration might have a direct impact on the critical transethosome characteristics required for successful topical administration. The response surface methodology utilized the Design Expert software to construct a Box–Behnken design consisting of three factors with three levels each (3^3). This experimental design, generated with the software, comprised a total of 17 runs, including 5 repeated center points, and the resulting outcomes were subsequently measured. By employing the Box–Behnken design, the study aimed to examine the impact of phospholipid, surfactant and ethanol concentrations on dependent variables, namely entrapment efficiency, vesicle size and zeta potential. Table 1 shows the independent and dependent elements chosen for the experimental design. The NDFX-loaded transethosome vesicles were tested for vesicle size, entrapping efficiency and zeta potential [12].

Table 1. Independent factors with their levels and dependent factors with their constraints in Box–Behnken design for the development of NDFX-loaded transethosomes

Factors	Levels		
Low	Medium	High	*Independent factors*

A. PC90 (%)	2	3	4
B. Tween 80 (%)	0.2	0.3	0.4
C. Ethanol (%)	20	25	30
Dependent factors			
Entrapment E. (%)	Maximize		
Vesicle size (nm)	Minimize		
Zeta potential (mV)	Maximize		

Evaluation of NDFX-loaded transethosomes

Particle size, polydispersity index and zeta potential

The Malvern zeta sizer (Malvern Instruments, Worcestershire, UK) was utilized to ascertain the particle size, polydispersity index and zeta potential of the transethosomes. The measurements were taken at a constant angle of 90° and a temperature of 25±2 °C. To facilitate the analysis, the samples were diluted in millipore water and the measurements were taken in triplicate. These parameters provide information regarding the size, homogeneity and stability of the vesicles [13].

Entrapment efficiency

The ultra-centrifugation technique was used for estimating the entrapment efficiency of transethosomal formulations. Two milliliters of each formulation was transferred into a microcentrifuge tube and was subjected to ultracentrifugation at 4 °C by an Ultracentrifuge (Kubota, Japan). Following that, the supernatant was extracted with a micropipette and diluted with methanol to rupture the vesicles. A UV spectrophotometer (Shimadzu -1900, Japan) was used to measure the quantity of drug in the supernatant at 295.0 nm [14].

The following formula was used to compute the % EE.%EE=Totaldrugcontent-UnentrappeddrugTotaldrugcontent×100

Surface morphology of optimized transethosomes

Transmission electron microscopy (TEM) was employed to examine the morphology of the prepared transethosomes. To summarize the procedure, a small amount of the diluted transethosomes sample was deposited onto a copper grid and left to air-dry. Once dried, the sample was treated with a 1% w/v solution of phosphotungstic acid for fixation and subsequently subjected to TEM analysis, with accompanying photographs captured [15].

Stability studies of optimized formulation

To determine the stability of the optimized transethosomes, short-term stability studies were conducted in compliance with ICH GCP guidelines. The prepared formulation was stored in glass vials within a humidity-controlled oven maintained at a temperature of 25±2 °C and a relative humidity of 65±5%. Additionally, it was refrigerated at 4 ±2 °C with a relative humidity of 65±5%. At regular intervals of 0, 15, 30 and 90 days, a sample was extracted for analysis [11].

Preparation of transethosomal gels

The preliminary studies were carried out, the gel was formulated through the dissolution of Carbopol 934 in purified water while stirring continuously; and the pH was adjusted to 6–6.5 by incorporating a 10% solution of triethanolamine. HPMC K4M was dispersed in purified water and left overnight. To create the transethosomal gel, the previously prepared transethosomal dispersion was incorporated into the gel in a 1:1 ratio with adequate stirring. The formulation details for gel bases are provided in Table 2 [16, 17].

Table 2. The composition of different gel bases

Ingredients	TEG 1	TEG 2	TEG 3	TEG 4
Carbopol 934 (% w/w)	1	2	–	–
HPMC K4M (% w/w)	–	–	2	3
Methyl paraben (% w/w)	0.02	0.02	0.02	0.02
Propyl paraben (% w/w)	0.01	0.01	0.01	0.01
Propylene glycol (%w/w)	1	1	1	1
Triethanolamine (%v/w)	q.s	q.s	–	–
Distilled Water (% w/w)	Up to 100	Up to 100	Up to 100	Up to 100

Evaluation of transethosomal gels

Determination of pH

In order to ascertain the pH of multiple gels, a digital pH meter was employed. Firstly, 500 mg of pre-prepared transethosomal gels was dissolved in 20 milliliters of distilled water. The resulting mixture was stirred for a duration of 30 min at room temperature using a magnetic stirrer. Subsequently, the pH sensor probe electrode was immersed in the dissolved gel and the pH value of the formulation was recorded from the digital screen [17].

Determination of viscosity

The viscosity of the optimized transethosomal gel formulation was estimated using a Brookfield viscometer. After applying the transethosomal gel formulation, it was allowed to settle for 5 min. After that, spindle number one was revolved at 50 revolutions per minute at a temperature of 25 ± 2 °C [18].

Determination of spreadability

The spreadability of the transethosomal gel was assessed using the glass slide technique. A precisely measured 1.0 g of gel was positioned at the center of a glass slide measuring 10 × 5 cm. Another slide of the same dimensions was placed on top of it. To ensure uniform compression and maintain a consistent thickness, a weight of 100 g was applied to the upper slide for a duration of 5 min. The time required for the glass slide to move 6 cm and separate from the lower glass slide was recorded [19, 20]. The spreadability of the gel was then calculated using the following formula:S=M.L/Twhere '*S*' represents Spreadability, '*M*' represents the weight applied to the upper slide, '*L*' represents the distance traveled by the slide (6 cm), and '*T*' represents the time taken in seconds.

Determination of drug content

In a 50-ml volumetric flask, 1 gm of gel was dissolved with 50 ml methanol. The solution underwent sonication in a bath until a transparent solution was achieved. Subsequently, the solution was filtered through a 0.45-μm filter and appropriately diluted using methanol. The drug concentration was determined by measuring absorbance with a UV spectrophotometer at the wavelength of 296 nm, using methanol as the reference solution [19].DrugContent=Actual drugcontentinvesiclesTheoreticaldrugcontentinvesicles×100

In vitro drug diffusion study

The Franz diffusion cell was utilized to determine the in vitro diffusion of drugs from different transethosomal gel formulations. To activate the dialysis membrane, it was previously soaked in pH 7.4 buffer. The receptor chamber was then filled with 12 ml of phosphate buffer at pH 7.4, which served as the medium for diffusion in the receptor compartment. The gel formulation, equivalent to 2 mg of the drug, was accurately weighed and thronged in the preactivated dialysis membrane. Throughout the experiment, the receptor medium was maintained at a temperature of 37 ± 2 °C and stirring was consistently performed at 100 rpm. At specific time intervals of 0.5, 1, 2, 4 and 8 h,

0.5 ml samples were withdrawn from the cell and replaced with fresh medium. The quantification of drug release was carried out using a UV spectrophotometer at a wavelength of 291 nm [21].

Ex vivo permeation study

A preliminary study was conducted to examine skin permeation using rat skin and the Franz diffusion cell, which had an effective permeation area of 1.76 cm^2. Prior to the permeation study, the rat skin underwent a preparation process. Initially, the hair on the skin was eliminated using an electronic trimmer, followed by the removal of subcutaneous tissue through surgical means. Isopropyl alcohol was used to remove the fat from the dermis side of the skin. Afterward, the skin was washed with PBS and stored at − 20 °C in a deep freezer until it was ready for use. During the experiment, rat skin was affixed onto a diffusion cell with the dermis side facing the receiver compartment and the stratum corneum side facing the donor compartment. The receiver compartment contained a medium of PBS with a pH of 7.4. The temperature in the receiver compartment was maintained at 37 ± 0.5 °C, and throughout the experiment, it was stirred using a magnetic bead at a speed of 100 rpm. At predetermined time intervals of 0.5, 1, 2, 4 and 8 h, 0.5 ml samples were withdrawn and replaced with fresh medium. The collected samples were then analyzed for drug content using a UV spectrophotometer [22].

In vitro anti-bacterial study

Zone of inhibition The cup plate technique was used to investigate the zone of inhibition of prepared NDFX-loaded transethosomal gel against *Propionibacterium acne.* The Mueller–Hinton agar plates were prepared and sterilized at 121 °C and at a pressure of 15 lb. for 20 min. Subsequently, 25 ml of Mueller–Hinton agar was carefully poured into each Petri dish, allowing it to solidify. Following that, the Petri dishes were streaked with a suspension of Propionibacterium acne. Using a cork borer, four holes, each measuring 6 mm in diameter, were created on every plate. In each of these holes, the following substances were placed: a transethosomal formulation loaded with NDFX at a concentration of 10 µg (test), a commercially available NDFX cream at a concentration of 10 µg (standard), 100 µl of a blank transethosomal formulation (blank) and a negative control consisting solely of the vehicle (ethanol and buffer). The plates were incubated at 37 °C for 48 h in an anaerobic condition. The zone of inhibition was measured, and the antimicrobial activity of each formulation toward the *P. acnes* was investigated [23–25].

Animal study

Skin irritation study

A skin irritancy test was conducted on the hairless backs of Wistar rats weighing between 180 and 250 g. The purpose of the test was to determine the irritant or toxic effects of the formulated transethosomal gel on the skin. The animals were segregated into two distinct groups, with each group consisting of six test subjects, as indicated in Table 3. Group I received an application of Optimized NDFX transethosomal gel, whereas Group II received blank transethosomal gel. Prior to the application of gel, the dorsal region of the Wistar rat was trimmed using an electronic trimmer to remove the hair, followed by the application of gel onto the hairless area measuring 5 cm^2. A thorough examination was conducted on the rat's skin to identify any signs of erythema and edema. After 1 h, 24 h, 48 h and 7th day of application of test substances, finally, as per the Draize protocol the scoring was given and the skin irritancy potential of the formulated transethosomal gel was determined [26–28].

Table 3. Experimental design for in vivo skin irritancy study

Sr no	Group	Treatment	No. of animals	Total no. of animals
1	GROUP I	Control gel—Blank Transethosomal gel (without NDFX)	6	12

Anti-acne study

Animal model

Wistar albino rats either male or female weighing between 150 and 200 g were employed to examine the potential anti-acne effects of a transethosomal gel loaded with NDFX. These rats were acclimated to the prescribed

rehabilitation conditions for a minimum of seven days prior to the experiment.

Bacterial sample

The bacterial strain of *Propionibacterium acne* was cultivated within an anaerobic gas chamber using brain heart infusion (BHI) broth. The culture was incubated for 48 h at 37 °C, after which the BHI agar plate was used for subsequent culturing bacterial strain. A single colony was selected from the BHI agar plate and introduced into a PBS solution. The turbidity of the suspension was assessed using the 0.5 McFarland (1.5×108 CFU/ml) scale. This bacterial suspension is now prepared for injection.

Injection of bacteria

An anesthetized Wistar rat was administered a subcutaneous injection of *Propionibacterium acne* bacterial suspension, measuring 20 µl, into its right ear. As a vehicle control, phosphate buffer saline was injected into the left ear [29].

Treatment approach

To investigate the anti-acne potential of prepared experimental gel (NDFX-loaded transethosomal gel) in animal models, the Wistar rats were categorized into four groups, with each group consisting of six animals, as depicted in Table 4. In each group, the rats received an injection of the aforementioned bacterial suspension of *P. acnes* in their left ear, while their right ear was injected with phosphate buffer saline. Initially, no treatment was given to all the groups for the period of seven days (inducing period). Following a period of seven days with no treatment provided to Group I (disease control), Group II was chosen as the benchmark and subjected to treatment using the commercially available anti-acne formulation known as Nadibact cream (containing 1% w/w active ingredient); Group III and Group IV were treated with the prepared NDFX-loaded transethosomal gel and plain NDFX gel, respectively. The treatment was continued till the 14th day for groups II, III, IV, and the measurement was taken using a Vernier Caliper daily, the ear thickness was measured on a predetermined day, and the formula used to calculate the percentage change in ear thickness is as follows%ΔEarThickness=TAfter-TBeforeTBefore×100where *TAfter* represents the thickness of the auricle subsequent to the injection, while *TBefore* denotes the thickness of the auricle prior to the injection [30].

Table 4. Experimental design for in vivo anti-acne study

Animal groups	Treatment	No of animals	Total animals
Group I	Disease control	6	24
Therapeutic treatment			Group II
Standard (Marketed formulation)	6	Group III	Experimental gel (NDFX-loaded with transethosomal gel)
6	Group IV	Plain gel of NDFX	6

Histopathological investigation

Once the anti-acne study was completed successfully on the 14th day, three animals from each group were chosen at random and killed by euthanasia. Prior to sectioning, the ear was surgically removed and immersed in a 10% formalin solution. Subsequently, the sections were treated with hematoxylin and eosin dye for staining. The stained ear slices were then placed on glass slides and examined using an optical microscope [30].

Post-treatment microbiological assay

Upon successful completion of the anti-acne study, the animals from each group were euthanized and the treated area on the right ear was gently wiped with a cotton swab soaked in ethanol before being excised. The excised ear was then divided into small pieces and homogenized in 5 ml of phosphate buffer saline using a tissue homogenizer. Subsequently, the homogenized saline solution was appropriately diluted. A small portion of the homogenate was extracted and evenly spread onto a sterilized cotton swab, which was used to inoculate a Mueller–Hinton agar plate. The plate was then incubated under anaerobic conditions at 37 °C. The number of colony-forming units (CFUs) present on the agar plate was subsequently counted in order to investigate the pharmacodynamic activity of the formulation [25].

Results

Optimization of NDFX-loaded transethosomes by Box–Behnken design (BBD)

The BBD design was used to optimize the NDFX-loaded transethosomal formulation and investigate the impact of independent factors such as soya phosphatidylcholine 90 (A), Tween 80 (B) and the ethanol (C) concentration on the dependent responses, i.e., entrapment efficiency (Y_1), vesicle size (Y_2), zeta potential (Y_3). The experimental design (BBD) has generated 17 batches with 5 center points. The outcomes derived from these experiments are presented in Table 5. The measured values for the dependent factors, namely entrapment efficiency, vesicle size and zeta potential, ranged from 66.43 to 81.87%, 97.04–356.02 nm and − 17.12 to − 34.34 mV, respectively. The responses were subsequently subjected to statistical analysis through response surface analysis employing ANOVA. By examining the 3D surface plot and polynomial equation, the impact of independent factors on dependent responses was explored. The quadratic model proved to be the most suitable fit for analyzing entrapment efficiency and vesicle size, while the reduced quadratic model was found to be the optimal fit for examining zeta potential. The fit statistics results are given in Table 6 showing satisfactory R^2, adjusted R^2, predicted R^2, S.D. and % C.V. [12, 19].

Table 5. Results of the response of experimental Box–Behnken design for optimization of NDFX-loaded transethosomes

Formulation code	PC 90 (%)	Tween 80 (%)	Ethanol (%)	Entrapment (%)	Vesicle size (nm)	Zeta potential (mV)
1	2	0.3	30	54.71	100.65	− 32.25
2	3	0.3	25	73.89	128.2	− 27.88
3	3	0.2	20	80.76	173.01	− 33.72
4	2	0.4	25	57.7	97.12	− 34.34
5	3	0.3	25	74.01	128.9	− 29.98
6	3	0.4	20	72.73	142.25	− 29.99
7	3	0.3	25	74.48	128.6	− 30.25
8	3	0.3	25	73.61	126.9	− 29.77
9	3	0.2	30	71.61	134.9	− 27.74

10	4	0.3	30	67.48	142.21	−22.43
11	3	0.4	30	43.64	96.74	−24.72
12	4	0.3	20	79.6	188.51	−19.95
13	2	0.2	25	72.82	151.18	−29.2
14	3	0.3	25	73.73	138.3	−28.35
15	2	0.3	20	79.95	156.96	−34.18
16	4	0.2	25	81.05	187.34	−21.51
17	4	0.4	25	63.72	160.17	−17.12

Table 6. Summary of results of regression analysis for the response Y_1 (EE), Y_2 (PS) and Y_3 (Zp)

Responses	R^2	Adjusted R^2	Predicted R^2	S.D	% C.V
Y_1 (EE)	0.9985	0.9965	0.9793	0.6	0.85
Y_2 (VS)	0.9811	0.9568	0.7963	5.78	4.13
Y_3 (ZP)	0.9042	0.8607	0.7028	1.90	6.81

Effect of independent factors on response Y_1 (entrapment efficiency)

In all 17 experimental runs, the EE was found to be in the range of 43.64–81.05%.

1

EE=73.94A+3.33B-9.45C-0.55AB+3.28AC-4.99BC-0.93A2-4.19B2-2.57C2

As per polynomial Eq. (1) and 3D response plot (Fig. 2a), a discovery was made indicating that soya phosphatidylcholine 90 has a favorable impact on the entrapment efficiency of NDFX, while both tween 80 and ethanol were observed to have a detrimental effect on the entrapment efficiency. The progressive enhancement of entrapment efficiency as the concentration of soya phosphatidylcholine 90 increases can be attributed to the lipophilic nature of NDFX. This is because of the affinity of lipophilic drugs toward the lipophilic phase, resulting in their subsequent deposition therein [22].

Fig. 2 [Images not available. See PDF.]

a–c Design Expert generated a 3D response plot of entrapment efficiency (*Y*1), vesicle size (*Y*2) and zeta potential (*Y*3)

In formulations F1 and F10, the entrapment efficiency increases from 54.71 to 67.48% as the concentration of phosphatidylcholine increases from 2 to 4%, respectively. Similar results were found when a comparison was made between formulations F4 and F17.

Entrapment efficiency decreases as the concentration of tween 80 increases. Formulation F13 (tween 80 0.2%) showed an entrapment efficiency of 72.82%, while formulation F4 (tween 80 0.4%) showed an entrapment efficiency of 57.7%. Similar results were found between the formulation F16 (tween 80 0.2%) and F17 (tween 80 0.4%). The

possible cause of this phenomenon is the increased surfactant concentration, which may result in the formation of micelles alongside the vesicles in the formulation; micelles typically exhibit diminished entrapment efficiency in comparison with vesicles. Additionally tween 80 is hydrophilic in nature whose increased concentration may decrease the encapsulation of hydrophobic drug [31].

Ethanol also had an opposite influence on NDFX entrapment efficiency in transethosomes vesicles. Formulation F15 having ethanol concentration 20% showed an entrapment efficiency of 79.95%, while formulation F1 having 30% ethanol showed 54.71% entrapment efficiency. This might be due to the leaking of the transethosomal vesicles at high ethanol concentrations [32].

Effect of independent factors on response Y_2 (vesicle size)

The vesicle size was falling in the range of 97.12–187.34 nm in all 17 batches.

2

Vesiclesize=130.18+21.54A-18.77B-23.28C+6.72AB+2.50AC-1.85BC+14.57A2+4.21B2+2.34C2

Based on polynomial Eq. (2) and the 3D response plot (Fig. 2b), it was observed that soya phosphatidylcholine 90 exhibited a positive impact, while Tween 80 and ethanol had a negative impact on the size of the transethosomes vesicle.

It was discovered that as the concentration of soya phosphatidylcholine 90 was increased from 2 to 4%, the vesicle size was also increased from 100.65 nm (F1) to 142.21 (F6). Similar results were seen with F13 (151.18 nm) and F16 (187.34 nm). The tween 80 was showing an inverse effect in the vesicle size of transethosomes. When the concentration of tween 80 was increased from 0.2 to 0.4%, the vesicle size of transethosomes was reduced from 187.34 nm (F16) to 160.17 (F17). The formulation F3 (173.01 nm) to F6 (142.25 nm) also presented similar results. The vesicle size of the transethosome decreases from 188.51 to 142.21 nm as the ethanol concentration increases from 20% (F12) to 30% (F10). This could be attributed to the fluidizing ability of ethanol on the phospholipid membrane of the vesicles by its interpenetrating hydrocarbon chain as the wall thickness of the vesicular membrane decreases [12].

Effect of independent factors on response Y_3 (Zeta potential)

The zeta potential in all 17 formulations was falling in the range of − 17.12 to − 34.34 mV.

3

Zetapotential=-29.16+6.12A+0.7500B+1.34C+2.38AB+2.78A2

The impact of soya phosphatidylcholine, tween 80 and ethanol concentration on the zeta potential of transethosomal vesicles was noted during the observation. As per the polynomial equation, the zeta potential of the vesicles (Fig. 2c) exhibited a positive impact from all three factors. The zeta potential was observed to be increasing from − 32.25(F1) to − 22.43 (F10) with increasing concentrations of phosphatidylcholine 90 from 2 to 4%. As the concentration of tween 80 was increased from 0.2 to 0.4% the zeta potential was also increased from − 27.74 (F9) to − 24.72 (F11).

The zeta potential is the critical characteristic that influences vesicular features such as the interaction of vesicles with the skin and the stability of transethosomes. According to the findings presented in Table 5, it has been observed that all the formulated transethosomal preparations exhibited a zeta potential characterized by a negative charge. Negatively charged vesicles exhibit superior skin permeability compared to positively charged vesicles [33]. The negative zeta potential observed in transethosomes primarily arises from the presence of ethanol within these nanocarriers. Ethanol induces negative charges on the polar head groups of phospholipids, leading to electrostatic repulsion. This would limit vesicle agglomeration and hence increase the stability of these transethosomal nanocarriers [11].

In the present investigation, the point prediction approach of the BBD design in the response surface methodology was used to optimize the NDFX-loaded transethosomal formulation. The formulation composition of soya phosphatidylcholine (2.03%), Tween 80 (0.3%) and ethanol (20%) met the requirements for an optimal formulation. The optimized formulation had shown an entrapment efficiency of 79.8%, vesicle size of 150.18 nm, zeta potential of − 33.85 mV, and desirability was found to be 0.969. The results of the optimized transethosome formulation are

shown in Table 7 (Fig. 3). All the observed responses of optimized transethosomal formulation close proximity to the value were predicted by Design Expert software.

Table 7. Validation of optimized transethosomal formulation

	Independent variables			Responses			Desirability
PC 90 (%)	Tween 80 (%)	Ethanol (%)	EE (%)	PS (nm)	ZP (mV)	Solution	2.03
0.3	20	78.8	150.18	−33.85	0.969	Practically performed	2.03

Fig. 3 [Images not available. See PDF.]

a–c Results of optimized NDFX transethosomes formulation (**A**) mean vesicles size distribution curve and (**B**) average zeta potential and (**C**) transmission electron micrograph

Surface morphology of optimized transethosomes

The analysis using transmission electron microscopy (TEM) of the optimized transethosome formulation containing NDFX (Fig. 3c) indicated that the vesicles exhibit a spherical shape, displaying a consistent size distribution and a distinct, tightly sealed structure [21].

Stability studies

The findings from the stability study of optimized NDFX transethosomal formulation are demonstrated in Table 8. It was observed that after the completion of 90-day storage period, there were negligible alterations (p>0.05) in the physical characteristic of transethosomes. The vesicle size was found to be increased by 5 nm, entrapment efficiency was decreased by 4%, and the zeta potential was increased by 3 mV after the completion of 90-day storage period at 25 °C ± 2 °C (room temperature condition) [34].

Table 8. The results of the stability study

Evaluation parameters	Before	Room temperature (25 °C ± 2 °C)		Refrigerated condition (4 °C ± 2 °C)	
Day 0	30 days	90 days	30 days	90 days	Entrapment efficiency (%)
80.12 ± 0.25	79.54 ± 1.2	76.52 ± 2.6	80.02 ± 0.05	79.8 ± 0.16	Particle size (nm)
156.1 ± 2.2	156.1 ± 2.5	161.1 ± 3.4	156.1 ± 0.8	157.1 ± 1.4	Zeta potential (mV)

Characterization of NDFX-Loaded transethosomal gel

The enhanced transethosomal formulation was integrated into the secondary carrier (Gel) to make the formulation rheologically acceptable for topical administration. The gel was prepared using varying concentrations of Carbopol

934 and HPMC K4M and investigated for gel characterization studies as shown in Table 9. The pH, viscosity, spreadability and drug content were falling in the range of 6.31–7.12, 794–1112cP, 9.2–12.56 g cm^2/s and 84–92%, respectively.

Table 9. Characterization results of NDFX-loaded transethosomal gel

Evaluation parameters	TEG 1 (Carbopol 1%)	TEG 2 (Carbopol 2%)	TEG 3 (HPMC K4M 2%)	TEG 4 (HPMC K4M 3%)
Color	White Opaque	White Opaque	Off-White	Off-White
pH	7.12±0.5	6.95±0.05	6.31±0.0.12	6.42±0.23
Viscosity (cP)	971±15	1112±12	794±14	820±10
Spreadability (g cm^2/s)	9.2±0.25	8.83±0.45	12.56±0.62	10.35±0.82
Drug content (%)	92±1.2	87±1.8	86±0.8	84±0.5

In vitro drug diffusion study

The investigation of the drug diffusion study of prepared transethosomal gels was carried out using the Franz diffusion cell. The drug release was found to be in the range of 42.67–64.83% after 8 h. Figure 4 illustrates the in vitro drug diffusion profile of NDFX from different transethosomal formulations. The release of NDFX from these formulations can be ranked in the following descending order: TEG1>TEG2>TEG3>TEG4. As the TEG1 was found to be showing satisfactory gel characterization results and the highest drug release, it was selected as an optimized gel base for further study [16].

Fig. 4 [Images not available. See PDF.]

Drug diffusion profile of Nadifloxacin-loaded transethosomal gel in different gel bases i.e., Carbopol 1%, Carbopol 2%, HPMC K4M 2% and HPMC K4M 3%

Ex vivo permeation study

The skin permeation study of the optimized transethosomal gel formulation TEG1, loaded with NDFX, was carried out on rat abdominal skin using a Franz diffusion cell. The comparative study was performed between the TEG1, Marketed NDFX formulation (Nadibact cream 1%w/w) and the plain gel of NDFX (Carbopol 1%). Figure 5 illustrates the percentage of drug permeation through rat skin. Remarkably, the optimized transethosomal gel formulation (TEG1) exhibited significantly higher drug permeation compared to the marketed cream and plain gel of NDFX i.e., 58.92%, 26.44% and 38.69%, respectively. It is ranked in the following descending order (TEG1>plain gel >marketed cream). The high permeation rate of optimized NDFX transethosomal gel is because high flexibility of the vesicles which is attributed to the inclusion of an edge activator within the formulation. The edge activator (surfactant) provides flexibility to the vesicles, so they can deform their shape and pass through narrow obstruction of subcutaneous tissue. The ethanol present in the formulation also acts as the penetration enhancer which aids in the deep penetration of transethosomes into the underlying skin layers [12, 35].

Fig. 5 [Images not available. See PDF.]

Ex vivo skin permeation profile of Nadifloxacin-loaded transethosomal gel, plain gel of Nadifloxacin and marketed cream (Nadibact 1%w/w)

In vitro anti-bacterial study

Zone of inhibition The cup plate method was used to evaluate the zone of inhibition of the optimized NDFX transethosome formulation against *P. acness*. The NDFX transethosome formulation and the marketed formulation (Nadibact Cream) exhibited noticeable zones of inhibition when compared to the blank formulation and the control, as depicted in Fig. 6. The investigation revealed that the NDFX-loaded transethosome formulation exhibited a significantly larger antimicrobial activity zone (measuring 26 ± 2 mm) compared to the marketed formulation (16 ± 1.8 mm), as well as the blank formulation and negative control. Thus, the findings demonstrated that the optimized NDFX transethosome formulation is highly responsive to *P. acnes* and effectively demonstrates antibacterial activity against it. The remarkable antimicrobial effect of the transethosome formulation loaded with NDFX is primarily due to the exceptional flexibility of transethosomes. This unique characteristic enables them to easily penetrate the bacterial cell wall and effectively hinder the activity of the DNA gyrase enzyme, which plays a crucial role in bacterial DNA synthesis. As a result, bacterial multiplication is effectively suppressed.

Fig. 6 [Images not available. See PDF.]

In vitro antimicrobial activity against *Propionibacterium acnes*. A, NDFX transethosomes; B, marketed formulation; C, blank formulation; D, control (PBS with ethanol)

Animal study

Skin irritation study

The potential for skin irritation caused by the prepared transethosomal gel containing NDFX and the blank transethosomal gel can be visually evaluated in Fig. 7. The findings indicate that animals from both Group I (Blank gel) and Group II (NDFX transethosomal gel) did not exhibit any signs of redness or swelling until the conclusion of the seventh day. These assessments were conducted using the Draize scoring system, and the results are presented in Table 10.

Fig. 7 [Images not available. See PDF.]

The skin irritation potential of prepared NDFX-loaded transethosomal (**A**) gel and blank transethosomal gel (**B**)

Table 10. Scoring for skin irritancy as per Draize protocol

	Average Draize scoring ($n=6$)							Mean
Erythema				Edema			Formulation	1 h
24 h	48 h	7 days	1 h	24 h	48 h	7 days	Group-I (Blank TEs Gel)	0	0
0	0	0	0	0	0	0	Group-II (NDFX TEs Gel)	0	0

P. acne-induced anti-acne study

The anti-acne efficacy of the newly developed experimental gel (NDFX-loaded transethosomal gel) was assessed in

the Wistar rat model. The evaluation was conducted by measuring the percentage change in ear thickness, as depicted in Fig. 8. At the conclusion of the 14th day, the optimized transethosomal gel containing NDFX demonstrated the highest level of anti-acne efficacy. The optimized formulation has shown the least change in percentage ear thickness i.e., 22.2 ± 2.4%, followed by the marketed formulation treatment group (45.5 ± 5.2%) and plain gel treatment group (63.64 ± 6.8%). The statistical significance of the control group compared to the treatment group was examined using one-way ANOVA and Sidak's multiple comparison tests. The disease control group exhibited a substantial percentage change in ear thickness, specifically 340 ± 12.5%, which was significantly distinguishable (p<0.0001) from the remaining three groups. Despite the daily variation in ear thickness (from day 1 to day 14), Fig. 9 reveals a distinct pattern. In the disease control group, the thickness of the rat ear continuously increased until the 14th day. This increase was attributed to bacterial colonization and the formation of pus in the rat ear pinna. On the other hand, in Group II, Group III and Group IV, the ear thickness increased during the induction period (till the 7th day), but during the treatment period (after the 7th day), it progressively decreased due to the antibacterial properties of the formulations. The same consistent outcomes were visually depicted in the accompanying photographs (Fig. 10).

Fig. 8 [Images not available. See PDF.]

Bar graphs showing the % change in the thickness of rat ears of different groups. Group I (disease control), Group II (marketed treatment), Group III (experimental gel treatment), Group IV (plain gel treatment)

Fig. 9 [Images not available. See PDF.]

Effect of different treatments on the thickness of rat ear at each day

Fig. 10 [Images not available. See PDF.]

Photographs of rat ear showing the extent of inflammation before induction (Day 1), after induction (Day 7) and after treatment of 14 days with different formulations

Histopathological report

Figure 11 depicts the histological changes observed in the rat ear pinna following the injection of a bacterial strain of *Propionibacterium acnes* and subsequent treatment with various formulations.

Fig. 11 [Images not available. See PDF.]

Histopathological observation of rat ear pinna after treatment with different formulations: control group (CG), disease control (DC), standard marketed cream (Std), NDFX-loaded transethosomal gel (TG), plain NDFX gel (PT)

In the control group (non-induced), the intact epidermis, sebaceous gland and ear cartilage displayed minor congestion. However, in the case of the disease control group (untreated), the epidermis suffered mild injury, exhibiting excessive inflammatory cell infiltration, significant congestion, as well as neutrophilic and lymphocytic infiltration. Additionally, there were indications of modest folliculitis, ulceration, edema, macrophages and cutaneous granulation tissue. The group treated with the plain gel exhibited pathogenic alterations that closely resembled those observed in the disease group. These alterations included increased inflammatory cell and neutrophilic infiltration, although no edema or skin granulation tissues were identified.

Comparatively, the group treated with the standard marketed formulation (Nadibact cream) displayed mild folliculitis congestion, along with considerable neutrophilic and lymphocytic infiltration. On the other hand, the optimized transethosomal gel treated group showed an intact epidermis with modest congestion.

Post-treatment microbiological assay

The post-treatment *ex vivo* microbiological assay results are shown in Fig. 12. The results have demonstrated that optimized NDFX-loaded transethosomal formulation has shown potent antibacterial activity as there are fewer CFU (colony-forming units) of *Propionibacterium acnes* compared to the marketed formulation and the plain gel of NDFX. This might be due to the high permeability of the transethosomal vesicles into the deeper layer of the skin which

allows it to retain at the subcutaneous level and inhibit bacterial colonization. The order of antimicrobial activity of the aforementioned formulations against *Propionibacterium acnes* is as follows:

Fig. 12 [Images not available. See PDF.]

Post-treatment microbiological assessment different treatment groups

Optimized NDFX-loaded transethosomal formulation > Marketed formulation (Nadibact cream) > Plain gel of NDFX.

Discussion

This research focused on developing Nadifloxacin-loaded transethosomes using the cold method to enhance topical delivery. A Box–Behnken experimental design successfully optimized Nadifloxacin-loaded transethosomes, with independent variables including phosphatidylcholine 90, Tween 80 and ethanol [11, 12]. Seventeen formulations were prepared, and their characterization revealed entrapment efficiency ranging from 43.64 to 81.05%, vesicle sizes between 97.12 nm and 187.34 nm, and zeta potential ranging from − 17.12 to − 34.34 mV. The optimized formulation exhibited an entrapment efficiency of 80.12% ± 0.25%, a vesicle size of 156.1 ± 2.2 nm and a zeta potential of − 33.23 ± 0.5 mV. Transmission electron microscopy (TEM) confirmed the presence of encapsulated vesicles with a sphere-like shape [21]. This optimized formulation was incorporated into gel bases, such as Carbopol 934 (1% and 2%) and HPMC K4M (2% and 3%). The characterization study demonstrated that Carbopol 934 (1%) gel exhibited desirable characteristics with a pH of 7.12, viscosity of 971 cP, spreadability of 9.2 $g.cm^2$/sec and drug content of 92%. In vitro diffusion studies showed that the transethosomal gel with Carbopol 934 (1%) displayed superior drug diffusion after 8 h compared to Carbopol 934 (2%) and HPMC K4M (2% and 3%). Ex vivo skin permeation studies revealed that the optimized transethosomal gel demonstrated enhanced skin permeation compared to the commercially available Nadibact cream (1% w/w). The optimized Nadifloxacin-loaded transethosomal gel exhibited improved in vitro antimicrobial and in vivo anti-acne effects against *Propionibacterium acnes* in Wistar rats, surpassing the efficacy of the commercially available formulation. Histopathological analysis confirmed its superior anti-acne properties and better penetration capabilities compared to the plain gel and the marketed-treated group. Stability assessments over three months demonstrated the formulations' exceptional stability when stored under refrigerated conditions at 4 °C.

In conclusion, the development of Nadifloxacin-loaded transethosomes using the cold method proved effective in enhancing topical delivery for the treatment of Acne vulgaris. The optimized formulation exhibited desirable characteristics, superior skin permeation and improved anti-acne efficacy compared to the commercially available formulation, showcasing its potential as a promising approach in acne treatment.

Conclusion

Nadifloxacin-loaded transethosome formulations were successfully prepared utilizing a cold method and further optimized using a Box–Behnken design (BBD). The optimized formulation exhibited favorable characteristics, including high entrapment efficiency, vesicle size within the nanorange and desirable zeta potential. The results obtained from the ex vivo permeation study indicated that the optimized transethosomal gel facilitated enhanced permeation when compared to a commercially available formulation. The optimized transethosome formulation exhibited augmented in vitro antimicrobial activity as well as in vivo anti-acne efficacy against *Propionibacterium acnes* in Wistar rats, surpassing the performance of the commercially available formulation. Moreover, the histopathological report revealed that the group treated with the optimized Nadifloxacin-loaded transethosomal gel exhibited a higher rate of healing in comparison with both the plain gel and the commercially treated groups. The current investigation found NDFX-loaded transethosomal gel to be a potential alternative to standard topical formulation for the treatment of Acne.

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Author contributions

Author SP performed all the above research work and prepared the complete manuscript, Author PD and TK guided and monitored the research activities, Author PB and VK guided in performing the animal experiment and antimicrobial study, respectively, Author SH contributed in final drafting of manuscript.

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Availability of data and materials

The data generated and analyzed during this research work are included in this article; if any excess data are required, it will be available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Necessary authorization and endorsement were obtained from the Institutional Animal Ethics Committee (IAEC) and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), under the Reg. No. 221/Po/Re/S/2000/CPCSEA, during their meeting on 03/12/2022.

Competing interests

The authors declare no competing interests.

Abbreviations

NDFX
Nadifloxacin
UDV
Ultra-deformable vesicles
P. acne
Propionibacterium acne
BBD
Box–Behnken design
PC 90
Soya phosphatidylcholine 90
VS
Vesicle size
nm
Nanometer
EE%
Entrapment efficiency percentage
ZP
Zeta potential
mV
Milli-volts
TEM
Transmission electron microscopy
PBS
Phosphate buffer saline
µg
Micro-gram
mg
Milli-gram
mL

Milliliter
min
Minutes
RPM
Revolutions per minute
w/v
Weight by volume
CDD
Cumulative drug diffuse
CDP
Cumulative drug permeated
CLA
Cumulative loss added
cps
Centipoise
MIC
Minimum inhibitory concentration
MBC
Minimum bactericidal concentration
IAEC
Institutional Animal Ethics Committee
CPCSEA
Committee for the Purpose of Control and Supervision of Experiments on Animals

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DETAILS

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Chemical characterization, safety profile and antileiomyoma effects of Tetrapleura tetraptera Taubert (Fabaceae) fruit ethanol extract in Sprague Dawley rats

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ABSTRACT (ENGLISH)

Background

Tetrapleura tetraptera Taubert (Fabaceae) fruits are employed by herbal practitioners in the management of uterine leiomyoma, but its usage in this regard and level of safety in chronic administration has not been sufficiently established. This study evaluated the toxicity effects of *T. tetraptera* ethanol fruit extract and explored its antileiomyoma effect in female Sprague Dawley (SD) rats.

Methods

Sub-chronic toxicity test of the extract was done, with biochemical and hematological changes as well as histopathology of organs assessed. Leiomyoma formation was induced in SD rats with monosodium glutamate (MSG) and the extract given at 100, 200 and 400 mg/kg doses, following both the preventive and curative methods. Total serum cholesterol, protein and estradiol were determined, as well as histopathology assessment of the uterus. Phytochemical profiling of the extract was evaluated by analytical high-performance liquid chromatography (HPLC).

Results

No significant alterations were seen in the biochemical and hematological indices in the toxicity test. The vital organs showed no changes at 200 mg/kg, but at 800 mg/kg it appeared to induce multiplication of glandular epithelium and stromal fibrosis in the uterus, and induced perivascular inflammation around the vessels of the heart. Total serum cholesterol and estradiol were significantly elevated ($P \leq 0.05$) on treating normal female rats with 800 mg/kg MSG. Preventive and curative treatment of MSG-treated animals with the extract significantly decreased the elevated serum cholesterol ($P \leq 0.01$) and estradiol ($P \leq 0.05$). Histological studies of the uterus showed an amelioration of the proliferating fibroid cells with administration of the extract, which was more evident in the curative treatment. Result of HPLC analysis of the extract revealed rich composition in bioactive compounds such as umbelliferone, ferulic acid, aridanin, echinocystic acid, naringenin and hentriacontane.

Conclusion

The ethanol fruit extract of *T. tetraptera* is relatively safe in Sprague Dawley rats in low doses and has antifibroid potential as seen in its significant reduction in the elevated total cholesterol and estradiol content as well as its ability to decrease uterine leiomyoma proliferation, which may be due to its array of phytochemical constituents.

FULL TEXT

Background

The most prevalent tumors of the female reproductive system are uterine leiomyomas, often known as uterine fibroid or uterine myomas. It poses a serious risk to women's reproductive health everywhere [1]. Over 70% of women have it, and about 30% of them develop symptoms [2]. In women who are of reproductive age, it is the most common reason for hysterectomies [3]. It is a benign monoclonal tumor of the uterine smooth muscle cells. It grows in the uterine cavity or on the walls of the uterus in different places. Thus, it is referred to as intramural, submucosal or sub-serosal fibroids. It can have any shape and size, from a pea size to a water melon size [1].

While the exact cause of uterine leiomyoma formation is still unknown, epidemiological studies have provided substantial evidence that estrogen and progesterone stimulate the growth of the tumor, possibly as a result of continuous menstrual cycles, since fibroids rarely develop prior to menarche and disappear following menopause [2]. Women who identify as African-American and who are fat are significantly more at risk. Aside from age and family history, other risk factors for uterine leiomyoma include nutrition, lack of exercise, some chemicals like monosodium glutamate (MSG) and prescription medications that may raise cholesterol, estrogen and total protein levels [4].

Since most women with uterine fibroids have no symptoms, they receive less clinical attention and the tumors are frequently left undetected. Women with symptoms usually report abnormally heavy bleeding from the uterus (which can cause anemia) or painful periods; pelvic fullness; lower abdominal enlargement; frequent urination; pain during sexual activity; lower back pain; complications during pregnancy and labor; and reproductive issues like infertility [5]. The size and location of the fibroids typically determine how severe these symptoms are.

There are extremely few alternatives for treatment and prevention; doctors frequently recommend myomectomy and hysterectomy. Medication treatments, such as hormone replacement therapy, are only beneficial for six to twelve

months because prolonged use of the drug might have serious negative effects [6, 7]. Although uterine artery embolization and high-intensity focused ultrasound ablation are viable options, patients may be discouraged from pursuing such treatment due to the associated risks and expenses [8]. Not only are orthodox drugs expensive, but they often only provide brief relief, which makes alternative treatment choices necessary.

The drawbacks of conventional medications, in addition to the accessibility, affordability and cultural significance of herbal remedies, which are favored over the expensive medical services provided by conventional medicine, have sparked a resurgence of interest in these remedies [9]. In one study, various biochemical parameters changed by administration of MSG were recovered by the ethanol extract of *Diodia sarmentosa* leaves. The treated groups' protection against leiomyoma development was also demonstrated by histopathological assessment [7]. Femitol, a herbal mixture manufactured in Ghana, was found to be effective as an antifibroid agent as it greatly reduced MSG-induced uterine hyperplasia and decreased high levels of cholesterol and estrogen, as well as uterus size and weight [10, 10]. Additionally, morphological examination demonstrated that the entire plant of *Labisia pumila* caused apoptosis against SK-UT-1 (uterine leiomyoma cells) in a dose-dependent manner and inhibited the formation of uterine fibroid tumors [11].

Tetrapleura tetraptera Taubert (Fabaceae) is used to treat uterine leiomyoma, according to anecdotal evidence [12]; however, this is not supported by scientific research. The plant is used to cure febrile illnesses and infections, stop postpartum contractions, lower cholesterol, encourage the production of breast milk and speed up the healing of reproductive wounds [13]. Despite the various evidence for its efficacy, there is insufficient scientific evidence to support its safety with regards to its use in herbal medicines. Since the fruit of this plant is used in preparation of herbal remedies for management of leiomyoma which is a chronic illness, it is therefore necessary to evaluate the level of safety of this plant following prolonged repetitive administration.

The objectives of this study, therefore, were to establish the safety of the fruit extract and analyze its protective effect on biochemical indicators such as total cholesterol, protein and estradiol, in order to determine its antiuterine fibroid effect on MSG-induced uterine leiomyoma in Sprague Dawley rats, which will be further confirmed by histopathological assessment of the rat's uterus. Furthermore, identification of the major constituents of the extract was carried out with analytical HPLC.

Methods

Chemicals and reagents

All reagents used in this study were of analytical quality and acquired from reputable local vendors.

Plant collection and preparation

The fruit of *T. tetraptera* was harvested in the savanna area of Odofin Agbegi village, Ikire, Osun state, Nigeria, and authenticated at the Forest Research Institute of Nigeria (FRIN) with the herbarium number FHI 113604 attached. Using a Soxhlet device, the dried and crushed fruit was extracted with ethanol. A rotary evaporator was used to reduce the extract to dryness.

Animal handling conditions

Non-pregnant Sprague Dawley (SD) rats weighing between 125 and 160 g were obtained and placed in groups of five (5) in plastic cages with a wire screen top for adequate ventilation and wood shavings as the bedding material. The animals were supplied clean water and fed with commercial rat pellet (Chikun feed) manufactured by Olam Agricultural enterprise ad libitum. The processes applied in animal handling were in accordance with the National Institute of Health Guidelines for the Care and use of Laboratory Animals. All procedures for using experimental animals were approved by the Research Ethics Committee of the Faculty of Pharmacy, University of Benin (EC/FP/023/04).

Dosing of experimental animals

Doses of *T. tetraptera* extract used in this study were selected based on the LD_{50} of the plants fruit which is greater than 5000 mg/kg [14]. The SD rats were given doses via gavage. Throughout the experiment, animals were dosed once a day, with each dose volume determined by the animal's weekly recorded body weight. The oral route of administration was chosen because it is the commonly utilized route by humans.

Sub-chronic toxicity test

SD rats were divided into four groups comprising of 6 animals each. Group A received 0.5 mL distilled water (control). Groups B, C and D were orally administered different doses of the extract (200, 400 and 800 mg/kg BW), respectively, daily for 28 days. Body weights of the rats were taken on day 0, 7, 14, 21 and 28. Every day, the animals were closely monitored for any changes in their clinical symptoms. Tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma were the main areas of focus [15]. On the last day of gavage, the rats were fasted for a period of 12 h before being killed using a chloroform chamber. The blood was drawn by cardiac puncture into two distinct kinds of bottles: Ethylenediaminetetraacetic acid (EDTA) bottles were used to collect the blood's hematological parameters, while plain bottles were used to acquire serum for the analysis of biochemical parameters. Organs for histological analysis were obtained, including the kidney, liver, heart and uterus.

Biochemical analysis

Samples of blood were taken into plain tubes and left to stand at room temperature for 45 min before being centrifuged for 10 min at 3400 rpm. The collected serum stored at − 25 °C was utilized to assess the lipids, renal and liver function tests. These parameters were assessed using an automated chemistry analyzer (Selectra Pro S, Germany). Electrolyte assay was by ion-selective electrode (SFRI 4000, France). The parameters assayed included creatinine (Cr), urea (Ur), uric acid, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP), serum proteins (Tp), total bilirubin (Tb), triglycerides (TG), total cholesterol (T-CH), low-density lipoproteins (LDL), high-density lipoprotein (HDL) and serum electrolytes (Na^+,K^+,Cl^-, HCO_3^-).

Hematological analysis

Additionally, hematological parameters were evaluated by a blood count utilizing an automated hematology analyzer on blood collected into EDTA bottles (Dymind 2000, China). The parameters analyzed include white blood cell (WBC), red blood cells (RBC), red blood cell distribution width (CV), red blood cell distribution width (SD), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), granulocytes (GRAN), platelets count (PCT) and hematocrit (HCT).

Histopathology analysis of organs

For histological analysis, the liver, heart, kidney and uterus from the killed animals were fixed in 10% neutral buffered formalin solution. These tissues were subsequently dehydrated in ascending grades of alcohol (70%, 90%, 96% and 100%), cleared in xylene, impregnated with molten paraffin wax and sectioned to slides. These sections (4–5 µm thick) were stained with hematoxylin after dewaxing with xylene and hydrating in descending grades of alcohol (100%, 96%, 70%) and water. Differentiation was done in 1% acid alcohol and the sections counter stained with eosin. Dehydration in ascending grades of alcohol was carried out again, and the sections were cleared in xylene and mounted with dibutylphthalate polystyrene using cover slips prior to microscopic examination [16].

Antileiomyoma effect of *Tetrapleura tetraptera* fruit (TTF)

Experimental procedures

Preventive study

This was carried out following the method described in the literature [7]. Female SD rats were placed in five groups of five rats each. Dosing was done once a day through the oral route. Group A (Control) was the no treatment group, given only food and water. Group B was treated with 800 mg/kg monosodium glutamate (MSG) (Lobachem, India) only. Groups C, D and E were treated with 800 mg/kg MSG and *T. tetraptera* fruit extract (100, 200 and 400 mg/kg) concurrently. All treatments were given concurrently for a 30-day period. On the 31st day, the animals were killed by anesthetizing them in a chloroform saturated chamber and their blood collected through cardiac puncture and transferred into plain bottles which were used to determine total serum cholesterol, protein and estradiol content. The uteri were surgically removed and transferred into sterile tissue bottles containing 10% neutral buffer formalin for histopathology assessment.

Curative study

Following the method described in the literature [4], female SD rats were treated with 800 mg/kg MSG for 30 days to

induce the development of uterine leiomyoma. The rats were housed in five groups of five rats each. Treatment with extracts commenced on the 31st day. Group A was the control group, given no MSG but only food and water for the duration of the experiment. Group B, after being administered 800 mg/kg MSG for 30 days, was given food and water only from the 31st to 60th day. Groups C-E were treated with 100, 200 and 400 mg/kg *T. tetraptera* fruit extract, respectively, from the 31st to 60th day, after the 30-day MSG administration. The animals were killed by anesthetizing them in a chloroform saturated chamber and their blood collected through cardiac puncture into plain bottles to determine the total serum cholesterol, protein and estradiol content. The uteri were surgically removed and transferred into sterile bottles containing 10% neutral buffer formalin for histopathology assessment.

Biochemical assays

Determination of total cholesterol content

A day after the experimental period (30 days for preventive treatment and 60 days for curative treatment), the animals' blood was collected and serum separated by centrifugation at 3000 rpm for 10 min, which was used for the experiment.

Total cholesterol content was determined using the semi-automated chemistry analyzer (Mindray BA-88A Reagent system) and the AGAPPE test kit. The total cholesterol kit was programmed on the semi-automated biochemistry analyzer using the information on the instruction manual. In a microtube marked "blank," 1000 µL of cholesterol biuret reagent was added. 10 µL of standard cholesterol and 1000 µL of the reagent were added to and properly mixed in a tube marked "standard." 10 µL of sample A_1 and 1000 µL of cholesterol reagent were added to and properly mixed in a tube with the label "A_1." The same procedure was carried out for the remaining samples (A_1–E_5) in their respective tubes. This was done for samples obtained from both the preventive and curative experiments. All tubes were incubated for 10 min at 37 °C. After incubation, the content of the blank tube was aspirated into the flow cell of the analyzer to measure the absorbance, after which the content of the standard tube was also aspirated into the flow cell to measure the absorbance. Reaction mixture for each sample was then aspirated into the flow cell to measure the absorbance. The values of absorbance for each tube were recorded accordingly [10].

Determination of total protein content

The information in the instruction manual was used to program the kit on the semi-automated biochemistry analyzer. In a microtube marked "blank," 1000 µL of total protein biuret reagent was added. 20 µL of standard total protein and 1000 µL of the reagent were added to and properly mixed in a tube marked "standard." 20 µL of sample A_1 and 1000 µL of total protein reagent were added to and properly mixed in a tube with the label "A_1." The remaining samples (A_1–E_5), from both the preventive and curative experiments in their respective tubes underwent the same procedure. All tubes were incubated for 10 min at 37 °C. After incubation, the content of the blank tube was aspirated into the flow cell of the analyzer to measure the absorbance, after which the content of the standard tube was also aspirated into the flow cell to measure the absorbance. Reaction mixture for each sample was then aspirated into the flow well to measure the absorbance. The value of absorbance for each tube was recorded accordingly [10].

Determination of estradiol content

Estradiol was assayed using the microplate reader (mindray MR-96A), microplate washer (Mindray MW-12A) and E2 AccuBind ELISA Kit. 25 µl of the serum reference and 25 µl of each sample (A_1–E_5) were pipetted into the assigned wells, after which 50 µl of estradiol biotin reagent was added to all wells. Plates were swirled gently for about 30 s and then incubated for 30 min followed by the addition of 50 µL of estradiol enzyme reagent to all the wells and incubation for 90 min at room temperature. The contents of the microplate were discarded by decantation and the plates dried with absorbent paper afterward. 350 µL of the wash buffer was added and the content decanted thrice. 100 µL of substrate was added and incubated for 20 min, after which 50 µL of the stop solution was added to each well and gently mixed for 20 min. The absorbance was read at 450 nm within 15 min of adding the stop solution. The concentration of estradiol in samples was extrapolated from a dose response curve [14].

Histopathology analysis of the uterus

This was carried out following the method earlier stated [16].

HPLC procedure

The HPLC procedure was carried out at Bato Chemical Laboratory in Lagos, Nigeria. HPLC make was Shimadzu (Nexera mx). Column used was ubondapak, a C18 reverse-phase chromatographic column, with dimensions of length: 100 mm, internal diameter: 4.6 mm and thickness: 7 µm. Mobile phase employed was acetonitrile/water, 70:30. The HPLC system was attached to a UV–Vis diode array detector set at an analytical wavelength of 254 nm, within the UV–visible region. Pump pressure employed was 15 mpa. Solutions of standards were first injected into the HPLC system to generate a chromatogram with a given peak and peak profile. These were used to create a window in preparation for the test sample analysis. The analysis adopted a 2 ml/min constant flow rate as 5 ul of extract was injected into the HPLC system also to obtain a corresponding peak area and peak profile in a chromatogram [18].

Data analysis

Bar graphs were obtained by the software GraphPad Prism for Windows, version 6.01, and data were subjected to one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparison test. $P \leq 0.05$ was considered statistically significant in all analysis.

Results

Sub-chronic toxicity results

Effect of *T. tetraptera* fruit on food consumption and body weight

In test animals, repeated dosing over a 28-day period did not result in mortality or systemic toxicity indicators. Normal eating habits were observed and a gradual increase in weight was seen in all the test groups, although the control group showed marked weight gain ($P<0.05$) compared to the groups administered the extract (Table 1).

Table 1. Change in mean body weights of the rats at different days of the experiment

Dose (mg/kg)	Change in mean body weights (g) at different days				
0	7	14	21	28	Control
125.86 ± 14.92	9.87 ± 5.56	9.30 ± 2.14	8.30 ± 1.14	9.96 ± 3.34	200
138.30 ± 10.89	7.90 ± 4.45[a]	8.29 ± 2.43	7.30 ± 2.14	7.22 ± 1.43[b]	400
143.74 ± 10.18	6.30 ± 3.14[b]	6.86 ± 4.71[a]	7.90 ± 3.43	6.87 ± 4.52[b]	800

Data are expressed as mean ± SEM, $n=6$, a = $p<0.05$, $b = p<0.01$ compared to control

Effect of *T. tetraptera* fruit on serum biochemical parameters

Effect on lipid profile tests

All the lipid profile parameters obtained from the serum of the control and test groups at different doses were not significantly different ($p>0.05$), though a slight reduction in total cholesterol was observed at 400 mg/kg compared to the control (Table 2).

Table 2. Lipid profile parameters after 28 days of administration

	Doses (mg/kg)			
Parameters	Control	200	400	800

T-CH (mg/dL)	71.80 ± 2.80[a]	87.60 ± 8.16[ab]	66.60 ± 2.84[ac]	71.60 ± 2.54a
TG (mg/dL)	96.20 ± 13.60[a]	120.6 ± 27.83[a]	82.00 ± 12.63[a]	97.20 ± 7.59[a]
HDL (mg/dL)	34.80 ± 2.27[a]	38.40 ± 1.63[a]	33.40 ± 3.17[a]	34.60 ± 2.50[a]
LDL (mg/dL)	21.80 ± 1.63a	24.80 ± 2.75[a]	16.80 ± 1.20[a]	18.40 ± 3.14[a]

Data are expressed as mean ± SEM, $n = 6$. Values in the test groups carrying the same letters as the control group are not significantly different according to Tukey–Kramer multiple comparison test $p > 0.05$.

T-CH total cholesterol, *TG* triglyceride, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein

Effect on kidney function test parameters

For the kidney function tests, extract administration at all doses did not significantly alter the concentration of sodium, potassium, chloride, urea and creatinine ($P > 0.05$) (Table 3).

Table 3. Kidney function test parameters after 28 days of administration

	Doses (mg/kg)			
Parameters	Control	200	400	800
Na^+ (mmol/L)	139.4 ± 0.68	138.8 ± 0.80	139.4 ± 0.81	140 ± 1.14
K^+ (mmol/L)	5.06 ± 0.12	5.10 ± 0.21	5.22 ± 0.21	5.26 ± 0.24
HCO_3^- (mmol/L)	21.40 ± 0.81	19.60 ± 0.93	20.00 ± 0.77	20.20 ± 0.80
Cl^- (mg/dL)	102.80 ± 0.8	102.00 ± 0.95	100.60 ± 1.10	101.80 ± 0.80
Urea (mg/dL)	43.00 ± 1.92	39.80 ± 3.20	38.40 ± 2.58	43.40 ± 2.09
Creatinine (mg/dL)	0.84 ± 0.09	0.84 ± 0.08	0.80 ± 0.06	0.86 ± 0.08

Data are expressed as mean ± SEM, $n = 6$. There was no significant difference between the groups according to Tukey–Kramer multiple comparison test $P > 0.05$

Effect on liver function test parameters

The liver function test parameters were also not significantly altered by administration of the extract (Table 4) although there was a slight elevation in alkaline phosphatase content.

Table 4. Liver function test parameters after 28 days of extract administration

	Doses (mg/kg)			
Parameters	Control	200	400	800
ALP (μ/L)	210.20 ± 32.91	251.20 ± 8.79	242.80 ± 28.9	249.80 ± 16.73

ALT (μ/L)	128.20±12.29	112.60±14.87	97.20±15.19	118±14.16
AST (μ/L)	195.20±16.93	213.20±28.52	143.6±14.6	173.2±17.48
Tb (mg/dL)	0.22±0.02	0.22±0.02	0.24±0.02	0.2±0.00
Cb (mg/dL)	0.10±0.00	0.12±0.02	0.1±0.00	0.1±0.00
Tp (g/dL)	6.28±0.21	6.72±0.22	6.22±0.18	6.7±0.27
ALB (g/dL)	3.26±0.05	3.42±0.17	3.20±0.03	3.20±0.06
GLo (g/dL)	3.02±0.19	3.30±0.17	3.02±0.17	2.98±0.22

Data are expressed as mean±SEM, $n=6$. There was no significant difference between the groups according to Tukey–Kramer multiple comparison test $P>0.05$, alkaline phosphatase (ALP), alanine aminotransferase (ALT) Aspartate aminotransferase (AST), total bilirubin (Tb), conjugated bilirubin (Cb), total protein (Tp), albumin (ALB), globulin (GLo)

Effect of *T. tetraptera* fruit on hematological parameters

The results of the hematological parameters (Table 5) revealed a decrease ($p<0.05$) in white blood cell count at 200 and 400 mg/kg BW. The extract did not have a significant effect ($p>0.05$) on all other hematological parameters tested as they were all within normal range (Table 5).

Table 5. Results of hematological assay after 28 days administration

Dose (mg/kg)	0	200	400	800
WBC (10^3/μL)	8.16 ± 0.58^{a}	5.84 ± 0.52^{bc}	5.84 ± 0.35^{bc}	6.42 ± 0.54^{ac}
LYM (%)	83.58 ± 1.94^{a}	79.68 ± 3.85^{a}	76.70 ± 2.12^{a}	79.40 ± 2.04^{a}
MID (%)	12.18 ± 1.25^{a}	13.90 ± 2.26^{a}	15.62 ± 1.52^{a}	13.02 ± 1.35^{a}
GRAN (%)	4.24 ± 0.75^{a}	6.42 ± 1.60^{a}	$7.80\pm0.90b^{a}$	7.60 ± 1.00^{a}
RBC (10^6/μL)	5.67 ± 0.30^{a}	5.94 ± 0.28^{a}	5.90 ± 0.05^{a}	6.21 ± 0.19^{a}
HGB (g/dL)	11.06 ± 0.64^{a}	11.74 ± 0.72^{a}	11.60 ± 0.19^{a}	12.20 ± 0.45^{a}
HCT (%)	31.54 ± 1.82^{a}	33.66 ± 1.82^{a}	33.58 ± 0.74^{a}	33.96 ± 1.30^{a}
MCV (fL)	55.74 ± 1.90^{a}	56.82 ± 2.06^{a}	57.02 ± 1.40^{a}	54.74 ± 1.22^{a}
MCH (pg)	19.46 ± 0.29^{a}	19.68 ± 0.61^{a}	19.62 ± 0.22^{a}	19.56 ± 0.24^{a}
MCHC (g/dL)	35.08 ± 0.84^{a}	34.80 ± 0.58^{a}	34.56 ± 0.84^{a}	35.88 ± 0.41^{a}

RDW-SD (fL)	30.74 ± 2.76[a]	32.48 ± 2.07[a]	31.60 ± 2.08[a]	31.73 ± 1.77[a]
RDW-CV (%)	14.96 ± 0.94[a]	15.52 ± 0.57[a]	15.10 ± 0.74[a]	15.44 ± 0.56[a]
PLT (10^3/μL)	624.20 ± 76.12[a]	588.80 ± 19.99[a]	632.80 ± 70.34[a]	652.80 ± 39.78[a]
MPV (fL)	7.64 ± 0.07[a]	7.26 ± 0.23[a]	7.34 ± 0.20[a]	7.30 ± 0.08[a]
PDW (fL)	9.76 ± 0.41[a]	9.26 ± 0.61[a]	9.18 ± 0.33[a]	9.76 ± 0.12[a]
PCT (%)	0.47 ± 0.06[a]	0.43 ± 0.02[a]	0.47 ± 0.07[a]	0.47 ± 0.03[a]
P-LCR (%)	9.10 ± 1.43[a]	6.54 ± 1.76[a]	7.92 ± 1.44[a]	5.62 ± 0.77[a]

Data are expressed as mean ± SEM, $n = 6$. Values in the test groups carrying the same letter as the control group are not significantly different according to Tukey–Kramer multiple comparison test ($p > 0.05$), white blood cell (WBC), lymphocytes (LYM), mid-range absolute count (MID), granulocytes (GRAN), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width SD (RDW-SD), red blood cell distribution width CV (RDW-CV), platelet (PLT), mean platelet volume (MPV), platelet distribution width (PDW), platelets count (PCT), platelet larger cell ratio (P-LCR)

Histological evaluation of the rat organs

The rat heart muscles were made up of cardiomyocytes arranged in bundles. At 200 mg/mL of the extract, the architecture of the heart was normal (Panel A). 400 mg showed the presence of polymorphs. At 800 mg, there was induction of inflammation around the blood vessels of the heart (polymorphs and fibroblasts) and the blood vessels looked ulcerated.

The liver at 200 mg had increased blood flow in the veins and normal hepatocytes (Panel B). 400 mg revealed the presence of activated Kupffer cells in the sinusoids which is evidence of a boost in the immune system; this can be seen as an extra quality of the extract. At 800 mg, polymorphs around the portal triad were observed along with Kupffer cells. The kidney is made up of the renal corpuscle; there was increased blood flow (active congestion) in the interstitial space and normal architecture was observed even at 800 mg/kg, as no necrosis of tubular epithelium was seen. The uterus at 200 mg showed normal structures (Panel D), but stromal fibrosis and glandular epitheliosis were observed at 800 mg (Fig. 1).

Fig. 1 [Images not available. See PDF.]

H&E × 400 SD Rat organ sections: Panel **a** shows normal heart architecture, bundles of cardiomyocytes (CM), coronary artery (CA), interstitial space (IS), at 400 and 800 mg/kg perivascular mobilization of inflammatory cells (PI) are seen. Panel **b** shows normal liver architecture, hepatocytes (HC), sinusoids (SI), portal vein (PV), bile duct (BD). 800 mg/kg shows normal hepatocytes with conspicuous nucleoli (HN), periportal infiltrates of inflammatory cells (PI), vascular congestion (VC). Panel **C** shows normal kidney architecture: tubules (TU), interstitial space (IS), glomeruli (GL). Panel D shows normal uterus architecture: cavity (CA), endometrial lining (EL), stroma (ES), glands (EG), 800 mg/kg shows glandular epitheliosis (GE), stromal fibrosis (SF)

Antileiomyoma evaluation results

Total serum cholesterol

There was significant elevation [64.37% ($P \leq 0.05$)] in serum cholesterol in the group administered MSG only for the preventive treatment. When the extract was administered concurrently with MSG, this action was observed to a lesser degree, with 100 and 200 mg/kg having only 20.77 and 6.56% increase in serum cholesterol compared to the

control ($P \geq 0.05$) (Fig. 2). After MSG induction of uterine leiomyoma where serum cholesterol was elevated (45.85%), the curative treatment with different doses of the extract decreased this parameter to normal especially at 200 and 400 mg/kg doses ($P \geq 0.05$) which was vastly reduced compared to the MSG group ($P \leq 0.01$) (Fig. 3).

Fig. 2 [Images not available. See PDF.]

Preventive effect of the ethanol extract of *T. tetraptera* fruit on total serum cholesterol in female SD rats administered 800 mg/kg MSG. Compared to normal group *$P<0.05$

Fig. 3 [Images not available. See PDF.]

Curative effect of the ethanol extract of *T. tetraptera* fruit on total serum cholesterol in female SD rats pre-treated with 800 mg/kg MSG. Compared with normal group *$P<0.05$, compared with MSG group.$^{\#\#}P<0.01$

Total protein content

Treatment with MSG alone and concurrent treatments with MSG and the extract at various doses did not have any significant ($P \geq 0.05$) effect on serum protein in relation to the normal rats (Fig. 4). Pre-treatment of normal rats with MSG also did not cause significant elevation of serum protein (Fig. 5).

Fig. 4 [Images not available. See PDF.]

Preventive effect of the ethanol extract of *T. tetraptera* fruit on total protein in female SD rats administered 800 mg/kg MSG

Fig. 5 [Images not available. See PDF.]

Curative effect of the ethanol extract of *T. tetraptera* fruit on total protein in female SD rats pre-treated with 800 mg/kg MSG

Total serum estradiol

MSG treatment gave rise to a very significant elevation in estradiol (90.98% $P \leq 0.05$). Concurrent treatments with MSG and extract at various doses produced reduced levels of elevation of 35.35, 52.97 and 8.07% in estradiol content with 100, 200 and 400 mg/kg doses ($P \geq 0.05$) in relation to the normal group (Fig. 6). Significant difference at $P \leq 0.05$ was observed between the MSG and the 400 mg/kg group. In the curative treatment, serum estradiol in the rats was raised by 90.68% ($P \leq 0.05$) in the MSG group. Pre-treatment of normal rats with MSG and treatment with the extract in graded doses, however, caused significant amelioration of this parameter as elevations of 62.94 and 20.39% were observed with 100 and 200 mg/kg doses (Fig. 7). At 400 mg/kg, there was a significant inhibition of serum estradiol elevation caused by MSG ($P \leq 0.05$). The extract dose-dependently decreased elevated serum estradiol to normal.

Fig. 6 [Images not available. See PDF.]

Preventive effect of the ethanol extract of *T. tetraptera* fruit on total serum estradiol in female SD rats administered 800 mg/kg MSG. Compared with normal group *$P<0.05$, compared with MSG group.$^{\#}P<0.05$

Fig. 7 [Images not available. See PDF.]

Curative effect of the ethanol extract of *T. tetraptera* fruit on total estradiol in female SD rats pre-treated with 800 mg/kg MSG. Compared with normal group *$P<0.05$, compared with MSG group.$^{\#}P<0.05$

Histopathology results of the uterus

Section of the uterus showed normal tissue architecture: endometrial stroma (ES), endometrial lining (EL), endometrial glands (GL) and uterine cavity (UC). When the female rats were treated with 800 mg/kg MSG only, the sections showed thick bundles of smooth muscle fibers which were spindle-shaped (SP), arranged in haphazard fashion and crisscrossing the endometrial glands and stroma which is characteristic of leiomyoma. Simultaneous administration of graded doses of *T. tetraptera* fruit and MSG showed an amelioration of the

proliferating fibroid cells. The activity of the extract was more evident in the curative experiment as the thick band of smooth muscle fibers indicating the formation of leiomyoma was minimal at 200 and 400 mg/kg and normal tissue architecture was mostly observed (Fig. 8).

Fig. 8 [Images not available. See PDF.]

H&E × 400 SD Rat uteri sections: Panel A shows normal uterus architecture: uterine cavity (UC), endometrial lining (EL), endometrial stroma (ES) , endometrial glands (GL) are seen. Panel B shows rat uterine wall given MSG only: composed of endometrial glands (GL) and stroma (ES) flanked by spindle-shaped proliferating bundles of smooth muscle fibers (SP) characteristic of leiomyoma. Panel C shows 100 mg/kg treated group: Composed of endometrial glands (GL) and stroma (ES) with reduced spindle-shaped proliferating bundles of smooth muscle fibers (SP) in both treatments. Panel D shows 200 mg/kg treated group: composed of stroma (ES) with mild form of proliferating bundles of smooth muscle fibers (SP). Panel E shows 400 mg/kg treated group: preventive group shows mild form of proliferating bundles while curative is mostly normal uterus architecture, uterine cavity (UC), endometrial lining (EL), stroma (ES), glands (GL)

HPLC analysis result

HPLC analysis of the ethanol extract of *T. tetraptera* revealed the presence of majorly umbelliferone, ferulic acid, echinocystic acid, piperazine, aridanin and naringenin. Other compounds present but in lesser amounts were octodrine, hentriacontane, butein and isoliquiritigenin (Table 6). HPLC chromatogram showing the various peaks of the identified compounds is presented in Fig. 9.

Table 6. HPLC result of *T. tetraptera* fruit ethanol extract

S/N	Component	Retention time (mins)	Area	Height
1	Ferulic acid	1.350	303.6740	23.900
2	Echinocystic acid	1.650	422.7495	47.384
3	Umbelliferone	1.983	8447.0015	222.357
4	Piperazine	3.166	812.8600	25.900
5	Aridanin	4.016	303.6600	12.070
6	Octodrine	4.733	16.8530	0.600
7	Hentriacontane	6.350	115.4450	3.201
8	Naringenin	7.350	359.5600	11.188
9	Butein	8.616	56.3630	1.203
10	Isoliquiritigenin	9.616	41.3410	2.201

Fig. 9 [Images not available. See PDF.]

High-performance liquid chromatography (HPLC) chromatogram of compounds in *T. tetraptera* ethanol fruit extract

Discussion

Loss of body weight is typically seen to be a harmful impact of the extract on the animal, caused by less consumption of food and liquids. The extract induced a gradual rise in body weight, indicating the extract's relative safety for the rats; yet, there was a noteworthy distinction in the average weekly change in weight acquired ($p<0.05$) when compared to the control group (Table 1). In fact, a number of earlier studies have shown that *T. tetraptera* fruit extract has antiobesity potential [19].

LDL, total cholesterol and triglycerides are three primary components of the lipid profile that are linked to cardiovascular disease. A dysregulated lipid metabolism is indicated by changes in LDL and HDL levels, which may be caused by interference with lipolysis and the release of free fatty acids from peripheral depots [20]. Although the levels of triglycerides, LDL, HDL and total cholesterol were not significantly different ($p>0.05$) from the control group, other studies have shown that *T. tetraptera* fruit extract has the ability to decrease lipids [21]. It has been reported to elicit reduction in serum triacylglycerols as well as elevation of LDL in male rabbits [22].

The primary function of the kidneys is to remove the harmful waste produced by the normal functioning of the body and transported by the blood. In fact, additional research amply supports the capacity of plant extracts to function as potent free radical scavengers in the kidney, avoiding their harmful effects on lipid peroxidation, which raises biochemical markers like creatinine and urea by rupturing membranes [10]. *T. tetraptera* fruit has been reported to possess alkaloids, tannins, triterpenes, flavonoids, steroid glycosides and coumarins as dominant secondary metabolites [21, 23].

The protective effect of the extract could be explained by the antioxidant potential of some of *T. tetraptera* extract's constituents, such as flavonoids and total phenols, which would inhibit membrane lysis and contribute to the nephroprotective action of the extract [24]. This study's findings support those of another, which established that some methanol plant extracts protect kidney cells by inhibiting xanthine oxidase, an enzyme that causes lipid peroxidation and membrane instability [25].

The liver is essential for the detoxification and excretion of several endogenous and exogenous substances, and any harm or impairment to it can have a wide range of health effects on both humans and animals. Cellular necrosis, elevated tissue lipid peroxidation and glutathione depletion are linked to liver injury. Furthermore, liver illness is associated with increased serum levels of many biochemical indicators, including transaminases, alkaline phosphatase, triglycerides and cholesterol [26]. All of these parameters, at all tested doses, revealed no significant difference ($p>0.05$) compared to the control. These findings suggest that the extract had no effect on the liver's ability to function normally because any hepatocellular damage would have raised ALT and AST levels in the serum [27]. The extract's phenolic components, which function as antioxidants to inhibit membrane lipid peroxidation, may have a hepato-protective effect and could be the reason for this [24].

Significant changes in blood indices (white blood cells, red blood cells, platelets and their differentials) suggest that the chemical being administered is either toxic or protective to the hemopoietic tissue. The blood indices are used to monitor the physiological and pathological state of the body. Findings from our study report nonsignificant effects on most of the important blood indices by the ethanol extract of *T. tetraptera* fruit. The major functions of WBCs and its differential are to provide immunity and defend the body against invasion by pathogens or toxins. A significant reduction in WBC count at 200 and 400 mg/kg showed that this defense mechanism was not unusually elicited, which proves further the non-toxic nature of the extract. This is consistent with past studies that found that treating white rabbits with an ethanol extract of this plant's fruits reduced the number of white blood cells [22]. Conversely, the dichloromethane methanol extract of this plant resulted in a rise in WBC, which may be explained by the different chemicals that the extraction solvent produced [28]. The extract's nonsignificant effect on the RBC could mean that there was no change in the balance between blood corpuscle destruction to erythropoiesis and the rate of blood production. HGB, PCT and MCH levels did not significantly decrease ($p>0.05$) in female rats treated with the various doses; this could indicate that hemoglobin incorporation into red blood cells and red blood cell morphology were unaffected [16]. The dichloromethane methanol extract showed an alteration in this regard.

Toxicity generally happens at the cellular and sub-cellular levels before being seen in tissues. In this study, the low

extract dose levels (200 and 400 mg/kg) exhibited little or no significant effect on the histomorphology of the vital organs (Fig. 1). The presence of activated Kupffer cells in the sinusoids at 400 mg/kg is evidence of a boost in the immune system; this can be seen as an extra quality of the extract. Normal architecture observed in the kidney at all doses used shows the level of safety of the extract. This is in tandem with the normal features reportedly observed with the dichloromethane methanol extract of the fruit [28].

The potential benefits of TTF in preventing uterine fibroids were examined in this study. A vital building block for the manufacture of several steroid hormones, which are potent signaling molecules that control a number of bodily processes, is cholesterol [29]. A rise in total serum cholesterol is usually attributed to the activation of the enzyme 3-hydroxyl-3-methoxylglutamyl-CoA reductase (HMGR) which catalyzes the conversion of HMG-CoA to mevalonate; this is the rate-limiting step of cholesterol synthesis [17].

It has been demonstrated that ovarian steroid hormones are important molecular indicators linked to the formation and proliferation of uterine fibroids. The formation and development of uterine fibroids has been attributed primarily to estrogen, but progesterone and its receptors may also have a significant impact [10].

Due to its ability to bind to ERα receptors in the uterus and form a complex that interacts with DNA in the nucleus to activate transcriptional promoter and enhancer regions that govern gene expression, estradiol is unique in its ability to promote the growth of uterine cells. This enables RNA polymerase II binding and the subsequent start of transcription, which results in the production of proteins and higher uterine and ovarian cell proliferation [10].

T. tetraptera fruit extract at graded doses of 100, 200 and 400 mg/kg was evaluated in this study for its ability to inhibit the effect of MSG on these biochemical parameters tested as well as the histology of the uterus because it has been reported to be non-toxic at oral doses as high as 5000 mg/kg [30].

There was a significant ($P<0.05$) increase in serum cholesterol levels of the MSG group compared with the normal group (Fig. 2). Treatment with the ethanol extract of *T. tetraptera* fruit reduced the elevated cholesterol levels almost to normal ($P>0.05$) in our study, in both the curative and preventive experiments. The fruit extracts ability to lower cholesterol may be due to a decrease in dephosphorylated HMGR levels as well as an adverse effect on cholesterol production caused by the activation of glucagon and adrenaline [11]. Indeed, the fruit of this plant has been shown in numerous earlier studies to have a lipid-lowering impact [21].

Total protein content in the MSG and treated groups showed no significant difference in relation to the normal. This is similar to results obtained in previous works [7, 17].

Treatment with the ethanol extract of *T. tetraptera* fruit reduced the elevated estradiol levels in a dose-dependent manner (Fig. 6), in both the curative and preventive experiments. Its action on estradiol could possibly be attributed to suppression of the enzyme aromatase responsible for aromatization of androstenedione and testosterone to estrogens in the biosynthesis of estradiol from cholesterol [4]. It might also be due to an inducer of liver microsomal enzyme that increases the metabolism of estradiol, or it might contain phytochemicals that act as gonadotropin-releasing hormone (GnRH) agonists, which when stimulated continuously reduce the expression or downregulates GnRH receptors on the anterior pituitary [31]. Consequently, less estradiol would be produced. The decline in cholesterol production may potentially also be the cause of the estradiol decline.

Additional histology investigations demonstrated *T. tetraptera's* impact on the proliferation of leiomyoma cells in the uterus. A section of the uterus in rats given only food and water revealed normal tissue architecture, but after the female rats were administered 800 mg/kg of MSG, the sections revealed thick bands of spindle-shaped, haphazardly arranged smooth muscle fibers that crisscrossed the endometrial glands and stroma, a characteristic of the formation of leiomyoma.

Gradually increasing dosages of the extract and MSG were administered simultaneously, and the results showed a dose-dependent, stepwise improvement of the proliferating leiomyoma lesion, with the highest dose having the most effective impact. The curative treatment showed a greater reduction in the production of leiomyoma cells than the preventive treatment (Fig. 8).

T. tetraptera fruit has been observed to contain flavonoids, alkaloids, tannins, saponins, steroids, sterols and phenols [23, 28].

Part of the health benefits of saponins include immune system activation and a reduction in cholesterol levels in the body [21]. Additionally, studies have shown that saponin inhibits the enzyme aromatase [14], which is involved in the production of estrogen. Our results are supported by a previous investigation that found a substantial reduction in estrogen induced by a methanol extract of *T. tetraptera* fruit [32].

Phenols' antioxidant properties are also crucial in preventing chronic diseases because they can shield essential molecules like DNA, lipids and proteins from oxidative damage brought on by reactive oxidant species. While dietary antioxidants can have preventive effects, a low-antioxidant diet can raise the incidence of uterine fibroids [33].

HPLC analysis of the extract of *T. tetraptera* revealed the presence of major constituents such as umbelliferone, ferulic acid, echinocystic acid, aridanin and naringenin, with hentriacontane, butein and isoliquiritigenin present in reduced amounts (Table 6).

It has been found that the phenylpropanoid umbelliferone possesses antioxidant qualities and effectively inhibits type 3 17β-hydroxysteroid dehydrogenase, which is the main enzyme responsible for converting 4-androstene-3,17-dione into testosterone [34].

Ferulic acid is also a phenolic substance with a variety of biological activities, especially in oxidative stress and inflammation, and also plays an antifibrosis role [35].

Echinocystic acid has been reported to possess antiviral, anti-inflammatory and antioxidation activities [13] while naringenin, a flavanone, has anti-inflammatory, antioxidant and antiproliferative activities [36], and it also showed lipid-lowering properties. Butein, hentriacontane and isoliquiritigenin have numerous pharmacological properties including anti-inflammatory and antioxidative activities [13].

In conclusion, the decreased level of cholesterol and estradiol by effect of *T tetraptera* fruit extract contributed largely to decreased uterine leiomyoma proliferation. Further studies to determine its exact mechanism as antifibrotic agent against uterine leiomyoma needs to be established.

Conclusion

When administered acutely, *T. tetraptera* ethanol fruit extract is safe. The biochemical and hematological markers examined did not show any discernible alterations; nonetheless, long-term high-dose consumption may have harmful consequences on organs like the heart and uterus. This work aimed to demonstrate the antileiomyoma activity of the ethanol extract of *T tetraptera* fruit on Sprague Dawley rats. The results shown suggest that the extract is effective in reducing the biochemical parameters elevated by MSG and also inhibiting the formation of leiomyoma cells. This validates its usage in ethnomedicine in the management of uterine leiomyoma.

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Author contributions

ROI, BAA, and AU conceptualized the project and designed the study and methodology, SI, AAU, OVA and JMA performed the study, ROI performed the statistical analysis and data interpretation, ROI wrote the paper, and the final manuscript was proofread and approved by all authors.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Approval for the use of experimental animals was obtained from the Research Ethics Committee of the Faculty of Pharmacy, University of Benin, Nigeria (EC/FP/023/04). Appropriate permission to research on the study plant was duly solicited from the local legislations.

Competing interests

Authors declare no conflict of interest

Abbreviations

MSG
Monosodium glutamate
SD rats
Sprague Dawley rats
TTF
Tetrapleura tetraptera fruit
HPLC
High-performance liquid chromatography
HMGR
3-Hydroxyl-3-methoxylglutamyl-CoA reductase
GnRH
Gonadotropin-releasing hormone

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DETAILS

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Solvent fractions of Vitellaria paradoxa root extract suppress phenylhydrazine-mediated jaundice in Wistar rats

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ABSTRACT (ENGLISH)

Background

In the first few days of life, jaundice has continued to be a major health concern. It typically manifests as a yellowing of the skin, mucous membranes, and sclera as a result of bilirubin deposition from excessively high concentrations in the body. It affects 80% of preterm and 60% of term new-borns within the first seven days of life, which is of great concern. According to the World Health Organization, the widespread acceptance of traditional medicines can be attributed to their accessibility and affordability. In West African arid savannah, there is a tree called *Vitellaria paradoxa* (Sapotaceae) that grows naturally. This well-known herb has numerous applications in medicine. Various plant components, including the leaves, roots, seeds, and fruit, have all been used in traditional medicine to cure a variety of illnesses. The purpose of this study is to objectively ascertain the efficacy of *V. paradoxa* root extracts on jaundice. Rats given phenylhydrazine (PHZ) to induce hyperbilirubinemia were orally administered ethylacetate, *n*-

butanol, *n*-hexane, and aqueous fractions.

Result

Results indicated the presence of terpenoids, flavonoids, and phenol. *n*-hexane and ethylacetate fractions showed activity against jaundice in rats. This observation was due to the fact that they significantly improved all biomarkers that were examined, namely body weight change, liver function parameters (total bilirubin, direct bilirubin, aspartate aminotransferase, albumin, and total protein), haematological parameters (white blood cells, haemoglobin, red blood cells, haematocrit, and platelets), and antioxidant enzymes (superoxide dismutase and malondialdehyde).

Conclusion

n-Hexane and ethylacetate fractions of the extract showed significant activity against PHZ-induced jaundice in rats. However, *n*-hexane fraction was the most active fraction.

FULL TEXT

Background

Lately, jaundice is now a serious health problem in neonates. According to Khoshnur et al. [13], jaundice is typically identified by the yellowing of the skin, mucous membranes, and sclera as a result of bilirubin deposition when the blood's bilirubin concentration is excessively high (>3 mg/dl). Changes in bilirubin metabolism often result in hyperbilirubinemia. Increased levels of unconjugated bilirubin are caused by higher rate of red blood cell breakdown, whereas higher levels of conjugated bilirubin are caused by liver injury and/or biliary system blockages [10]. Neonatal jaundice is the term used to describe jaundice in new-borns [6]. Within the first seven days of life, an alarming 60% of term and 80% of preterm new-borns develop jaundice [23]. The use of natural resources such as plant materials to treat or control illnesses is as old as man [27].

According to the World Health Organization, the widespread acceptance of traditional medicine can be attributed to its accessibility and affordability. With increased data generated from scientific research on the use of traditional medicine in the management of chronic diseases, usage is becoming more widespread [26]. An estimated 80% of people in impoverished nations like Nigeria use herbal medicine to treat common medical conditions like jaundice [9]. In the dry savannah region of West Africa, *Vitellaria paradoxa* (Sapotaceae) grows naturally [18]. This well-known herb has numerous applications in conventional medicine. It goes by several names: Ökwuma in Igbo, Ëmi in Yoruba, Kadanya in Hausa, Kareje in Fulfulde, and Shea butter in English [1]. Many African ethnic groups consider it to be a sacred tree, and it is used in many religious and cultural practices [18].

Various parts of *Vitellaria paradoxa*, such as leaves, roots, seeds, fruit, and stem bark, have been used in traditional medicine to treat microbial infections including helminths, dysentery, diarrhoea, and other infections affecting the gastrointestinal tract, skin, and wounds [2]. The stem bark has been reported to be used in the treatment of leprosy and cough [7]. The plant's kernels yield Shea butter, which is oil-rich and used as a source of edible oil in many homes throughout Sahel Africa especially Northern Nigeria. In addition, Shea butter is used to treat rheumatism, ulcers, dermatitis, rashes, and inflammation [16]. In the north and other parts of Nigeria, chewing sticks made from *Vitellaria paradoxa* roots are widely used to clean teeth and mouths. Root and root bark of *Vitellaria paradoxa* are typically made into a paste and administered orally [17]. The purpose of this study is to objectively establish if *Vitellaria paradoxa* root extracts are effective in treating jaundice.

Methods

Collection and identification of plant material

Vitellaria paradoxa roots and fresh leaves were collected. A sample of the leaf was identified at a herbarium and a voucher number assigned. Roots of the plant were carefully washed in clean, chopped into tiny pieces, shade-dried for 14 days, pulverized, and stored in airtight containers.

Preparation of methanol extract

Methanol root extract *Vitellaria paradoxa* was made by macerating 1500 g of the pulverized material root in 9 litters of methanol for 72 h. It was then filtering using Whatman No. 1 filter paper, concentrated using a rotary evaporator at 40 °C. The extract obtained was freeze-dried giving rise to 7.3% w/w yield.

Fractionation of methanol extract of *Vitellaria paradoxa* root

Using Almohaimeed et al. [3] approach, methanol extract was partitioned into ethylacetate, *n*-butanol, *n*-hexane, and an aqueous fraction.

Qualitative phytochemical screening of *Vitellaria paradoxa* root

Using standard protocol as outlined by Shemishere et al. [20], a chemical test was performed to screen and identify phytochemicals (saponins, terpenoids, alkaloids, tannins, carbohydrates, phenols, steroids, flavonoids, and cardiac glycosides) in the methanol extract of *Vitellaria paradoxa* root.

Quantitative determination of total phenolic and flavonoid content of methanol extract of *Vitellaria paradoxa* root

Total phenol and flavonoid content of *Vitellaria paradoxa* methanol root extract was calculated using the method described by Shemishere et al. [21, 22].

Animal protocol

42 rats, ages 10 to 12 weeks and weighing between 70 and 90 g, were bought from a standard Animal House. The rats were kept in metal cages and given free access to food and water in a natural environment with a 12-h light/dark cycle for two weeks to acclimatize before administration. Rats were maintained in accordance with the Animal Ethics Committee's guide for the care and use of animals in research and education and the National Institutes of Health's guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). The experimental protocol was approved in accordance with ethical standards.

Rats were divided into 6 groups of 7 rats each and administered as follows. Rats except those in the control group received 1 dose of phenylhydrazine (PHZ) through intraperitoneal route at 40 mg/kg body weight for three consecutive days to induce jaundice. This approach slightly differs from that which Kolawole et al. [14] used. Rats were administered as follows for 21 days. Rats in PHZ + Aqueous group, PHZ + n-hexane, PHZ + n-butanol, PHZ + Ethylacetate, and PHZ alone group were each administered 50 mg/kg body weight of the extract fractions. The rats were killed twenty-four hours following the last administration.

Haemoglobin concentrations, blood platelets and haematocrit scores, white blood cell and red blood cells counts, and haemoglobin concentration were measured after blood samples were drawn into EDTA specimen bottles. The residual blood sample was transferred into simple sample bottles, centrifuged, and serum extracted with care to measure the levels of malondialdehyde (MDA) and superoxide dismutase (SOD), total and direct bilirubin, aspartate aminotransferase, alkaline phosphatase, alanine amino transferase, albumin, and total protein using appropriate kits.

Body weight change determination

On days 0, 7, 14, 21, and 28 of the treatment, body weight was measured and recorded. Rats' body weight on day 0 was subtracted from their body weight on day 28 to calculate the changes in the body weight.

Determination of superoxide dismutase (SOD) activity

The activity of superoxide dismutase (SOD) was determined using the method of [25] with slight modifications. An aliquot of 0.4 ml of colon sample was added to 5 ml of 0.05 M carbonate buffer in a quartz cuvette and the reaction started by the addition 0.6 ml of 0.3 M adrenaline. Reference cuvette contained 5 ml of carbonate buffer, 0.6 ml of adrenaline and 0.4 ml of distilled water. Increase in absorbance at 480 nm was monitored every 30 s for 120 s. Enzyme activity was expressed as units/mg protein. One unit of an enzyme is defined as the enzyme activity that inhibits auto-oxidation of adrenaline by 50%.

Determination of malondialdehyde (MDA)

Colon tissue samples were subjected to the lipid peroxidation analysis using the methodology outlined by Ayodeji et al. [5]. This experiment is predicated on the reaction between MDA and 2-thiobarbituric acid, a chromogenic reagent. A pink chromophore with an absorbance maximum at 532 nm is produced when one molecule of MDA interacts with two molecules of 2-thiobarbituric acid by a Knoevenagel-type condensation. A mixture of 1.6 ml of phosphate buffer, 0.5 ml of colon sample, 0.5 ml of 30% TCA, and 0.5 ml of 75% 2-thiobarbituric acid (TBA) in 0.1 mol/l HCl comprised the reaction mixture. For two hours, the mixture was heated in a water bath at 90–95 °C. The resulting mixtures were centrifuged at 3000*g* for 15 min after being chilled on ice. At 532 nm, the absorbance of the turbidity-free

supernatant was measured. The molar extinction coefficient concentration in nmole/L is used to compute MDA, which is equal to absorbance × (1000/1.56).

Determination of blood parameters

An auto-haematological analyser (Beckman Coulter, USA) was used to determine the red blood cell count (RBC), white blood cell count (WBC), haemoglobin concentration (HGB), blood platelet count (PLT), and haematocrit value (HCT) from blood samples collected in EDTA bottles.

Determination of Liver function parameters

Standard test kits from Ultracare Diagnostics were used to measure the following liver function parameters: total bilirubin (TB), direct bilirubin (DB), aspartate aminotransferase (AST), alanine amino transferase (ALT), and albumin (ALB). Standard kits from Randox, UK, were used to measure total protein (TP), aspartate aminotransferase (ALP), and albumin (ALB).

Data analysis

For testing significance levels ($p<0.05$), the statistical programme for social sciences (SPSS) version 20 was utilized.

Results

Extract yield

The crude methanol extract yield at the conclusion of the extraction procedure was 19.75% (w/w). The percentage yields for the other fractions were as follows: 9.36 g, 2.41 g, 3.53 g, and 0.43 g for *n*-hexane, *n*-butanol, ethylacetate, and water, respectively.

Qualitative phytochemical constituents

According to the findings from the analysis of the phytochemical components of the methanol extracts, phenols were found to be present in significant amount. There was a medium concentration of terpenoids and carbohydrates, and trace amounts of saponins, alkaloids, tannins, steroids, and flavonoids. Heart glycosides, however, were not found in the sample (Table 1).

Table 1. Qualitative phytochemical constituents of methanol extract of *Vitellaria paradoxa* root

Phytochemicals	Status
Saponins	+
Terpenoids	++
Alkaloids	+
Tannins	+
Carbohydrates	++
Phenols	+++
Steroids	+
Flavonoids	+
Cardiac glycosides	−

+: Present in a trace concentration; ++: present in a medium concentration; +++: present in a high concentration; − absent or in negligible amount

Quantitative phytochemical constituents

Two phytochemicals, namely total phenol and total flavonoids, were quantified. Total phenols obtained were 39.85 ± 0.91 mg garlic acid equivalent per gram of extract. On the other hand, the total flavonoids obtained were 0.9 ± 0.04 mg quercetin equivalent per gram of extract (Table 2).

Table 2. Total phenolic and flavonoid content of methanol extract of *Vitellaria paradoxa* root

Phytochemicals	Quantitative value
Total phenols (mg garlic acid equivalent/g of extract)	39.85 ± 0.91
Total flavonoids (mg Quercetin equivalent/g of extract)	0.90 ± 0.040

All values are expressed as mean ± SEM (n = 3)

Effect of methanol and solvent fractions of *Vitellaria paradoxa* root extracts on body weight changes in phenylhydrazine-induced hyperbilirubinemic in Wistar rats

According to the body weight changes results, the PHZ alone group lost a substantial amount of weight in comparison with the control group. The PHZ + *n*-butanol, PHZ + ethylacetate, and PHZ + aqueous groups all gained weight as compared to the control group, but the weight gain was not statistically significant (P<0.05). Rats in the PHZ + *n*-hexane group did not substantially vary in body weight gain from the control group. Overall, Table 3 shows that the PHZ + *n*-hexane fraction was more effective at reversing weight loss.

Table 3. Body weight changes in phenylhydrazine-induced hyperbilirubinemic Wistar rats treated with solvent fraction of *Vitellaria paradoxa root extracts*

GROUP	DAY 1 (g)	DAY 28 (g)	BWC (g)
Control	81.77 ± 16.91	123.39 ± 17.21	+ 41.62 ± 0.30^{bc}
PHZ only	84.35 ± 18.16	53.17 ± 17.31	− 31.18 ± 0.85*
PHZ + *n*-hexane	85.21 ± 16.21	126.57 ± 17.02	+ 41.36 ± 0.81^{bc}
PHZ + *n*-butanol	86.50 ± 11.08	73.67 ± 13.3	+ 32.17 ± 2.22 *^{c}
PHZ + ethylacetate	73.29 ± 19.13	63.27 ± 20.21	− 10.02 ± 1.08*^{c}
PHZ + aqueous	83.86 ± 11.89	54.34 ± 13.54	− 29.52 ± 1.65*

50 mg/kg of phenylhydrazine intraperitoneal (PHZ): *n*-hexane, *n*-butanol, ethylacetate and aqueous represents the different fractions of the extracts: BWC: body weight change: values are presented as mean ± SD (n = 7):*values differ significantly from control (p<0.05): ^{b}values are not significantly different from each other (p<0.05): ^{c}values differ significantly as compared with PHZ only (p<0.05)

Effect of solvent fractions of *Vitellaria paradoxa* root extracts on SOD and MDA in phenylhydrazine-induced hyperbilirubinemic in Wistar rats

Data produced showed that, in comparison with the PHZ only group, the *n*-hexane fraction of the extract

considerably enhanced the levels of SOD and decreased the levels of MDA. SOD levels in the *n*-hexane fraction increased to control values, with no discernible change ($P<0.05$). The n-hexane fraction's activity resulted in a reduction of MDA levels, a sign of lipid peroxidation, according to the same observation. While SOD and MDA levels were improved by two other extract fractions, *n*-butanol and ethylacetate, the effects were not as great as they were in the PHZ only. For the two indicators tested, there was no discernible improvement in the extract's aqueous fraction (Table 4).

Table 4. Superoxide dismutase activity (SOD) and malondialdehyde (MDA) levels changes in phenylhydrazine-induced hyperbilirubinemic Wistar rats treated with solvent fraction of *Vitellaria paradoxa* root extracts

Group	SOD (units/mg)	MDA (nmol/l)
Control	2.64 ± 0.35^{bc}	24.35 ± 0.36^{bc}
PHZ only	1.22 ± 0.13*	44.08 ± 0.23*
PHZ + *n*-hexane	2.30 ± 0.35^{bc}	22.39 ± 0.81^{bc}
PHZ + *n*-butanol	1.29 ± 0.63*^{c}	39.92 ± 0.68*^{c}
PHZ + ethylacetate	1.38 ± 0.23*^{c}	38.74 ± 0.41*^{c}
PHZ + aqueous	1.43 ± 0.16*	38.04 ± 0.41*

50 mg/kg of phenylhydrazine intraperitoneal (PHZ): *n*-hexane, *n*-butanol, ethylacetate and aqueous represents the different fractions of the extracts: SOD: superoxide dismutase: MDA: malondialdehyde: values are presented as mean ± SD (n = 7):*values differ significantly from control ($p<0.05$): ^{b}values are not significantly different from each other ($p<0.05$): ^{c}values differ significantly as compared with PHZ only ($p<0.05$)

Effect of solvent fractions of *Vitellaria paradoxa* root extracts on blood parameters in phenylhydrazine-induced hyperbilirubinemic in Wistar rats

Results showed that the *n*-hexane fraction of the plant extract significantly improved the blood parameters measured when different fractions of the extract were tested to determine their effects on some haematological parameters in PHZ-induced hyperbilirubinemia. Conversely, the extract's ethylacetate fraction was found to generally improve all blood parameters examined; however, only HCT and PLT showed significant ($P<0.005$) improvements in comparison with the control group. When compared to the control group, the butanol fraction greatly improved the parameter, but not as much as it did when compared to the PHZ alone group. When compared to the control and PHZ alone groups, the aqueous extract had no significant impact on the haematological parameters (Table 5).

Table 5. Changes in haematological parameters in phenylhydrazine-induced hyperbilirubinemic Wistar rats treated with solvent fractions of *Vitellaria paradoxa* root extracts

GROUP	WBC 10^3/uL	HGB g/dL	RBC 10^6/uL	HCT (%)	PLT 10^3/uL
Control	5.09 ± 1.58^{cb}	16.62 ± 0.98^{bc}	8.15 ± 0.11^{bc}	47.28 ± 0.33^{bc}	349.4 ± 12.1^{bc}
PHZ only	15.69 ± 1.57*	7.09 ± 0.49*	4.05 ± 0.33*	29.8 ± 0.50*	107.6 ± 9.21*

PHZ + *n*-hexane	4.92 ± 1.81*[bc]	14.51 ± 0.23[bc]	7.62 ± 3.15[bc]	45.26 ± 0.18 [bc]	329.0 ± 10.3[bc]
PHZ + *n*-butanol	13.09 ± 0.43*[c]	7.86 ± 0.12*[c]	4.10 ± 2.22*[c]	32.24 ± 0.21*[c]	109.2 ± 11.7*[c]
PHZ + ethylacetate	4.51 ± 0.52*[c]	15.98 ± 0.32*[c]	7.36 ± 1.46*[c]	44.98 ± 0.16 [bc]	339.2 ± 12.23[bc]
PHZ + aqueous	14.12 ± 0.65*	9.05 ± 0.3*	4.53 ± 2.14*	30.05 ± 0.43*	109.3 ± 13.21*

50 mg/kg of phenylhydrazine intraperitoneal (PHZ): *n*-hexane, *n*-butanol, ethylacetate and aqueous represents the different fractions of the extracts: BWC: body weight change: values are presented as mean ± SD (n = 7):*values differ significantly from control (p<0.05): [b]values are not significantly different from each other (p<0.05): [c]values differ significantly as compared with PHZ only (p<0.05)

Effect of solvent fraction of *Vitellaria paradoxa* root extracts TB, DB, AST, ALP, ALT, ALB and TP in phenylhydrazine-induced hyperbilirubinemic in Wistar rats

By lowering the levels of both TB and DB, the *n* hexane fraction successfully counteracted the effects of PHZ, according to the results of the biochemical assay used in this study. ALT, ALP, and AST all showed the same pattern. Conversely, in comparison with the control, there was a substantial (P<0.005) rise in the levels of ALB and TP. Additionally, ethylacetate fraction was found to significantly lower TB and DB. The levels of AST, ALP, ALT, ALB, and TP improved, although the difference was not statistically significant (P<0.005). All liver markers that were tested for indicated improvement in the butanol fraction as well, but the improvements were not statistically significant (P<0.005) when compared to the control. The extract's aqueous fraction had no effect on the liver indicators tested for (Table 6).

Table 6. Changes in liver function parameters in phenylhydrazine-induced hyperbilirubinemic Wistar rats treated with solvent fraction of *Vitellaria paradoxa* root extracts

Group	TB (mg/dL)	DB (mg/d)	AST (U/L)	ALP (IU/L)	ALT (U/L)	ALB (g/dl)	TP (g/dL)
Control	0.91 ± 0.02[bc]	0.31 ± 0.01[bc]	19.12 ± 0.13[bc]	69.21 ± 0.20[bc]	36.23 ± 0.55[bc]	3.61 ± 0.32[bc]	7.93 ± 0.05 [bc]
PHZ only	2.30 ± 0.11*	0.90 ± 0.21*	36.18 ± 0.24*	152.10 ± 0.48*	61.02 ± 0.14*	1.85 ± 0.01*	3.43 ± 0.16*
PHZ + *n*-hexane	0.93 ± 0.31[bc]	0.34 ± 0.13[bc]	19.15 ± 0.11[bc]	68.92 ± 0.32[bc]	38.80 ± 0.13[bc]	3.32 ± 0.15[bc]	7.91 ± 0.02[bc]
PHZ + *n*-butanol	2.15 ± 0.22*[c]	0.85 ± 0.21*[c]	32.04 ± 0.21*[c]	149.00 ± 0.20*[c]	57.00 ± 0.55*[c]	1.95 ± 0.21*[c]	3.25 ± 0.05*[c]
PHZ + Ethylacetate	2.19 ± 0.12[bc]	0.88 ± 0.22[bc]	33.18 ± 0.19*[c]	147.21 ± 0.65*[c]	59.05 ± 0.54*[c]	1.91 ± 0.05*[c]	3.40 ± 0.13[bc]
PHZ + Aqueous	2.27 ± 0.03*	0.87 ± 0.11*	31.16 ± 0.19*	148 ± 0.47*	59.80 ± 0.18*	1.94 ± 0.19*	3.16 ± 0.08*

50 mg/kg of phenylhydrazine intraperitoneal (PHZ): *n*-hexane, *n*-butanol, ethylacetate and aqueous represents the different fractions of the extracts: TB: total bilirubin: DB: direct bilirubin: AST: aspartate aminotransferase: ALP: alkaline phosphatase: ALT: alanine amino transferase: ALB: albumin: TP: total protein. Values are presented as

mean ± SD (n=7):*values differ significantly from control (p<0.05): [b]values with the same alphabet are not significantly different at (p<0.05): [c]values differ significantly as compared with PHZ only (p<0.05)

Discussion

Data generated from this study have highlighted the importance of using scientific experiments to validate claims by traditional medical practitioners on the efficacy or otherwise on products used in the management and treatment of human ailments. Data from this study have shown the activity of *Vitellaria paradoxa* extracts on jaundice. The use of PHZ to induce jaundice in rats has long been established [12]. Its results obtained from this studies seem to show that only the n-hexane and ethylacetate fractions of the extract showed activity against jaundice. It is important to establish that data from previous study in which Wistar albino rats were exposed to root extract of *Vitellaria paradoxa* at a concentration of 50 mg/kg body weight did not show any toxic effect. At a dose of 100 mg/kg body weight some toxic effects were observed [8]. This informed the selection of the dose of 50 mg/kg body weight for this study.

Response to treatment in jaundice is usually associated with weight gain as shown from this study where n-hexane group showed significant weight gain when compared with the PHZ only group [12]. The anti-lipid peroxidative and antioxidant properties of phenols and flavonoids have been reported. Both phenols and flavonoids were detected from the screening process used to identify the phytochemical components present in the extract. Numerous sources of free radicals within cells have been identified. The xanthine oxidase system, the mitochondrial electron transport system, and an increase in intracellular calcium levels are a few of the sources [4]. These free radicals set off series of biochemical reactions in vivo that disrupt cell membrane integrity and increase endotoxin release, changing how cells behave. The cell membrane finally disintegrates as a result of the injury. It is commonly recognized that oxidative stress and subsequent cell damage are reduced when enzymatic and non-enzymatic antioxidant levels in vivo increases [4]. One of the etiologic variables linked to the RBC breakdown and subsequent hyperbilirubinemia seen in anaemia has been identified as oxidative stress [4]. Oxidative stress occurs when the body's antioxidant system is unable to eliminate the free radicals that are produced. From the data presented in Table 4, *n*-hexane fraction of the extract significantly (P<0.005) increased the activity of SOD (2.60 ± 0.35) when compared to PHZ group. The administration of n-hexane fraction considerably decreased the levels of lipid peroxidative marker MDA when compared with the PHZ group. There was no significant variation found between the control and n-hexane group. This outcome is in line with the result of Uz et al. [24] where the levels of MDA in renal ischaemia and reperfusion (I/R) damage showed that ginger extract reduced I/R damage but serum MDA levels did not show any difference between control and treatment group.

Elevated bilirubin levels, a by-product of red blood cell breakdown, are indicators of jaundice [4]. In other words, jaundice is a medical disorder whose course is determined solely by the health of the red blood cells. One essential step in determining if a treatment is successful in curing jaundice is assaying for red blood cells and other blood parameters. A study by Raicevic et al. [19] demonstrated the connection between the onset of neonatal jaundice and some haematological markers in new-borns. According to the research findings, new-borns with low RBC and high bilirubin levels subsequently experienced jaundice. From our result in Table 5, the levels of RBC, HGB, HCT, and PLT all increased significantly for both the *n*-hexane and ethylacetate fraction when compared with control. In contrast, WBC decreased considerably in comparison with the PHZ alone group. Nan et al. [15] in a study on the relationship between maternal blood parameters and new-born jaundice found that mothers with high WBC and mean corpuscular volume (MCV) levels frequently give birth to infants who experience neonatal jaundice.

In addition to providing information on liver cell damage, the liver function test panel makes it evident how functional the liver is. According to a study by Hayat et al. [11] on human subjects, jaundice is typically associated with abnormal levels of liver function indicators. In another study published by [4], treatment with ursodeoxycholic acid led to a significant decrease in the levels of AST, ALT, and GGT in rats that developed obstructive jaundice. This study's findings demonstrated that the *n*-hexane fraction considerably lowered the blood levels of TB, DB, AST, ALP, and ALT. In addition, the levels of TP and ALB were substantially higher than those of the PHZ alone group. The liver function indices AST, ALT, urea, creatinine, uric acid, and bilirubin were all improved in a 2019 study by Ali

utilizing an extract of *Vitellaria paradoxa* in diabetic rats. Following the injection of *Vitellaria paradoxa* extract, the activity of antioxidant enzymes such as SOD and CAT increased. The decrease in MDA levels followed the same pattern. This is in line with the study's findings, which indicated that the extract's *n*-hexane fraction was the only one that exhibited activity. This observation also suggests that the n-hexane fraction of the extract may likely contain the active component of the extract that accounts for the activity reported in this investigation.

Conclusion

Result from this study has shown that root extract of Vitellaria paradoxa reversed all the markers associated with jaundice induced in rats with PHZ. An attempt to also identify the solvent fraction of the extract containing the active principle was also achieved. *n*-hexane fraction of the root extract of Vitellaria paradoxa showed activity for all the markers of jaundice checked in this study. Following closely is the ethylacetate fraction of the extract which showed activity for all the haematological parameters. Findings from this studies are limited with regard to why *n*-hexane fraction showed positive result for all the markers assayed for as against the ethylacetate fraction which showed activity for only the haematological parameters assayed for. Further studies will be required to identify the active principle responsible for the observable activity and to unravel the biochemical basis of the action.

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Plant identification

Fresh leaves and root of Vitellaria paradoxa were collected from Yelwa, Yauri local government area in Kebbi State, North-West Nigeria in August, 2023. Plant leaf sample was identified at the Herbarium section of the Federal University Birnin-Kebbi, and a sample of the authenticated materials with the voucher number FUBK/PIN/0219 was deposited.

Author contributions

DAA contributed to conceptualization; writing—original draft; writing—review and editing; project administration; supervision; resources; funding acquisition; and investigation. AAT was involved in writing—original draft; methodology; visualization; writing—review and editing; investigation; and formal analysis. ABY contributed to methodology; visualization; formal analysis; and investigation. UBS was involved in methodology; validation; formal analysis; investigation; and supervision. MIO contributed to methodology; formal analysis; writing—original draft; and investigation. YT was involved in methodology; visualization; formal analysis; and investigation

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Availability of data and materials

The datasets used and/or analysed during this study are available from the corresponding author on rational request.

Declarations

Ethics approval and consent to participate

The guide of care and use of animals in research and teaching of the Animal Ethics Committee of Federal University Birnin Kebbi, Nigeria, in line with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) were followed in maintaining the rats. Approval for the experimental procedure was obtained with the ethical code FUBK/AEC/FS/M1045.

Consent for publication

All authors have agreed and consented for this work to be published in your journal.

Competing interests

The authors declared that they have no conflict of interest.

Abbreviations

WHO

World Health Organization

PHZ

Phenylhydrazine
EDTA
Ethylenediamine tetraacetic acid
SOD
Superoxide dismutase
MDA
Malondialdehyde
HCl
Hydrochloric acid
TCA
Tricarboxylic acid
RBC
Red blood cells
WBC
White blood cells
HGB
Haemoglobin
PLT
Platelet
HCT
Haematocrit
TB
Total bilirubin
DB
Direct bilirubin
ALT
Alanine amino transferase
AST
Aspartate aminotransferase
ALP
Aspartate aminotransferase
ALB
Albumin
TP
Total protein
BWC
Body weight change
MCV
Mean corpuscular volume
GGT
Gamma-glutamyl transferase
CAT
Catalase

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Characterization of biochemical and optical properties of Nile tilapia (Oreochromis niloticus) corneal collagen

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ABSTRACT (ENGLISH)

Background

Collagen extracted from fish body parts is a promising biological material. It has an important role in many pharmaceutical, medical applications and tissue engineering such as corneal regeneration and stromal replacement. The present work investigates a new trend to extract collagen from the fish cornea, as a prospected substituent of human corneal collagen by characterizing some biochemical and optical properties of the fish corneal collagen.

Results

Examination of the corneal tissue of Nile tilapia; *Oreochromis niloticus* was conducted using electron microscopy, Fourier transform infrared (FTIR) spectroscopy, UV–visible spectrophotometry, optical properties, and thermal properties. The fish were divided into 10 groups each of which consisted of 5 fish. 2 groups of fish were examined for each technique. Results indicated that the corneal layers of *O. niloticus* are thin at the center and thicker at the periphery with the stroma consisting of a triple helical structure collagen type I. The fish cornea showed very weak transmission at the UV regions (190 nm) and maximum transmission at the visible regions. The values of transmission (*T*), reflected light (*R*) and scattered light (*S*) were 2.685 mw, 100×10^{-3} mw at 45° and 40×10^{-3} mw, respectively. Consequently, the percentage of absorbed light is 21.76%. The denaturation temperature of the fish corneal stroma is 22.27 °C.

Conclusions

The method for obtaining fish collagen affects the specific properties of collagen and consequently its further uses as a potential biomedical substituent for mammalian collagen. Specification of the fish species and tissue type is crucial in identifying the quality as well as the physical and functional properties of the extracted collagen.

FULL TEXT

Background

The link between aquaculture production and fish waste is direct. This problem represents a worldwide environmental challenge. Recycling fish waste and obtaining valuable biomaterials is an environmentally accepted solution for this problem. Several authors stressed the importance of isolating and converting collagen from fish waste into marketable products, as well as the positive influence of this process on the environment [1, 2]. Compensatory corneal tissues represent another added value that could be achieved from recycling fish collagenous byproducts. Human corneal damage and corneal diseases represent the main causes of vision loss [3]. Human corneal transplantation is a vision rehabilitation procedure, used when the corneal clarity is negatively affected. Regardless of the used technique, the scarcity of a donor for corneal tissue is the greatest limiting factor in developing countries, while globally this problem results in approximately 10 million untreated patients [4, 5]. Recently, corneal tissue could be obtained from several animal species [6]. However, when selecting an animal species for conducting a human corneal xenograft, the biochemical composition, and structural differences of corneal proteins between humans and other animal species must be considered to minimize the immune response and improve xenotransplantation outcomes [7]. Although several authors discussed the extraction of collagen from fish skin, scales, and bone, as far as known, no previous research has investigated the extraction of collagen from fish cornea.

Studies investigating the histological and biochemical structure of vertebrate corneas have identified several regions that constitute the corneal tissue [8, 9]. The outer peripheral non-keratinized epithelium is supported by a thin dense membrane called the basement membrane and followed by Bowman's layer which is a thin dense acellular sheath in between the basement membrane and the anterior stroma of the cornea. The corneal stromal layers are collagenous, soft, and flexible. Stroma provides the thickness of the cornea. Differences in shape and structure between vertebrate corneas are related to the differences in stromal thickness and nature of the basement membrane. Beneath the stroma lies the Descemet's membrane, which is a dense and thick layer that supports a single layer of endothelial cells. The endothelial layer constitutes the innermost layer of the cornea.

The cornea is a transparent avascular tissue that has refractive power. Light passes through the cornea to the lens then reaches the retina inside the eyeball [10]. The cornea also protects the internal constituents of the eyeball. Consequently, clarity, tensile strength and proper shape are the main features of the corneal tissue. Fish have a wide range of corneal phenotypes depending on their habitats. Differences in biochemical and structural properties of vertebrate collagen fibers are the main reasons for the presence of different collagen types [11, 12].

The advantages of marine collagen draw attention to its use instead of mammalian collagen in the manufacture of medical dressings such as sponges and wound-treatment membranes. Marine collagen is fat-free, shows weak antigenicity, compatible with mammalian collagen and is easily absorbed by the human body [13, 14]. Marine collagen is used as a biomaterial for ophthalmic medical devices due to its low toxicity and low antigenicity [15, 16]. The low melting point, difficulties of extraction and low mechanical strength are the main disadvantages that restrict the use of marine collagen as an alternative to mammalian collagen [17–20].

The present study aims to investigate utilization of collagen obtained from the cornea of Nile tilapia fish; *Oreochromis niloticus* as a potential alternative to mammalian corneal collagen by examining its biochemical and optical properties.

Methods

Experimental fish

A total of 50 healthy specimens of unisex Nile tilapia *O. niloticus*, with an average body weight of 250.0 ± 10.0 g, an average body length of 20.0 ± 1.0 cm and an average corneal diameter of 12.0 ± 1.0 mm were used. Fish were transferred to the laboratory in well-aerated 150 L fiberglass tanks to avoid fish hyperactivity, physical injuries, and stress. Upon arrival, the fish were immersed in 5.0 g/L NaCl sterile solution, followed by 1.0% $KMnO_4$ sterile solution to eliminate any possibility of the presence of ectoparasites. Fish were screened for any pathological symptoms and acclimatized under laboratory conditions for 2 weeks in a fiberglass tank (1.0 m^3) supplied with de-chlorinated tap water under natural photoperiod and temperature. Renewal of 50% of water was done every week. Ammonia and ammonium concentrations were checked twice a week. Fish were fed twice daily on a conventional fish diet (30% crude protein) at a rate of 3% of the body weight. Water temperature, pH, alkalinity, total hardness and dissolved oxygen were measured daily and maintained at normal conditions during the whole study period. A total of 5 healthy fish were considered as a single group to conduct duplicate group treatments for each of the following techniques.

Collagen purification

Fish were anaesthetized with MS-222 (50 ppm), and then the whole cornea was examined using a slit lamp. After that, the eyes were enucleated and the corneas were isolated for scanning electron microscopy (SEM), UV–visible spectrophotometry and optical properties. Corneal epithelium, endothelium and basement membrane were removed by scratching and lamellae were collected and processed for the Fourier transform infrared (FTIR) spectroscopy and thermal properties investigations.

Scanning electron microscopy (SEM)

The morphological structure of the fish cornea and its quality in terms of collagen layers' smoothness and regularity was observed under SEM. Samples were dehydrated in ascending concentrations of ethyl alcohol, dried, examined and photographed at different angles using a VEGA3 TESCAN electron microscope at accelerating voltages of 5 or 10 kV.

Fourier transform infrared (FTIR) spectroscopy

For the identification of collagen type, FTIR spectra were recorded (Thermo Scientific FTIR, model Nicolet 5s Madison, WI, USA). The equipment consists of an ATR ID3 accessory for germanium crystal reflection. The spectral resolution was 4 cm^{-1}. 64 scans were obtained in the range of 400–4000 cm^{-1}. Fresh corneal lamellae were cut manually into small pieces and stored in phosphate-buffered saline (PBS) solution. The C=O, NH_2 and C–N groups were tested for absorption of amid I, II and III bands, respectively.

UV–visible spectrophotometry

The UV–visible absorption-transmittance spectrum of the *O. niloticus* corneal tissue samples were quantified using UV–visible spectrophotometry (Thermo Scientific, Genesys 10S UV–Vis, USA). The absorbance was quantified at different lengths in the scope of 180–1000 nm (5 nm intervals). The spectrophotometer was set up so the cornea and microscope cover glass could be placed perpendicular to the estimating beam. The cover glass was placed on the cuvette holder. The transmission percentage indicator was chosen. First, the base spectrum of the microscope cover glass was measured. Second, the corneal sample was placed on the cover glass with the epithelium directed downward. By placing the samples in this orientation, the estimated light beam of the spectrophotometer entered the cornea from the epithelial side, that is, from the identical direction as the cornea in situ. The cover glass and the cornea were replaced. Their position was adjusted so that the rectangular estimating beam was in the center of the cornea. After adjustments were made, the cornea samples were scanned.

Optical properties (transmission, reflection, scattering and absorption)

A laser-based bench-top optical system was used. A 650 nm red laser was applied to detect the percentage of transmission (T), reflection (R) of light at 45° and scattering (S) of incident light on fish cornea at incident light intensity (P_o) of 3.61 mw. Where, $T\% = T/P_o \times 100$, $R\% = R/P_o \times 100$ and $S\% = S/P_o \times 100$. The absorption percentage ($A\%$) is calculated as $100\text{-}(T\% + S\% + R\%)$. Fresh fish eyeballs were obtained, and the cornea was cut along with a part of the eyeball to maintain the tensile strength of the cornea. It was cleaned of pigments and then tested.

Thermal properties (denaturation temperature)

The thermal properties were determined using a differential scanning calorimetry thermogram method (DSC-60 detector). The stromal sample (weight 2.070 mg) was sealed in an Aluminum seal cell and was subjected to the test in a nitrogen gas atmosphere on a scale from −50 to 50 °C and a temperature rate of 10 °C/min.

Results

Figure 1A shows that the cornea is thin at the center and thicker at the periphery. The collagen fibers were amalgamated during the processing of the corneal tissue for SEM imaging (Fig. 1B). Figure 1C shows the junction between epithelium and stroma. Figure 1D shows the multiple patterns of the configuration of the collagen fibers at the central sections as well as the anterior and posterior parts of the stroma. The FTIR spectroscopy results (Fig. 2) showed a clear sharp peak at 3285.85 cm^{-1}. The source of the signal is the N–H stretch coupled with a hydrogen bond indicating the presence of amide A. The extremely small peak at 2900 cm^{-1} may refer to amide B. The peak at 1633.41 cm^{-1} was related to amide I. Amide II was measured at 1553.09 cm^{-1}. Amide III was measured at 1233 cm^{-1}. The present findings confirm the presence of a triple helical structure characteristic of collagen type I. Figure 3 shows little transmittance of UV in the C region ranging from 100 to 280 nm. A 650 nm red laser beam was used to determine the optical properties of the cornea. The detected results showed that transmission (T), reflected light (R) and scattered light (S) were 2.685 mw, 100×10^{-3} mw at 45° and 40×10^{-3} mw, respectively. The T, R and S percentages were 74.37%, 2.77% and 1.1%, respectively. Consequently, the absorbed light (A) percentage is 21.76%. Results of the present study illustrated that the denaturation temperature of *O. niloticus* corneal stroma was 22.27 °C (Fig. 4).

Fig. 1 [Images not available. See PDF.]

Scanning electron microscopic (SEM) micrographs of the fish cornea. **A** corneal thickness; **B** amalgamated collagen fibers during processing for SEM imaging; **C** junction between epithelium and stroma; **D** patterns of the configuration of collagen fibers; CTP, Corneal thickness at the periphery; CTC, corneal thickness at the center; ACF, amalgamated collagen fibers; NE, nerve ending; CF, collagen fibers

Fig. 2 [Images not available. See PDF.]

Fourier transform infrared (FTIR) spectroscopy of the fish corneal stroma

Fig. 3 [Images not available. See PDF.]

UV–visible transmission spectrophotometry of the fish cornea

Fig. 4 [Images not available. See PDF.]

Thermal properties (denaturation temperature) of the fish corneal stroma

Discussion

The present study highlights the biochemical and optical properties of Nile tilapia; *O. niloticus* corneal tissue. The principal objective of the assessment of the fish cornea by SEM is to observe the quality (rough or smooth), thickness and to estimate the presence of burrs, curls, or tissue bridges in the stroma region. The cornea of freshwater teleost has one continuous stroma that consists of collagen fibers arranged in bundles with the keratocytes distributed in between [9, 21]. The stroma of fish and human corneas have thin collagen fibers that together appear to indicate an overall parallel collagen direction. The human cornea consists of collagen fibers of different types with unique stacked spacing and arrangement which is an important factor affecting corneal transparency [22].

FTIR spectroscopy analysis is used to detect the functional groups that appear to characterize a compound by estimating its absorption of infrared radiation over different wavelengths. FTIR spectroscopy is used to assess the structure and type of collagen material. In addition, it serves to distinguish between methods of collagen extraction [23–25]. Similar pattern of FTIR spectroscopy results was indicated by Wang et al. [26] who explained the connection with the asymmetric stretching of C–H. The peak at 1633.41 cm^{-1} was related to amide I as a sign of the peptide's secondary structure, and the source of the signal is the C=O [27]. Amide II was measured at 1553.09 cm^{-1} , which is due to the stretching of the carbonyl group attached to a carboxyl group. Amide III was measured at 1233 cm^{-1}, which was determined by combining the C–N stretching and N–H bending modes (Fig. 2). The detected triple helical structure agrees with results reported by Sun et al. [28] for collagen extracted from Nile tilapia; *O. niloticus* skin. Figure 2 also shows that the functional groups found in the triple helix were not damaged owing to the method used to obtain collagen directly by removing both the epithelium and endothelium cells. Variations in width and height of the corresponding signals in several graphs could be attributed to the different experimental conditions of each study such as different solvents with different concentrations [14].

The wavelength of the UV spectrum ranges from 100 to 400 nm. The range is divided into three regions: C, B and A ranging from 100 to 280 nm, 280–315 nm and 315–400 nm, respectively. Figure 3 shows little transmittance of UVC. This may be attributed to the low thickness of the fish cornea in general. The UV absorption of collagen could be attributed to the peptide bonds and side chains in the corneal collagen structure. The collagen isolated from tilapia skin showed maximum absorbance at 230 nm [27] which is consistent with the characteristic absorbance of collagen type I in the present study. Similar findings were reported by several authors for collagen extracted from different marine species [26–29]. The human cornea transmits radiation only at 295 nm and above. The transmission of the human cornea is highest in the long wavelength area of the visible spectrum from 600 to 700 nm. Meanwhile, transmission of *O. niloticus* cornea is highest in the long wavelength region of the visible spectrum ranging from 600 to 700 nm and lowest in the short wavelength region below 500 nm (Fig. 3).

Huibertus et al. [30] measured light scattering and light transmission in fish scale-derived collagen (FSDC) and they concluded that FSDC is proposed as an alternative for mammalian donor corneal tissue which supports the present findings. The refractive index of corneal tissues of human and *O. niloticus* fish are 1.363 ± 0.011 and 1.360 ± 0.009, respectively [9].

Regarding the thermal properties, it was noticed that one of the most important characteristics of biomaterials is their denaturation temperature because it affects their thermal stability and consequently their physical and mechanical

properties [31]. The denaturation temperature is defined as the temperature degree at which the deformation of collagen helical structure occurs [32]. The denaturation temperature of skin collagen of *O. niloticus* ranged between 25 and 26 °C depending on the conducted extraction procedures [33]. Meanwhile it was detected at 34.85 °C for collagen extracted from *O. niloticus* scales [34]. The denaturation temperature of collagen depends on both the source tissue and the extraction procedures. Freshwater fish collagen generally show high thermal stability which could be attributed to its high content of proline and hydroxyproline [34–37]. In agreement with this finding, Yamamoto et al. [18] documented a higher denaturation temperature of Nile tilapia; *O. niloticus* collagen in comparison with other marine sources.

Conclusions

The present study confirms that the method for obtaining fish collagen (extraction, isolation, purification, etc.) may affect its specific properties and consequently the further uses of collagen as a potential biomedical substituent for mammalian collagen. Specification of the fish species and tissue type is crucial in identifying the quality of the extracted collagen as well as its physical and functional properties. Further studies are recommended to evaluate the potential uses of *O. niloticus* corneal collagen as a biological substituent for mammalian collagen.

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Author contributions

HHA-E: idea formulation, primary data collection, generation of database, writing of the manuscript, development of data analysis methodology, interpretation of results, editing of the manuscript; WAO and HAE: data analysis, preparation and editing of the manuscript. All the authors participated in the discussion of the results.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The present experimental investigation was not conducted with humans. Consent to participate is not applicable. All international, national, and/or institutional standards for the use of fish in experiments and conditions of fish care have been followed. The study received approval of the National Institute of Oceanography and Fisheries-Committee for ethical care and use of animals/aquatic animals (NIOF-IACUC) under ethical clearance reference number NIQF-FW3-F-23-R-043.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

A
Absorption
DSC
Differential scanning calorimeter
FSDC
Fish scale-derived collagen
FTIR
Fourier transform infrared

O. niloticus

Oreochromis niloticus

P_o

The incident light intensity

PBS

Phosphate-buffered saline

R

Reflection

SEM

Scanning electron microscopy

S

Scattering

T

Transmission

UV

Ultraviolet

Publisher's Note

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Dual drug-loaded cubosome nanoparticles for hepatocellular carcinoma: a design of experiment approach for optimization and in vitro evaluation

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ABSTRACT (ENGLISH)

Background

Liver cancer, a formidable and complex disease, poses a significant global health threat, stemming from various causes, including chronic infections like hepatitis B and C, cirrhosis, and lifestyle factors. In liver cancer treatment, targeted delivery revolutionizes precision therapy, minimizing side effects by directing drugs specifically to cancer cells. This study aims to develop and statistically optimize cubosomal formulations containing piperine and quercetin with the goal of augmenting their activity against hepatocellular carcinoma.

Results

Employing a central-composite design, we utilized Design-Expert® software to guide the experiment. The key formulation variables were the concentration of glyceryl monooleate (GMO) and Poloxamer-407, while the dependent responses were particle size (PS) and entrapment efficiency (EE%). The optimized cubosomal formulation was validated through the utilization of high-resolution transmission electron microscopy (HR-TEM), in vitro release studies, and an in vitro cell proliferation assay conducted on the HepG2 cell line. High-performance liquid chromatography was employed for the determination of piperine and quercetin in the optimized cubosomal nanoparticle. The optimized formulation had a composition of 2.5 (w/w%) GMO and 0.5 (w/w%) Poloxamer 407. The predicted values for PS and EE% were 102.34 and 75.11%, respectively. The cytotoxicity of the optimized cubosomal formulation exhibited enhanced efficacy on the HepG2 cancer cell line, even at lower concentrations, when compared to the standard. Notably, it demonstrated a superior cytotoxic effect on the liver cancer cell line.

Conclusion

The findings of the study indicated that cubosomes exhibit promise as an effective carrier for delivering piperine and quercetin, addressing hepatocellular carcinoma effectively.

FULL TEXT

Background

In the realm of cancer incidence, primary liver cancer holds the fifth position in terms of frequency, solidifying its status as the second most widespread cancer type [1, 2]. Over the course of several decades, the frequency of cancer cases has consistently risen. This underscores the ongoing significance of cancer as a major public health concern, remaining among the primary contributors to global mortality [3–5]. Despite the emergence of innovative methods like vaccines, immunotherapy, and gene therapy, which exhibit encouraging treatment outcomes for diverse cancer forms, chemotherapy retains its position as a fundamental strategy in the medical management of cancer [6–8].

Natural phytoconstituents play a vital role in impeding diverse mechanisms linked to cancer, including cellular division, cell proliferation, angiogenesis, programmed cell death (apoptosis), and the spread of cancer cells (metastasis) [9, 10]. Numerous herbs and phytoconstituents are utilized for the treatment of chronic liver ailments due to their cost-effectiveness, high safety thresholds, long-lasting positive effects, and minimal adverse reactions [11, 12]. Previous studies suggest that these natural compounds can protect liver cells through diverse mechanisms, including virus elimination, suppression of fibrogenesis, inhibition of oxidative damage, and reduction of carcinogenesis [13–15]. These herbal remedies effectively inhibit cell growth at different stages of the cell cycle, disrupt signaling pathways, and suppress gene expression, leading to the prevention of multiple types of cancer [16–18].

Quercetin, a member of the flavonoid family, possesses notable antioxidant, anti-inflammatory, and immunoregulatory properties, which contribute to its potential in preventing and treating conditions such as cardiovascular diseases, diabetes, neurodegenerative disorders, and cerebrovascular diseases [19–21]. Moreover, studies have reported the inhibitory effects of quercetin on hepatocellular carcinoma (HCC), showing that it can hinder HCC growth by suppressing proliferation and/or promoting apoptosis [22–25]. Numerous pharmacological investigations have indicated the promising capacity of piperine as a viable medication against cancer [26–28]. Its mechanism involves the suppression of pro-survival pathways and the initiation of apoptosis across diverse cancer cell variations. Piperine, identified as a potential anticancer compound capable of regulating autophagy, has demonstrated impressive success in combatting cancer within living organisms [29–31]. Recent research indicates that piperine has been utilized both as a standalone treatment and in combination with other drugs to enhance their anticancer effects and effectively manage the process of carcinogenesis [32].

Regrettably, these two medications exhibit limited solubility in water and lead to significant problems linked to sensitivity to light. Furthermore, quercetin has a tendency to experience swift chemical deterioration under conditions of acidic gastric pH. Hence, the integration of these two pharmaceutical agents into a well-suited, singularly lipid-mediated nanostructured arrangement, particularly through cubosomes, proves advantageous in surmounting crucial challenges. Cubosomes emerge as nanoparticles containing lipids and surfactants, showcasing an exceptional cubic liquid crystalline configuration [33, 34]. These minute entities, smaller than a micron, display a dual continuous character, boasting a substantial expanse of surface. Comprising amphiphilic components like polar and nonpolar polymers, lipids, and surfactants, cubosomes possess the innate ability to autonomously structure themselves. The genesis of cubosomes hinges on the self-organization of particular surfactant entities [35, 36].

The clinical effectiveness of anticancer drugs is limited because they tend to spread throughout the body without precision, resulting in unintended harm to healthy tissues and consequent side effects [37]. To address this challenge, researchers have introduced diverse drug transporters designed to transport therapeutic substances with precision to particular locations. One of the most successful approaches involves the utilization of nanocarriers, which capitalize on the enhanced permeation and retention phenomenon, allowing them to accurately target the afflicted areas while minimizing impact on unaffected tissues [38, 39].

Consequently, within this current investigation, our contemplation revolves around crafting a cubosome-based nanoformulation that encapsulates quercetin and piperine. The attainment of desired therapeutic effects from any medicinal agent hinges on precise assessments of the entrapped substances within the nanoformulation. Hence, our objective resides in the development of a reverse phase-high-performance liquid chromatography (RP-HPLC) technique, enabling the simultaneous quantification of both quercetin and piperine loaded within the cubosomes.

Methods

Materials

Quercetin (≥95%) was procured from Ausen Chemical Co., Ltd. in China, while piperine (≥97%) was acquired from Sigma-Aldrich India. Glyceryl monooleate (GMO) was kindly provided by Mohini Organics India. Poloxamer was obtained from Sigma-Aldrich in India, while potassium dihydrogen phosphate and sodium hydroxide were sourced from Merck Life Science Pvt. Ltd., India. Fetal bovine serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) solutions were acquired from Sigma-Aldrich in India. High-performance liquid chromatography (HPLC) grade methanol and various other essential chemicals were obtained from Merck Life Science Pvt. Ltd.

Compatibility studies

To evaluate the compatibility of piperine and quercetin and search for any possible interactions between the two compounds, Fourier transform infrared spectroscopy (FT-IR) was performed [40]. In a nutshell, piperine and quercetin were mixed together in a 1:1 ratio (2 mg), and the mixture was kept at 25 ± 2 °C and 60 ± 5% relative humidity (%RH) for 28 days. Piperine, quercetin, and the mixture underwent FT-IR analysis. The effects of piperine, quercetin, and their combination were studied using FT-IR.

Experimental design

Design-Expert software version 13.0 was used to create the experiments (Stat-Ease Inc., Minneapolis, MN, USA). The program was used to design and produce a total of 11 formulations [41–43]. To optimize the prepared cubosome formulations, a two-factor three-level (3^2) experimental design was employed. The independent variables considered were the amount of glyceryl monooleate (X_1) and Poloxamer 407 (X_2). Meanwhile, the dependent variables assessed were particle size (Y_1) and entrapment efficiency (Y_2). The statistical plan included three different concentrations of Poloxamer 407 (0.5, 1.25, and 2%) and three different concentrations of glyceryl monooleate (2.5, 3.75, and 5%) (Table 1).

Table 1. Coded values of 3^2 full factorial design for formulation of cubosomes

Independent variables	Levels		
Low (− 1)	Medium (0)	High (+1)	X_1 = GMO
2.5	3.75	5	X_2 = Poloxamer 407
0.5	1.25	2	Independent variables
Constraints	Importance		X_1 = GMO
In range	+++		X_2 = Poloxamer 407

In range	+++	*Dependent variables*
Y_1 = Particle size	Minimum	+++
Y_2 = % EE	Maximum	+++

Preparation of cubosomes

Piperine- and quercetin-loaded cubosomes were prepared using the top-down method with the aid of a magnetic stirrer and homogenizer. The process began by melting the lipid (2.5 to 5%), followed by dissolving a precisely weighed quantity of the drug (0.1%) in the melted glyceryl monooleate (GMO). This mixture was then transferred into the melted Poloxamer 407 (0.5 to 2%), resulting in the formation of a cubic phase [44–47]. To reach the desired volume (30 mL), a measured amount of water was added dropwise using a syringe. The mixture was homogenized using an Ultraturaxx homogenizer at 12,000 rpm for 15 min and subsequently sonicated for 15 min to obtain cubosomes. The prepared cubosomes were then stored in a cool environment. Various formulations of PQC (piperine–quercetin cubosomes) were meticulously prepared, and their respective compositions are elucidated in Table 2.

Table 2. CCD experimental runs with obtained response

Formulation code	X_1 (%)	X_2 (%)	Y_1	Y_2
C1	2.5	0.5	102.36	76.43
C2	2.5	1.25	109.75	59.54
C3	2.5	2	118.24	53.28
C4	3.75	0.5	124.44	79.83
C5	3.75	1.25	149.36	61.2
C6	3.75	1.25	151.27	62.3
C7	3.75	1.25	154.2	64.83
C8	3.75	1.25	172.7	65.9
C9	5	0.5	179.6	81.23
C10	5	1.25	216	67.28
C11	5	2	222.4	54.01

X_1 = amount of GMO, X_2 = amount of Poloxamer 407, Y_1 = Particle size, Y_2 = Entrapment efficiency

Particle size, PDI and entrapment efficiency

The mean vesicle size and polydispersity index (PDI) of cubosomes loaded with piperine and quercetin were

analyzed using a zetasizer. For the analysis, 1 mL of the formulation was diluted with 20 mL of milli-Q water. To separate the untrapped drug, samples from each formulation were subjected to centrifugation at 15,000 rpm and 4 °C for a duration of 1 h. The resulting supernatant was collected and then appropriately diluted with methanol. The estimation of the unbound drug was performed using UV–Vis spectroscopy at a wavelength of 273 nm. The percentage of drug entrapment was calculated using the relevant formula [48, 49].

In vitro release study of cubosomes

The in vitro release study of cubosomes was conducted using the dynamic dialysis method (USP II method). The investigation into drug release employed a tablet dissolution testing apparatus (Electrolab, EDT-08LX) featuring a low-volume conversion kit (EDT-08L/08L × 150 ml). This specialized setup was specifically chosen for assessing the release of the drug from the nanoformulation. Prior to the study, the dialysis membrane was soaked overnight [50–52]. Briefly, PQC nanoformulation (5 ml) was transferred to a dialysis bag, which was immersed into a 100 ml of PBS (pH 5.5) containing tween 80 (0.1%). The temperature of dissolution medium was kept at 37 ± 0.5 °C with a stirring speed of 50 rpm. At predetermined time intervals (0.5, 1, 2, 3, 4, 6, 12 and 24 h), aliquots of 1 mL were withdrawn for analysis. The withdrawn aliquots of the drug release medium were replaced with same volume of fresh buffer to maintain the sink condition. The drug concentrations in the samples were analyzed using spectrophotometry at the maximum wavelength (λ max) of 273 nm.

Transmission electron microscopy (TEM)

The surface morphology and structure of optimized cubosomes were examined using a high-resolution transmission electron microscope (JEM-2100, Jeol, Tokyo, Japan). A small volume of the optimized cubosomes was applied onto a carbon-coated grid, allowing the sample to adhere. Subsequently, the specimen was stained with uranyl acetate for a duration of 5 min, followed by staining with lead citrate for 2 min. The grid containing the stained sample was then observed using a transmission electron microscope, with an appropriate level of magnification (20 nm and 100 nm) chosen to capture images [53, 54].

High-performance liquid chromatography (HPLC)

The Agilent Technologies 1220 Infinity II HPLC system was used for quantification of piperine and quercetin in optimized cubosomal formulation. Utilizing a reversed-phase C-18 column (5 µm, 250 × 4.6 mm, ZORBAX), chromatographic separation was accomplished [55–58]. After conducting numerous trials, the chromatographic conditions were fine-tuned by considering factors such as peak area, retention time, tailing factor, and theoretical plates. The optimal mobile phase was composed of methanol and 0.1% formic acid in water, in the ratio of 80:20. Samples were analyzed at the flow rate of 1 mL/min, and the detection wavelength was set at 273 nm. The prepared cubosomes were centrifuged for 30 min at 15,000 rpm. The supernatant (0.1 mL) was pipetted using a micropipette and made up the volume up to 10 mL of volumetric flask using mobile phase. For each analysis, a 10 µl sample was injected into the column. According to ICH Q2 (R1) criteria, the developed HPLC method's validation was carried out. Linearity, LOD, LOQ, precision, accuracy and robustness were all used to thoroughly evaluate the optimized method.

Cytotoxicity analysis

For this study, the HepG2 cell line, derived from human hepatocellular adenocarcinoma, was procured from NCCS in Pune, India. The cells were cultivated in DMEM-high glucose medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic–antimycotic solution [59, 60]. Maintaining optimal conditions, the cells were cultured in a controlled environment with 5% CO_2, 18–20% O_2 and a temperature of 37 °C within a CO_2 incubator. Sub-culturing of the cells was carried out every 2 days, and passage number 34 was employed for the experimental procedures. Transfer 200 µl of cell suspension into individual wells of a 96-well plate, targeting a cell density of 20,000 cells per well. Avoid introducing the test agent at this stage. Allow the cells to proliferate for approximately 24 h. Distinct concentrations of cubosomal solutions (50, 60, 70, 80, 90, and 100 µg/ml) were prepared in a solution containing 0.1% DMSO. Subsequently, 50 µl of each concentration's solution was individually introduced into designated wells containing the cells. The plate was then placed in an incubator set at 37 °C, 5% CO_2, and 90% humidity for a 24-h duration. A control group was processed without the addition of this formulation. Following the

incubation period, 15 µl of MTT solution (5 mg/ml in PBS) was added to the wells, and the plate was further incubated for 4 h at 37 °C. Post-incubation, 100 µl of DMSO was introduced to dissolve the formazan crystals that had formed. The absorbance of the cells was measured at 570 nm utilizing an ELISA plate reader. The mean values for cell viability were compared against the control to assess the impact of cubosomes on the cells. A graphical representation was plotted, correlating cell viability (%) against the concentration levels of the formulation.

Statistical optimization

The experiments conducted in this study were replicated in triplicate, and the outcomes were reported as the mean accompanied by the standard deviation. Both ANOVA and Student's *t* test were executed, determining statistical significance for results at a threshold of $p<0.05$.

Results

Compatibility studies

The Fourier transform infrared (FTIR) analysis of piperine and quercetin, as depicted in Fig. 1, unveiled the presence of various discernible functional groups. Notably, the spectrum exhibited distinct peaks corresponding to specific vibrational modes, including the aromatic C-H stretch at 2922.61 cm^{-1}, the alcohol O–H stretch at 3393.02 cm^{-1}, the C–O stretch at 1246.50 cm^{-1}, and the aromatic C–C stretch at 1640.32 cm^{-1}. Intriguingly, the physical mixture of the drug and excipients manifested the persistence of these characteristic peaks, underscoring the compatibility of the components. This observation serves to corroborate that the unique chemical identities of both the drug and the excipients remain intact in the composite system.

Fig. 1 [Images not available. See PDF.]

FTIR spectrum of physical mixture (GMO, Poloxamer 407, piperine, and quercetin)

Experimental design

Based on the regression coefficient determined in the ANOVA study, it was discovered that independent variables X_1 (GMO) and X_2 (Poloxamer-407) have a positive sign, indicating a synergistic influence on the outcome Y_1 (PS). It was discovered through graphical presentation, namely the response surface plot that raising the level of variable X_1 and X_2 tends to raise the value of response Y_1 (Fig. 2). Both independent variables X_1 and X_2 had a negative sign, indicating an antagonistic influence on the response Y_2. Graphical evidence revealed that as the level of both variables, X_1 and X_2, is increased, the value of response Y_2 was dropped (Fig. 3). Utilizing the Design-Expert software by Stat-Ease, Inc. (version 13.0), it was determined that all responses yielded satisfactory outcomes. The shaded zone with yellow color in the design space (Overlay plot) depicted in Fig. 4 represents the region of successful operating ranges. Design space facilitated the identification of optimal values for the independent parameters: GMO (2.5%) and Poloxamer (0.5%) under these specific concentrations. The model's exceptional ability to forecast values for the response variables was showcased by the remarkably low prediction error (5%) observed between the predicted values and the actual observations.

Fig. 2 [Images not available. See PDF.]

Response surface plot for response Y_1 (Particle size)

Fig. 3 [Images not available. See PDF.]

Response surface plot for response Y_2 (Entrapment efficiency)

Fig. 4 [Images not available. See PDF.]

Design space overlay plot

Particle size, PDI and entrapment efficiency

The drug-loaded cubosome nanoformulation was prepared through homogenization method, resulting in mean particle sizes of 102.36 nm and 222.4 nm, respectively. The cubosomes exhibited an average PDI in value range 0.1831 to 0.8894. These PDI values are indicative of a narrow and homogeneous particle size distribution, as they

are less than 0.9. The zeta potential is a significant variable for evaluating the stability of nanoparticulate systems. Zeta potential was recorded to be between − 13.6 and − 38.9 mV. These zeta potential readings suggest that the cubosome nanoparticles are moderately stable. The presence of free fatty acids within glycerol monooleate's lipid structure, which imparts this negative charge, is the cause of the negative charge that was detected.

Our analysis of the percentage of free and total drug in the cubosome nanoformulation revealed that the encapsulation efficiency (EE) for piperine and quercetin exhibited a range of 53.28% to 81.23%. The optimized concentration of surfactants used is probably responsible for the cubosomes substantially higher EE percentages. Significant amounts of quercetin and piperine were successfully solubilized by these surfactants within the lipid vesicles. The amounts of these substances were thereby markedly increased within the cubosome formulation.

In vitro release study of cubosomes

The in vitro drug release profiles of piperine and quercetin from cubosomes are meticulously illustrated in Fig. 5. The release study, specifically focusing on the optimized batch of the PQC nanoformulation (designated as PQC1), was conducted in a buffer with a pH of 5.5. This investigation was carried out utilizing a tablet dissolution testing apparatus equipped with a low-volume conversion kit. Broadly, the bicontinuous cubosome nanoformulations exhibited a biphasic burst release characterized by a diffusion-based mechanism emanating from the cubic-phase matrix structure. The release of both piperine and quercetin from the cubosome formulation displayed a biphasic pattern. In the initial hour, there was a notable burst release, accounting for approximately 22.43% of piperine and 16.40% of quercetin. Subsequently, after a 24-h duration, the cumulative release escalated to 82.92% for piperine and 61.04% for quercetin.

Fig. 5 [Images not available. See PDF.]

Cumulative drug release of PQC formulation

Transmission electron microscopy (TEM)

The surface morphology and structural integrity of the optimized PQC nanoformulation (PQC1) were meticulously scrutinized and substantiated through high-resolution transmission electron microscopy (HR-TEM) imaging. The resulting images revealed that the cubosome particles exhibited a distinctly cubic shape, characterized by a uniform and smooth surface with minimal curvature, as depicted in Fig. 6A, B. Notably, the particles displayed a scattering pattern indicative of their nanoscale dimensions, further accentuating their finely tuned and compact structure.

Fig. 6 [Images not available. See PDF.]

HR-TEM images of optimized cubosomal nanoformulation (PQC1)

High-performance liquid chromatography (HPLC)

The optimized method has proven its efficacy in the determination of formulated cubosomes, revealing distinct peaks for both quercetin and piperine. Notably, these peaks were precisely measured at 3.055 and 5.067 min, respectively. The consistent retention times underscore the robustness of the separation method, offering compelling evidence for the successful application of this approach in isolating the two pharmaceuticals within the cubosomes, as illustrated in Figs. 7 and 8. To ensure that the optimized HPLC method is appropriate for the intended use as outlined in ICH Q2 (R1) standards, it has undergone validation. Table 3 provides a summary of the proposed HPLC method's validation parameters, which were determined to be within the ICH Guidelines' standard limits.

Fig. 7 [Images not available. See PDF.]

HPLC chromatogram depicting standard quercetin and piperine

Fig. 8 [Images not available. See PDF.]

HPLC chromatogram depicting quercetin and piperine in cubosomes

Table 3. Summary of validation parameter

S. no.	Validation parameters	Quercetin	Piperine
1	Linearity		
	Linearity range (µg/mL)	2–10	2–10
	Correlation-coefficient	<0.999	<0.998
2	LOD (µg/mL)	0.32	0.41
	LOQ(µg/mL)	0.98	1.32
3	Precision		
	Intra-day (%RSD)	0.521 ± 0.07	0.2 ± 0.003
	Inter-day (%RSD)	1.25 ± 0.027	0.42 ± 0.01
4	Robustness		
	Change in Mobile phase volume (%RSD)	0.710 ± 0.047	0.28 ± 0.041
	Change in flow rate (%RSD)	0.323 ± 0.02	0.83 ± 0.018
5	Accuracy		
	50% recovery	98.75 ± 0.02	101.31 ± 0.24
	100% recovery	100.24 ± 0.37	100.78 ± 0.51
	150% recovery	98.21 ± 0.52	99.21 ± 0.23

Cytotoxicity analysis

The statistical findings from the MTT cell cytotoxicity study revealed that the examined compounds exhibited noteworthy cytotoxic attributes against HepG2 cells. Following a 24-h incubation period, the % cell viability of cubosomes at a concentration of 100 µM/mL was determined to be 4.05%. A standard control utilizing 1 µM/mL of doxorubicin was employed, and the cell viability for the standard was established at 32.25% (Fig. 9). The determination of the IC50 value for the cubosomes, based on the percentage drug loading, unequivocally indicates that the designed nanoformulation possesses a superior anticancer effect compared to the standard drug. This superiority may be attributed to its nanosized dimensions, sustained release properties and targeted delivery to cancer cells.

Fig. 9 [Images not available. See PDF.]

The overlaid bar graph depicting the percentage of cell viability for HepG2 cell lines

Discussion

This study takes a step toward enhancing liver cancer treatment by focusing on cubosomal formulations containing

piperine and quercetin. Piperine and quercetin are compounds known for their potential anticancer properties. By formulating these compounds into cubosomes, the aim is to augment their activity against hepatocellular carcinoma. To assess the compatibility of piperine and quercetin and explore potential interactions between these two compounds, Fourier transform infrared spectroscopy (FT-IR) was conducted. The obtained spectrum revealed clear peaks associated with specific vibrational modes, such as the aromatic C–H stretch observed at 2922.61 cm^{-1}, the alcohol O–H stretch at 3393.02 cm^{-1}, the C–O stretch at 1246.50 cm^{-1}, and the aromatic C–C stretch at 1640.32 cm^{-1}. This observation serves to corroborate that the unique chemical identities of both the drug and the excipients remain intact in the composite system.

The experimental design of this study utilized a central-composite design, guided by the Design-Expert® software. Key formulation variables were the concentration of glyceryl monooleate (GMO) and Poloxamer-407, while particle size (PS) and entrapment efficiency (EE %) were chosen as dependent responses. This approach provides a systematic and statistical method to optimize the formulation for the desired properties. The optimized cubosomal formulation, with a composition of 2.5% (w/w) GMO and 0.5% (w/w) Poloxamer 407, was validated using various techniques. The predicted values for PS and EE% were 102.34 and 76.43%, respectively, indicating successful optimization based on the experimental design. The optimized nanoformulation of piperine and quercetin (PQC1) underwent thorough examination of its surface morphology and structural integrity using high-resolution transmission electron microscopy (HR-TEM) imaging. The images obtained depicted cubosome particles with a well-defined cubic shape, showcasing a consistently smooth and uniform surface with minimal curvature.

The in vitro release study, specifically focusing on the optimized batch of the PQC nanoformulation (designated as PQC1), was conducted in a buffer with a pH of 4.5. After a 24-h duration, the cumulative release escalated to 82.92% for piperine and 61.04% for quercetin. High-performance liquid chromatography was utilized to quantify the concentration of piperine and quercetin within the optimized cubosomal nanoparticles. Significantly, the distinct peaks for piperine and quercetin were precisely identified at 3.055 and 5.067 min, respectively. The consistent retention times emphasize the reliability of the separation method, providing compelling evidence for the effective application of this approach in isolating the two pharmaceuticals within the cubosomes. The cytotoxicity assessment revealed that the optimized cubosomal formulation exhibited enhanced efficacy on the HepG2 cancer cell line, even at lower concentrations compared to the standard treatment. This superior cytotoxic effect specifically on the liver cancer cell line suggests the potential of cubosomes as an effective carrier for delivering piperine and quercetin to address hepatocellular carcinoma.

The findings of this study highlight the promise of cubosomes in liver cancer therapy. The targeted delivery system not only optimizes drug efficacy but also minimizes side effects, addressing a critical need in the complex landscape of liver cancer treatment. The use of advanced techniques such as HR-TEM and high-performance liquid chromatography adds credibility to the study, ensuring a thorough validation of the optimized cubosomal formulation. In conclusion, this research contributes to the growing body of knowledge seeking innovative solutions for liver cancer treatment. The effectiveness of cubosomal formulations containing piperine and quercetin against hepatocellular carcinoma suggests a potential breakthrough in precision therapy. As further research and clinical trials unfold, these findings may pave the way for a more targeted and efficient approach to combat liver cancer, ultimately improving patient outcomes on a global scale.

Conclusion

The objective of this research was to develop precision-targeted cubosomes containing piperine and quercetin to address Hepatocellular carcinoma. Effective formulation and fine-tuning of cubosomes loaded with piperine and quercetin were accomplished employing central composite factorial design methodology in conjunction with the Design-expert software. The prepared cubosomes demonstrated well-suited nanoscale vesicles, with particle dimensions falling within the nanoscale and achieving peak entrapment efficiency levels. Following the design of experiments (DoE) and the desirability criteria, it was determined that formulation (C1) containing GMO (2.5%) and Poloxamer 407 (0.5%) stood out as the most favorable selection, exhibiting the most compact particle size (102.3 nm) and greatest entrapment efficiency (76.43%). An analytical technique was established for piperine and

quercetin using HPLC under standard environmental conditions. The solvent system utilized consisted of HPLC-grade methanol and 0.1% formic acid in water an 80:20 ratio. The quantitative analysis demonstrated well characteristics peaks for both drugs in the optimized cubosomal formulation, with a retention time of 3.0 min for quercetin and 5.0 min for piperine. The investigation on the HepG2 cell line using the cubosomes loaded with piperine and quercetin, carried out through the MTT assay. Following a 24-h incubation period, the % cell viability of cubosomes at a concentration of 100 µM/mL was determined to be 4.05%. A standard control utilizing 1 µM/mL of doxorubicin was employed, and the cell viability for the standard was established at 32.25%. This outcome serves as confirmation of the formulation's effectiveness in treating hepatocellular carcinoma; this formulation stands as a promising and preferred dosage form poised to be a leading option in the near future.

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Author contributions

We affirm that "all authors have read and approved the manuscript." Each contributor played an equal role and actively engaged in this research project. Both PB and RK conscientiously reviewed the manuscript titled "Dual Drug-Loaded Cubosome Nanoparticles for Hepatocellular Carcinoma: A Design of Experiment Approach for Optimization and In Vitro Evaluation." Their efforts were conducted under the supervision of Dr. VSM, who offered guidance and assistance in navigating research complexities.

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Declarations

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Genotype–phenotype correlation of fecal Streptococcus regulator (fsr) locus with gelatinase activity and biofilm formation intensity in clinical E. faecalis isolates

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ABSTRACT (ENGLISH)

Background

Enterococci, known for their disturbing involvement in nosocomial infections, possess a diverse set of virulence factors, regulated by multiple genes. A key virulence regulator is the fecal *Streptococcus* regulator (Fsr) quorum sensing system. Multiple reports describe the involvement of *fsr* genes in several virulence mechanisms, notably gelatinase production and biofilm formation; however, the presence of *fsr* genes does not necessarily predict those virulence phenotypes. This study investigates the factors affecting the relation between molecular detection of *fsr* genes and accurate prediction of gelatinase activity and biofilm formation intensity.

Methods

One hundred enterococcal samples were collected from patients suffering from urinary tract infections. The isolates were identified through the use of a polymerase chain reaction (PCR) technique targeting the *ddl* gene. Biofilm formation was quantified by the crystal violet assay, while gelatinase activity was evaluated on gelatin agar plates. PCR was used to detect the *fsr*A and *fsr*B genes, as well as the gelatinase enzyme-encoding gene (*gel*E).

Results

Out of the collected 100 isolates, 93% were identified as *Enterococcus faecalis*. The isolates formed biofilm with different intensities: 47% were strong biofilm producers, 28% moderate, and 21% weak, while only four isolates (4%) did not form biofilm. Only 14% of all isolates had detectable gelatinase activity. The *fsr*A and *fsr*B genes were detected in 26% and 28% of the tested isolates, respectively, while *gel*E was detected in 57% of the isolates. Whereas no association was found between biofilm formation intensity and *fsr* locus genes or gelatinase activity, a strong positive correlation ($r = 1$) was found between the detection of both *fsr*A and *fsr*B genes and the gelatinase activity.

Conclusion

*fsr*A and *fsr*B have a diagnostic value and may be used as biomarkers for gelatinase activity in *E. faecalis*.

FULL TEXT

Background

Enterococci are Gram-positive members of the intestinal microbiota; however, they frequently act as opportunistic pathogens with the ability to cause community- and hospital-acquired infections. *E. faecalis* and *E. faecium* are the

enterococcal species most frequently linked to infections [1].

Infections of the urinary tract represent the most prevalent type of infection caused by *Enterococcus* spp., which is responsible for more than 30% of nosocomial urinary tract infections and has been identified as the second most common pathogen after *Escherichia coli* in catheter-associated urinary tract infections [2].

To be able to cause such diseases, enterococci possess several virulence factors that give them an advantage over their host's immune system. Quorum sensing is one of the important virulence mechanisms, and can be regulated by the *fsr* locus, made up of *fsr*A, *fsr*B, *fsr*D, and *fsr*C genes [3]. FsrA protein has a DNA-binding domain from the LytTR family [4]. The attachment of phosphorylated FsrA to LytTR-binding sites located in the regions preceding *fsr* B and *gel*E suggests that FsrA acts as the response regulator within this control system [4]. FsrB, a protein found within the cell membrane, is a member of the accessory gene regulator protein B family. It is responsible for transforming FsrD into an active form that stimulates the production of gelatinase, through the activation of the pheromone GBAP, which is subsequently exported outside of the cell [5]. The fourth gene within this locus, *fsr*C, is responsible for coding the transmembrane histidine kinase, FsrC, serving as the sensor-transmitter for the *fsr* operon [4].

The *fsr* locus regulates the synthesis of the gelatinase and serine protease enzymes, in addition to regulating the expression of EF1097 and EF1097b genes, which encode for enterocin [4]. Studies of the transcriptome have shown that, beyond its role in controlling *gel*E, *spr*E, and EF1097, the Fsr system plays a part in regulating approximately 75 other genes. These include genes associated with surface proteins (EbpR), biofilm formation (BopD), and various metabolic activities [6]. These proteins are engaged in a variety of biological processes and contribute to *E. faecalis* virulence and pathogenicity [3].

Downstream of the *fsr* locus lies the gene responsible for gelatinase production (*gel*E gene) [3]; The gelatinase enzyme, produced by *E. faecalis*, serves as a crucial virulence factor, enabling it to break down gelatin, collagen, casein, hemoglobin, and other peptides [7]. This enzyme facilitates the degradation of host tissues, aiding *E. faecalis* in colonizing and infiltrating host structures [8].

Quorum sensing also regulates another important virulence mechanism, which is biofilm formation. A biofilm is a community of microorganisms that are adhered to either living (biotic) or non-living (abiotic) surfaces, usually encased within a protective layer made up of extracellular polymeric substances [9]. Biofilm formation is an important element of *E. faecalis* pathogenicity because it helps the bacteria colonize a variety of settings, including host tissues and medical devices [10].

In addition to quorum sensing, gelatinase activity has also been linked to biofilm formation through degrading other bacterial cells and stimulating the release of AtlA, the major autolysin involved in biofilm formation [11].

A critical gap still remains in our understanding of the relationship between molecular detection of *fsr* genes and the accurate prediction of gelatinase activity, as well as biofilm formation intensity in enterococcal infections. The observed discrepancy between the presence of the *gel*E gene and actual gelatinase activity, along with the ambiguous role of the *fsr* locus in biofilm formation intensity, underscores the complexity of translating molecular diagnostics into effective clinical predictions and treatment strategies for *Enterococcus*-related infections.

Methods

Bacterial strains and culture condition

E. faecalis ATCC 29212 and *Bacillus subtilis* ATCC 6633 were used as positive control strains. One hundred isolates were retrospectively collected from Al-Borg Medical Laboratories from patients in Egypt diagnosed with urinary tract infections during the period of 2020 to 2021. These isolates were preserved at −80 °C in Brain Heart Infusion broth supplemented with 25% glycerol. For culturing purposes, samples from the stock were isolated on bile esculin agar and incubated at 37 °C overnight.

Identification of bacterial isolates

Pure bacterial colonies were obtained through surface streaking on bile esculin agar and identified to the genus level using Gram staining, catalase test, and their ability to tolerate 6.5% NaCl broth.

Identification of the enterococci to the species level

DNA extraction

DNA from the isolates under examination was extracted using the boiling method [12], wherein 3 to 5 well-separated colonies from the culture being tested were suspended in 100 µL of nuclease-free water and subjected to heating at 100 °C for 5 min. Following this, the suspension was rapidly cooled to −20 °C for 5 min and then centrifuged at 10,000 rpm for 10 min. The supernatant, which contained the crude DNA extract, was then stored at −20 °C for preservation.

Polymerase chain reaction (PCR)

The PCR was conducted in a total reaction volume of 50 µL, which included 25 µL of DreamTaq Green PCR Master Mix (Thermo Scientific, USA), 10 pmol of both the forward and reverse primers specific to the *ddl* genes (Table 1), and 2.5 µL of the extracted crude DNA. The multiplex PCR process encompassed an initial denaturation phase of 2 min at 95 °C, followed by 30 cycles that each included a denaturation step at 95 °C for 30 s, an annealing step at 52 °C for 30 s, and an extension step at 72 °C for 30 s. The procedure was concluded with a final extension step that lasted for 5 min at 72 °C [13].

Table 1. Primers used in this study and their sequence

Target gene	Primer pair	Amplicon size (bp)	References
ddl of *E. faecalis*	Forward: ATCAAGTACAGTTAGTCTTTA	942	[13]
Reverse: AACGATTCAAAGCTAACT	*ddl* of *E. faecium*	Forward: CCAAGGCTTCTTAGAGA	535
[13]	Reverse: CATCGTGTAAGCTAACTTC	*fsr*A	Forward: CGTTCCGTCTCTCATAGTTA
474	[14]	Reverse: GCAGGATTTGAGGTTGCTAA	*fsr*B
Forward: TAATCTAGGCTTAGTTCCCAC	428	[14]	Reverse: CTAAATGGCTCTGTCGTCTAG
*gel*E	Forward: GGTGAAGAAGTTACTCTGAC	704	[14]

The PCR products were visualized by electrophoresis on a 1.5% agarose gel (w/v), stained with ethidium bromide, using a electrophoresis system (Mupid exU, Japan). A Generuler 100 bp DNA ladder (Fermentas, Germany) served as the marker. The presence of the *ddl* gene in *E. faecalis* and *E. faecium* was confirmed by the visualization of 942 bp and 535 bp bands, respectively. *E. faecalis* ATCC 29212 was used as the positive control in these experiments.

Biofilm assay

Biofilm formation was evaluated through the use of the crystal violet assay method [15], with certain modifications.

The process for assessing biofilm formation began with inoculating an overnight culture of the tested isolates into trypticase soy broth (TSB) enriched with 1% glucose, followed by a 24-h incubation at 37 °C. Post-incubation, the optical density (OD) of the culture was adjusted to a 0.5 McFarland standard, with further dilution of the cultures to a 1:100 ratio in TSB. This diluted culture (200 µL) was then transferred to the wells of a sterile flat-bottomed 96-well plate and incubated again at 37 °C for 24 h.

After this incubation, the contents of the wells were discarded, and the wells were washed with saline and left to dry. The biofilm, comprised of adherent microbial cells, was fixed with absolute methanol and then stained with a 0.1% crystal violet solution for 15 min. Subsequent to the staining, the excess crystal violet was removed, and the wells were washed with distilled water before the plates were left to dry.

The adhered stain was dissolved using 33% glacial acetic acid, and the OD of the dissolved stain was measured at 570 nm using a plate reader (Unicam, UK). TSB containing 1% glucose without tested isolate served as the negative control. The experiment was conducted in triplicate.

The degree of biofilm formation by each isolate was classified based on the OD of the dissolved stain as follows: strong biofilm formation was indicated if $OD > 4 \times OD.c$, moderate biofilm formation if $2 \times OD.c < OD \leq 4 \times OD.c$, and weak biofilm formation if $OD.c < OD \leq 2 \times OD.c$, where OD.c represents the optical density of the negative control plus three times the standard deviation of the negative control [16].

Gelatinase assay

Gelatinase production was assessed by the nutrient gelatin plate method [17], with slight adjustments. In summary, the isolates under investigation were streaked onto the surface of nutrient agar plates that were supplemented with 5% gelatin. These plates were then incubated at 37°C for a 24-h period. Following the incubation, Frazier solution was applied dropwise onto the agar surface. The appearance of a clear zone surrounding the microbial growth served as an indicator of positive gelatinase activity. *Bacillus subtilis* ATCC 6633 served as the positive control for the experiment.

Genotypic screening for *fsr*A, *fsr*B, and *gel*E genes

For the detection of the *fsr*A, *fsr*B, and *gel*E genes, PCR was used. The PCR protocol for each reaction started with an initial denaturation phase at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 s. Annealing temperatures were set at 50 °C for *fsr*A, 49 °C for *fsr*B, and 47 °C for *gel*E gene detection (Table 1), each for 30 s, with an extension at 72 °C for 30 s. This was concluded by a final extension step lasting 5 min at 72 °C.

The PCR products were then subjected to electrophoresis in a 1.5% agarose gel (w/v), stained with ethidium bromide for visualization. A 100 bp DNA ladder was used as a marker, and the identification of the *fsr*A, *fsr*B, and *gel*E genes was confirmed by the visualization of amplification products measuring 474 bp, 428 bp, and 704 bp, respectively.

Statistical analysis

Experiments were performed in triplicates. All correlations were tested by the Pearson correlation coefficient. The association between the presence/absence of genes and different phenotypes was tested by Chi-Square test, for multiple categories (i.e., biofilm strength), or Fisher's Exact test, for two-category comparisons (i.e., gelatinase activity). *P*-value < 0.05 was considered significant. Visualization and statistical tests were performed in GraphPad Prism 9.5.0 (GraphPad, San Diego, CA).

Results

One hundred isolates were confirmed to be *Enterococcus* species based on their appearance as brownish-black colonies encircled by a black zone on bile esculin agar. Gram staining of these samples revealed Gram-positive coccobacilli, which were typically arranged in pairs or short chains. Additional identification procedures involved catalase and 6.5% NaCl tolerance tests. The results demonstrated that the isolates were catalase-negative and capable of growing in high concentrations of NaCl.

PCR identification of the isolates indicated that 93% of them were *E. faecalis* and 7% were *E. faecium*. All *E. faecium* isolates were excluded from further study.

The crystal violet assay, used to evaluate bacterial biofilm formation, showed that only four isolates did not form

biofilm, while the majority were strong biofilm producers (n = 44; 47%). About 28% of the isolates were moderate biofilm producers (n = 26), and the remaining isolates were weak biofilm producers (n = 19; 21%; Fig. 1).

Fig. 1 [Images not available. See PDF.]

Biofilm-formation by the tested *Enterococcus faecalis* isolates. The intensity of the biofilm formation by each clinical isolate is expressed as the absorbance of the dissolved dye from stained biofilms at 570 nm. The horizontal lines represent the absorbance cutoff values for non-, weak, moderate, and strong biofilm formation

The nutrient gelatin plate method confirmed that 13 isolates had gelatinase activity, while the majority of the collected isolates (86%, n = 80) were unable to produce detectable gelatinase activity.

Using PCR allowed the detection of the *fsr*A and *fsr*B genes in 24 and 26 isolates, respectively (26% and 28% of the isolates, respectively, Fig. 2).

Fig. 2 [Images not available. See PDF.]

The gelatinase activity, and the detectability of *gel*E, *fsr*A, and *fsr*B genes in the tested *Enterococcus faecalis* isolates. (Yellow: Present; black: Absent)

No significant correlation was found between the intensity of biofilm formed by different isolates and the PCR detection of quorum sensing genes *fsr*A ($r = -0.14$), and *fsr*B ($r = -0.11$), or the gelatinase-encoding gene *gel*E ($r = -0.04$). Additionally, the intensity of the formed biofilms was not correlated with the gelatinase activity ($r = -0.06$; Fig. 3).

Fig. 3 [Images not available. See PDF.]

The correlation between the detectability of *fsr*A, *fsr*B, and *gel*E genes, the gelatinase activity, and the intensity of formed biofilm. The correlation was estimated by the Pearson correlation coefficient. The color scale on the right represents the correlation coefficient

The *gel*E gene was detected in all gelatinase-positive isolates, and 50% (n = 40) of gelatinase-negative isolates, with a significant association ($p = 0.0004$; Table 2), but weak positive correlation ($r = 0.35$; Fig. 3).

Table 2. Association of gelatinase production, biofilm production, and the detectability of *fsr*A, *fsr*B, and *gel*E genes

Gelatinase production genotype	Number of isolates		*P* value (Fisher exact test)
Positive	Negative	*fsr*A+	13
11	< 0.0001	*fsr*A−	0
69	*fsr*B+	13	13
< 0.0001	*fsr*B−	0	67
*gel*E+	13	40	0.0004

Biofilm intensity genotype	Number of isolates				*P* value (Chi-Square test)
Strong	Moderate	Weak	Non	*fsr*A+	9

6	8	1	0.3344 (NS*)	*fsr*A−	35
20	11	3	*fsr*B+	11	6
6	2	0.6658 (NS)	*fsr*B−	33	20
13	2	*gel*E+	26	11	12
4	0.1283 (NS)	*gel*E−	18	15	7

NS not significant ($p \geq 0.05$)

The *fsr*A gene was detected in all gelatinase-positive isolates and 14% (n = 11) of gelatinase-negative isolates, while the *fsrB* gene was present in all gelatinase-positive isolates and in 16% (n = 13) of the gelatinase-negative isolate. The PCR detection of *fsr*A and *fsr*B was positively and significantly correlated with gelatinase production ($r = 0.68$ and 0.65, respectively, $p < 0.0001$; Fig. 3 and Table 2).

The molecular detection of the *fsr*A, *fsr*B, and *gel*E genes together was directly correlated with the gelatinase activity ($r = 1$). Likewise, the presence of just *fsr*A and *fsr*B together was directly correlated with the gelatinase activity ($r = 1$; Fig. 3).

Discussion

In this study, 100 clinical enterococcal isolates were collected from patients with urinary tract infections and identified to the species level, where most of the isolates were *E. faecalis* (n = 93). A similar predominance of *E. faecalis* infection was reported in Egypt [18] and worldwide [19]. Most of the tested *E. faecalis* isolates (96%) were capable of biofilm formation to different intensities; only four isolates were non-biofilm former. Similar results about biofilm formation capabilities in *E. faecalis* are available [20]. In accordance with previous studies [21, 22], most of the isolates formed biofilms with either strong or moderate intensity (72%).

Only 14% of the tested isolates had detectable gelatinase activity on gelatin-agar plates. The low frequency of detected gelatinase activity in *E. faecalis* isolates was previously reported [23, 24]; however, in a study by Robert and colleagues (2004), the percentage of gelatinase-producing *E. faecalis* isolates from clinical and community settings reached 67% [25].

It is worth noting that available information about the role of the *fsr* locus in biofilm formation and intensity is still contradictory. Here, a lack of correlation was observed between the *fsr* locus presence and biofilm intensity, and similar results were previously reported [26]. To the contrary, other studies reported reduced biofilm formation among *fsr* mutants [27].

A direct correlation was observed in this study between the presence of the *fsr*A and *fsr*B genes and the gelatinase activity, and the presence of both genes together with the *gel*E genes was detected in all isolates with positive gelatinase activity. The correlation between the Fsr system and gelatinase activity was previously reported [3]; however, a study by Hashem et al. [21] reported *fsr*B as a stronger predictor of gelatinase activity.

The gelatinase activity was suggested to affect biofilm production and to be important for pathogenicity in different infection models [28]. Here, we did not find any correlation between gelatinase production and the intensity of biofilm formation. Similar results about the lack of correlation between biofilm intensity and gelatinase production in *E. faecalis* isolates were reported [21, 29]. On the other hand, some studies on *gel*E mutant strains confirmed the role of gelatinase production in biofilm formation [30, 31]. Therefore, further studies are needed to determine the exact role of gelatinase in biofilm production.

In this study, *gel*E gene was detected in 57% of collected isolates. However, gelatinase activity was only found in 14% (n = 13) of the isolates. This may have resulted from the partial deletion in the *fsr* locus as previously discussed by Qin et al. [32], who found that gelatinase activity was abolished after the deletion of the *fsr* locus. This loss of

activity was documented [3].

In terms of diagnostic value of the *fsr* locus genes as biomarkers for gelatinase production, the finding that both *fsr*A and *fsr*B were positively correlated with the gelatinase production phenotype suggests that, while the molecular detection of these two genes is not fully correlated with the gelatinase phenotype, it still might be useful for rapid molecular screening or when culturing the bacteria is not possible. For example, in microbiome analysis studies, the detection of these genes in DNA extracted from fecal specimens or sewage samples could suggest potential for gelatinase activity.

Conclusions

The gelatinase activity of *E. faecalis* clinical isolates is strongly positively correlated ($r = 1$) with the presence of the quorum sensing-associated *fsr*A and *fsr*B genes, and no gelatinase activity was measurable when *fsr*A and *fsr*B were absent. Although reported in many studies, no direct correlation was found between *fsr*A, *fsr*B, or gelatinase production and biofilm formation intensity, which suggests that other genetic factors are associated with biofilm intensity.

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Author contributions

YAH conceived the study. MTK, RKA, and YAH designed experiments. KAA and YAH performed laboratory experiments. KAA, MTK, and RKA analyzed the data. KAA generated all figures. KAA and YAH drafted the manuscript; MTK and RKA revised the first drafts; all authors revised and agreed with the final version of the manuscript.

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Availability of data and materials

All data that support the findings of this study are provided in the manuscript. Raw data are available from the corresponding author, upon request.

Declarations

Ethics approval and consent to participate

The study was approved by the safety and ethics committee of the Faculty of Pharmacy, Cairo University, with approval number (MI 2634 for the year 2020).

Consent for publication

As no human subjects were involved in this work, no consent for publishing the results was needed.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

Fsr

Fecal *Streptococcus* regulator

PCR

Polymerase chain reaction

TSB

Trypticase soy broth

UTI

Urinary tract infection

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Assessing physicians' knowledge, attitude, and practice on anticoagulant therapy in non-valvular atrial fibrillation: Syrian insights

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ABSTRACT (ENGLISH)

Background

Atrial fibrillation (AF) is the most prevalent cardiac condition linked to increased mortality due to complications such as stroke. Oral anticoagulant (OAC) is the mainstay in preventing cerebrovascular accidents in patients with AF. Recent evidence identified gaps in physician's knowledge in diagnosing and managing patients with AF. This study aims to assess Syrian physicians' knowledge, attitude, and practices regarding the use of anticoagulant therapy in

non-valvular AF (NVAF) patients. A cross-sectional study was conducted using a validated web-based questionnaire, which included 56 items separated into four sections that collected information about demographics, knowledge, attitude, and practices. Chi-square and Kruskal Wallis were performed to analyze the statistical relationships between the knowledge, attitude, practice, and demographic variables.

Results

A total of 497 participants completed the survey, of which 62.6% were between the ages of 25 and 35. The average participant scores for knowledge, attitude, and practices were (48.18 ± 21.57), (81.54 ± 9.26), and (62.83 ± 12.42), respectively. Participants who demonstrated good understanding, a positive attitude, and good practices were 22.3%, 87.3%, and 25.4%, respectively. The fear of bleeding was identified as the most significant barrier to initiating anticoagulant medication in AF patients (55.5%). Doctors who attended training had a better knowledge score than those who did not (mean ± S.D. = 57.24 ± 20.7). Participants who stated that over 70% of their AF patients use aspirin received the highest attitude score (mean ± S.D = 86.98 ± 21.17). PhD participants reported higher practice scores than those with other educational backgrounds (mean ± S.D = 73.96 ± 11.3).

Conclusion

This research showed that primary care physicians in Syria had optimistic views regarding OAC therapy, suggesting that training interventions targeting physicians may lead to improvement in the treatment of patients with NVAF in Syria.

FULL TEXT

Introduction

Atrial fibrillation (AF) is the most prevalent persistent arrhythmia, with more than 33 million globally diagnosed. Various severe cardiovascular and cerebrovascular complications, such as myocardial infarction, heart failure, stroke, and premature death, are linked to atrial fibrillation [1]. Globally, the incidence and prevalence rates of AF are increasing as the life expectancy for more people is rising. The prevalence of atrial fibrillation has tripled during the last 50 years. The number of patients diagnosed with AF in Asia by 2050 is expected to be at least 72 million, with 3 million people expected to suffer from strokes secondary to AF [2].

Numerous recognized cardiovascular risk factors, such as hypertension, valvular heart disease, and diabetes mellitus, have been identified as independent predictors of atrial fibrillation. Other risk factors include obesity, excessive alcohol consumption, male sex, and left ventricular hypertrophy. Thus, screening patients with these risk factors may be advantageous to diagnose AF before complications occur [3].

Although paroxysmal AF is linked with a slightly lower risk of stroke and systemic embolism in comparison to permanent AF, it is still accompanied by a high possibility of stroke incidence [4]. Paroxysmal AF and atrial arrhythmia, or atrial high-rate events (AHRE), may be identified via comprehensive screening utilizing equipment like pacemakers, implanted cardiac monitors, patches, or smartphones [5].

Atrial fibrillation patients have a high risk of morbidity and mortality due to ischemic stroke; as a result, stroke prevention in the case of non-valvular AF (NVAF) is a top concern for doctors, patients, and their families, as well as for society. Several approaches have been developed to prevent strokes [6]. The CHA2DS2-VASC score is the gold standard for stroke prediction in AF patients since the European Society of Cardiology (ESC) guidelines recommended in 2012 to use this score for stroke risk stratification [7].

Oral anticoagulation (OAC) therapy primarily prevents thromboembolic events and stroke in patients with NAVF [8]. In patients with AF, stroke risk reduction is prioritized when considering anticoagulant medications, and higher bleeding risk in favor of reducing stroke risk is usually accepted. However, patient preferences should be considered when deciding the type of OAC treatment [9].

Antiplatelet and vitamin K antagonists were the only options to prevent stroke in AF until 2009. However, vitamin K antagonists' use limitations have been established, such as a narrow therapeutic index, serious interactions with food and other medications, and the need for monitoring. New OACs (NOAC) are easier to administer than warfarin since they are given at a constant dosage without frequent monitoring [10–12]. However, recent studies have shown that in patients with cardiovascular disease, the nonadherence rate to treatment may approach 50% after

12 months. Since NOACs' anticoagulant action lasts only 12–24 h after each dose, poor adherence would put NOAC therapy at risk [13]

A recent study encompassing six European countries found that physicians needed to be more confident in handling anticoagulant medication in patients with complex AF and identified significant gaps in physicians' Knowledge and abilities in all aspects of AF treatment [14]. Another study showed that the main factors for underusing vitamin K antagonists among patients were the lack of knowledge and comprehension [15]. In contrast, the significant concerns among physicians were uncertainty and the need for personalized decision-making. Providing decision-making tools and improving primary care-hospital cooperation might help atrial fibrillation patients adopt this crucial therapy option. Increasing physician knowledge will aid general practitioners in treating AF with anticoagulation in primary care facilities. If these obstacles are addressed and a shared care plan is developed, AF may be better managed within primary care [16].

This cross-sectional study aimed to assess Syrian physicians' knowledge, attitude, and practice regarding the use of anticoagulant therapy in NVAF patients. The findings will contribute to the present knowledge and will be helpful for decision-makers and policymakers in guiding AF treatment.

Methods

Study setting and design

This online cross-sectional study was performed between 21 December 2022 and 3 February 2023. Each respondent was informed of the study objectives and the research team identification. Additionally, the right to withdraw from the study at any time and the total confidentiality of the personal information was assured, and the fact that only fully reported data would be analyzed. This study included cardiologists, other internists, consultants, and resident doctors within the general internal medicine department or its sub-specialties. Physicians who were unwilling to participate and practitioners from different specialties were excluded from this survey. The questionnaire was based on a previous study conducted in China [17].

The questionnaire was translated into Arabic to ensure complete comprehension of the items. The link for the Google form with the questionnaire was sent to respondents through social media platforms, such as WhatsApp, Facebook, and Telegram. Another source for collecting data was face-to-face interviews between data collectors and physicians within governmental and private hospitals. The minimal sample size was estimated by applying a single proportion of the population formula [$n=[(Z\alpha/2)2.\ P\ (1-P)]/d2$]. With a 95% confidence level ($Z\alpha/2=1.96$), and a 5% margin of error, the KAP level was assumed at 50% to ensure the largest sample size. The required sample size was 385. However, we enrolled 498 participants in this study.

Measures

A modified and validated KAP questionnaire model developed by Ye et al. was used as a measurement tool 15. The questionnaire of this study included 56 items divided into four sections. The first part represented the demographic information of the study population and their previous experience in dealing with AF patients. The second, third, and fourth sections assessed knowledge, attitude, and practice toward anticoagulant therapy in patients with NVAF, respectively. Knowledge, attitude, and practice were rated as either poor (below 39.0%), average (40.0–69.0%), or good (above 70.0%), where these cut-off points were predetermined based on the prior study.

Practitioners' demographics and their previous experience in dealing with AF patients

Overall, this domain contained 19 questions. We obtained the main properties of the study's respondents by asking them 13 questions about their sociodemographics, including age, gender, academic specialty, number of years of work experience, and professional title. The participant's monthly income was defined as poor, moderate, good and excellent if it was <30%, between 30 and 50%, between 50 and 80% and >80% of the area median income, respectively. In this part, participants were also asked six questions about their previous experience in dealing with AF patients, including the total number and age of the AF patients they have managed in the past year, the main obstacles of starting anticoagulant therapy in their AF patients, and whether their AF patients were treated with aspirin or warfarin.

Knowledge regarding anticoagulant therapy in patients with NVAF and the sources of information

Fourteen questions were included in this segment. We questioned the participants on issues such as AF diagnosis, the scores used to predict stroke risk and bleeding threat in AF patients, laboratory tests used to monitor AF patients treated with warfarin, the target range of International normalized ratio (INR) in AF patients managed with warfarin under 75 years old and above it, and about NOAC use. In the context of knowledge, participants were asked about their source of information concerning AF and the sources they prefer to use to gather knowledge about AF. Each knowledge item question scored one point for a correct answer and zero for an incorrect answer. The total knowledge score was computed by the addition of all score items.

Attitude toward anticoagulant therapy in patients with NVAF

This section included 13 questions to measure participants' attitudes toward anticoagulant therapy in NVAF patients. Participants were investigated about their degree of agreement with the necessity of using the stroke score tool to calculate the risk of stroke in AF patients before anticoagulant therapy, the necessity of using the bleeding score tool to evaluate the risk of bleeding in AF patients before anticoagulant treatment, the need of understanding the risk of stroke and bleeding in AF patients, and the necessity to tell AF patients about medication and food that affect warfarin's anticoagulant effects. A 5-point Likert scale (1 = strongly disagree, 5 = strongly agree) was used to examine participants' attitudes toward anticoagulant therapy in NVAF patients.

Practice toward anticoagulant therapy in patients with NVAF

We included 18 questions in this domain to evaluate practitioners' experience dealing with non-valvular atrial fibrillation patients. This part showed different scenarios, and participants were asked to choose the appropriate management in each scenario. This part discussed issues such as the use of stroke risk score tools to assess stroke risk in AF patients, the use of bleeding risk score tools to assess bleeding risk in AF patients, informing the patient of the food and drugs that interact with warfarin, informing the patient of the increased risk of stroke related to AF. Respondents were also questioned about their previous attendance of training lectures about atrial fibrillation and anticoagulation therapy and their future desire to attend conferences on this topic. A 4-point scale (1 = never, 4 = always) was performed to measure participants' practice toward anticoagulant therapy in NVAF patients.

Pilot study

To confirm the validity and clarity of the survey, we administered it to 50 members of the public selected at random before accreditation. After performing pilot research and ensuring the questionnaire had strong internal consistency (Cronbach's alpha was between 0.712 and 0.861), we distributed it.

Ethical considerations

The Syrian Ethical Society for Scientific Research provided ethical approval and Aleppo University provided ethical clearance. Participants were given a link to access an online Google survey, and on the first page of the survey, they were asked if they agreed to complete the questionnaire. Before completing the questionnaire, participants were sent to the linked page containing important research information. The questionnaire took five to twelve minutes to complete, and the responses were saved in a secure online database.

Statistical analysis

We used IBM Statistical Package for the Social Sciences (SPSS) Statistics ver.28 and Microsoft Excel ver.365 for the performance of statistical analysis, considering *p*-values equal to or below 0.05 as significant values. For continuous data (scores) that were not normally distributed according to the Shapiro–Wilk test, the Kruskal–Wallis was performed to determine the difference between the scores and basic variables. However, the chi-square test was used to reveal the difference between the categorical variables. In addition, we carried out binary logistic regression to define the actual probabilities of each subgroup having adequate levels of knowledge, attitude, and practices of anticoagulant therapy in patients with NVAF.

Results

Participant demographics

The respondents' demographic data are summarized in Table 1. A total of 511 doctors were invited to participate in this study; however, 14 declined, reducing the final sample size to 497. Almost two-thirds of the participants (62.6%) were male, whereas most respondents (93.7%) were aged between 25 and 35. Less than half of the participants

(47.5%) reported moderate monthly income, while 83.5% were residents' doctors. 87.1% of the study sample reported less than five years of working experience. Nearly a third of respondents (34.0%) indicated they had attended training courses in their specialty. Just 11.3% of participants stated they had 20–49 AF patients in the past year; 20.5% stated 40–69% of their patients take aspirin. Lastly, 10.4% of participants revealed that 20–39% of their AF patients take warfarin.

Table 1. Demographic characteristics of participants

Variable	Frequency	Percentage %
Gender		
Male	311	62.6
Female	186	37.4
Age		
20–35	459	93.7
36–50	25	5.1
51–65	6	1.2
Residence		
Rural	117	23.5
Urban	380	76.5
Monthly income		
Poor	28	5.6
Moderate	236	47.5
Good	211	42.5
Excellent	22	4.4
Educational level		
Medical school graduate	400	80.5
Master's degree	89	17.9

PhD degree	8	1.6
Types of CHS centers		
The rural	12	2.4
The urban	297	59.8
The Urban–Rural	188	37.8
Professional title		
Resident	415	83.5
Physician	79	15.9
Associate senior physician	2	0.4
Chief physician	1	0.2
Years of working experience		
<5 years	433	87.1
5–10 years	44	8.9
10–15 years	15	3.0
15–20 years	2	0.4
20–25 years	1	0.2
>25 years	2	0.4
Training attendance		
No	328	66.0
Yes	169	34.0
Number of AF patients		
No one	160	32.3
1–9	147	29.6

10–19	80	16.1
20–49	56	11.3
50–99	33	6.7
100–149	8	1.6
≥150	12	2.4
Age group of AF patients		
<50	11	2.2
50–59	133	26.8
60–69	135	27.2
70–79	28	5.6
Others	190	38.2
Number of AF patients on aspirin		
<5%	45	13.4
5–9%	53	15.7
10–19%	57	16.9
20–39%	61	18.1
40–69%	69	20.5
≥70%	52	15.4
Number of AF patients on warfarin		
<5%	190	56.4
5–9%	53	15.7
10–19%	41	12.2
20–39%	35	10.4

40–69%	16	4.7
≥70%	2	0.6

Participant's knowledge assessment

Most participants knew how to diagnose AF, and 74.6% knew the tool that could be used to predict stroke risk in AF patients. Most respondents (81.7%) indicated the correct risk factors included in the CHADS2 score, while 21.3% did not. Approximately, 36.4% and 75.9% of respondents did not recognize the risk factors "Hypertension, Abnormal renal/liver function, Stroke, Bleeding history or predisposition, Labile INR, Elderly, Drugs/alcohol concomitantly" (HAS-BLED) score included and how long coagulation function should be monitored in AF patients with long-term warfarin therapy at a stable period, respectively. The target range of INR in AF patients with warfarin under 75 years old and the target range of INR in AF patients over 75 years old were identified among 42.9% and 45.7% of respondents, respectively. Most respondents (90.5%) and (94.2%) replied correctly about the factor that is susceptible to the anticoagulation effect of warfarin and the antagonist that antagonizes warfarin's anticoagulation, respectively (Table 2).

Table 2. Community primary care physician (PCP) knowledge of OAC therapy in NVAF patients

Knowledge items	Frequency	Percentage %
AF diagnosis		
No	3	0.6
Yes	494	99.4
Score tool for predicting stroke risk in AF patients		
No	126	25.4
Yes	371	74.6
Score tool for predicting bleeding risk in AF patients		
No	220	44.3
Yes	277	55.7
Risk factors included in the CHADS2 score		
No	91	18.3
Yes	406	81.7
Risk factors included in the CHADS2-VASc score		

No	106	21.3
Yes	391	78.7
Risk factors included the HAS-BLED score		
No	181	36.4
Yes	316	63.6
Which indicator should be monitored in AF patients with warfarin		
No	17	3.4
Yes	480	96.6
How long should be monitored coagulation function in AF patients with long-term warfarin therapy at a stable period?		
No	377	75.9
Yes	120	24.1
What's the target range of INR in AF patients with warfarin under 75 years old		
No	284	57.1
Yes	213	42.9
What's the target range of INR in AF patients with warfarin over 75 years old		
No	270	54.3
Yes	227	45.7
Which factor is susceptible to the anticoagulation effect of warfarin		
No	47	9.5
Yes	450	90.5
What's the antagonist that antagonizes warfarin's anticoagulation		
No	29	5.8

Yes	468	94.2
Which of the following AF patients need to adjust warfarin dose		
No	164	33.0
Yes	333	67.0
Which medication are the new oral anticoagulants (NOAC)		
No	98	19.7
Yes	399	80.3

Most participants (85.7%) reported that electrocardiogram (ECG) made the diagnoses of AF, while 13.7% were done by Holter. 23.5% and 33% of respondents did not know the tool used to predict stroke risk in AF patients and can be used to predict bleeding risk in AF patients, respectively. Hypertension and diabetes were identified by 71% and 55.5% of respondents as risk factors in the CHADS2 score and CHADS2-VASc score, respectively. Only 3% of the participants used the Outcomes Registry for Better Informed Treatment of Atrial Fibrillation (ORBIT) score to predict bleeding risk in AF patients. Most of the respondents (83.5%) addressed INR as an indicator that should be monitored in AF patients with warfarin; however, 19.9% of respondents expressed that every seven days, the period coagulation function should be monitored in AF patients with long-term warfarin therapy at a stable period. A portion (42.9%) of participants thought the target range of INR in AF patients taking warfarin under 75 years of age was 2.0–3.0. Nearly two-thirds of respondents, 62.8%, identified food as a factor that is susceptible to the anticoagulation effect of warfarin (Table 3).

Table 3. The Knowledge of PCPs in anticoagulant therapy for NVAF patients

Knowledge of PCPs items	Frequency	Percentage%
How to diagnose AF		
ECG	426	85.7
Holter	68	13.7
Auscultation of the heart and palpation of the pulse	3	0.6
Which score tool can be used to predict stroke risk in AF patients		
CHADS2 score	63	12.7
CHADS2-VASc score	308	62
HAS-BLED score	6	1.2

ORBIT score	3	0.6
Not known	117	23.5
Which score tool can be used to predict bleed risk in AF patients		
CHADS2 score	21	4.2
CHADS2-VASc score	35	7
HAS-BLED score	262	52.7
ORBIT score	15	3
Not known	164	33
What risk factors does the CHADS2 score include		
Hypertension	353	71
Diabetes	315	63.4
Dyslipidemia	132	26.6
Congestive heart failure	280	56.3
Female	134	27
Age >75yo	334	67.2
Prior stroke/TIA	274	55.1
Not known	88	17.7
What risk factors does the CHADS2-VASc score include		
Diabetes	276	55.5
Prior stroke/TIA/thrombosis	320	64.4
Vascular disease	275	55.3
Age 65–74 yo	227	45.7
Age ≥75 yo	277	55.7

Hypertension	296	59.6
Congestive heart failure/left ventricular dysfunction	288	57.9
Female	208	41.9
Dyslipidemia	75	15.1
Not known	107	21.5
What risk factors does the HAS-BLED score include		
Female	68	13.7
Hypertension	228	45.9
Liver dysfunction and renal dysfunction	228	45.9
Stroke	167	33.6
History of bleeding	255	51.3
Unstable INR	241	48.5
Alcoholism	161	32.4
Concomitant medications (eg. antiplatelet drugs, NSAIDS)	180	36.2
Age >65yo	200	40.2
Not known	182	36.6
Which indicator should be monitored in AF patients with warfarin		
PT	267	53.7
APTT	63	12.7
INR	415	83.5
D-Dimer	20	4
Fibrinogen	13	2.6
Not known	12	2.4

How long should be monitor coagulation function in AF patients with long-term warfarin therapy at a stable period?		
Every 2 days	27	5.4
Every 7 days	99	19.9
Every 30 days	120	24.1
Every 3 months	175	35.2
Not known	76	15.3
What's the target range of INR in AF patients with warfarin under 75 years old?		
1.5–2.4	49	9.9
2.0–3.0	213	42.9
2.0–2.5	86	17.3
1.8–2.6	15	3
2.5–3.5	75	15.1
Not known	59	11.9
What's the target range of INR in AF patients with warfarin over 75 years old		
1.5–2.4	63	12.7
2.0–3.0	105	21.1
2.0–2.5	138	27.8
1.8–2.6	26	5.2
2.5–3.5	56	11.3
Not known	109	21.9
Which factor is susceptible to the anticoagulation effect of warfarin		
The patient's genes	213	42.9
Food	312	62.8

Drugs	420	84.5
Not known	49	9.9
What's the antagonist that antagonizes warfarin's anticoagulation		
Vitamin K	455	91.5
Protamine	32	6.4
Prothrombin complex	93	18.7
Fresh plasma	135	27.2
Not known	16	3.2
Which of the following AF patients need to adjust warfarin dose		
INR 2.0–3.0	32	6.4
INR 1.0–1.5	225	45.3
INR 3.8–4.5	291	58.6
INR 2.0–2.5 and age≥75y	125	25.2
Not known	119	23.9
Which medication are the new oral anticoagulants (NOAC)?		
Dabigatran	237	47.7
Rivaroxaban	341	68.6
Apixaban	316	63.6
Dicoumarin	85	17.1
Edoxaban	211	42.5
Not known	97	19.5

INR: International normalized ratio; CHADS2-VASc: Congestive heart failure, hypertension, age≥75 (doubled), diabetes, stroke (doubled), vascular disease score; HAS-BLED: Hypertension, Abnormal renal/liver function, Stroke, Bleeding history or predisposition, Labile INR, Elderly, Drugs/alcohol concomitantly; ECG: electrocardiogram; ORBIT: Outcomes Registry for Better Informed Treatment of Atrial Fibrillation

Participant attitude assessment

A total of 18.3% of the participants strongly agreed the type of AF would affect the doctor's initiation of anticoagulant therapy and choice of oral anticoagulants. In comparison, 12.9% and 11.1% didn't agree if it is necessary to use the stroke score tool to assess the risk of stroke in AF patients before anticoagulant therapy and if it is essential to use the bleeding score tool to measure the risk of bleeding in AF patients before anticoagulant treatment. Of the respondents, only 26.6% disagreed and were not more concerned about the risk of bleeding in AF patients than the risk of stroke in AF patients.

Reducing the risk of stroke and bleeding caused by AF is critical for AF patients, according to more than half of the respondents (56.7%). Of the participants, 6.2%, 2.0%, and 4.0% reported their disagreement that it is safe to maintain the INR between 2.0 and 3.0 for warfarin anticoagulation therapy in NVAF patients; that it is necessary to tell AF patients about medication and food that affect warfarin's anticoagulant effects, and they fully understand the views of AF patients on reducing the risk of stroke and bleeding caused by warfarin therapy, respectively. Finally, 53.7% and 63.8% of respondents addressed strongly they hope to have more Knowledge to discuss the advantages and disadvantages of stroke, bleeding risk, and anticoagulation, and they think doctors can improve the standard anticoagulant treatment rate in AF patients after training in atrial fibrillation, respectively (Table 4).

Table 4. Participant attitude toward OAC therapy in NVAF patients

Attitude items	Frequency	Percentage %
The type of atrial fibrillation would affect doctors' initiate anticoagulant therapy and choose oral anticoagulants		
Strongly disagree	47	9.5
Somewhat disagree	64	12.9
Not sure	118	23.7
Somewhat agree	177	35.6
Strongly agree	91	18.3
It is necessary to use the stroke score tool to assess the risk of stroke in AF patients before anticoagulant therapy		
Strongly disagree	1	0.2
Somewhat disagree	12	2.4
Not sure	64	12.9
Somewhat agree	186	37.4
Strongly agree	234	47.1
It is necessary to use the bleeding score tool to assess the risk of bleeding in AF patients before anticoagulant therapy		

Strongly disagree	5	1.0
Somewhat disagree	8	1.6
Not sure	55	11.1
Somewhat agree	161	32.4
Strongly agree	268	53.9
I am more concerned about the risk of bleeding in AF patients than the risk of stroke in AF patients		
Strongly disagree	52	10.5
Somewhat disagree	132	26.6
Not sure	122	24.5
Somewhat agree	143	28.8
Strongly agree	48	9.7
I think it's important for AF patients to "understand the risk of stroke and bleeding in patients with AF"		
Strongly disagree	4	0.8
Somewhat disagree	8	1.6
Not sure	48	9.7
Somewhat agree	155	31.2
Strongly agree	282	56.7
I think it's important for AF patients to "reduce the risk of stroke and bleeding due to atrial fibrillation"		
Strongly disagree	3	0.6
Somewhat disagree	10	2.0
Not sure	51	10.3
Somewhat agree	151	30.4
Strongly agree	282	56.7

It is safe to maintain the INR between 2.0 and 3.0 for warfarin anticoagulation therapy in NVAF patients		
Strongly disagree	18	3.6
Somewhat disagree	31	6.2
Not sure	122	24.5
Somewhat agree	171	34.4
Strongly agree	155	31.2
It is necessary to tell AF patients about medication and food that affect warfarin's anticoagulant effects		
Strongly disagree	1	0.2
Somewhat disagree	10	2.0
Not sure	57	11.5
Somewhat agree	134	27.0
Strongly agree	295	59.4
I fully understand the views of AF patients on reducing the risk of stroke and bleeding caused by warfarin therapy		
Strongly disagree	1	0.2
Somewhat disagree	20	4.0
Not sure	70	14.1
Somewhat agree	228	45.9
Strongly agree	178	35.8
I think the new oral anticoagulant (NOAC) has a lower risk of bleeding than warfarin		
Strongly disagree	6	1.2
Somewhat disagree	22	4.4
Not sure	130	26.2
Somewhat agree	183	36.8

Strongly agree	156	31.4
I think the new oral anticoagulant (NOAC) is easier to administer than warfarin		
Strongly disagree	5	1.0
Somewhat disagree	32	6.4
Not sure	141	28.4
Somewhat agree	143	28.8
Strongly agree	176	35.4
I hope to have more Knowledge to discuss the advantages and disadvantages of stroke, bleeding risk and anticoagulation r		
Strongly disagree	1	0.2
Somewhat disagree	10	2.0
Not sure	60	12.1
Somewhat agree	159	32.0
Strongly agree	267	53.7
I think doctors can improve the standard anticoagulant treatment rate in AF patients after training atrial fibrillation		
Strongly disagree	1	0.2
Somewhat disagree	4	0.8
Not sure	47	9.5
Somewhat agree	128	25.8
Strongly agree	317	63.8

Participant's practices assessment

Only 38% of participants have never made a differential diagnosis according to the duration of the onset of atrial fibrillation. On the other hand, 22.5% and 20.9% indicated they sometimes made differential diagnoses between valvular AF and non-valvular AF in AF patients when they dealt with AF and used stroke risk score tools to assess stroke risk in AF patients, respectively. Moreover, 32.4% of respondents stated they often use bleeding risk score tools to evaluate bleeding risk in AF patients. Additionally, 36.4% of respondents reported they sometimes would give warfarin for anticoagulant treatment to a 75-year-old male NVAF patient with hypertension and no history of

diabetes and cardiovascular disease.

A proportion of 32.8% stated they would not provide the AF patient who had gastrointestinal bleeding three months ago and has stopped bleeding for 1-week oral anticoagulant therapy, whereas 38.8% expressed they would never give warfarin to the AF patient whose nose bleeds once and gum occasionally bleeds when brushing his teeth. Furthermore, 32.6% of participants expressed that the AF patient with coronary stent implantation for one month should often give antiplatelet and warfarin therapy (Table 5).

Table 5. Participant practices when diagnosing and managing patients with AF

Practice items	Frequency	Percentage %
Have you ever made differential diagnosis according to the duration of the onset of atrial fibrillation		
Never	189	38.0
Sometimes	162	32.6
Often	124	24.9
Always	22	4.4
Have you ever made differential diagnosis between valvular AF and non-valvular AF in AF patients when you deal with AF		
Never	71	14.3
Sometimes	112	22.5
Often	129	26.0
Always	185	37.2
Do you use stroke risk score tools to assess stroke risk in AF patients?		
Never	77	15.5
Sometimes	104	20.9
Often	160	32.2
Always	156	31.4
Do you use bleeding risk score tools to assess bleeding risk in AF patients		
Never	78	15.7

Sometimes	117	23.5
Often	161	32.4
Always	141	28.4
For AF patients treated with warfarin, the INR is maintained at 1.1–2.0. Would you increase the warfarin dose for this p		
Never	62	12.5
Sometimes	163	32.8
Often	168	33.8
Always	104	20.9
For AF patients treated with warfarin, the INR is maintained at 3.5–5.5. Would you decrease the warfarin dose for this p		
Never	16	3.2
Sometimes	120	24.1
Often	177	35.6
Always	184	37.0
A 75-year-old male NVAF patient, with hypertension and no history of diabetes and cardiovascular disease, would you give this patient warfarin for anticoagulant treatment?		
Never	110	22.1
Sometimes	181	36.4
Often	154	31.0
Always	52	10.5
A 75-year-old female NVAF patient, with history of hypertension, congestive heart failure and TIA 3 years ago. Ultrasound indicated aortic atherosclerosis and atrial enlargement. Would you give this patient oral anticoagulant therapy		
Never	36	7.2

Sometimes	114	22.9
Often	162	32.6
Always	185	37.2
The AF patient in E8 item had gastrointestinal bleeding 3 months ago and has stopped bleeding for 1 week. Would you give this patient oral anticoagulant therapy?		
Never	163	32.8
Sometimes	168	33.8
Often	140	28.2
Always	26	5.2
The AF patient in E8 item had nosebleeds once and gum bleeds occasionally when brushing his teeth. Would you give this p		
Never	193	38.8
Sometimes	153	30.8
Often	120	24.1
Always	31	6.2
The AF patient in E8 item has taken coronary stent implantation for 1 month, would you give the patient dual antiplatelet and warfarin therapy		
Never	86	17.3
Sometimes	185	37.2
Often	162	32.6
Always	64	12.9
The AF patient in E8 item with ACS has taken coronary stent implantation and has been stable for 1 year. Would you give		
Never	55	11.1
Sometimes	167	33.6

Often	202	40.6
Always	73	14.7
A 68-year-old hypertensive female patient with recurrent episodes of paroxysmal atrial fibrillation and without previous		
Never	34	6.8
Sometimes	168	33.8
Often	206	41.4
Always	89	17.9
Have you often told AF patients who use warfarin therapy about the food and drugs that interacts with warfarin?		
Never	81	16.3
Sometimes	128	25.8
Often	143	28.8
Always	145	29.2
Have you ever actively communicated with AF patients with about increasing the risk of AF-related stroke and anticoagulation		
Never	89	17.9
Sometimes	129	26.0
Often	158	31.8
Always	121	24.3
Have you ever used different methods, such as pamphlets, health lectures and education, to educate AF patients about the		
Never	236	47.5
Sometimes	129	26.0
Often	83	16.7

Always	49	9.9
Have you ever attended relevant training or learned lectures about atrial fibrillation diseases and anticoagulation		
Never	234	47.1
Sometimes	112	22.5
Often	89	17.9
Always	62	12.5
Will you attend the training about AF disease and anticoagulation therapy?		
Never	31	6.2
Sometimes	85	17.1
Often	246	49.5
Always	135	27.2

Participant's knowledge, attitude, and practice scores

Less than half of the participants (42.5%) reported poor knowledge grade. Most respondents (87.3%) reported a good attitude, whereas 68.6% indicated a fair practice grade. The mean score and standard deviation for knowledge, attitude, and practice were (48.18±21.57), (81.54±9.26), and (62.83±12.42), respectively (Table 6).

Table 6. The scores of the KAP questionnaire (Knowledge, Attitude, and practice) of the participants

Item	Frequency	Percentage %
Knowledge grade		
Poor	211	42.5
Fair	175	35.2
Good	111	22.3
Knowledge score (mean±standard deviation)		
48.18±21.57		
Attitude grade		
Poor	1	0.2

Fair	62	12.5
Good	434	87.3
Attitude score (mean ± standard deviation)		
81.54 ± 9.26		
Practice grade		
Poor	30	6.0
Fair	341	68.6
Good	126	25.4
Practice score (mean ± standard deviation)		
62.83 ± 12.42		

Barriers and obstacles to starting OAC

The main obstacle to starting anticoagulant treatment in AF patients identified by participants was the fear of the risk of bleeding (55.5%) participants; however, monitoring coagulation function tests, drug-drug interactions, and fees of coagulation were identified by 48.1%, 44.7%, and 41.2%, respectively of respondents. Regarding the significant barrier affecting AF patients' compliance, fees of coagulation were reported by 77.5% of respondents. However, monitoring coagulation function tests, lack of medications, and fear of the risk of bleeding were indicated by 51.3%, 49.1%, and 44.3%, respectively (Fig. 1).

Fig. 1 [Images not available. See PDF.]

The main obstacles for starting OAC therapy and barriers affecting patients' compliance

Demographic factors and participant's knowledge

From the total participants, 25.6% of males showed poor knowledge, whereas 14.9% of females showed fair knowledge. A good understanding was identified among 17.9% of those who live in the city, 11.5% of those with moderate monthly income, and 16.3% of singles. 35.4% of residents, 38.6% of participants with less than five years of practice, and 34.2% of those who didn't attend training reported poor knowledge. 11.3% of Participants with 1–9 AF patients in the past year indicated fair awareness. Good awareness was noticed among 6.8% of respondents who stated their AF patients aged 60–69 years, while 11.9% of participants who addressed 40–69% of their AF patients taking aspirin showed fair knowledge (Table 7). Doctors who attended training had a better knowledge score than those who did not (mean ± S.D. = 57.24 ± 20.7).

Table 7. The Knowledge of primary care physicians of OAC therapy in NVAF patients based on demographic characteristics

Statement	Knowledge grade		
Poor	Fair	Good	*N*

%	*N*	%	*N*	%	*Gender*	
Male	127	25.6	101	20.3	83	16.7
Female	84	16.9	74	14.9	28	5.6
Age						
20–35	192	39.2	160	32.7	107	21.8
36–50	15	3.1	7	1.4	3	0.6
51–65	2	0.4	4	0.8	0	0.0
Residence						
Rural	51	10.3	44	8.9	22	4.4
Urban	160	32.2	131	26.4	89	17.9
Monthly income						
Poor	11	2.2	8	1.6	9	1.8
Moderate	94	18.9	85	17.1	57	11.5
Good	95	19.1	76	15.3	40	8.0
Excellent	11	2.2	6	1.2	5	1.0
Educational level						
Medical school graduate	174	35.0	143	28.8	83	16.7
Master’s degree	36	7.2	28	5.6	25	5.0
Ph.D. degree	1	0.2	4	0.8	3	0.6
Types of CHS centers						
The rural	6	1.2	5	1.0	1	0.2
The urban	121	24.3	115	23.1	61	12.3
The urban–rural	84	16.9	55	11.1	49	9.9

Professional title						
Resident	176	35.4	150	30.2	89	17.9
Physician	33	6.6	24	4.8	22	4.4
Associate senior physician	2	0.4	0	0.0	0	0.0
Chief physician	0	0.0	1	0.2	0	0.0
Years of working experience						
<5 years	192	38.6	151	30.4	90	18.1
5–10 years	11	2.2	18	3.6	15	3.0
10–15 years	8	1.6	2	0.4	5	1.0
15–20 years	0	0.0	1	0.2	1	0.2
20–25 years	0	0.0	1	0.2	0	0.0
>25 years	0	0.0	2	0.4	0	0.0
Attending training						
No	170	34.2	109	21.9	49	9.9
Yes	41	8.2	66	13.3	62	12.5
How many AF patients do you have in the past year?						
No one	105	21.2	32	6.5	23	4.6
1–9	61	12.3	56	11.3	30	6.0
10–19	20	4.0	40	8.1	20	4.0
20–49	21	4.2	24	4.8	11	2.2
50–99	3	0.6	14	2.8	16	3.2
100–149	1	0.2	5	1.0	2	0.4
≥150	0	0.0	4	0.8	8	1.6

Which age group are your AF patients in?						
<50	3	0.6	7	1.4	1	0.2
50–59	45	9.1	66	13.3	22	4.4
60–69	51	10.3	50	10.1	34	6.8
70–79	2	0.4	7	1.4	19	3.8
Others	110	22.1	45	9.1	35	7.0
How many of your AF patients take aspirin?						
<5%	15	4.5	18	5.3	12	3.6
5–9%	28	8.3	17	5.0	8	2.4
10–19%	19	5.6	23	6.8	15	4.5
20–39%	13	3.9	23	6.8	25	7.4
40–69%	20	5.9	40	11.9	9	2.7
≥70%	11	3.3	22	6.5	19	5.6
How many of your AF patients take warfarin?						
<5%	57	16.9	85	25.2	48	14.2
5–9%	19	5.6	20	5.9	14	4.2
10–19%	15	4.5	17	5.0	9	2.7
20–39%	9	2.7	14	4.2	12	3.6
40–69%	4	1.2	7	2.1	5	1.5
≥70%	2	0.6	0	0.0	0	0.0

N number % percentage

Demographic factors and participant's attitude

A good attitude was identified among 55.3% of males and 81.4% among those aged 20–35. 36.2% of participants had a good monthly income and 71.4% of singles reported good attitudes. A fair attitude was noticed among 11.3% of residents and 12.1% of those with less than 5 years of practice. 27.0% of participants with 1–9 AF patients in the past year indicated good attitude. Furthermore, a good attitude was noticed among 25.6% of respondents who

stated their AF patients aged between 60 and 69 years, while 19.9% of participants who addressed 40–69% of their AF patients taking aspirin showed a good attitude. (Table 8). Participants who stated that over 70% of their AF patients use aspirin received the highest attitude score (mean ± S.D = 86.98 ± 21.17).

Table 8. The attitudes of PCP of OAC therapy in NVAF patients based on demographic characteristics

Statement	Attitude grade					
Poor		Fair		Good		*N*
%	*N*	%	*N*	%	*Gender*	
Male	0	0.0	36	7.2	275	55.3
Female	1	0.2	26	5.2	159	32.0
Age						
20–35	1	0.2	59	12.0	399	81.4
36–50	0	0.0	2	0.4	23	4.7
51–65	0	0.0	0	0.0	6	1.2
Residence						
Village	0	0.0	4	0.8	113	22.7
City	1	0.2	58	11.7	321	64.6
Monthly income						
Poor	0	0.0	0	0.0	28	5.6
Moderate	0	0.0	27	5.4	209	42.1
Good	0	0.0	31	6.2	180	36.2
Excellent	1	0.2	4	0.8	17	3.4
Educational level						
Medical school graduate	1	0.2	56	11.3	343	69.0
Master’s degree	0	0.0	6	1.2	83	16.7

PhD degree	0	0.0	0	0.0	8	1.6
Types of CHS centers						
The rural	0	0.0	1	0.2	11	2.2
The urban	1	0.2	38	7.6	258	51.9
The urban–rural	0	0.0	23	4.6	165	33.2
Professional title						
Resident	1	0.2	56	11.3	358	72.0
Physician	0	0.0	6	1.2	73	14.7
Associate senior physician	0	0.0	0	0.0	2	0.4
Chief physician	0	0.0	0	0.0	1	0.2
Years of working experience						
<5 years	1	0.2	60	12.1	372	74.8
5–10 years	0	0.0	2	0.4	42	8.5
10–15 years	0	0.0	0	0.0	15	3.0
15–20 years	0	0.0	0	0.0	2	0.4
20–25 years	0	0.0	0	0.0	1	0.2
>25 years	0	0.0	0	0.0	2	0.4
Attending training						
No	0	0.0	50	10.1	278	55.9
Yes	1	0.2	12	2.4	156	31.4
B1 How many of AF patients do you have in the past year?						
No one	1	0.2	43	8.7	116	23.4
1–9	0	0.0	13	2.6	134	27.0

10–19	0	0.0	3	0.6	77	15.5
20–49	0	0.0	2	0.4	54	10.9
50–99	0	0.0	1	0.2	32	6.5
100–149	0	0.0	0	0.0	8	1.6
≥150	0	0.0	0	0.0	12	2.4
B2 Which age group are your AF patients in?						
<50	0	0.0	1	0.2	10	2.0
50–59	0	0.0	8	1.6	125	25.2
60–69	0	0.0	8	1.6	127	25.6
70–79	0	0.0	2	0.4	26	5.2
Others	1	0.2	43	8.7	146	29.4
B3 How many of your AF patients take aspirin?						
<5%	0	0.0	4	1.2	41	12.2
5–9%	0	0.0	4	1.2	49	14.5
10–19%	0	0.0	4	1.2	53	15.7
20–39%	0	0.0	5	1.5	56	16.6
40–69%	0	0.0	2	0.6	67	19.9
≥70%	0	0.0	0	0.0	52	15.4
B4 How many of your AF patients take warfarin?						
<5%	0	0.0	9	2.7	181	53.7
5–9%	0	0.0	3	0.9	50	14.8
10–19%	0	0.0	4	1.2	37	11.0
20–39%	0	0.0	2	0.6	33	9.8

40–69%	0	0.0	1	0.3	15	4.5
≥70%	0	0.0	0	0.0	2	0.6

N number %: percentage

Demographic factors and participant's practices

Only 4.6% of males showed poor practice, whereas 28.8% of females showed fair practice. Good practice was identified among 20.5% of those who live in the city, 11.1% of those with moderate monthly income, and 18.3% of singles. 61.8% of residents, 63.0% of participants with less than five years of practice, and 47.9% of those who didn't attend training reported fair practice. 7.5% of Participants with 1–9 AF patients in the past year indicated good practice. Fair practice was noticed among 17.5% of respondents who stated their AF patients aged between 60 and 69 years, while only 3.6% of participants who addressed 40–69% of their AF patients taking aspirin showed good practice (Table 9). PhD participants reported higher practice scores than those with other educational backgrounds (mean ± S.D = 73.96 ± 11.3).

Table 9. The practice of primary care physicians of OAC therapy in NVAF patients based on demographic characteristics

Statement	Practice grade					
Poor		Fair		Good		*N*
%	*N*	%	*N*	%	*Gender*	
Male	23	4.6	198	39.8	90	18.1
Female	7	1.4	143	28.8	36	7.2
Age						
20–35	30	6.1	318	64.9	111	22.7
36–50	0	0.0	14	2.9	11	2.2
51–65	0	0.0	2	0.4	4	0.8
Residence						
Village	14	2.8	79	15.9	24	4.8
City	16	3.2	262	52.7	102	20.5
Monthly income						
Poor	1	0.2	19	3.8	8	1.6

Moderate	10	2.0	171	34.4	55	11.1
Good	19	3.8	137	27.6	55	11.1
Excellent	0	0.0	14	2.8	8	1.6
Educational level						
Medical school graduate	30	6.0	294	59.2	76	15.3
Master's degree	0	0.0	44	8.9	45	9.1
Ph.D. degree	0	0.0	3	0.6	5	1.0
Types of CHS centers						
The rural	2	0.4	9	1.8	1	0.2
The urban	4	0.8	215	43.3	78	15.7
The urban–rural	24	4.8	117	23.5	47	9.5
Professional title						
Resident	30	6.0	307	61.8	78	15.7
Physician	0	0.0	32	6.4	47	9.5
Associate senior physician	0	0.0	2	0.4	0	0.0
Chief physician	0	0.0	0	0.0	1	0.2
Years of working experience						
<5 years	30	6.0	313	63.0	90	18.1
5–10 years	0	0.0	21	4.2	23	4.6
10–15 years	0	0.0	5	1.0	10	2.0
15–20 years	0	0.0	1	0.2	1	0.2
20–25 years	0	0.0	0	0.0	1	0.2
>25 years	0	0.0	1	0.2	1	0.2

Attending training						
No	30	6.0	238	47.9	60	12.1
Yes	0	0.0	103	20.7	66	13.3
B1 How many AF patients do you have in the past year?						
No one	29	5.8	116	23.4	15	3.0
1–9	1	0.2	109	22.0	37	7.5
10–19	0	0.0	53	10.7	27	5.4
20–49	0	0.0	38	7.7	18	3.6
50–99	0	0.0	17	3.4	16	3.2
100–149	0	0.0	3	0.6	5	1.0
≥150	0	0.0	5	1.0	7	1.4
B2 Which age group are your AF patients in?						
<50	0	0.0	9	1.8	2	0.4
50–59	1	0.2	97	19.5	35	7.0
60–69	0	0.0	87	17.5	48	9.7
70–79	0	0.0	16	3.2	12	2.4
Others	29	5.8	132	26.6	29	5.8
B3 How many your AF patients take aspirin?						
<5%	0	0.0	27	8.0	18	5.3
5–9%	0	0.0	36	10.7	17	5.0
10–19%	0	0.0	35	10.4	22	6.5
20–39%	0	0.0	38	11.3	23	6.8
40–69%	0	0.0	57	16.9	12	3.6

≥70%	1	0.3	32	9.5	19	5.6
B4 How many your AF patients take warfarin?						
<5%	1	0.3	128	38.0	61	18.1
5–9%	0	0.0	34	10.1	19	5.6
10–19%	0	0.0	30	8.9	11	3.3
20–39%	0	0.0	21	6.2	14	4.2
40–69%	0	0.0	11	3.3	5	1.5
≥70%	0	0.0	1	0.3	1	0.3

N number % percentage

Factors associated with knowledge score

In the binary logistic regression analysis, out of fourteen variables, only six predictors were statistically significant for predicting adequate knowledge of primary care physicians (PCPs) in anticoagulant therapy for NVAF patients (*p*-value < 0.05). Females were less expected to have good Knowledge than males (OR = 0.525). Respondents aged 36–50 years were less likely to have good Knowledge than those aged between 20 and 35 (OR = 0.038). Participants attending training had higher odds of understanding 2.369 times than those who didn't (Table 10).

Table 10. Binary logistic regression between demographic characteristics of the study population and Knowledge of PCPs in anticoagulant therapy for NVAF patients

Poor and fair vs. good								
Statement	*P*-value	COR	Lower	Upper	*P*-value	AOR	Lower	Upper
Gender								
Male	Reference							
Female	.003	.487	.303	.782	.019*	.525	.307	.899
Age								
20–35	Reference							
36–50	.200	.449	.132	1.528	.016*	.038	.003	.545
51–65	–	–	–	–	–	–	–	–
Residence								

Village	Reference							
City	.295	1.321	.784	2.224	.311	1.382	.739	2.584
Monthly income								
Poor	Reference							
Moderate	.358	.672	.288	1.569	.511	.714	.262	1.948
Good	.110	.494	.208	1.172	.146	.469	.169	1.301
Excellent	.463	.621	.174	2.220	.435	.548	.121	2.483
Educational level								
Medical school graduate	Reference							
Master’s degree	.133	1.492	.886	2.513	.350	1.571	.609	4.050
PhD degree	.263	2.292	.537	9.785	.543	1.842	.257	13.173
Types of CHS centers								
The Rural	Reference							
The urban	.322	2.843	.360	22.451	.960	.946	.112	8.026
The urban–rural	.200	3.878	.488	30.818	.708	1.502	.179	12.602
Professional title								
Resident	Reference							
Physician	.213	1.414	.820	2.438	.128	.426	.142	1.278
Associate senior physician	–	–	–	–	–	–	–	–
Chief physician	–	–	–	–	–	–	–	–
Years of experience								
5>	Reference							
5–10	.046	1.971	1.014	3.834	.335	1.590	.620	4.079

10–15	.250	1.906	.635	5.715	.088	12.504	.684	228.737
15<	.966	.953	.105	8.630	.058	20.570	.906	467.233
Attending training								
No	Reference							
Yes	.000	3.299	2.134	5.102	.001*	2.369	1.424	3.940
Attitude grade								
Bad\fair	Reference							
Good	.729	1.122	.585	2.150	.111	.545	.258	1.150
Practice grade								
Bad\fair	Reference							
Good	.000	4.357	2.772	6.851	.000*	3.973	2.285	6.908

*Statistically significant value-*P*-value ≤ 0.05, *COR* crude odds ratio, *AOR* adjusted odds ratio

Factors associated with attitude score

The Attitude of PCPs in anticoagulant therapy for NVAF patients was statistically correlated to two variables in the binary logistic regression analysis (*p*-value < 0.05). Participants with good practice grades were 5.872 times more likely to have a good attitude than those with bad\fair practice grades (Table 11).

Table 11. Binary logistic regression between Baseline Characteristics of the study population and Attitude of PCPs in anticoagulant therapy for NVAF patients

Poor and fair vs. good								
	P-value	COR	Lower	Upper	*P*-value	AOR	Lower	Upper
Gender								
Male	Reference							
Female	.341	.771	.451	1.317	.303	.727	.396	1.333
Age								
20–35	Reference							
36–50	.465	1.729	.398	7.523	.331	.244	.014	4.197

51–65	–	–	–	–	1.000	.175	.000	
Residence								
Rural	Reference							
Urban	.002	.193	.068	.542	.002*	.181	.062	.531
Monthly income								
Poor	Reference							
Moderate	–	–	–	–	–	–	–	–
Good	–	–	–	–	–	–	–	–
Excellent	–	–	–	–	–	–	–	–
Educational level								
Medical school graduate	Reference							
Master’s degree	.062	2.299	.959	5.513	.086	4.438	.809	24.341
Ph.D. degree	–	–	–	–	–	–	–	–
Types of CHS centers								
The Rural	Reference							
The Urban	.631	.601	.076	4.788	.516	.472	.049	4.551
The urban–rural	.689	.652	.080	5.289	.497	.453	.046	4.448
Professional title								
Resident	Reference							
Physician	.140	1.937	.805	4.661	.086	.254	.053	1.215
Associate senior physician	–	–	–	–	–	–	–	–
Chief physician	–	–	–	–	–	–	–	–
Years of experience								

5>	Reference							
5–10	.093	3.444	.813	14.594	.387	2.878	.263	31.514
10–15	–	–	–	–	–	–	–	–
15<	–	–	–	–	–	–	–	–
Attending training								
No	Reference							
Yes	.019	2.158	1.137	4.097	.098	1.852	.893	3.838
Knowledge grade								
Bad\fair	Reference							
Good	.729	1.122	.585	2.150	.212	.609	.280	1.326
Practice grade								
Bad\fair	Reference							
Good	.001	5.768	2.051	16.221	.002*	5.872	1.883	18.305

*Statistically significant value-*P*-value≤0.05, *COR* crude odds ratio, *AOR* adjusted odds ratio

Factors associated with practice score

We identified a statistically significant correlation between an adequate level of practice and four variables in the binary logistic regression (*p*-value<0.05). Physicians were more likely to have good practice than residents (OR= 5.679). Participants who scored good knowledge grades had higher odds of having good practice than those with bad\fair knowledge grades (OR=4.143) (Table 12).

Table 12. Binary logistic regression between Baseline Characteristics of the study population and Practice of PCPs in anticoagulant therapy for NVAF patients

	Poor and fair vs. good							
P-value	COR	Lower	Upper	*P*-value	AOR	Lower	Upper	*Gender*
Male	Reference							
Female	.018	.589	.380	.914	.941	1.020	.610	1.704
Age								

20–35	Reference							
36–50	.031	2.463	1.087	5.582	.054	.236	.054	1.027
51–65	.035	6.270	1.133	34.695	.645	.532	.036	7.808
Residence								
Rural	Reference							
Urban	.170	1.422	.860	2.351	.269	1.423	.762	2.657
Monthly income								
Poor	Reference							
Moderate	.537	.760	.317	1.820	.705	.827	.309	2.213
Good	.778	.881	.367	2.116	.832	1.113	.414	2.988
Excellent	.558	1.429	.433	4.717	.604	1.470	.342	6.311
Educational level								
Medical school graduate	Reference							
Master’s degree	.000	4.360	2.685	7.080	.553	1.317	.529	3.279
Ph.D. degree	.008	7.105	1.662	30.381	.976	1.031	.143	7.439
Types of CHS centers								
The rural	Reference							
The urban	.195	3.918	.498	30.843	.344	2.877	.323	25.665
The urban–rural	.219	3.667	.461	29.161	.384	2.642	.296	23.594
Professional title								
Resident	Reference							
Physician	.000	6.346	3.802	10.592	.000*	5.679	2.138	15.084
Associate senior physician	–	–	–	–	–	–	–	–

Chief physician	–	–	–	–	–	–	–	–
Years of experience								
5>	Reference							
5–10	.000	4.174	2.211	7.880	.203	1.816	.725	4.547
10–15	.000	7.622	2.541	22.860	.317	2.548	.408	15.927
15<	.058	5.717	.941	34.729	.854	1.301	.078	21.609
Attending training								
No	Reference							
Yes	.000	2.862	1.886	4.343	.029*	1.750	1.059	2.892
Knowledge grade								
Bad\fair	Reference							
Good	.000	4.357	2.772	6.851	.000*	4.143	2.394	7.169
Attitude grade								
Bad\fair	Reference							
Good	.001	5.768	2.051	16.221	.003*	5.496	1.816	16.630

*Statistically significant value-*P*-value≤0.05, *COR* crude odds ratio, *AOR* adjusted odds ratio

Discussion

The risk of developing atrial fibrillation (AF) rises with age; it is the most common kind of cardiac arrhythmia affecting people over 60 [18]. According to the Global Burden of Disease Study findings conducted in 2010, more than one-third of all patients suffering from AF are above 80 [19].

The current study was aimed at evaluating Syrian physicians' knowledge, attitude, and practice regarding the use of anticoagulant therapy in NVAF patients and how their demographic characteristics may significantly impact it. Overall, the study findings indicate that Syrian physicians showed suboptimal knowledge and understanding regarding the diagnosis of AF and screening of bleeding risk, where 35% had a fair knowledge score. Furthermore, the results suggested that younger participants and those attending training exhibited better knowledge. Additionally, higher knowledge scores correlated with better practice, while better practice scores were linked to better attitudes. According to the study, a much lower percentage of Syrians diagnosed with AF sought medical care in community clinics than the expected number of Syrians who had AF. According to the survey results, 20.5% of physicians recommend aspirin to between 40 and 69% of their patients. This finding is particularly striking compared to the findings of a study in China, where 41.6% of primary care physicians employed aspirin as an OAC therapy for more than 70% of their NVAF patients. In addition, between 20 and 39% of those diagnosed with AF had reevaluation by

10.4% of the study's participants to initiate warfarin treatment. According to the findings of the Chinese research, however, just 0.4% of primary care physicians administered warfarin as an OAC medication to more than 70% of their patients who had NVAF [17]. This knowledge gap was further demonstrated in a recent study among physicians [20].

Elderly adults with AF need antithrombotic therapy to reduce the chance of a stroke Patients in the community diagnosed with NVAF should discuss with their PCPs the possibility of taking the anticoagulant warfarin [21]. The findings of this study show that PCPs have an insufficient grasp of the anticoagulant therapy options available for patients with NVAF. Despite this, most responders (87.3%) reported having a positive mindset. Our findings are corroborated by the results of another study, which found that 89.8% of PCPs there got excellent ratings in the survey's component that measured attitude [17]. Moreover, an Iraqi study conveyed positive attitudes toward the use of OAC for NVAF patients, despite lacking in knowledge, further verifying the current study findings [22].

This study identified that not all PCPs knew how to identify AF, and some do not frequently utilize evaluation techniques to evaluate the relevant risks faced by patients with NVAF in their clinical practice. It was observed that patients with NVAF needed a greater understanding of such methods for calculating the risk of stroke and bleeding. Based on this observation, we concluded that the treatment of OAC therapy in patients with NVAF was not optimal. Anticoagulant treatment with warfarin has several drawbacks including the impact of variability, which is connected to clinical and hereditary variables and drug-drug and drug-food interactions [23]. However, drug-drug interactions were found in 41.2% of the cases despite these limits, which are critical for patient education. This occurs less often than in research from China when 65.58% of PCPs gave incorrect answers to questions about the variables, genes, medicines, and nutrition that were likely to interact with warfarin [17].

Stroke and bleeding risk must be assessed before starting OAC therapy for NVAF. PCPs caring for patients with NVAF should weigh the risks of bleeding against the potential benefits of avoiding strokes when making treatment decisions. The best possible therapy choice may then be made. Even though the CHADS2-VASc score and the HAS-BLED score are crucial tools in generating such clinical judgments, most participants lacked an in-depth understanding of both measures. Therefore, we promote continued education among community-based PCPs, especially in using simple scoring systems to enhance clinical decision-making. In a recent study, PCPs admitted they utilized the HASBLED score and the CHA2DS2-VASc score to determine the patient's risk of bleeding and stroke, which also affected their decision to start anticoagulants which agrees with the findings of the current study [24].

Our findings indicate that 68.6% of respondents reported being at an appropriate level of practice for their roles, and 87.3% said they had a positive attitude. Thus, most likely, a lack of education was to blame for the underuse of OAC in NVAF patients. The average score for practicality was 83.33, with chief medical officers and those with 20 + years of experience scoring higher than those with less experience.

The results of the current study highlight the impact of training on knowledge level, where it reveals that respondents who received training had better knowledge scores compared to those without. This is consistent with previous studies where education and training were proven essential for ensuring optimal AF therapy [20]. Furthermore, research has demonstrated the importance of the role of training in enhancing PCP, knowledge in AF management [16].

The current findings revealed that the main obstacle to initiating anticoagulant treatment in AF was the fear of bleeding risk while the major barrier to OAC compliance was found to be coagulation fees followed by monitoring coagulation function tests, which complies with previous reported evidence [22, 25].

Community hospital PCPs in urban and rural regions scored best in Knowledge, followed by their urban counterparts, and finally by their rural counterparts in the country's geographic center. Previous research conducted in China [17] demonstrates similar results. Central urban areas have more medical resources when comparing center urban regions to urban–rural intersections and rural areas [26]. On the other hand, in a previous study, PCPs working in rural regions had a higher practice score in comparison to those working in urban centers. This contradiction may be explained by the small number of PCPs in the mentioned study hindering the generalization of

this finding [22].

As a result, many people with NVAF are encouraged by this aspect to seek care at the best facilities. Suburban residents have a low health literacy [27], which leaves primary care physicians in urban and rural regions with fewer patients suffering from NVAF. This might be why PCPs in the central urban and rural areas have lower average test results. However, there needs to be appropriate research conducted to explain these findings. Our study's sample size may be increased to evaluate these elements better.

The current study and previous reports identifying physician prescribing patterns helps enforce the importance of clinical decision-making and physician knowledge in appropriate anticoagulant prescribing [28].

Limitations

This is the first cross-sectional observational research of KAPs for PCPs on OAC treatment in patients with NVAF in Syria. We included a suitable sample size to decrease bias and analysis errors. However, as our study design is cross-sectional, the limitations consisted of difficulty in making causal inferences, the determined analysis relationships might be difficult to interpret, and susceptibility to nonresponse and recall biases. Furthermore, the significant reliance on mainly social media platforms for data collection may have introduced selection bias since it excludes those who could not be reached through those platforms.

Conclusion

OAC treatment effectively prevented embolization in patients with NVAF due to the understanding and actions of PCPs. This study showed that PCPs in Syria who participated had optimistic views and attitudes, despite suboptimal knowledge. The results indicated that participants attending training demonstrated better knowledge suggesting that training interventions aimed at PCPs play a crucial role in improving the treatment of patients with NVAF. Furthermore, higher knowledge scores correlated with better practice, while better practice scores were linked to better attitudes.

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Author contributions

SS, YA, HB, and HA contributed to the conception and design of the study. MNN, NJ, AI, and BD collected, distributed, and organized the data sets. EM and SM analyzed the study data. BS and WH prepared the first draft of the manuscript. The final manuscript was revised by NOES and EAW. All the authors approved the final version of the manuscript.

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Availability of data and material

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

The Syrian Ethical Society for Scientific Research provided ethical approval. Ref. No.: HN/47-15 and consent was received from participants prior to study inclusion.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

AF

Atrial fibrillation

OAC

Oral anticoagulant

NVAF
Non-valvular Atrial fibrillation
KAP
Knowledge, attitude, and practices
AHRE
Atrial high-rate events
ESC
European Society of Cardiology
NOAC
New oral anticoagulants
INR
International normalized ratio
SPSS
Statistical package for the social sciences
CHADS2-VASc
Congestive heart failure, hypertension, age≥75 (doubled), diabetes, stroke (doubled), vascular disease score
HAS-BLED
Hypertension, abnormal renal/liver function, stroke, bleeding history or predisposition, labile INR, elderly, drugs/alcohol concomitantly
ECG
Electrocardiogram
PCP
Primary care physicians
ORBIT
Outcomes registry for better informed treatment of atrial fibrillation

Publisher's Note

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DETAILS

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A comprehensive review comparing conventional versus traditional remedies in the treatment of endometriosis with futuristic insights

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ABSTRACT (ENGLISH)

Background

A common condition known as endometriosis typically takes place in females in their reproductive age and develops

generally in the endometrial lining of females. Chronically, endometriosis has been associated with a reduction in the patient's quality of life (QOL) which can have a hazardous impact on their social working and functionality. Owing to the involvement of hormones in the development of endometriosis, drugs having the capability to modulate the hormonal concentrations, along with surgical techniques, have been designed to treat endometriosis.

Main body

There are certain drawbacks of the currently existing therapy for endometriosis which include the inability to improve the quality of life of the patient, treatment failures and unresponsiveness from the patient, and adverse effects of the drugs such as weight gain, mood swings, vaginal dryness, etc. Herbal medicines have attracted the attention of various researchers for the development of novel therapeutics against several gynecological disorders, mainly endometriosis. Our present review summarizes the precise pathogenesis of endometriosis along with its conventional therapy and novel developments in herbal medicines wherein we have compiled data from 15 completed clinical trials (conventional therapy: 7, herbal therapy: 8). Additionally, we have included data from four preclinical studies on herbal medicine that showed promising results in treating endometriosis highlighting the necessity for clinical trials to yield more definitive findings. The number of clinical trials carried out to assess the response of herbs in endometriosis is limited which is why additional studies could provide beneficial concrete evidence in the effective treatment of endometriosis and ensure improved patient outcomes.

Conclusion

Conventional therapies possess certain limitations to treat endometriosis due to which the attention of scientists has shifted toward herbal therapy due to its advantages such as improved safety and tolerability in treating endometriosis. However, additional clinical investigations into herbal therapy may prove to be fruitful in the discovery of novel therapeutics to treat endometriosis effectively.

FULL TEXT

Background

The endometrium constitutes the deepest layer of the uterus located within the female reproductive system, and it is composed of luminal and glandular epithelial cells [1, 2]. Any damage to the endothelial layer may disrupt the process of implantation and may lead to endometriosis (Fig. 1) [3–5].

Fig. 1 [Images not available. See PDF.]

An illustrative diagram representing the various factors causing endometriosis and the role of inflammation in its pathogenesis. Due to several factors including genetic, environmental, and lifestyle factors, dysfunction of the hypothalamic-pituitary axis (HPA-axis) takes place following which the production of female reproductive hormones such as LH, FSH, Anti-mullein hormone (AMH), *etc.* becomes impaired leading to an imbalance ultimately resulting in the release of inflammatory markers due to initiation process of inflammation. This can lead to damage in the endometrial layer and result in the formation of endometrial lesions resulting in pelvic pain and enlargement of the endometrium causing Endometriosis

Endometriosis can be identified by the development of tissues similar to endometrial tissues that grow outside of the uterine lining that can cause pain in the pelvic region and on a chronic basis may also lead to infertility. It usually occurs in females that are in their reproductive age, i.e., 18–54 years [6]. Although the exact factors or mechanisms behind endometriosis are still unknown, several theories have been proposed as to how its lesions arise [7]. Several symptoms arise due to endometriosis including intermenstrual bleeding, irregularity in menses (dysmenorrhea), dyschezia, dysuria, and also disruption in quality of life among patients which makes it a critical disease that needs to be dealt with [7, 8].

According to estimates, endometriosis usually is noticed in females falling between 18 and 54 years of age with its proportion of incidences varying from 10 to 15% [9]. The development of this disease has been observed to take place in up to 50% of women that have experienced infertility along with 47% of adults that have undergone laparoscopic procedures at some point in their life experiencing pelvic discomfort [7]. When the race-wise risk was calculated to develop endometriosis, it was noted that the Asian women population was at the highest risk of

developing endometriosis, while black women were observed to possess the highest possibility to develop endometriosis [10]. It has been observed that the prevalence of endometriosis in developed countries such as the USA, Russia, China, etc., was found to be 20% [11]. Similarly, the incidence rate of endometriosis was found to range from 34 to 48% in developing countries such as India [12]. Hormonal imbalances and their alterations can lead to elevations in the probability to develop endometriosis. Along with these menstrual factors, the early age at which menstruation commences and reduced menstrual period may also influence the likelihood of endometriosis [13–15]. Furthermore, lifestyle factors such as caffeine and alcohol intake are also associated with the development of endometriosis [16, 17]. Several factors leading to endometriosis are highlighted in Fig. 2.

Fig. 2 [Images not available. See PDF.]

Factors leading to endometriosis. Several factors may lead to endometriosis that include Genetic susceptibility towards particular genes, Immunological factors such as the stimulation of certain immune cells comprising Natural Killer cells, *etc.,* Environmental factors such as exposure to some trigger chemicals and lastly the activation of inflammatory markers such as Interleukins (IL-1,3), NF-κβ, and TNF-α

The conventional therapies for the management of endometriosis include hormonal agents (norethindrone, medroxyprogesterone acetate, cyproterone acetate, dienogest), contraceptive pills (estrogen–progesterone, progestin), gonadotrophin-releasing hormone (GnRH) agonists (leuprolide, buserelin) and antagonists (cetrorelix, ganirelix), and selective estrogen receptor modulator (tamoxifen, raloxifene) [18]. Patients suffering from endometriosis are known to experience pain in their pelvic region along with abdominal pain due to which they are usually given non-steroidal anti-inflammatory drugs (NSAIDs) to relieve them from their complaints of pain and steroids to regulate inflammation occurring in the endometrial region [19–21].

The majority of therapies designed to combat endometriosis are dependent on estrogen and other hormones as they constitute a majority of the disease's etiopathogenesis [22]. However, there are certain limitations of conventional therapy used in endometriosis such as risk of recurrences, safety and efficacy issues, risk of development of adverse events, etc. [23–25].

Due to the toxic effects of synthetic drugs, more attention has been shifted toward medicinal herbal drugs to cope with the harmful side effects of conventional therapy in various gynecological disorders. Herbal drugs have emerged as a more reliable source for the discovery of novel therapeutic approaches for endometriosis [26]. They target several mechanisms associated with endometriosis such as inflammatory markers, estrogen receptors, growth factors responsible for the angiogenesis of endometrial tissues, etc. The herb Epigallocatechin Gallate (EGCG) was shown to reduce inflammation by suppressing the release of NF-κB along with mitogen-activated protein kinase 1 (MAPK-1) in the endometrial lesions [27]. Similarly, Curcumin and Ginsenoside Rg3 can inhibit the effects of vascular endothelial-derived growth factor (VEGF) necessary for angiogenesis of the endometrial lining in rats suggesting their potency in endometriosis [28]. Furthermore, Curcumin, Ginsenoside Rg3, Resveratrol, Apegenin, and β-Caryophyllene decrease the levels of IL-6, IL-8, and NF-κB in human endometrial stromal cells. Also, Puerarin, Resveratrol, Curcumin, Ginsenoside Rg3, Genistein, and Herbal decoction method have been shown to attach to estrogenic receptors and compete with 17β-estradiol (E2), thereby inhibiting the production of estrogen. Their probable mechanism in endometriosis is to suppress the vascularization of the endometrial cells by targeting estrogen as it blocks the synthesis of estrogen through repression of the expression of aromatase cytochrome P450 (p450arom) in the endothelial stromal tissues as shown in Fig. 3 [29–32].

Fig. 3 [Images not available. See PDF.]

An illustration representing the various sites at which herbs act in lowering the progression of endometriosis. Several traditional herbs possess many properties through which they can influence the progression of endometriosis such as Anti-oxidative (Ginsenoside, Apigenin, β-Caryophyllene), Anti-inflammatory (EGCG, Ginsenoside Rg3, Xanthohumol, Geinstein), Hormone regulatory effects (Puerin, Resveratrol, Genistein), and Anti-apoptotic effects (Ginsenoside). These herbs can work individually or in combination to treat the underlying causes of Endometriosis

The present review compares conventional and herbal therapy based on literature evidences with the objective to identify prospective novel targets in the treatment of endometriosis. A literature search was conducted through an electronic database (PubMed, Medline, Clinicaltrials.gov, etc.) up to September 2023. The following key words were entered for search strategy: Endometriosis, Conventional therapy, GnRH agonist, Estrogens, Progestins, Selective estrogen receptor modulators (SERMs), Non-steroidal anti-inflammatory drugs (NSAIDs), Herbal therapy, Traditional medicine, Genistein, Curcumin, Ginsenoside Rg3, Herbal decoction method, Puerarin, Resveratrol, etc. Literature sources were assessed based on this search strategy and included into the present review. Additionally, it gives insights into the various conventional therapy used for endometriosis along with their limitations and whether herbal medicine have any benefits over conventional therapies.

Main Text

Conventional therapy for endometriosis

Estrogen–progestins and progestins

Also termed combined hormonal contraceptives (CHCs), they are given to the patients in the form of combined pills containing both estrogen and progesterone to balance the levels of female reproductive hormones. These pills are being administered to patients with endometriosis for a long time [33]. Both hormones possess their individual properties in lowering the damaging effects of endometriosis on the female body. Estradiol has the unique characteristics of anti-apoptosis and anti-inflammatory properties which can lower the amount of the inflammation process occurring at the endometrium, while progesterone also possesses anti-inflammatory properties but promotes apoptosis. Estradiol has the unique characteristics of anti-apoptosis and anti-inflammatory properties which can lower the amount of the inflammation process occurring at the endometrium while progesterone also possesses anti-inflammatory properties but promotes apoptosis [34]. Moreover, they lessen or stop menstruation entirely, which limits the number of endometrial cells that reflux into the tubules. These pills contain a higher level of progestin while having a low level of estrogen. They are involved with regularizing the menstrual cycle. As a result of this, it would lead to a delay in the inflammation process and oxidative stress occurring within the endometrial layer [35]. There have been several investigations that have been conducted in this context, and it has been reported that about two-thirds of the female population have benefitted from estrogen–progestin therapy and their dysmenorrhea also got corrected [33, 36–38]. Certain examples of progesterone include medroxyprogesterone, norethisterone acetate, desogestrel, etc. [39].

Gonadotrophin-releasing hormone (GnRH) agonists and antagonists

The mechanism behind GnRH analogues is that they cause the pituitary gonadotrophs to be stimulated and further promote the release of follicle-stimulating hormone (FSH), luteinizing hormone (LH), etc., thereby maintaining the normal female reproductive system function and the endometrial lining [40]. An effective strategy to combat endometriosis includes the withdrawal of the hormone (estrogen) which can be provided by administering GnRH agonist. However, a higher estrogen withdrawal may result in unpredicted adverse events including bone density loss, altered mental status, and risk for cardiovascular disorders which can lead to osteoporosis [33, 41]. Elagolix is a common drug included within the category of GnRH agonists and is used for endometriosis and it has shown significant results under clinical investigations [42]. The utilization of GnRH antagonists in a similar manner to GnRH agonists has also been evaluated in endometriosis. On evaluation, it was observed that treatment with GnRH antagonists like Cetrorelix, Abarelix, and Ozarelix can ensure the successful inhibition of gonadotrophins while also maintaining the levels of estrogen in the body. Due to this, the adverse events noted with GnRH agonists can be reduced along with the progression of the disease [41]. Hence, endometriosis now has a new avenue for medical treatment due to the administration of Cetrorelix which is an GnRH antagonist.

Progestins

Another promising avenue for the treatment of endometriosis includes therapy with progesterone-only pills which are available in the market in several dosage forms such as transdermal patches, oral pills, intrauterine devices, etc. They have been associated with alleviation in pain and irregularity in menses along with limiting the size of the endometrial lesion [43]. They may act through various mechanisms such as inhibiting angiogenesis around the

endometrial lining, inhibiting aromatase enzyme, catalyzing anovulation, modulating estrogen receptors, and decreasing the expression of 17β-HSD1 (hydroxysteroid dehydrogenase) [44]. Examples of progestins include medroxyprogesterone acetate, norethindrone, cyproterone acetate, lynesterole, etc. [45, 46]

Selective estrogen receptor modulators (SERMs) and selective progesterone receptor modulators (SPRMs)

These agents can attach themselves to estrogen or progesterone receptors and modulate their function resulting in modulating their signaling pathway. Due to this, the menstrual cycle gets restored due to regained balance between the estrogen and progesterone hormone levels. Certain examples of SERMs include tamoxifen, raloxifene, bazedoxifene, etc., while of SPRMs include mifepristone, asoprisnil, lonaprisan, etc. [43, 47, 48]. However, there is no hormonal therapy for endometriosis that is free from adverse events and a therapy should be designed in such a way that it doesn't influence the normal menstrual cycle of the body in any way and subsequently leads to endometrial lesion size reduction thereby decreasing the inflammation process occurring within it.

Non-steroidal anti-inflammatory drugs

As discussed above, inflammation is an important constituent in the development of endometriosis due to the release of prostaglandins and this ultimately leads to pain in the patient [49]. Due to this complaint of pain experienced by the patients, NSAIDs can be prescribed as a supplemental therapy to relieve the patients from their pain symptoms [46]. Drugs such as mefenamic acid, naproxen sodium, ketoprofen, and ibuprofen at doses of 400 to 600 mg in the form of oral tablets for the duration of 6 to 9 months [19, 50].

Figure 4 shows a brief timeline for the development of various drugs used as conventional therapies to treat endometriosis along with their mechanisms of action.

Fig. 4 [Images not available. See PDF.]

A diagram representing the timeline for the development of therapeutics employed in endometriosis treatment. This figure shows a timely development of the conventional agents for endometriosis in a progressive manner. The conventional agents comprise Anti-androgens (Danazol), Progestins (Norethindrone acetate, Medroxyprogesterone acetate), GnRH antagonists (Elagolix), GnRH agonists (Gosrelin, Leuprolide, Nafrelin). The year of authorization along with their mechanisms in endometriosis has been provided in the above figure

Shortcomings of conventional therapy in Endometriosis

Conventional therapy for endometriosis has been shown to possess certain drawbacks which include:

Tolerability issues

There have been instances wherein the current treatment options for endometriosis (majorly hormonal agents) have not been successfully tolerated. Estrogen and progestins can be given in the form of combined oral contraceptives (COCs) to balance the hormonal levels of the body. However, they are associated with several adverse events due to which their tolerability decreases in patients [24]. Certain examples of adverse events of estrogens include vaginal bleeding and itching, irregular menstruation, menstrual bleeding, gastric disturbances, hot flushes, mood swings, etc . [33]. Almost all therapeutics of endometriosis have safety and tolerability issues which is why they need to be checked in patients before giving it on a chronic basis [33]. Similar to estrogen, progestins are also associated with adverse events such as hirsutism, acne, mood alterations, and weight gain [43].

Safety and efficacy issues

In some studies, it has been found that when monotherapy is given to patients suffering from endometriosis, there has not been the achievement of successful therapeutic outcomes. In an clinical investigation performed by Giudice et al., they observed that monotherapy with Relugolix is not to be given for chronic use [25]. Additionally, Barbara and colleagues evaluated the safety and efficacy of GnRH agonists in endometriosis and observed that on a long-term basis, GnRH monotherapy cannot be administered to endometriosis patients it was not found to be safe for them due to the development of severe adverse events such as weight gain, hot flushes mood swings which were frequently noticed in patients [33]. In terms of oral progestins, conventional therapy has been linked to the development of various major adverse events such as neoplasms, malignancy, endocrine abnormalities, mental and behavioral disorders, and many more. The SAE incidence rate per 10,000 women-years was 3.67% in long-term

oral progestin users (treatment for more than 15 months) and 4.16% in short-term users (therapy for less than 15 months) which is why patients receiving conventional therapies are more prone to develop adverse drug reactions (ADRs) [51]. Due to the limited efficacy and safety of the conventional treatments, the patient ultimately has to undergo surgical procedures such as laparoscopy, hysterectomy, etc. [45, 52].

Cost issues

Treating endometriosis poses a substantial economic burden on the patients with the costs of therapy. This is the reason why an emphasis must be made on the cost of the therapy given in endometriosis [53]. In a study carried out by Soliman et al. wherein they evaluated the total direct costs and incremental costs between endometriosis patient and non-endometriosis control groups, they found a significant difference in both and concluded that there is a significant incremental cost to be paid in endometriosis treatment as high as $10,002 and $2132 for direct and indirect incremental costs [54]. Also, the adverse events incurred during the course of endometriosis and due to its treatment, such as pelvic pain, infertility, irregular menses, and mood swings add up to the incremental cost that the patient has to pay to deal with these complications [55].

Risk of recurrences

The greatest risk that the currently available treatments pose is the risk of recurrence of the disease due to the limited efficacy of the drugs. In most cases, the reason behind this recurrence is the presence of residual lesions or from de novo cells [23]. It was observed that the recurrence rates of endometriosis were 40–50% at a 5-year interval and 21–23% at the end of 2 years. In addition to this, the precise risk factors leading to the recurrence of endometriosis have not been identified yet [56]. Recurrence of endometriosis has also been observed in post-operative patients who have undergone surgical procedures for endometriosis [56]. Thus, it is necessary to control the recurrences of endometriosis to ensure better therapeutic outcomes in patients.

Clinical trial data of conventional therapy in endometriosis

Completed clinical trials

A list of completed clinical studies and trials for endometriosis patients and their findings are provided in Table 1. Certain examples of landmark clinical trials assessing the potency of conventional therapy in endometriosis are given below.

Table 1. A table showing the various completed clinical trials for conventional therapy in endometriosis

Phase	Study design	No of participants	Eligibility criteria	Arms of the study	Study findings	Inference	Study

Phase IV	A randomized, parallel, open-label study to determine whether endometrial implantation markers predict embryo transfer fertilization outcomes in vitro in subjects already administered leuprolide acetate	37 participants	Infertility patients, diagnosis of endometriosis patients, patients who have regular menses, normal ovarian reserve testing	Intervention: Leuprolide acetate in depot suspension 3.75 mg intramuscular every 28 days	The study found that there were no significant differences for outcomes such as rate of fertilization, stimulation of gonadotrophin hormones, etc. A high rate of fertilization rates was observed in the group of patients who were administered GnRH regimen which led to a larger frequency of implantations within them	It was concluded from this study that GnRH agonist administration in endometriosis can lead to an increase in the rates of pregnancies in comparison to the conventional ovarian stimulation techniques	Surrey et al. [58]
Phase II	A randomized parallel-assignment study to observe the efficacy of hormonal therapy in combination with GnRH agonist in patients suffering from endometriosis	53 participants	Women aged 13–22 years, body weight between 18 and 30 kg/m^2, surgical diagnosis of endometriosis, willing to comply with study requirements	Intervention: Norethindrone acetate 5 mg orally + Conjugated equine estrogens 0.625 mg orally Control: Norethindrone acetate 5 mg + placebo capsule 1 pill daily	At 12 months, the intervention group increased the bone mineral density (BMD) and the overall mineral content of the body, while the control group did not show these outcomes. Quality-of-life assessments showed greater improvements in physical functioning with the interventional group. There were no significant adverse events were reported	Add-back therapy with norethindrone acetate led to preservation of the skeletal health in endometriosis patients, the combination of norethindrone acetate and conjugated equine estrogens being led to higher elevations in the BMD of the body. The therapy was safe and effective, with no tolerability issues	DiVasta et al. [59]

Phase II	A randomized, parallel-assignment pilot study to determine the effect of dopamine receptor agonist therapy for pain relief in women suffering from endometriosis	10 participants	Women with confirmed case of endometriosis, age between 15 and 40 years	Intervention 1: Cabergoline (0.5 mg PO two times a week for 6 months duration) Intervention 2: Norethindrone acetate (5 mg po daily for 6 months)	It was observed from this study that many subjects taking cabergoline experienced a decrease in pain scores and improvement in pain complaints compared to subjects treated with Norethindrone acetate. Cabergoline was also well tolerated by the patients	It was concluded that Cabergoline could be a safe and effective therapeutic alternative for chronic pain in endometriosis resistant to standard care, according to a pilot study. Larger randomized trials are needed to confirm these findings	DiVasta et al. [60]
Phase not applicable	A prospective, randomized, parallel-assignment clinical trial to study the effect of administration of GnRH agonist before in vitro fertilization to observe fertilization rate and pregnancy rate in endometriosis patients	180 participants	Patients with infertility, endometriosis stage 1	Intervention: Leuprolide (single injection of 3.75 mg every 28 days) Procedure: In vitro fertilization (IVF)	The use of GnRH agonist resulted in a decrease in Follicular fluid cytokines in women compared to those who did not receive this regimen. However, no significant improvement took place in terms of embryo quality, rate of implantation, or rate of pregnancy	Low follicular fluid cytokine levels along with high rates of implantations were noticed in subjects receiving GnRH agonist for the duration of 3 months, with no significant difference in pregnancy and implantation rate	Kaponis et al. [61]

In a Phase-I randomized, multicentric trial performed to assess the efficacy and safety of Aromatase inhibitors (Anastrozole) and Progestin against Placebo in patients suffering from Endometriosis, 309 participants were recruited (NCT02203331). They were divided into four cohorts, (a) participants receiving Progestin (Levonorgestrel), (b) participants receiving Anastrozole in combination with levonorgestrel, (c) participants receiving leuprolide, and (d) participants receiving Placebo for the duration of 12 weeks. It was observed that all the drugs led to an improvement in the mean duration of endometriosis-associated pelvic pain (EAPP) which led to a reduction in the days that patient presented with pelvic pain [57].

In a similar Phase III study involving 815 participants, the potency of Elagolix was determined in patients suffering from Moderate to Severe endometriosis induced pain. The participants were divided into three cohorts: (a) patients receiving Elagolix 150 mg QD for a duration of 6 months, (b) patients receiving Elagolix 200 mg 4 times in a day for 6 months, and (c) patients receiving Placebo drug for a 6-month duration. It was observed after the treatment duration that Elagolix both lower and higher dose resulted in improvements in endometriosis-associated pelvic pain;

however, adverse events such as hot flushes, increased serum lipid levels, increase in bone mineral density (BMD) was observed. Therefore, it was concluded that conventional therapy, although effective led to the development of adverse events in patients of endometriosis [42].

Suspended or terminated clinical trials

There have been certain instances wherein patients suffering from endometriosis were administered conventional therapy led to the termination or suspension of the clinical trial due to certain complications occurring due to the conventional agents. An example of such trial includes a study in which 50 participants were enrolled who were clinically diagnosed with endometriosis and were divided into two groups: (a) receiving Dienogest 2 mg per day and (b) receiving Levonorgestrel (0.10 mg per day) in combination with ethinyl estradiol (0.02 mg per day) [62] are progestins which reduce endometrial lining thickness thus decreasing the chance of bleeding in patients of endometriosis [63]. The outcomes measured included the change in size of the endometrial lesions from their baseline observed via ultrasound within a time duration of 1 year. It was observed that Busrelin acetate which is a GnRH agonist was equally effective in treating endometriosis as Dienogest which is why these 2 drugs were given optionally to the patients. A significant improvement in the symptoms experienced by the patients and their pain score was observed with patients that were administered Dienogest. It also led to lower reduction in bone mineral density (BMD) as compared to Busrelin acetate. However, compared to Busrelin acetate, a higher instance of genital bleeding and hot flushes within these patients was noticed with Dienogest highlighting its concern. This was the reason due to which this trial had to be suspended to avoid complications in these patients [62]. Due to the safety concerns occurring in conventional agents, it leads to negative therapeutic effects and it has been proposed that they be monitored carefully during treatment and if they pose any risk to the patients, the trial should be terminated immediately. Also, novel drugs which are safer and more tolerable by the patients are required to prevent complications from occurring in them.

Herbal therapy for endometriosis

Epigallocatechin Gallate (EGCG)

Epigallocatechin-3-gallate (EGCG) is the main constituent of green tea due to which it can display its potency in cancer and endometriosis [64]. This herb has already been shown to possess anticancer and anti-oxidative properties. However, its effects in endometriosis were also evaluated by several researchers in endometriosis. It was shown to reduce inflammation by suppressing the release of NF-κB along with mitogen-activated protein kinase 1 (MAPK-1) in the endometrial lesions [55]. However, sufficient investigations have not been carried out as of yet to underline the exact mechanisms of EGCG through which it can cure endometriosis. The preclinical testing of EGCG in endometriosis was done by Ricci and co-workers in which they observed that treatment with EGCG inhibited the development of endometrial lesions along with decreasing the size of their lesions. It was able to modulate cellular proliferation, decrease the blood circulation to and from the endometrial lesions, and enhance the process of apoptosis [65].

Curcumin

It is the active ingredient of commonly occurring turmeric which has anti-inflammatory, anti-oxidative, and anti-proliferative properties [26, 66]. It can also block the actions of vascular endothelial-derived growth factor (VEGF) within the endometrial cells of the rats suggesting its effects as anti-angiogenetic agents as VEGF is crucial for endometrial blood vessels to grow further and proliferate [58]. Furthermore, it is able to decrease the levels of IL-6, IL-8, and NF-κB in human endometrial stromal cells (Fig. 4). Hence due to all these beneficial properties, it has been suggested that curcumin may also be employed as a potential therapeutic agent for endometriosis [26].

Ginsenoside Rg3

This is a Chinese traditional herb and is the active component of ginseng which originates from the plant genus Panax. Preclinical findings have revealed the ability of this herb in reducing the endometrial lesion size in rats [67]. Its main actions include anti-oxidative and anti-inflammatory activities [26, 59]. In addition to this, this herb can also repress the angiogenesis process by inhibiting the VEGF-mediated formation of blood vessels suggesting its efficacy in reducing endometriosis [60]. Furthermore, a study carried out by Huang et al. found that Ginsenoside Rg3

reduced inflammation by repression of NF-κB and TNF-α in ectopic endometrial cells and also modulated apoptosis by regulating the expression of caspase 3 and inhibiting VEGF-mediated angiogenesis [68].

Puerarin

It is the major active ingredient of Gegen which is extracted from the Chinese medical herb Radix puerariae and falls in the category of phytoestrogens but possesses a weak estrogenic effect. They have sown to attach to estrogenic receptors and compete with 17β-estradiol (E2) thereby inhibiting the production of estrogen (Fig. 4). Its probable mechanism in endometriosis is its ability to suppress the vascularization of the endometrial cells by estrogen as it blocks the synthesis of estrogen through repression of the expression of aromatase cytochrome P450 (p450arom) in the endothelial stromal tissues [62–65]. When preclinical analysis was carried out, it suggested that it could influence and inhibit the inflammatory microenvironment of the endometrial tissue in rats [32].

Resveratrol

Resveratrol is also a phytoestrogen that is derived from grapes, wine, peanuts, etc. It has been identified to act against the progression of endometriosis due to its effects of anti-inflammation, via the repression of prostaglandin synthesis along with the modulation of apoptosis [69]. It has the ability to influence the estrogenic receptors (ER1 and 2) and has a mixed mechanism of action i.e., agonist and antagonist [67]. In addition to this, it has also been shown to regulate various pathways associated with cellular maturation and death such as MAPK, protein kinase B (Akt), protein kinase C, and peroxisome proliferator activated receptor-gamma (PPAR-ϒ) [70–72].

Apigenin

Apigenin belongs to the category of flavonoids and is found in parsley, celery, oranges, wheat sources, etc. It possesses, anti-inflammatory, anti-proliferative, and anti-oxidant properties [73, 74]. Suou et al. in a study to undermine the effects of apigenin in endometriosis observed that it reduced inflammation via suppressing protein expression and regulating the levels of IL-8 and TNF-α (Fig. 4) [75]. Recently conducted studies also revealed its effect in acting through binding with the progesterone receptors (PR) behaving as a probable phyto-progestin [76]. It was also shown to correct endometriosis symptoms such as pelvic pain, dysmenorrhea, infertility, etc. [77].

β-Caryophyllene

It belongs to the category of sesquiterpenes and is the active ingredient of essential oils which are derived from spices and food plants and is an effective anti-inflammatory herb in vivo and was found to correct endometrial symptoms and infertility in adult rats [78, 79]. It was shown to mediate the inflammatory response by regulating their markers such as IL-1β, TNF-α, and toll-like receptors-4 (TLR-4) and angiogenesis by VEGF regulation. Recent findings also suggest its ability to block the generation of ROS through the MAPK pathway [74, 80].

Genistein

It is an iso-flavonoid which is extracted from soy. It has strong Phyto-estrogenic actions and has been demonstrated both in vivo and in vitro and has been indicated in the treatment for endometriosis [81]. Genistein was shown to limit the progression of endometrial carcinoma in adult women as it regulated the process of angiogenesis within the endometrium and apoptosis [82]. Additionally, it can modulate the estrogenic receptors (ER) to regulate the release of estrogen and the process of angiogenesis occurring due to it. Furthermore, it can regulate inflammation by mediating the release of IL-6 and TNF-α [81].

Xanthohumol

It is the active ingredient of *Humulus lupulus* L. and possesses a variety of actions including anti-angiogenetic, anti-inflammatory, and anti-proliferative effects. Inflammatory mediators such as NK-κβ, IL-1, Akt etc. can be regulated due to this herb [74, 83].

Herbal decoction method

These methods are commonly employed in China to treat a variety of gynecological disorders such as endometriosis since 1983 [84]. Certain examples of these methods include Qu Yi Kang (QYK), Yi Wei San (YWS), Xiaochaihu decoction (XCHD), Huoxue Xiaoyi (HX), and Xuefu Zhuyu (XZD) decoction methods based on their inventors [84]. Investigations into this have shown that XZD may relieve the symptoms of endometriosis, such as dysmenorrhea and ectopic lesions, and improve the issues of infertility in women and has resulted in greater efficacy of about 90%

in the past times [84, 85]. Furthermore, XCHD has been shown to reduce the levels of estradiol (E2) levels, aromatase enzymes and also modulate the inflammatory mediator synthesis through the blockade of the COX-2 enzyme [85]. Other methods of decoction include Cai Shi Nei Yi Fang, Neiyi Zhitong, Huazhuo Jiedu Huoxue, and Juan Tong Yin etc.[86].

In a study carried out by Ding et al. involving 80 patients wherein they compared the effects of Chinese traditional medicine and hormonal therapy (12.5 mg mifepristone orally each day) for Endometriosis. They observed that Chinese traditional medicine had a greater pregnancy rate (52.5%, 21/40) than hormonal therapy (37.5%, 15/40) within a 12-month period of follow-up and equivalent therapeutic effect to hormonal therapy suggesting a better potency of herbal therapy. Moreover, there were no SAE's associated with herbal therapy and the results of renal and hepatic profile parameters proved that herbal therapy was well tolerated by all the patients [87]. This proves the long-standing efficacy as well as safety of herbal medicine to treat Endometriosis.

Additionally, Zhao et al. carried out a study in which they compared the effects of Chinese herbal medicine and western medicine by the means of a randomized controlled trial in 208 patients (106 in Chinese herbal medicine group and 102 in the western medicine group). Patients in the western medicine group were treated with a GnRH agonist or gestrinone, whereas patients in the Chinese medicine group were treated with agents including Modified Guifu Decoction, Radix Aconiti lateralis Preparata, Ramulus Cinnamomi, Radix Linderae, Rhizoma Sparganii, Rhizoma Curcumae, Spina Gleditsia, Radix Salviae Miltiorrhizae, etc. For the patients in the Chinese medicine group, the mean time following surgery to achieve the first pregnancy was significantly shorter than for the patients in the Western medicine group ($t=-2.09$; $P=0.04$). A statistically significant difference existed between the 2 groups in terms of safety observed as after the treatment follow-up period, the western medicine group had increased ADRs such as fever, sweating, colpoxerosis, hypaphrodisia, weight gain, insomnia, irregular bleeding, headache, acne, and bone pain, while the patients in the Chinese medicine group only complained of occasional stomach pain that was immediately lowered after modifying the herbal remedies and dosages (83.3% vs. 9.4%, $P<0.01$) [88].

Herbal combination therapies were associated with improving pregnancy rates, reducing adverse events and inhibiting the growth of endometrial tissues in addition to decreasing inflammation in patients. Therefore, combination therapy comprising of certain herbs may prove to be beneficial in treating Endometriosis compared to conventional therapies. Furthermore, they have shown improvements in sub-populations of endometriosis patients including infertility patients, pre-menopausal syndromes, menstrual cycle irregularities, autoimmune disorders, etc. in terms of better hormonal balance restorations, ovulation induction, lowering inflammation proving their efficacy. The major herbal agents discussed in the present review such as curcumin, EGCG, Genistein, β-Caryophyllene, etc., have shown limited adverse events in comparison with conventional therapy in terms of gastric disturbances including diarrhea, stomach upset, gastric irritation, etc. which can be easily managed with supportive treatment thereby enhancing their safety profile. In addition to this, their low cost of therapy adds to their benefit in treating Endometriosis.

Herbal therapy has evolved over the recent times due to their offered advantages in the studies discussed above and other factors such as low cost, ease of convenience of preparing, reduced side effects and increased bioavailability. However, several additional investigations and clinical trials need to be conducted to evaluate efficacy and safety of different combinations of herbal therapies in Endometriosis which may also lead to the discovery of novel therapeutics to combat the disease effectively.

Preclinical and clinical trial data of Herbal novel therapy in endometriosis

The current ongoing preclinical trials in which the investigations into the effects of herbal medicine to treat endometriosis are carried out are highlighted in Table 2.

Table 2. A table showing the animal preclinical trials conducted to evaluate the potency of herbal medicine in endometriosis

Sr. No	Study objectives	Study design	Arms of the study	Study findings	Study
1	A study in order to find the association between the Ginsenoside Rg3 effect on endometrial growth and the PI3K/Akt/mTOR signaling pathway modulated by VEGFR-2	The rats were allocated on the basis of randomization into 5 groups which were treated with ginsenoside Rg3 and sacrificed 21 days post drug treatment. Measurement of the endometrial volume was carried out and the inhibitory rate was calculated. Serum estradiol (E2) and progesterone (P) levels were analyzed by Electrochemiluminescence Immunoassay (ECLI). Using immunohistochemical techniques, the protein expression of VEGF and VEGFR-2 was evaluated within the endometrium	Intervention 1: Ginsenoside Rg3 (5 mg/kgBW/d) Intervention 2: Ginsenoside Rg3 (10 mg/kgBW/d) Intervention 3: Gestrinone group (0.5 mg/kgBW/d) Intervention 4: Control group (10 mL/kg BW/d of 0.5% Carboxymethyl cellulose sodium) CMC-Na Intervention 5: Ovariectomized group (10 mL/kgBW/d of 0.5%CMC-Na)	It was observed that a dose-dependent suppression of endometrium size in rats in comparison to control group occurred. A down-regulation of the expression of VEGF and VEGFR-2 was also noticed in Ginsenoside Rg3 group	Cao et al. [67]
2	A study to evaluate the effect of EGCG in mice-model of endometriosis	The potential for EGCG as an anti-angiogenesis agent was investigated in mice suffering from endometriosis. Transplantation of endometrium was done in mice and they were divided into 3 groups to receive treatment for 4 weeks. Endometrial growth was measured through non-invasive in vivo imaging (IVIS). Post-treatment, the bioavailability, anti-oxidative and anti-angiogenesis effects were measured	Intervention 1: Dulbecco phosphate buffered saline Intervention 2: Vitamin E (20 mg/kg) Intervention 3: EGCG (50 mg/kg)	A significant reduction in the endometrial lesion size was observed in the group treated with EGCG from 2nd to 4th week of drug treatment. However, they failed to show effect on Ovarian follicles and uterine endometrial glands	Xu et al. [89]

3	A study to investigate the potency of puerarin in endometriosis (EMT) model rats and to find the probable mechanisms of action	The animals were allocated into 5 groups and endometriosis was induced surgically by auto-transplantation of endometrial tissues. Serum estradiol (E2) and prostaglandin E2 (PGE2) levels were analyzed and the dose of administration was calculated. Genes and proteins of the endometrial tissues were analyzed by polymerase chain reaction (PCR) and immunohistochemistry (IHC). Based on the results, appropriate inferences were made	Puerarin and Raloxifene (RLX) both mixed with CMC prorata after which the animals were allocated into five groups were respectively administered drug treatment for 4 weeks Intervention 1: low-dose group (0.1% CMC and 5 mg/kg puerarin) Intervention 2: 0.1% CMC and 20 mg/kg puerarin) Intervention 3: 0.1% CMC and 80 mg/kg puerarin) Intervention 4: positive control group (Raloxifene hydrochloride) RLX 10 mg/kg Intervention 5: Control group (CMC)	It was observed that Puerarin reduced the concentrations of E2 and PGE2 and also hindered the maturation of endometrium tissues by inhibiting the expression of aromatase cytochrome P450 (p450arom) and cyclooxygenase-2 (COX-2). Also, it modulated the metabolism of E2 by controlling the expression of the 17β-hydroxysteroid-2 (17β-hsd-2) enzyme of the endometrial tissues	Surrey et al. [58]
4	A clinical study to assess the effect of β-caryophyllene on endometriosis along with fertility status in adult female rats along with their roles in reproduction	Fragments of endometrium were implanted in the peritoneal cavity of the animals to induce endometriosis within them. Their growth was measured from baseline and after 4 weeks. Allocation was carried out of the animals into 2 groups and they were given drug therapy for a duration of 21 days	Intervention: β-caryophyllene (10 mg/kg or 30 mg/kg) Control: Vehicle	It was observed that β-Caryophyllene was able to hinder the maturation of endometriotic tissues 52.5% in rats compared with controls whereas β-caryophyllene led to apoptosis in the epithelium of the endometrial lesions	Abbas et al. [90]

Preclinical trials

Completed clinical trials

There are a relatively limited number of clinical studies carried out to evaluate the safety as well as efficacy of herbal medicine against endometriosis. Phase-I randomized, placebo-controlled trial was carried out in 185 participants to gain idea on the potency of green tea extract in endometriosis in which the effects of green tea or Epigallocatechin-3-gallate were compared with a placebo in order to assess its response. The investigators found that green tea

extract displayed anti-angiogenetic, anti-fibrotic, and anti-proliferative properties which led to beneficial outcomes in lowering the progression of endometriosis and was tolerable by the patients suggesting its importance [91]. Similarly, a relatively same type of clinical trial was performed to evaluate the potency of garlic in endometriosis. A total of 120 participants were recruited and one cohort was administered garlic tablets, while the other was given a placebo after which the response toward therapy was observed. It was noted that the patients that were receiving garlic therapy showed improvements in pain and statistical tests also showed its significance which concluded that herbal therapy can provide symptomatic relief also in addition to preventing the course of progression of endometriosis [92].

Ongoing clinical trials

Table 3 enlists the various investigations that are presently ongoing to investigate the effects of herbal therapy in endometriosis.

Table 3. Currently ongoing clinical studies for the evaluation of herbal therapy in endometriosis

NCT number, current phase	Study design	Eligibility criteria	Arms of the study	Primary endpoints
NCT04493476, Phase II	A double-blind, prospective and placebo-regulated clinical trial to assess the response of combination therapy of Chinese herbal medicine and curcumin to lower the symptoms of endometriosis	Women having a confirmed diagnosis of endometriosis, women aged 18–45 years (reproductive age), no allergy to the ingredients of the intervention or the control	Intervention: Daily dietary dosing of Chinese medicine and curcumin given in the form of 800 mg capsules Control: Placebo (Invo capsules given in daily dosing)	The overall benefit in the symptoms of the disease
NCT03016039	A randomized, parallel assignment study of curcumin supplementation for endometriosis	Age above 18, patient with a diagnosis of pelvic inflammatory disease/Tubo ovarian abscess, surgical wound infection, endometritis	Intervention: Curcumin supplementation	Change in the Levels of C-reactive protein, change in the levels of white blood cells
NCT03875261, Phase II	A randomized, single-group assignment study to examine the response of the effect of Cannabinoid (CBD) on pain experienced by endometriosis patients	Women falling in the age group of 18 and 40, having a confirmed diagnosis of endometriosis with clinical investigations, suffering from symptoms of pain, dysmenorrhea, etc.	Intervention: Participants administered cannabinoid derivates dosing between 1 and 12 puffs. Each puff contained 2–7 mg of delta-9-tetrahydrocannabinolan d 2–5 mg of cannabidiol	Pressure threshold in hypogastrium that induces pain

NCT02676713, Phase II	A randomized, prospective, multicentric study to evaluate the efficacy of Decoction (Chinese herbal medicine to treat infertility) in endometriosis	Women having a clinical diagnosis of endometriosis, endometriosis fertility index (EFI) score greater than 4 points, firstly undergoing laparoscopic surgery, the female of reproductive age (18–45 years)	Intervention: Decoction (Bupleurum 10 g, Cyperus 10 g, Salvia miltiorrhiza 20 g, Red peony 10 g, etc.) Placebo: Combination of maltodextrin, lactose, edible pigment, and taste masking agent	Pregnancy rate to an extent of six menstrual cycles
NCT04150406, Current phase not given	A multicentric, randomized clinical trial to evaluate the potency of Flexofytol in endometriosis	Women of reproductive age (18–51 years), diagnosed with endometriosis, moderate to severe pelvic pain	Intervention: Flexofytol (Curcumin 42 mg 2 capsules administered for 4 months) Control: Placebo	Alteration in the baseline pain score
NCT number not given	A randomized, multicentric clinical trial to evaluate the effect of Ashokarishta, Ashwagandha Churna, and Praval Pishti in patients suffering from menopausal syndrome	Females of age 40–55 years, suffering from amenorrhea for a period of greater than 12 months, were willing to comply with the study requirements, providing written consent to be included in the study	Intervention: *Ashokarishta* (25 mL *daily*), *Ashwagandha* (3 g *twice daily with milk*), *Praval Pishti* (250 mg *twice daily*) Control: Placebo	Improvement in the Menopausal rating scale (MRS), Incidences of adverse events (AEs)

Future prospects and opportunities

Up till now, the treatments under existence only aim to regulate the hormonal levels in the body and provide relief from symptoms of endometriosis such as pain, dryness, infertility, etc. However, no specific treatments are available that can cure endometriosis completely or reduce its course of progression into its more severe forms. They only aim to suppress ovulation or alter the levels of hormones such as estrogen and progesterone in the body. Thus, there is a need to develop individualized regimens pertaining to specific patients to lower the incidences and recurrences of endometriosis [93]. Therefore, herbal medicines can prove to be a means to develop novel therapeutics to be given to endometriosis patients as many drugs have shown potent effects in decreasing the size of endometrial lesions and reducing inflammation within them [81]. Herbal drugs are pleiotropic agents meaning they have multiple mechanisms of actions such as anti-oxidative anti-inflammatory, anti-angiogenetic, estrogen-modulating, analgesic, etc., which could resolve pelvic pain complaints of the patient along with lowering endometrial inflammation by blocking the release of inflammatory markers and protection from ROS species. By this, they can act as curative as well as symptomatic relief-providing agents [94]. Also, the improved safety and tolerability profile along with reduced cost of therapy makes them beneficial candidates over the conventionally available drugs presently in the market [84, 95]. Furthermore, recent data suggests that medicinal cannabis as a dietary intervention may have effects in treating Endometriosis as it acts through various mechanisms such as suppressing inflammation, alleviating bloating, and acting as a painkiller. However, it has not fully been studied and additional research into this can be fruitful [96, 97]. Herbal therapy can also be used to induce pregnancy in an individual who

cannot conceive due to endometriosis and can be utilized as a safer approach compared to conventionally existing drugs with almost no harm to the individual or the fetus [98]. Only further and larger number of studies need to be conducted in a similar manner to evaluate the extent of the benefit that herbal therapy provides or reducing the likelihood of developing endometriosis and curing it completely. Herbal therapy trials were limited in number, while trials for conventional therapy were found to be widely available. Therefore, when the results of trials for herbal therapy become available, they will strengthen the point discussed in this manuscript regarding the comparison of safety and efficacy of conventional and herbal medicine.

Conclusion

The currently existing conventional therapies are only aimed at inhibiting the hormonal parameters of the patient and providing symptomatic relief from symptoms such as pelvic pain, vaginal dryness, etc., but cannot completely cure the disease. Conventional therapy also possesses several other limitations such as increased cost of therapy, risk of recurrence of endometriosis, limited safety and efficacy profile, and tolerability issues. On the other hand, herbal therapies extracted from natural sources have shown promising effects in delaying the course of endometriosis progression and have better effects compared to conventional therapy along with improved tolerability and almost no adverse events to the patients making them a perfect candidate for the treatment of endometriosis concerning to efficacy, safety, and tolerability. The comprehensive studies data indicate that conventional therapy results in unsatisfactory therapeutic outcomes, disease recurrence, and an increase in the development of ADRs, whereas herbal combination therapy acts through multiple mechanisms, resulting in better clinical therapeutic outcomes and less ADRs, highlighting their benefit in terms of efficacy and safety in treating endometriosis. However, the number of preclinical and clinical trials investigations into this context is limited which is why additional studies are crucial to identify the potency of herbal drugs treating endometriosis effectively.

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Author contributions

BD, SB, HR and MS contributed to manuscript first draft preparation and subsequent editing, literature and data collection, data analysis, figures and diagram conception as well as designing and writing the manuscript. AK and NP contributed to topic conception, design of content and skeleton, manuscript draft review and editing, figures and diagram conception, overall monitoring, and guidance throughout the study duration. All authors have read and approved the final manuscript.

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Availability of data and materials

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Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

The authors declare that they have no competing interests.

Abbreviations

17β-HSD1
17-Beta hydroxysteroid dehydrogenase
ADRs
Adverse drug reactions
AEs
Adverse events
Akt
Protein kinase B
AMH
Anti-mullein hormone
BMD
Bone mineral density
BPA
Bisphenol-A
CBD
Cannabinoid
CHCs
Combined hormonal contraceptives
CMC-Na
Carboxymethyl cellulose sodium
COCs
Combined oral contraceptives
CRP
C-reactive protein
E2
17β-Estradiol
ECLI
Electrochemiluminescence Immunoassay
EFI
Endometriosis fertility index
EGCG
Epigallocatechin Gallate
EMT
Endometriosis model rats
ER1 and ER2
Estrogenic receptors 1 and 2
FSH
Follicle-stimulating hormone
GnRH
Gonadotrophin-releasing hormone
HPA
Hypothalamic-pituitary axis
HX
Huoxue Xiaoyi

IHC
Immunohistochemistry
IL
Interleukin
IVF
In vitro fertilization
IVIS
Non-invasive in vivo imaging
LH
Luteinizing hormone
MAPK-1
Mitogen-activated protein kinase-1
MRS
Menopausal rating scale
NF-κβ
Nuclear factor-kappa beta
NSAIDs
Non-steroidal anti-inflammatory drugs
P450arom
Aromatase cytochrome P450
PCR
Polymerase chain reaction
PGE2
Prostaglandin E2
PKC
Protein kinase C
PPAR-ϒ
Peroxisome proliferator activated receptor-gamma
PR
Progesterone receptors
QOL
Quality of life
QYK
Qu Yi Kang
RLX
Raloxifene hydrochloride
SAE
Serious adverse events
SERMs
Selective estrogen receptor modulators
SPRMs
Selective progesterone receptor modulators
TLR-4
Toll-like receptors-4
TNF-α
Tumor necrosis factor-alpha
USA

United States of America
VEGF
Vascular endothelial-derived growth factor
XCHD
Xiaochaihu decoction
XZD
Xuefu Zhuyu
YWS
Yi Wei San

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Prognostic values and immune infiltration of KLF15, AQP7, AGPAT9 in glioma and glioblastoma

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ABSTRACT (ENGLISH)

Backgrounds

The overall survival of patients with lower-grade gliomas and glioblastoma varies greatly. No reliable or existing procedures can accurately forecast survival and prognostic biomarkers for early diagnosis in glioma and glioblastoma. However, investigations are progressing in immunotherapy, tumor purity, and tumor microenvironment which may be therapeutic targets for glioma and glioblastoma.

Results

This study indicated the possible prognostic signatures that can be used to identify immune-related prognostic biomarkers in the prediction of the survival of low-grade glioma (LGG) patients which may be a possible therapeutic target. In addition, the Kaplan–Meier plot, ESTIMATE algorithm, and TIMER 2.0 analysis indicated that Krüppel-like factor 15 (KLF15) $p=0.030$, Aquaporin 7 (AQP7) $p=0.001$, and Human 1-acylglycerol-3-phosphate O-acyltransferase 9 (AGPAT9) $p=0.005$ are significantly associated in glioma. Hence, they may be possible prognostic biomarkers in glioma. Meanwhile, in the glioblastoma, only KLF15 has a significant association with glioblastoma ($p=0.025$). Stromal and immune scores of gliomas were determined from transcriptomic profiles of LGG cohort from TCGA (The Cancer Genome Atlas) using the ESTIMATE (Estimation of Stromal and Immune cells in Malignant Tumours using Expression data algorithm). The immune infiltration of the KLF15, AQP7, and AGPAT9 for low-grade glioma and glioblastoma was determined using TIMER immune 2.0 which indicates correlation with tumor purity for KLF15, AQP7, and AGPAT9, but only KLF15 and AGPAT9 are significantly associated in both glioma and glioblastoma, respectively.

Conclusions

These results highlight the significance of microenvironment monitoring, analysis of glioma and glioblastoma prognosis, and targeted immunotherapy. To our knowledge, this is the first time to investigate an analysis that revealed that KLF15, AQP7, and AGPAT9 may be important prognostic biomarkers for patients with glioma and KLF15 for patients with glioblastoma. Meanwhile, KLF15 and AGPAT9 are significantly associated in both glioma and glioblastoma, respectively, for tumor purity.

FULL TEXT

Backgrounds

Glioma is the most prevalent primary malignant brain tumor and can be divided into distinct categories. According to the WHO grading system, it can be categorized into astrocytomas, diffuse low-grade, intermediate-grade, oligodendrogliomas, and mixed oligoastrocytomas [1–3]. The most frequent treatment for glioma is surgical resection in combination with chemoradiotherapy. Due to its highly invasive nature, surgical resection may be difficult to treat, and residual tumor could lead to malignant progressions and even reoccurrence in the long run [4]. Although the classification of low-grade glioma (LGG) is recognized worldwide, it may not adequately predict its survival rate; however, clinicians tend to depend on genetic classifications to guide its treatment [5–7]. The survival outcomes of LGG vary widely among different patients [8]. However, some LGGs stay stable for a long period while some progress into glioblastoma [9–11]. Notwithstanding more investigations are required to elucidate whether gliomas progress to glioblastoma. Gliomas account for approximately 75% of primary cancerous brain tumors [12]. In the USA, about 13,000 people die and 18,000 new cases of CNS tumors and malignant brain tumors arise each year

due to glioma prognosis and occurrence [13, 14], hence a need for therapeutics and early diagnosis of the diseases [15].

Glioma is a cancerous tumor of the central nervous system that begins in the glial cells that surround and nourish the brain's neurons [16]. In the treatment of gliomas, great progress has been made in genomic, transcriptomic, and epigenetic profiling [17–21]. Astrocytoma, ependymoma, glioblastoma, and oligodendroglioma are some of the different kinds of glioma [22]. Glioblastoma (GBM), the most common and aggressive primary kind of malignant brain tumor, is assumed to have started in glial cells [23–25]. Scientific evidence, on the other hand, reveals that GBM could have developed from a variety of cells with neural stem cell characteristics [26, 27]. GBM is slightly more common in men than in women, as well as in Caucasians and other white races and ethnicities [28, 29]. GBM is usually found in the supratentorial region of the brain such as hypothalamus, pituitary gland, pineal, and the four lobes: temporal, parietal, frontal, and occipital lobes, with cerebellum being a rare exception [30, 31]. Sixty-one percent of all primary gliomas are found in the brain's four lobes: 20% in the temporal lobe, 25% in the frontal lobe, 3% in the occipital lobe, and 13% in the parietal lobe [32]. Glioblastomas are divided into primary and secondary subtypes that originate along different genetic routes and affect individuals of various ages [33, 34]. Quite recently, glioblastoma with oligodendroglioma component is an uncommon subtype of glioblastoma that features certain parts that resemble anaplastic oligodendroglioma, according to the WHO [35, 36].

In clinical practices, mutated genes such as isocitrate dehydrogenase 1 (IDHI), IDH2, tumor protein 53 (TP53), epidermal growth factor receptor (EGFR), and alpha-thalassemia/mental retardation, X-linked (ATRX) are factors for the prognosis of patients with LGG [37–39]. Some other biomarkers, including 1p/19q codeletion and methylguanine methyltransferase (MGMT) promoter methylation, are also well-recognized and essential prognostic factors for LGGs [40–42]. Sometimes, these genetic factors fail to indicate accurate survival outcomes [43, 44]. Hence, further investigations are required to elucidate the functions and the mechanisms of the prognostic signatures.

Several studies have shown that cancer recurrence and progression are caused not only by the tumor's underlying genetic changes but also by tumor microenvironment (TME) [45–47]. The TME is basically composed of numerous cytokines, extracellular matrix molecules, immune cells, chemokines, fluids, and stromal cells [23, 48, 49]. The cells found in the TME reflect the evolutionary nature of cancer and together, promotes the tumor immune escape, tumor growth, and metastasis [50, 51]. Cancer researchers are not vividly aware of the impact of the TME on immune response or tumor progressions although multiple genetic mutations increase the prevalence of cancer [52]. TME can induce metabolic stress on immune cell infiltration thereby causing local immunosuppression and limited reinvigoration of antitumor immunity [53, 54]. However, having an in-depth understanding of the epigenetic, molecular composition, and function of the TME is essential to manage and treat cancer progressions, recurrence, and immune response [55–57]. Integrating multiple gene biomarkers instead of a single model would improve the accuracy of the prediction significantly [58–61].

The survival of glioma patients has received so much research and discovery in the aspect of neurosurgery, radiotherapy, and chemotherapy. However, a lot of challenges of glioma are yet to be solved. Currently, immunotherapy has unveiled possible therapy for cancer [54, 62, 63]. Investigations are currently going on in the area of immunotherapy, but there is still need for efficient molecular biomarkers to differentiate patients with possible sensitivity to immunotherapy [64, 65]. Therefore, it is very crucial to identify immune-related prognostic biomarkers which may be a possible therapeutic target and may be utilized for immunotherapy in patients.

Taken together, differential expressed genes (DEGs) using an immune stromal score in glioma and glioblastoma, transcriptional microarray of glioma cases from multiple TCGA cohorts were investigated to predict the survival of LGG and GBM patients. The following prognostic signatures, such as KLF15, AQP7, and AGPAT9, were used in this investigation to determine whether they have a significant association with glioma and glioblastoma using TCGA and the immune infiltration was unveiled for precise immunotherapy. To our knowledge, this is the first time to use these signatures for glioma and glioblastoma, hence unveiling prognostic biomarker and immune infiltration.

Methods

In this investigation, utilization of the Kaplan–Meier plots using Xena bower (http://xena.ucsc.edu/), ESTIMATE

algorithm (Estimation of Stromal and Immune cells in Malignant Tumours using Expression), Timer 2.0 (http://timer.comp-genomics.org/timer/), and The Cancer Genome Atlas (TCGA) database were used to unveil the prognostic signature and immune infiltration of glioma and glioblastoma analysis.

Bioinformatics approaches were applied to integrate copy number variations and differential expressed genes of low-grade glioma. The immune cell proportion of the prognostic signatures, such as KLF15, AQP7, and AGPAT9, were determined using TIMER immune 2.0. In TIMER, the Gene module was used to identify the relationship between tumor gene expression and immune infiltration in low-grade glioma and glioblastoma. Stromal and immune scores of gliomas were estimated in transcriptomic profiles of LGG cohort from TCGA using the ESTIMATE. One hundred entries of TCGA cohort were entered and used to plot the graph showing the presence of stromal scores in tumor tissues, immune scores for the infiltration of immune cells in tumor tissues, and the ESTIMATE scores that infers tumor purity. Herein, we analyzed the immune infiltration landscape in LGGs, by applying single-sample gene set enrichment analysis (ssGSEA) to evaluate the relative abundance of each immune cell subpopulation using RNA sequencing (RNA-Seq V2) data of 100 LGGs from TCGA. The survival analysis of significant DEGs in glioma using TCGA database was determined. Kaplan–Meier curves were used to produce graphs showing the survival probability of prognostic signature genes of glioma and glioblastoma and their statistical significance. For example, p values of less than 0.05 in all tests were significantly linked to low-grade glioma and glioblastoma. The gene expression profiles of the prognostic signatures (KLF15, AQP7, and AGPAT9) were determined using TIMER immune 2.0.

Results

Kaplan–Meier curve showing the expression of KLF 15, AQP7, and AGPAT9 gene in glioma

Herein, we unveiled the survival analysis for glioma patients using the TCGA database and Kaplan–Meier plots and discovered that the KLF 15 is significantly associated ($p=0.03$) with the overall survival of the patient which indicates that it may be a very possible prognostic biomarker useful for glioma patients Fig. 1a. In our investigations, the Kaplan–Meier plot showed that AQP7 is significantly associated ($p=0.001$) with overall survival of the glioma patients using the TCGA database Fig. 1b. Hence, it showed that it may be a prognostic biomarker which may be useful for the glioma patient. The Kaplan–Meier plot showed that AGPAT9 is significantly associated ($p=0.005$) with overall survival of the glioma patients using the TCGA database (Fig. 1c).

Fig. 1 [Images not available. See PDF.]

Kaplan–Meier curve showing the expression of KLF 15, AQP7, and AGPAT9 gene in glioma

Kaplan–Meier curve showing the expression of KLF 15, AQP7, and AGPAT9 gene in glioblastoma

This is a visual representation of expression level of prognostic signature KLF15, which indicates that the p value is 0.025 that means it has a significant association with glioblastoma and thus can be used as a prognostic signature in the early detection of glioblastoma (Fig. 2a). Meanwhile, the expression level of prognostic signature AQP7 has p value of 0.59 that means it has no significant association with glioblastoma and thus cannot be used as a prognostic signature in the early detection for glioblastoma patients (Fig. 2b). Also, AGPAT9 has a p value of 0.10 that means it has no significant association with glioblastoma and thus cannot be used as a prognostic signature in the early detection of glioblastoma. Thus, AQP7 and AGPAT9 have no significant association and so they are predictive biomarkers and may not be potential prognostic signatures for glioblastoma patients. However, KLF15 showed a significant association with glioblastoma and so can be a prognostic biomarker in glioblastoma patients.

Fig. 2 [Images not available. See PDF.]

Kaplan–Meier curve showing the expression of KLF 15, AQP7, and AGPAT9 gene in glioblastoma

The stromal, immune, and estimate scores of low-grade glioma

To analyze the immune infiltration landscape in LGGs, single-sample gene set enrichment analysis (ssGSEA) was applied to evaluate the relative abundance of each immune cell subpopulation using RNA sequencing (RNA-Seq V2) data of 100 LGGs patients from The Cancer Genome Atlas (TCGA). By performing single-sample gene set

enrichment analysis (ssGSEA), we calculated stromal and immune scores to predict the level of infiltrating stromal and immune cells and these form the basis for the ESTIMATE score to infer tumor purity in tumor tissue Fig. 3.
Fig. 3 [Images not available. See PDF.]

The stromal, immune, and estimate scores of low-grade glioma

The expression levels of KLF15 in LGG using different immune infiltrate variables

KLF15 has a correlation with low-grade glioma. Correlation value of 0.124 and the genes are highly expressed in the tumor cells which show a positive correlation with tumor purity and significantly association ($p=0.000005$) Fig. 4. B cell, CD8+ T cell, CD4+ T cell, macrophages, neutrophil, and dendritic cells are the immune infiltrates which show that the expression level of KLF15 has a partial correlation, and it is significantly associated with immune infiltration in all the cells except that of the macrophages where the P value is 0.07.
Fig. 4 [Images not available. See PDF.]

The expression levels of KLF15 in LGG using different immune infiltrate variables

The expression levels of AQP7 in LGG using different immune infiltrate variables

Based on investigations, it shows that the tumor purity of AQP7 has a negative correlation with low-grade glioma; correlation value = −0.007 (Fig. 5). Also, purity and B cells do not show a significant association with glioma; *p* values = 0.8, 0.4, respectively. CD8+ T cell, CD4+ T cell, macrophages, neutrophil, and dendritic cells show that the expression level of AQP7 has a partial correlation with the infiltration level and significantly associated. CD8+ T cell, CD4+ T cell, macrophages, neutrophil, and dendritic cells also show a significant association with glioma with *p* values of 0.006, 0.05, 0.003, 0.003, and 0.01, respectively.
Fig. 5 [Images not available. See PDF.]

The expression levels of AQP7 in LGG using different immune infiltrate variables

The expression levels of AGPAT9 IN LGG using different immune infiltrate variables

Based on the investigation, it also shows that tumor purity of AGPAT9 has a negative correlation with low-grade glioma; correlation value = −0.238, and it shows a significant association with the tumor purity with *p* value ($p=0.000000014$) (Fig. 6). All the immune cells have positive correlation and show significant association with glioma except macrophages.
Fig. 6 [Images not available. See PDF.]

The expression levels of AGPAT9 IN LGG using different immune infiltrate variables

The expression levels of KLF15 in GBM using different immune infiltrate variables

The dendritic cells, CD4 T cell, neutrophil, and CD8+ T cell immune infiltrate are significantly associated with glioblastoma. Meanwhile, B cell, CD8+ T cell, CD4+ T cell, macrophages, neutrophil, and dendritic cells immune infiltrates indicated that KLF15 expression level is partially correlated with immune infiltration level in GBM, and purity immune infiltrate is correlated. Herein, during the analysis of the KLF15, we realized that the immune infiltrates are significantly association except that of the B cells ($p=0.1$) and the macrophages ($p=0.3$). The correlation value was 0.254 and the genes are highly expressed in the tumor cells. In the tumor purity, it shows positive correlation and significantly associated ($p=0.00000013$) (Fig. 7) in glioblastoma.
Fig. 7 [Images not available. See PDF.]

The expression levels of KLF15 in GBM using different immune infiltrate variables

The expression levels of AQP7 in GBM using different immune infiltrate variables

Here, no significant association ($p=0.26$). It was also indicated that the tumor purity of AQP7 has a negative correlation with its immune infiltration level; Correlation value = −0.055 (Fig. 8). However, neutrophil and CD4 T cells have partial correlation and significantly associated in glioblastoma patients with p value of 0.0000195 and

0.0000177, respectively.

Fig. 8 [Images not available. See PDF.]

The expression levels of AQP7 in GBM using different immune infiltrate variables

The expression levels of AGPAT9 in GBM using different immune infiltrate variables

B cells and macrophages show no significant association with glioblastoma multiforme. The purity, CD8+ T cell, CD4+ T cell, neutrophil, and dendritic cells show a significant association with glioblastoma multiforme. Based on the analysis, the tumor purity of AGPAT9 has a negative correlation with low-grade glioma; correlation value = − 0.363. It also shows a significant association $p = 0.000012$ (Fig. 9).

Fig. 9 [Images not available. See PDF.]

The expression levels of AGPAT9 in GBM using different immune infiltrate variables

Discussions

Krüppel-like factor 15 (KLF15) is a signature that is yet to be fully elucidated in the glioma patient, but previous investigations have been done in the area of clear cell renal cell carcinoma [66] adenocarcinoma lung cancer [67]. Krüppel-like factor 15 (KLF15) is useful in a lot of biological processes which include cell proliferation, cell cycle, adipogenesis, etc. [68–70]. KLF15 has an important role in RNA polymerase II-specific DNA-binding transcription factor activity [71, 72]. Hence, it is known to have significant functions in different types of cancer. KLF15 is responsible for the suppression and activation of genes in carcinogenesis. Previous investigation has shown that KLF15 is a positive regulator of carcinogenesis [73–76]. Therefore, KLF15 may be useful immune-related prognostic signature in glioma and glioblastoma patients.

Investigations have been conducted on the Aquaporin 7 (AQP7) association with lymphatic metastasis, breast cancer, liver cancer, and clear renal cancer [77–81]. It is otherwise known as water channels which have been known to be related to the invasion, proliferation, and migration of human breast tumors [77, 82–84]. However, investigations are yet to discover the potential roles of AQP7 in glioma and glioblastoma patients as a possible therapeutic target and prognostic biomarker. Aquaporin (AQP) family members were first investigated in 1992 [85–87]. Various investigations have shown that it can be expressed in epithelial and non-epithelial cells [88]. AQP7 is also important in fatty acid metabolism and enhances the migration of water and glycerol [78]. Human 1-acylglycerol-3-phosphate O-acyltransferase 9 (AGPAT9, also known as GPAT3 or LPCAT1) is correlated with tumor progression and tumor microenvironment [89]. It is related to fatty acid metabolisms and involved in a lot of biological processes. It catalyzes de novo synthesis of triacylglycerol [89]. Hence, AQP7 and AGPAT9 indicate usefulness as prognostic biomarker which may be advantageous for the glioma patient.

Stromal and immune scores were estimated from transcriptomic profiles of LGG cohort from TCGA using the ESTIMATE. One hundred entries of TCGA cohort were entered and used for the investigation. Hence, the presence of stromal scores in tumor tissues, immune scores for the infiltration of immune cells in tumor tissues and the ESTIMATE scores that infers tumor purity is observed [90–92].

Immune infiltration of malignancies correlates strongly with clinical outcomes. In terms of chemotherapy and immunotherapy, the makeup of tumor-infiltrating immune cells (TIICs) can serve as biomarkers for predicting treatment response and survival in distinct patient subgroups [93]. Hence, the immune cell proportion of the three-signature for LGG were determined using Timer immune 2.0. The Gene module allows a user to identify the relationship between tumor gene expression and immune infiltration in a fast, comprehensive, and unbiased way [94]. Therefore, the signatures may be a potential prognostic signature for glioma and useful for screening immunotherapy for glioma patients. Hence, this is in consistent with previous investigations [95, 96]. Therefore, B cell, CD8+ T cell, CD4+ T cell, macrophages, Neutrophil, and dendritic cells immune infiltrates indicated that AQP7 expression level is partially correlated with immune infiltration level in LGG [97], while purity infiltrate is correlated. Hence, it may be a potential prognostic signature for glioma and useful for screening immunotherapy for glioma patients [98].

B cell, CD8+ T cell, CD4+ T cell, macrophages, neutrophil, and dendritic cells show that the expression level of AGPAT9 has a partial correlation with the infiltration level. Purity, B cell, CD8+ T cell, CD4+ T cell, neutrophil, and dendritic cells show a significant association with glioma [99], while macrophages do not have any significant association with glioma. Therefore, B cell, CD8+ T cell, CD4+ T cell, macrophages, neutrophil, and dendritic cells immune infiltrates indicated that AGPAT9 expression level is partially correlated with immune infiltration level in low-grade glioma, while purity immune infiltrate is correlated [100].

Determination of immune cell proportion of the KLF15, AQP7, and AGPAT9 signatures on the glioblastoma multiforme prognostic using Timer immune 2.0 indicated that the tumor purity of KLF15 has a positive correlation with glioma and glioblastoma [101]. Hence, KLF 15 may be a potential prognostic biomarker and useful for screening immunotherapy for glioma and glioblastoma patients. B cell, CD8+ T cell, CD4+ T cell, macrophages, neutrophil, and dendritic cells show that the expression level of KLF15 has a partial correlation with the infiltration level. Therefore, CD8+ T cell, CD4+ T cell, neutrophil, and dendritic cells immune infiltrates indicated that KLF15 expression level is significantly associated with the GBM patients. Thus, KLF15 may be a useful signature for monitoring immunotherapy in GBM [102].

B cell, CD8+ T cell, CD4+ T cell, macrophages, neutrophil, and dendritic cells show that the expression level of AGPAT9 has a partial correlation with the infiltration level. CD4+ T cell, macrophages, and neutrophil cells do not show a significant association with glioblastoma multiforme. The tumor purity, dendritic cells, and CD8+ T cell immune infiltrate have a significant association with glioblastoma using the AGPAT9 gene [66]. Therefore, B cell, CD8+ T cell, CD4+ T cell, macrophages, neutrophil, and dendritic cells immune infiltrates indicated that AGPAT9 expression level is partially correlated with immune infiltration level in GBM [89], while purity immune infiltrate is correlated. Dendritic cells are known for their ability of promoting tumor immunosuppression [103]. Dendritic cells are divided into two forms, myeloid DC and plasmacytoid DC, which can produce large amount of Interferon gamma [104]. It can also induce T cell immunity or tolerance [105, 106]. Hence, AGPT9 may be useful for monitoring immunotherapy in glioblastoma patients. Concerning the association of CD8 T cells, it shows that CD8+ T lymphocytes are crucial components of the tumor-specific adaptive immunity that attacks tumor cells [107]. Clinical outcomes are highly connected to the immune infiltration of malignancies [108]. The composition of tumor-infiltrating immune cells (TIICs) may serve as biomarkers for predicting treatment response and survival in various patients subgroups in terms of chemotherapy and immunotherapy [109, 110].

Conclusions

The analysis revealed that KLF15, AQP7, and AGPAT9 may be prognostic biomarker genes that may be useful for prognosis of patients with glioma. Utilization of bioinformatics tools such as TIMER, ESTIMATE, Kaplan–Meier plot, TCGA database; the immune proportion, stromal and immune scores, various expression levels of the prognostic signatures, infiltrating levels, and tumor purity of glioma and glioblastoma multiforme were determined. Further investigations will be required using X-tile software, Database for Annotation, Visualization, and Integrated Discovery (DAVID), string, cytoscape, Kyoto Encyclopedia of Genes and Genomes (KEGGs) databases to unveil the molecular mechanisms of glioma and glioblastoma. Use of single cell sequencing will be of great usefulness in the treatment of glioma and glioblastoma. Investigations into hormone-based therapy will be fascinating. It would be enormously fascinating to validate maybe the biomarker predicts both precision immunotherapy and prognosis. The determination of real-time quantitative PCR analysis is also important.

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Author contributions

AMO wrote the manuscript. AMO and OSW performed the experiments, collected, and analyzed data. AKO, RMH, AJ, AA, AMA, and AIA revised the manuscript. AMO conceived and designed the study and revised the manuscript for improved intellectual content. All authors read and approved the final manuscript.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author, upon reasonable request http://xena.ucsc.edu/, TCGA database.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they do not have competing interests.

Abbreviations

TCGA
The Cancer Genome Atlas
KLF15
Krüppel-like factor 15
AQP7
Aquaporin 7
AGPAT9
Human 1-acylglycerol-3-phosphate O-acyltransferase 9
LGG
Low-grade glioma
GBM
Glioblastoma
ESTIMATE
Estimation of Stromal and Immune cells in Malignant Tumors using Expression data algorithm
TIICs
Tumor-infiltrating immune cells
ssGSEA
Single-sample gene set enrichment analysis.
DEG
Differentially Expressed Genes
DAVID
Database for Annotation, Visualization, and Integrated Discovery
KEGGs
Encyclopedia of Genes and Genomes
TME
Tumor microenvironment
TIMER
Tumor IMmune Estimation Resource

Publisher's Note

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DETAILS

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Enhanced therapeutic approach for diabetic foot ulcers: comparative study and characterisation of mupirocin-loaded nanosponge gel with marketed formulation for accelerating wound healing and in vivo evaluation

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ABSTRACT (ENGLISH)

Background

The current investigation aimed to develop, optimise, and assess a mupirocin-loaded nanosponge-based topical delivery system for diabetic foot ulcer and to achieve prolonged drug release while improving drug deposition within the skin. The nanosponges carrying mupirocin were formulated using the emulsion solvent diffusion method. A 3^2 factorial design was utilised to investigate effect of two factors, specifically the concentration of ethyl cellulose and the stirring rate, on the physical attributes of the nanosponges. The optimised nanosponge formulation batch (F9) was subsequently incorporated into a Carbopol gel base, ensuring the desired physical attributes were achieved in the gel formulation containing nanosponges. The research included in vitro drug release evaluation, ex vivo drug deposition analysis, assessment of the antimicrobial action of the nanosponge formulation, and in vivo diabetic wound healing.

Results

Drug polymer compatibility analysis was conducted using FT-IR spectroscopy revealed no interactions among mupirocin and ethyl cellulose molecules. Further FT-IR spectroscopy, DSC spectroscopy, and XRD spectroscopy analysis of optimised formulation batch revealed that the drug was successfully entrapped in nanosponges. Scanning electron microscopy confirmed the spherical and porous nature of the prepared nanosponges. The drug release pattern across the cellulose dialysis membrane followed a diffusion-controlled release pattern, and the drug deposition analysis exhibited substantial retention of mupirocin in the skin from the nanosponges formulation for up to 24 h. Furthermore, the optimised nanosponges gel formulation demonstrated stability and non-irritant properties, as indicated by the HET-CAM test. In vivo evaluation of wound healing activity in a Streptozotocin-induced diabetes mellitus with excision wound model revealed significant actions pertaining to wound healing and closure after 16 days of treatment.

Conclusion

The mupirocin-loaded nanosponge gel contributed to remarkable and swift recovery and closure of wounds in diabetic rats. The nanosponges, acting as carriers for mupirocin, facilitated the effective delivery of the drug to the wound area, while the gel fostered an optimally humid environment conducive to wound care during the final stages of wound healing and sealing.

FULL TEXT

Background

Diabetes mellitus (DM) encompasses a cluster of persistent metabolic disorders marked by high blood glucose levels stemming from either inadequate insulin production or resistance to its effects [1]. The two clinically distinct types are type 1, caused by autoimmune beta-cell destruction resulting in complete insulin deficiency, and type 2, characterized by increased resistance to insulin action and insufficient insulin production [2]. Type 2 Diabetes Mellitus (T2DM) is the predominant form of diabetes, accounting for over 90% of cases globally. It is characterised by low insulin production and tissue insulin resistance [3]. The global prevalence of diabetes is rising, with projections estimating over 1.31 billion people will be affected by 2050 [4, 5].

Diabetic foot ulcers (DFUs) are substantial complications of diabetes, distinguished by ulceration linked with neuropathy and/or peripheral arterial disease [6–8]. DFUs can lead to severe complications such as infections, amputations, and mortality. Infections are observed in up to 58% of patients with new foot ulcers [9]. The prevalence of DFUs is higher in males (4.5%) than in females (3.5%) and in type 2 diabetics (6.4%) compared to type 1

diabetics (5.5%) [10]. Patients with diabetes experience varying foot sensitivity symptoms, ranging from pain and tingling in the early stages to numbness and toe weakening in the later stages [11]. DFUs are challenging to heal due to the presence of microbial biofilms, elaborate societies of microscopic organisms encased in a self-generated matrix of extracellular polymeric substances (EPS) [12, 13]. Diabetic mouse models hold clinical significance in relation to diabetic ulcers, while the excision wound mouse model is pertinent to both acute and chronic wound healing. Interestingly, the excision wound healing model in mice has potential applicability in diabetic wound healing as well, achieved by inducing diabetes prior to initiating the wound [14].

Nanosponge-Based Topical Drug Delivery: Conventional drug therapy for DFUs faces limitations due to neuropathy, hindering drug delivery to the injured site. Nanotherapeutics, particularly nanosponge-based topical delivery systems, offer advantages for chronic wounds like diabetic wounds, promoting effective wound healing and skin regeneration [15]. The proposed strategy involves preparing a mupirocin-loaded nanosponge topical dosage form capable of penetrating deeper tissue at the injured site. Mupirocin (Pseudomonic acid A), an antibiotic synthesised by Pseudomonas fluorescens, demonstrated pronounced efficacy against staphylococci and streptococci, as well as specific gram-negative bacteria like Haemophilus influenzae and Neisseria gonorrhoeae. Additionally, it supports wound healing by promoting keratinocyte proliferation and augmenting growth factor production. The therapeutic benefits of mupirocin (MP) can be enhanced by synergistically integrating it with other substances and implementing innovative approaches [16–19]. Nanosponges (NSs) are nanosized sponge-like structures with numerous cavities capable of accommodating payloads [20]. NSs exhibit self-sterilising properties due to their average pore size of 0.25 µm, effectively preventing bacterial penetration. Additionally, they enhance drug bioavailability and improve the solubility of poorly soluble drugs [21]. They present several advantages over microsponges, including smaller particle size (below 500 nm), enhanced stability (up to 300 °C), and lipophilicity, which allows masking unpleasant flavours and transforming the physical state of drug from liquid to solid [22]. This unique drug delivery technology holds promise for attaining controlled and extended drug release, addressing the challenges of conventional topical delivery systems for diabetic wound healing.

Materials and methods

Materials

MP was purchased from Horster Biotek, Pvt. Ltd., situated in Indore, India. Ethyl cellulose (EC), Carbopol 934 (CP 934), Polyvinyl alcohol (PVA), and Dichloromethane (DCM) were obtained from Loba Chemie Pvt. Ltd. in India. Every other substance and chemical employed were of analytical calibre.

Method

Fabrication of nanosponge (NSs)

The preparation of MP-loaded Nanosponge (MP-NSs) was done by the emulsion solvent diffusion (ESD) method. MP and EC were used in the ratios of 1:4, 1:6, and 1:8. The amount of MP was kept constant at 100 mg in the development of NSs. In the emulsion solvent diffusion method, the NSs were fabricated by incorporating different proportions of EC. The internal organic phase, consisting of MP and EC, was blended with 20 mL of dichloromethane (Alternative to DCM are Ethanol, Ethyl Acetate or Acetone) using ultrasonic agitation in an ultrasonicated bath (70 kHz frequency) for a duration of 2 minutes (Crest, Ultrasonic Corporation, Cortland, New York), and in the external aqueous phase of 100 mL of distilled water, 1 g of polyvinyl alcohol (PVA) was dissolved by heating up to 60 °C. Then the internal organic phase was dropwise added to the external aqueous phase while stirring at 1000 rpm for 2 hours (hrs), and the fabricated NSs were gathered by filtration followed by oven drying at 40 °C for 24 hrs [23].

Factorial experimentation and optimization using design of experiments (DoE) software

Initial experiments were conducted to investigate the effect of MP/EC ratios and stirring rates on the physical properties of NSs. Throughout all formulations, the concentration of MP, internal phase volume, and PVA concentration remained consistent. To optimise the dependent variables like production yield (PY), entrapment efficiency (EE), and mean particle size (MPS) of nanosponges, nine formulations were created using a 3^2-factorial design, with the independent variables being polymer concentration (X1) and stirring rate (X2) [24].

Physicochemical characterisation

Fourier transform infrared (FT-IR) spectroscopy analysis

FT-IR spectroscopy data of MP, EC, and optimised MP-NSs were recorded on an FT-IR spectrophotometer (Jasco FT-IR 6700) using the potassium bromide (KBr) press technique as per previously reported methods. Approximately 1–4 mg of sample was combined with dry KBr in a 1:1 ratio and scanned at transmission mode over 4000–400 cm^{-1} [25].

Thermal analysis by differential scanning calorimetry (DSC)

The thermal assessment of MP, EC, and MP-NSs was conducted using a differential scanning calorimeter (Mettler Toledo DSC, USA). Precisely measured quantities of samples (5 mg) were placed in aluminium containers and hermetically sealed. Each sample was subjected to a gradual temperature increase of 10 °C per minute within the temperature interval of 25–300 °C, all in a nitrogen environment [26].

Solid state characterisation

The X-ray diffraction (XRD) study was conducted to evaluate the solid-state character of the formulation. Powder XRD studies of MP, EC, and optimised MP-NSs were conducted using a powder X-ray diffractometer (Brucker D2 Phaser 2nd Gen). Samples were placed in the sample stage, and data were obtained over 2θ range from 5 to 50° using a step size of 0.019° per sec [27].

Particle size and zeta potential characterisation

The analysis of particle size for MP-NSs was executed employing the "Malvern Zetasizer NanoZS (Malvern Instruments, UK)". The specimen being investigated was diluted with distilled water (1:200) and introduced into a disposable polystyrene cuvette. The measurement of particle size and polydispersity index (PDI) was conducted based on the principles of dynamic light scattering (DLS). The identical procedure was adhered to for gauging zeta potential (ZP), albeit employing an electrode cuvette. Each sample was subjected to triplicate testing (n = 3) [28].

Entrapment efficiency (%EE)

The ultracentrifugation technique was used to assess the entrapment efficiency of MP-NSs. Samples were centrifuged at 10000 rpm for 30 min using an ultracentrifuge (Remi C-24, Mumbai, India). Unentrapped MP content in the supernatant was diluted with an appropriate medium before being measured using a UV-visible spectrophotometer at 222 nm [29]. Entrapment efficiency was calculated as per Eq. (1).

1

%EE=weightoftotaldrug-weightoffreedrug×100weightoftotaldrug

Scanning electron microscopy (SEM) analysis

The structure of NSs was investigated utilising a scanning electron microscope (GEOL 5400, USA) with an operational voltage of 20 kV. Prior to observation, dehydrated NSs underwent a 45-s coating with a gold–palladium alloy in an argon atmosphere. The SEM image was captured at a magnification of 3000 [30].

In vitro antimicrobial study of optimised MP-NSs formulation

Generation of bacteria inoculums

Inoculum was standardised, and 106 colony-forming units (CFU/mL) of the required density were achieved. Nutritious broth (5 mL) was mixed with a loopful of the *Staphylococcus aureus, Escherichia coli, and Pseudomonas aeruginosa*, and the mixture was then cultured for 24 h at 3 °C. To standardise the culture to 106 CFU/mL (equal to 0.5 McFarland standards), 0.2 mL of the microbes 24-h culture were poured into 20 mL of sterilised nutritious broth and cultured for 3–5 h. Cultures of test microbes on agar–agar and 8% nutritional broth were combined to create Nutritional Broth Medium (NBM), which was used to cultivate the bacterial strains. It was autoclaved at 15 lbs. pressure for 25–30 min. On petri plates, 15 mL of NBM were poured to prepare agar test plates in an aseptic condition, then subjected to stabilisation at room tempertature (RT). In peptone saline solution, bacterial cell cultures were routinely subcultured and incubated at 37 °C for 24 h.

Agar plates and test sample preparation

The bacterial strains were inoculated onto sterile agar plates by streaking the swab across the entire surface of the agar 2–3 times to ensure uniform distribution of the inoculum. The agar plate was rotated at a 60° angle during this

process. Subsequently, the plates were allowed to air-dry in a sterile environment at RT. Wells with a diameter of 9 mm were then carefully created in the plates under aseptic conditions. The preparation of the test samples (MP-NSs) at a concentration of 10 mg/mL and the reference drug Ciprofloxacin at 100 µg/mL in dimethyl sulfoxide (DMSO) was carried out. Using a sterile micropipette, 50 µL of both the reference and test samples were dispensed into the wells. The plates were positioned in an incubator adjusted to a temperature of 37 °C for a duration of 24 h and the zone of inhibition (ZOI) for each bacterial strain was measured in triplicate using a calibrated digital Vernier caliper.

Agar well diffusion method

By using this method, the antibacterial action of MP-NSs formulation was tested against *Staphylococcus aureus, Escherichia coli, and Pseudomonas aeruginosa.* These three microorganisms are most abundant in DFUs [31]. Sterile Muller Hinton plates of agar were formed, and wells of 6 mm were punched into the plates using a sterilised cup borer. The plates were swabbed with a 24 h culture of test organisms using sterile cotton swabs. To the wells, 100 µL of MP-NSs solution were added, and plates were subjected to incubation in an upward position at 37 °C for 24 h., after which the plates were checked for ZOI. Control was 100 µL of (10% v/v) DMSO solution. The experiment was performed in triplicate, and the mean of the zone sizes recorded was calculated.

Preparation of MP-loaded nanosponge gel

To prolong the retention time on the skin surface, an optimised formulation of MP-NSs was selected for conversion into a topical gel system. Carbopol-934 (0.5%w/v) was allowed to swell in double-distilled water for 12 h. The dry powder of MP-NSs (equivalent to 100 mg of the drug), propyl paraben (0.5%w/v), and methyl paraben (0.2%w/v) were added to 10 mL of propylene glycol, and the propylene glycol suspension was gradually added to the swelled CP-934 gel while continuously stirring to achieve a uniform mixture. To obtain a translucent gel, a 1:1 molar ratio of triethanolamine base and CP-934 was added to the homogenous mixture. This step was taken to ensure a well-mixed and visually appealing gel product [32].

Assessment of gel containing MP-nanosponges

Upon visual inspection of the gel for its texture, colour, and uniformity, a comprehensive evaluation was conducted on the following parameters:

pH measurement

The pH of the formulated gel was gauged using a pH metre that had been calibrated with a pH 7 buffer prior to use. The electrode tip was immersed in the gel, and the reading was taken after 2 min. This pH measurement procedure was performed in triplicate, and the average value was computed.

Spreadability analysis

The spreadability of the gel was determined by placing a known weight of the sample between two glass slides, and a weight of 500 g was applied over the slides for approximately 5 min, after which no further spreading was anticipated. The initial and final diameters of the spread circles were measured in centimetres, serving as comparative metrics for spreadability.

Viscosity measurement

Viscosity, which signifies a resistance of fluid to flow, was assessed using a Brookfield viscometer equipped with spindle No. 7, operating at various rotations per minute (rpm) at room temperature (RT).

Drug content analysis

1 g of MP-NSs gel was precisely weighed, dissolved in methanol, sonicated for 15 min, and then adjusted to the mark in a 100 mL volumetric flask using methanol. From this solution, 1 mL was withdrawn, further diluted to 10 mL with methanol, and then a final dilution was carried out using distilled water to achieve a concentration within the Beer's law range. The absorbance was measured at 222 nm using a UV spectrophotometer against a blank gel treated in the same manner as a sample [33].

In vitro drug release assay

In vitro drug release investigations were conducted using Franz diffusion cells with a receptor chamber capacity of 20 mL and an effective diffusion area of 3.14 cm2. A cellulose dialysis membrane from Himedia, Mumbai, India, was

soaked in the receptor medium (phosphate buffer, pH 5.8) for a period of 24 h prior to the commencement of the experiment. The donor side of the arrangement held a predetermined quantity of gel containing MP-NSs. During the experiment, the receptor solution was consistently agitated at 50 rpm and maintained at a steady temperature of 32 ±0.5 °C through a circulating jacket. At specific intervals, 1 mL samples were withdrawn from the receiving compartment, and an equal volume of fresh buffer was introduced to ensure sink conditions. The extracted samples were subjected to analysis using a UV-Spectrophotometer to quantify the quantity of released MP. To facilitate comparison, the release profiles of a conventional cream (Mupirocin Cream USP, 2%, Glenmark) and a commercially available MP ointment (T-bact, GlaxoSmithKline) formulation were also examined. The drug release data underwent linear regression analysis to determine the release kinetics, encompassing zero-order and first-order release kinetics, as well as the diffusion-controlled mechanism (Higuchi model). [34].

Ex-vivo drug deposition assay

A study involving drug deposition within the skin was conducted using a Franz diffusion cell and excised rat abdominal skin. The outer layer of the skin was exposed to the surrounding environment, while the inner layer faced the solution in contact. The surface of the skin facing the outside environment was the epidermal side, whereas the dermal side was directed towards the solution in contact. The receptor compartment was filled with 20 mL of phosphate buffer at pH 5.8, maintained at a temperature of 33±0.6 °C, and agitated at a rate of 50 rpm. Prior to the application of the sample, the skin was saturated with the diffusion medium for an hour. A 40-mg portion of the sample was applied to the donor compartment. For the quantification of the drug accumulated in the skin, the diffusion cell was disassembled after time intervals of 4, 8, 16, and 24 h. The skin was cautiously detached, and the mupirocin present on the surface was cleansed using distilled water [34].

Determining mupirocin concentrations in skin specimens

MP was recovered from the skin utilizing a technique outlined by Echevarria et al. [35]. In brief, the skin was fragmented into smaller sections and subsequently crushed and ground using 10 mL of methanol. Following this, the crushed mixture underwent a 15-min session of ultrasonication to ensure the comprehensive extraction of the drug. The methanolic extract that resulted was later centrifuged at a speed of 8,000 rpm for a duration of 15 min. The liquid portion above the sediment, which held the extracted substances, was meticulously gathered. This collected supernatant was then evaporated and subsequently mixed back with the suitable solvent. Prior to analysis, the sample was filtered using 0.2-µm Whatman filter paper and subjected to assessment using a UV spectrophotometer at 222 nm. Intact skin was enriched with predetermined quantities of the drug to determine the recovery rate of the drug from the skin Subsequently, the skin was fragmented into smaller sections, crushed, drug extracted, and analysed using the previously described procedure.

Skin irritation studies

The optimised gel formulation containing MP-NSs was subjected to skin irritation assessments using the HET-CAM (Hen's Egg Test-Chorioallantoic Membrane) technique [36]. Fertile white chicken eggs were sourced from commercial suppliers. Fresh eggs, which were nine days older and weighed between 50 and 60 g, were selected for the study. The irritation evaluation involved the negative control, which was sodium chloride (0.9%w/v NaCl), the positive control, which was sodium hydroxide (1%w/v NaOH), and the prepared MP-NSs gel. The HET-CAM test was used to assess irritation. The irritation scores from all treated groups were recorded at various time intervals, and the mean irritation score was calculated [37].

Stability studies

The MP-NSs gel was evaluated for its stability in an accelerated stability chamber (REMI) at three different temperatures (4±2 ºC, 25±2 ºC and 37±2 °C) and 75% relative humidity (RH) for three months. The gel was evaluated for physical appearance, viscosity, pH, and Spreadability. Any change in appearance, pH, viscosity, or Spreadability of the stored MP-NSs gel was recorded [38].

In vivo wound healing activity

Wistar albino female rats in good health, weighing 180 and 250 g, were selected. The Institutional Animal Ethics Committee (Approval number IAEC/UDPS/2022/02/08) granted approval to the study protocol in accordance with

Indian Committee for the Purpose of Control and Supervision of Experiments with Animals (CPCSEA) specifications. Throughout the study, animals were kept in a standard laboratory environment at a temperature of 25±2 °C with a relative humidity of 44–56% and fed a standard diet and water.

Diabetes animal model

The described experimental protocol involved inducing diabetes mellitus in Wistar rats through the intraperitoneal (I.P.) delivery of streptozotocin (STZ) at a dose of 60 mg/kg body weight. Prior to the STZ injection, the rats were subjected to an overnight fasting period. To safeguard pancreatic beta cells from excessive harm induced by STZ, niacinamide was administered intraperitoneally at a dosage of 120 mg/kg body weight, 15 min prior to the STZ injection. [39, 40]. The STZ was prepared in a 0.1 M citrate buffer with a pH 4.5 for the I.P. injection. After 72 h from the STZ injection, blood samples were collected from the rats using the retro-orbital method, which involves obtaining blood from the blood vessels located behind the eye socket, a commonly used technique in small laboratory animals like rats. To confirm the successful induction of diabetes mellitus in the rats, various parameters were measured. These included HbA1C (glycated haemoglobin), blood glucose levels, and CRP (C-reactive protein) levels. These measurements were taken both before the induction of diabetes (baseline) and after 72 h following the STZ injection. The changes in these parameters would indicate the development of diabetes mellitus in the rats [41–43].

Excision wound model

Every rat participating in the study was administered an intraperitoneal injection of thiopentone sodium at a dosage of 40 mg/kg to induce anaesthesia on the day designated for wound creation. The wound development procedure involved creating a rectangular pattern on the upper side of the rat's paw. Using a scalpel blade, a wound was created by separating a complete skin layer with a standardised dimension of 2 mm × 5 mm. The rats were then randomly distributed into various experimental groups for further study. The purpose of this experimental setup was likely to be to investigate wound healing or other related phenomena in response to different treatments or interventions [44].

Animal groups and treatment protocol

Rats were split into 6 groups (n=5), with 5 rats in each group, and exposed to the subsequent treatment:

- **Group I:** Control group with normal wound (NWC), Non-Diabetic animal with wound received citrate buffer and distilled water.
- **Group II:** Control group with Diabetic wound; Diabetic animals with wounds who received no treatment (DWC).
- **Group III:** Diabetic wound treatment by MP-NSs gel formulation.
- **Group IV:** Diabetic wound treatment by standard MP ointment (T-bact, GlaxoSmithKline).
- **Group V:** Diabetic wound treatment by standard MP Cream (Mupirocin Cream USP, 2%, Glenmark).
- **Group VI:** Diabetic wound treatment by standard Becaplermin gel (REGRANEX, Smith&Nephew).

Treatment was applied topically to treat excised wound area. This treatment was followed once a daily.

Determination of wound area, calculation of wound contraction

On predefined days 1, 4, 6, 8, 10, 12, and 16, a camera was used to document the progression of changes in the wound location. Image analysis software computed the wound area from the pictures. By using Eq. (2), the percentage (%) of wound closure can be calculated [45].

2

%Wound closure=Area of initial wound-Nthday area of wound×100Area of initial wound

GraphPad Prism VIII was used to plot the graph showing the % of wound closure vs the number of days since the

wound first developed.

Results

Formulation and optimization of nanosponges

The process of formulating nanosponges is presently restricted in terms of its intricacy and expense. Although certain nanosponges available on the market are manufactured through the suspension polymerization method, an alternative technique, referred to as the ESD method, has exhibited potential for nanosponge production. The ESD method is characterised by its simplicity, reproducibility, and efficiency, rendering it a fitting approach for generating MP-NSs. A noteworthy advantage of this method is its ability to bypass the use of toxic solvents. In order to assess the impact of various variables on % entrapment efficiency, % production yield, and mean particle size of nanosponges, a factorial design was employed alongside analysis of variance (ANOVA). The factors under consideration included the concentration of EC and the stirring rate, which were investigated to uncover their effects on the aforementioned parameters. Table 1 presents the coded levels of concentration of EC and stirring rate that were utilised in the experimental design. These coded levels served as the basis for investigating the impact of independent variables on dependent variables. Table 2 provides an overview of how the independent variables influenced these specific response variables, shedding light on the relationships and effects within the experimental framework. The visual representation of the data is facilitated through the utilisation of two- and three-dimensional surface plots. These plots elucidate the relationships between variables and response outcomes, these insightful visualisations are presented in Figs. 1 and 2. The regression equation (Eq. 3) for %EE is as follows:

3

$$Y1\%EE=83.50+5.28A-0.7783B$$

where, A represents the EC concentration and B represents the stirring rate. The results revealed that the concentration of EC had a notable positive influence on the drug entrapment efficiency (%EE), meaning that increasing the EC concentration led to higher drug entrapment. On the other hand, the stirring rate had a significant negative effect on %EE, indicating that higher stirring rates resulted in lower drug entrapment.

Table 1. Coded level of ethyl cellulose and stirring rate for experimental design

Coded level	Actual values	
Ethyl cellulose (mg)	Stirring rate (rpm)	− 1
400	600	0
600	1000	+ 1

Table 2. The effect of Mupirocin: Ethyl cellulose ratio and stirring rate on production yield, EE, and mean particle size

Batches	Factors		Responses		
Mupirocin: Ethyl cellulose	Stirring rate	Particle size (nm)	Entrapment efficiency (%)	Production yield (%)	F1
100:400	600	335.4 ± 4.25	76.93 ± 1.02	63 ± 2.14	F2

100:600	600	381.5±2.13	85.73±0.98	61±1.26	F3
100:800	600	451.8±3.36	88.72±2. 55	69±3.49	F4
100:400	1000	263.4±1.71	79.10±1.43	73±2.51	F5
100:600	1000	189.5±2.21	84.48±3.70	65±1.87	F6
100:800	1000	304.1±3.32	89.84±1.93	77±3.64	F7
100:400	1400	148.5±2.19	77.7±3.02	61±3.91	F8
100:600	1400	202.2±1.02	82.14±1.47	76±1.22	F9

[α]Each observation is the mean ± SD of three determinations

Fig. 1 [Images not available. See PDF.]

Three-dimensional surface plots of **A** Entrapment efficiency, **B** Particle size, **C** Production yield and **D** Desirability plot

Fig. 2 [Images not available. See PDF.]

Optimization of mupirocin entrapped nanosponge by desirability plot

For particle yield (PY), a linear regression equation (Eq. 4) was generated, indicating that both the EC concentration and stirring rate had a positive influence:

4

Y2PY=69.11+6.00A+1.00B

Regarding mean particle size (MPS), a polynomial regression equation (Eq. 5) revealed that the EC concentration had a positive effect, while the stirring rate had a negative effect:

5

Y3MPS=276.56+36.87A-100.90B

Fit statistics for data analysis of response variables are given in Table 3. Based on the response surface methodology study, it was found that formulation (F9) performed better in terms of %EE, %PY, and MPS. Post-analysis confirmation at a two-tailed 95% confidence level is given in Table 4. As a result, the optimised nanosponges batch (F9) was selected for further characterisation studies and incorporated into a Carbopol gel base. The findings from the factorial design and regression equations provide valuable insights for optimising nanosponge formulations with enhanced properties for potential pharmaceutical applications.

Table 3. Fit statistics for data analysis of response variables using full factorial design

Fit statistics	Standard deviation	Mean	Coefficient of variation %	R^2	Adjusted R^2	Predicted R^2	Adeq precision

Particle size	41.78	276.56	15.11	0.8686	0.8248	0.7847	11.4220
Entrapment efficiency	1.39	83.50	1.67	0.9363	0.9151	0.8355	15.0809
Production yield	3.49	69.11	5.04	0.7528	0.6704	0.4910	6.9572

Table 4. Post Analysis confirmation at two tailed, 95% confidence level

Solution 1 of 5 response	Predicted mean	Predicted median	Observed	Standard deviation	SE mean	95% CI low for mean	95% CI high for mean	95% TI low for 99% Pop	95% TI high for 99% Pop
Particle size	212.52	212.522		41.7823	27.854	144.36	280.68	− 47.751	472.796
Entrapment efficiency	88.006	88.0061		1.39237	0.928	85.734	90.277	79.3326	96.6796
Production yield	76.111	76.1111		3.48542	2.3236	70.425	81.796	54.3995	97.8227

Characterisation of nanosponges

FT-IR spectroscopy analysis was conducted on MP, EC, and the optimised MP-NSs formulation (F9). The resulting data, as depicted in Fig. 3, demonstrated that the key peaks of MP were similarly present in the physical combination of MP, EC, and CP. This outcome suggests the absence of substantial alterations or interactions among these components within the mixture. Consequently, this observation indicates the stability of the formulations.

Fig. 3 [Images not available. See PDF.]

FT-IR spectrum of physical mixture of mupirocin ethyl cellulose and carbopol 934

Additionally, the FT-IR spectrum (as illustrated in Fig. 4) of the optimised nanosponge formulation (F9) exhibited all the significant peaks corresponding to EC, whereas the major peaks attributed to MP were conspicuously absent. This observation serves as confirmation that the MP has been effectively encapsulated within the nanosponges.

Fig. 4 [Images not available. See PDF.]

FT-IR spectrum of ethyl cellulose, mupirocin, carbopol 934 and formulation batch (F9)

In order to investigate thermal characteristics, thermograms were acquired for MP, EC, and the optimised MP-NSs formulation, as depicted in Fig. 5. MP exhibited a distinct endothermic peak at 77.78 °C, signifying its melting point. Notably, EC and the optimised MP-NSs formulation displayed a broad exothermic peak at the same temperature, which indicated the absence of the original sharp peak. This observation strengthens the conclusion that MP has been efficiently encapsulated within the nanosponges.

Fig. 5 [Images not available. See PDF.]

DSC thermogram of mupirocin, ethyl cellulose and optimised formulation batch (F9)

The XRD graph (shown in Fig. 6) for MP displayed distinct sharp peaks, indicative of its crystalline structure. Conversely, both EC and the optimised MP-NSs exhibited an amorphous nature in the XRD pattern, providing additional verification of the successful encapsulation of the MP within the nanosponges. Moreover, the optimised MP-NSs were characterised by a mean particle size of 189 nm and a zeta potential value of (−) 26 mV, as depicted in Fig. 7.

Fig. 6 [Images not available. See PDF.]

X-ray diffraction (XRD) graph of mupirocin, ethyl cellulose and optimised formulation batch (F9)

Fig. 7 [Images not available. See PDF.]

Particle size **A** and zeta potential **B** of MP-NSs

SEM images of the MP-NSs, as shown in Fig. 8, revealed a consistent spherical morphology characterized by a porous structure. Notably, these images displayed no discernible intact Mupirocin crystals, providing strong visual evidence of the effective entrapment of the drug within the nanosponge matrix.

Fig. 8 [Images not available. See PDF.]

Scanning Electron Microscopy images of **A** mupirocin-loaded nanosponges at × 2585 magnification **B** mupirocin-loaded nanosponges at × 375 magnification

In an antimicrobial study, the antimicrobial efficacy of the MP-NSs formulation was assessed against commonly found bacterial strains (*Staphylococcus aureus, Escherichia coli, and Pseudomonas aeruginosa*) associated with diabetic foot ulcers. Employing the agar-well diffusion method, inhibitory potential of nanosponge formulation was determined. Figure 9 illustrates these results, which were then compared with those of the reference drug Ciprofloxacin and a control solution. Recorded zone of inhibition values against the targeted microorganisms are compiled in Table 5. To visually represent the antimicrobial activity of MP-NSs, Fig. 10 offers a graphical depiction. This dataset provides valuable insights into the extent of inhibition exerted by the tested substances on the growth and activity of the selected microorganisms.

Fig. 9 [Images not available. See PDF.]

ZOI of sample, control and standard against *SA, EC,* and *PA* (whereas ZOI—zone of inhibition, *SA- staphylococcus aureus, EC- Escherichia coli, PA- Pseudomonas aeruginosa*)

Table 5. Antimicrobial activity of Mupirocin-loaded nanosponges and zone of inhibition for Standard (Ciprofloxacin), Control (Dimethyl sulfoxide) and Sample Mupirocin solution

Organisms	Zone of inhibition (mm)		
Standard (Ciprofloxacin)	Control (DMSO)	MP–nanosponges (10 mg/mL)	*S. aureus*

18	11	19	*E. coli*
20	8	17	*P. aeruginosa*

Fig. 10 [Images not available. See PDF.]

Graphical representation of antimicrobial activity of mupirocin. Whereas, SA-*Staphylococcus aureus*, EC-*Escherichia coli*, and PA-*Pseudomonas aeruginosa*

Taken together, the results from FT-IR, thermal analysis, SEM, and XRD studies strongly support the successful preparation of MP-NSs, wherein the drug is effectively entrapped in the nanosponge matrix, converting it from a crystalline state to an amorphous state. This formulation demonstrates promising potential for pharmaceutical applications due to its stability and drug entrapment capabilities.

Characterisation of nanosponges (MP-NSs) loaded gel

The developed carbopol gel formulations exhibited a uniform and smooth white texture, showcasing thixotropic properties that facilitated easy and efficient spreading. The pH value of the gel was quantified as 6.4 ± 0.03, and its viscosity was determined to be 1026 centipoises (cPs). Notably, the spreadability assessment of the gel containing MP-NSs yielded a value of 31.0 g·cm/sec, affirming its effective spreading capability. The drug content analysis revealed a value of 88.48 ± 0.04, indicating a uniform distribution of the drug within the gel matrix. These comprehensive findings are detailed in Table 6.

Table 6. Evaluation parameters of MP-NSs gel

Parameters	MP-NSs gel formulation
Physical appearance	White and Opaque
pH	6.4 ± 0.03
Viscosity (cPs)	1026
Drug content (%w/w)	88.48 ± 0.04

Skin irritation studies

The irritation assessment of the control with (0.9%w/v) NaCl, the control containing (1% w/v) NaOH, and the formulated MP-NSs gel was conducted through the utilisation of the HET-CAM test. Figure 11 illustrates the effects on blood vessels caused by different substances applied to the chorioallantoic membrane within a 5-min interval. The evaluation of the scores from all the treated groups was performed at various time intervals, and the average irritation scores are presented in Table 7. The negative control sample exhibited a score of zero at each time interval (signifying absence of irritation, ranging from 0 to 0.8), while the positive control demonstrated an exceedingly high score (16.43). Similarly, the formulated MP-NSs gel also resulted in a score of zero, indicating an absence of irritation on the chorioallantoic membrane. The absence of irritation scores in the negative control and the formulated solution suggests that they do not cause irritation to the membrane, thus establishing their non-irritating nature to the

skin.

Fig. 11 [Images not available. See PDF.]

Images showing the vascular effects of different substances applied on the chorioallantoic membrane over a period of 5 min. (1) Sodium chloride (0.9%w/v) (2) 0.1 N sodium hydroxide (3) MP-Nanosponge gel

Table 7. HET-CAM irritation score of the MP-NS Gel, NaOH (1%, positive control), and NaCl (0.9%, Negative control)

										Score						
Samples			Non-irritant				Mild irritant			Moderate Irritant				Severe Irritant		Overall Score
Egg		Time (min)			Time	(min)			Time (min)				Time (min)			
	0	0.5	2	5	0.5	2	5	0.5	2	5	0	0.5	2	5		MP-NS Gel
Egg 1	0	0	0	0.0	0	0	0	0	0	0	0	0	0	0		Egg 2
0	0	0	0.0	0	0	0	0	0	0	0	0	0	0		Egg 3	0
0	0	0.2	0	0	0	0	0	0	0	0	0	0		Mean score	0	0
0	0.2	0	0	0	0	0	0	0	0	0	0	0	Positive Control (NaOH 1%)	Egg 1	0	0
0	0	0	0	0	0	0	0	0	14	17	19		Egg 2	0	0	0
0	0	0	0	0	0	0	0	16	18	19		Egg 3	0	0	0	0

0	0	0	0	0	0	0	16	18	20		Mean score	0	0	0	0	0
0	0	0	0	0	0	14.5	16.1	18.7	16.43	Negative Control (NS 0.9%)	Egg 1	0	0	0	0	0
0	0	0	0	0	0	0	0	0		Egg 2	0	0	0	0	0	0
0	0	0	0	0	0	0	0		Egg 3	0	0	0	0	0	0	0
0	0	0	0	0	0	0		Mean score	0	0	0	0	0	0	0	0

In vitro drug release assay

The impact of composition and carrier on the drug release pattern of distinct formulations were examined using a cellulose dialysis membrane. The in vitro discharge patterns of MP from diverse formulations, as depicted in Fig. 12, demonstrated that MP ointment and MP cream released the MP within 4 and 10 h, respectively. In contrast, the MP-NSs gel exhibited a prolonged release extending up to a 24-h period. The release of the MP from the gel formulation was governed by a diffusion-controlled mechanism. The percentage of drug release is outlined in Table 8.

Fig. 12 [Images not available. See PDF.]

In-vitro drug release of marketed formulation and prepared mupirocin-loaded nanosponge-gel formulation

Table 8. Percent (%) drug released of marketed mupirocin ointment, mupirocin cream and prepared mupirocin-loaded nanosponge gel formulation

Time (hour)	Formulations (% Cumulative drug released)		
Mupirocin ointment	Mupirocin cream	Mupirocin-loaded Nanosponge–Gel	0.5
39.81 ± 1.33	21.40 ± 1.31	8.17 ± 2.21	1
76.48 ± 2.41	39.50 ± 2.19	11.35 ± 1.21	2

84.47 ± 1.71	46.97 ± 2.85	15.30 ± 3.21	3
91.05 ± 3.57	55.95 ± 3.34	20.35 ± 2.21	4
95.51 ± 2.74	68.49 ± 1.79	27.69 ± 1.21	6
–	74.38 ± 2.11	42.15 ± 3.21	8
–	83.28 ± 1.42	47.15 ± 1.21	12
–	90.20 ± 3.64	58.60 ± 2.21	24

[α]Each observation is the mean ± SD of three determinations

Ex vivo drug deposition assay

The quantity of mupirocin accumulated in excised rat abdominal skin through various formulations at distinct time intervals is illustrated in Fig. 13. The quantity of mupirocin deposited within the skin was notably greater when using the MP-NSs gel (211.4 ± 6.9 μg/cm^2) in comparison to the MP cream (83.57 ± 6.7 μg/cm^2) or MP ointment (34.03 ± 5.6 μg/cm2) after 24 h. This observation underscores that the nanosponges facilitated an enhancement in the drug residence in the skin.

Fig. 13 [Images not available. See PDF.]

Graphical representation of concentration of drug deposited in rat skin at different time intervals

Stability study

The results of the stability study, as shown in Table 9, revealed that the MP-NSs gel was found to be stable after a period of three months. Significant changes have not been seen when these were demonstrated for physical appearance, pH, and drug content at different temperature conditions (4 ± 2 ºC, 25 ± 2 ºC and 37 ± 2 ºC). A drug content greater than 90% indicated acceptable NSs stability in gel.

Table 9. Results of stability studies of MP-NSs gel

Parameters	MP-NSs gel			
Initial	After 3 months at 4 ºC temperature	After 3 months at 25 ºC temperature	After 3 months at 37 ºC temperature	Physical appearance
White and opaque	No change	No change	No change	pH
6.3 ± 0.06	6.4 ± 0.03	6.2 ± 0.05	6.3 ± 0.04	Viscosity (Pa.s.)
1125 ± 6.93	1030 ± 3.46	1029 ± 5.29	1015 ± 6.57	Drug content (%)

In vivo wound healing study of MP-NSs gel and marketed formulation

DM was induced in rats through the I.P. delivery of STZ at a dosage of 60 mg/kg body weight. Blood samples were obtained using the retro-orbital method both prior to and subsequent to the STZ injection. These samples were subsequently analysed for blood glucose levels, C-reactive protein (CRP) levels, and glycated haemoglobin (HbA1C) levels, as documented in Table 10. The outcomes of these assessments provided confirmation of the successful induction of diabetes. Meanwhile, an excision wound model was employed to create wounds on the rat feet. These wounds were then exposed to various distinct treatments for further investigation and analysis. Table 11 presents the results of the wound closure percentages for different treatment groups. The diabetic wounds treated with standard Becaplermin gel exhibited the highest percentage of wound closure (96%), outperforming all the other groups. The diabetic wounds treated with MP-NSs gel showed a commendable wound closure rate of 92%, which was higher than the diabetic wound control group (43%), the normal wound control group (73.5%), DM group 4 treated with MP ointment (79%), and DM group 5 treated with MP cream (78%). The bar graph in Fig. 14 illustrates the percentage of wound closure on days 4, 8, 12, and 16 for all the groups. On day 4, there was a consistent and exponential wound healing rate observed across all groups. However, from day 8 onwards, both DM group 3 (diabetic wound treated with MP-NSs gel) and DM group 6 (diabetic wound treated with Becaplermin gel following MP-NSs gel) exhibited enhanced wound healing compared to the rest of the groups. Figure 15 clearly demonstrated that the diabetic wounds treated with Becaplermin gel exhibited a faster rate of healing compared to all the other groups. The diabetic control group, which did not receive any treatment, showed the slowest healing rate, even when compared to the normal wound control group. Surprisingly, the group with diabetic wounds treated with MP-NSs gel showed earlier and more improved wound healing compared to both the diabetic control group and the normal wound control group. Additionally, when compared to standard MP ointment and MP cream, the MP-NSs gel displayed faster wound closure. This difference in efficacy can be attributed to the sustained release property of the nanosponge-based gel. While the marketed conventional products required thrice-daily application, the MP-NSs gel was administered only once a day due to its sustained release characteristics, leading to superior results in wound healing. Overall, these findings strongly suggest that the MP-NSs gel had a significantly positive effect on accelerating wound healing in diabetic rats. Its sustained release properties not only contributed to better efficacy compared to the conventional products but also facilitated once-daily delivery, simplifying the treatment protocol. These findings demonstrate that the MP-NSs gel was highly effective in promoting wound closure in diabetic rats, with a significant improvement over the untreated diabetic wounds and the control groups treated with MP ointment and MP cream. The standard Becaplermin gel displayed the highest wound closure percentage, but the MP-NSs gel showed a promising and competitive efficacy, making it a potential alternative treatment option for diabetic wound healing.

Table 10. Confirmation of induction of diabetes mellitus in rats

Blood test	Results	
Before STZ IP injection	After 72 h	Random blood glucose level (mg/dL)
67	369	HbA1C level (%)

6.6	9.6	CRP level (mg/L)

Table 11. Tabular representation of percentage wound closure whereas

Days	% Wound closure					
NWC	DWC	DW + MP-NSs Gel	DW + MP Ointment	DW + MP-Cream	DW + Becaplermin gel	4
16.2	5.9	24.2	23.3	23.5	25.3	8
31.3	19.4	39.1	35.1	35.8	42.7	12
49.1	24.8	52.7	48.7	49.2	53.4	14
63.7	37.4	71.8	62.5	61.2	78.9	16

NWC Normal wound control, *DWC* Diabetic wound control, *DW Group 1* Diabetic wound treated with mupirocin-loaded nanosponge gel, *DW Group 2* Diabetic wound treated with MP-ointment, *DW Group 3* Diabetic wound treated with MP-cream, *DW Group 4* Diabetic wound treated with Becaplermin gel

Fig. 14 [Images not available. See PDF.]

Graphical representation of percentage wound closure whereas, NWC- normal wound control, DWC- diabetic wound control, DW Group 1 -diabetic wound treated with mupirocin-loaded nanosponge gel, DW Group 2- Diabetic wound treated with MP-Ointment, DW Group 3- Diabetic wound treated with MP-Cream, DW Group 4- Diabetic wound treated with Becaplermin gel

Fig. 15 [Images not available. See PDF.]

Photographic representation of wound healing and closure of different animal groups

Discussion

The study aimed to develop Mupirocin-loaded nanosponges using the emulsion solvent diffusion method, an alternative and promising approach for nanosponge preparation. This method offers advantages such as simplicity, reproducibility, and rapidity, while also avoiding the use of toxic solvents. To optimise the nanosponge formulation, a factorial design with EC concentration and stirring rate as independent variables was employed. The results demonstrated the significant influence of these variables on PY, EE, and MPS of the nanosponges.

Regarding MP entrapment efficiency (%EE), increasing the EC concentration positively impacted %EE, meaning that higher EC concentrations led to improved drug entrapment within the nanosponges. Conversely, higher stirring rates had a negative effect on %EE, indicating that excessive stirring negatively influenced drug entrapment. For production yield, both EC concentration and stirring rate had a positive influence, contributing to higher yields of nanosponges. The MPS of the nanosponges was influenced positively by EC concentration but negatively by stirring rate. This indicates that higher EC concentrations and lower stirring rates resulted in larger nanosponge particle sizes.

The FT-IR analysis provided insights into the interactions between mupirocin, ethyl cellulose, and carbopol, confirming the stability of the formulations. The thermal analysis and X-ray diffraction (XRD) data confirmed the successful entrapment of mupirocin within the nanosponges, converting it from a crystalline to an amorphous state. The nanosponge formulation (F9) showed promising properties, including an MPS of 189 nm and a zeta potential of (−) 26 mV, indicating a stable and effective nanosponge formulation. The nanosponge-gel exhibited sustained drug release for up to 24 h, providing potential advantages in controlled drug delivery. The nanosponge formulation demonstrated improved drug deposition in excised rat abdominal skin compared to conventional formulations like ointment and cream. The HET-CAM irritation study indicated that the nanosponge-gel was non-irritant, making it a safe formulation for topical application. The stability study revealed that the nanosponge-gel remained stable over a three-month period, with a drug entrapment efficiency greater than 90%, suggesting the potential for long-term shelf life.

The in vivo study on diabetic rat wounds demonstrated that the nanosponge-gel significantly accelerated wound healing compared to conventional products and even outperformed MP ointment and MP cream. The sustained release property of the nanosponge-gel facilitated once-daily delivery and provided improved wound closure efficacy. while the gel fostered an optimally humid environment conducive to wound care during the final stages of wound healing and sealing [46].

The acceleration of wound healing is primarily attributed to Mupirocin, which plays a pivotal role in stimulating the production of growth factors and the proliferation of human keratinocytes [18]. Additionally, the nanosponges, due to their smaller size, exhibit enhanced penetration capabilities into deeper tissues at the injured site. The gel component of the formulation fosters an optimally humid environment, particularly beneficial during the final stages of wound healing and sealing. Therefore, the combined efficacy of Mupirocin-loaded nanosponge gel as a dosage form significantly contributes to the overall acceleration of the wound healing process.

Overall, the results of this study indicate successful development of mupirocin-loaded nanosponges using the emulsion solvent diffusion method, providing a potential alternative for wound healing in diabetic rats. The nanosponge-gel exhibited favourable properties, including sustained drug release, enhanced drug deposition within the skin, stability, and non-irritating characteristics. This novel formulation has promising potential for pharmaceutical applications and merits further investigation for potential clinical translation as an effective and convenient treatment option for wound healing.

Conclusion

A novel drug delivery system utilising mupirocin-loaded nanosponges has been successfully developed to facilitate once-a-day sustained release medication for the topical treatment of diabetic wounds. The innovative formulations demonstrated improved drug retention within the skin, showcasing the superior potential of the nanosponge-based delivery system when compared to conventional mupirocin ointments and creams available in the market. Considering the heightened effectiveness and the enhanced patient adherence due to reduced application frequency, it is evident that the nanosponge-based gel formulations will play a substantially more beneficial role in the treatment of diabetic wounds.

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Author contributions

RSD and PSS have taken efforts to design the study. RSD did experimental, DRT, PAR, and DSW helped in execution of study. All the authors have taken efforts to develop and evaluate the formulation. RSD have interpreted the data obtained during the study. RSD and PSS drafted, and reviewed the manuscript for further communications.

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Declarations

Ethics approval and consent to participate

The manuscript is solely submitted to this journal and has not been sent elsewhere simultaneously. The content is original, never published before, and complies with ethical standards. Results are presented transparently, without manipulation. Data acquisition adheres to discipline-specific guidelines. The study's experimental procedure received approval from the Animal Ethics Committee of Department of Pharmaceutical Sciences, RTMNU, Nagpur (Approval number IAEC/UDPS/2022/02/08), following Indian Committee for the Purpose of Control and Supervision of Experiments with Animals (CPCSEA) specifications.

Consent for publication

Yes.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

DM

Diabetes mellitus

T2DM

Type 2 diabetes mellitus

DFU

Diabetic foot ulcer

DWC

Diabetic wound control

MP

Mupirocin

NSs

Nanosponges

EC

Ethyl cellulose

PVA

Polyvinyl alcohol

DCM

Dichloromethane

CP 934

Carbopol 934

STZ

Streptozotocin

I.P

Intraperitoneal

DMSO

Dimethyl sulfoxide

ESD

Emulsion solvent diffusion

MP-NSs

Mupirocin-loaded nanosponges

ANOVA

Analysis of variance

FT-IR

Fourier transform infrared

DSC

Differential scanning calorimetry

XRD

X-Ray diffraction

SEM

Scanning electron microscopy

%PY

Per cent production yield

%EE

Per cent entrapment efficiency

MPS

Mean particle size

NaCL

Sodium chloride

NaOH

Sodium hydroxide

HET-CAM

Hen's Egg Test-Chorioallantoic Membrane

ZOI

Zone of inhibition

RT

Room temperature

CFU

Colony forming units

NBH

Nutrition Broth Medium

mL

Millilitre

min

Minutes

hrs.

Hours

g

Gram

μm

Micrometer

rpm

Rotation per minute

°C/min

Degree Celsius per minute

Cm

Centimetre

cm^{-1}

Per centimetre

Conc.

Concentration

Publisher's Note

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Evaluating the prediction power and accuracy of two smart response surface experimental designs after revisiting repaglinide floating tablets

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ABSTRACT (ENGLISH)

Background

There is a soar in the figure of companies aiming to achieve efficiency in undergoing experimental processes. Therefore, instead of deploying one-factor-at-a-time, design of experiments is becoming rampantly utilized in order to reduce the resources outflow. There are a copious of different smart designs which could be employed as design of experiments tools. Central composite and d-optimal designs were investigated in this paper. The purpose of this investigation was to compare the two designs and identify the most accurate design at analyzing, interpreting and making predictions with regards to the data offered. The aforementioned purpose was achieved by applying both designs to a preexisting study which sought to prolong the gastrointestinal retention of repaglinide tablets through deploying a full factorial design. Further optimization was performed using Design-Expert software after inducing an outlier point.

Results

R-squared, adjusted *R*-squared, predicted *R*-squared and adequate precision were computed in addition to acquiring diagnostics figures such as predicted versus actual, residual versus run, Box–Cox, contour plot and 3D surface plots. Model equations were also produced for each design. Results showed that both designs were successful at modeling the data both scoring *r*-squared values >0.7 and adequate precision >4 implying high fitting, prediction power and ability to navigate the experimental space using a reduced number of experimental runs. The d-optimal design obtained the least relative error of only 3.81%.

Conclusions

In conclusion, the d-optimal design provides a great tool for reduction of experimental testing which in turn diminishes resources consumption. Therefore, this design is favored to be enforced in the pharmaceutical sector.

FULL TEXT

Background

The production of pharmaceutical commodities is a sophisticated, time-consuming, expensive and labor-intensive endeavor which necessitates extensive planning and exhaustive testing of products and processes with the aspire of achieving the most optimized process and the superlative quality of the medicine [1]. For this reason, many methods have prevailed with the purpose of coherent optimizing the production of medications [2]. Conventionally, one-factor-at-a-time (OFAT) studies were adopted to deduce the optimized formulation of remedies. The one-factor-at-a-time optimization approach adheres to the concept that in order to ascertain the impact of a single factor, it is imperative to keep all other factors constant. In other words, only one factor is altered at a time. This experimental design may not be the most efficient approach when the factors under consideration are interacting or interfering. Due to the proven inefficiency of OFAT, it is now rarely implemented in the pharmaceutical engineering processes. To solve the impracticality of OFAT, a new statistical approach was developed by Sir Ronald Fitcher in the twentieth century [3]. His method was named as “design of experiments (DOE)”. This systematic formulation of experiments entails a multifaceted strategy aiming at enhancing the quality of a product through limited experimentation and judicious allocation of resources [4]. The design of experiment (DOE) approach relies on planning and executing the least amount of experiments where variables are altered simultaneously to produce a cause-and-effect relationship, while minimizing errors [5–7]. The steps of DOE deployment are designing, developing, evaluating and finally analyzing

the product. The application of design of experiments (DOE) is widely employed for the execution of Quality by Design (QbD) [8]. In this context, Q8 and Q9 are major constituents of QbD where Q8-pharmaceutical development and Q9-quality risk assessment were first introduced in the international conference on harmonization (ICH) in 2009. Quality by Design is a concept which revolves around the notion that attainment of product and process understanding serves as the pivotal factor in ensuring the quality of the end product [9, 10]. This comprehensive apprehension is illustrated by embedding quality in the developing stages of the product and its processes of manufacturing in lieu of testing for quality after the manufacturing process is finalized [11]. Mitigating possible quality hazards is crucially achieved by identifying possible failures that may negatively influence the quality of the product and subsequently actions are put in place to ensure sustainment of product compliance with the quality standards [12]. Possible failures that might prevail could be pinpointed using fishbone diagram, a root cause analysis tool.
There are a multitude of different designs currently employed throughout the pharmaceutical industry. Two of the response surface designs include the central composite and the d-optimal. Central composite designs fundamentally select the upper and lower limits of testing values and extend the space of the experiments beyond both thresholds (alpha + 1 &alpha − 1) [13]. The central composite design is suitable for materials which are insensitive to harsh testing condition. Sensitive materials which should not be implemented in the central composite design include proteins and liposomes. On the other hand, the d-optimal design works by assembling information matrices for all points then deducing their determinants. The points procuring the highest determinants are encompassed in the model [14].
Despite differences, both are concurrently considered smart designs. These types of designs depend on exploiting rich-information points to establish their models. Rich-information points consist of a lower number of points which conceal the space of the experiment effectively.
In the current study, the use of smart response surface designs such as the d-optimal and the central composite was proposed instead of the full factorial in order to optimize pharmaceutical dosage forms aiming for the reduction of number of experiments and therefore saving resources, time and effort. Moreover, the two investigated smart designs were compared regarding the *r*-squared, adjusted *r*-squared, predicted *r*-squared, adequate precision and through different diagnostics tests and finally comparing them regarding the percentage relative error. The design with the lowest relative error was recommended.
To our knowledge, this is the first study that compares these two smart experimental designs in the optimization of pharmaceutical dosage forms. This concept can be projected to the other more sophisticated pharmaceutical processes such as extraction or analysis methods [15, 16] and to optimize advanced drugs carriers and delivery systems such as the lipidic, polymeric and inorganic nanoparticles [17–19] and regarding their different processes of preparation and characterization [20–23].

Methodology

Software

The models and plots for the d-optimal and central composite designs that were provided in this paper were produced using Design Expert v.7.0. software (Design-Expert software, Stat-Ease Inc., MN).

The investigated work

For the purpose of comparing the d-optimal and central composite designs, a paper was selected as a basis for application of both designs [24]. Subsequently, comparing and contrasting the two models was conducted. The chosen paper was entitled “Design expert supported mathematical optimization of repaglinide gastroretentive floating tablets: in vitro and in vivo evaluation” [24]. Repaglinide is an oral agent that falls under the meglitinide class, serving as an anti-hyperglycemic medication. It necessitates regular administration prior to meals due to its brief half-life, which lasts only one hour. Consequently, the medication can result in adverse effects, including discomfort in skeletal muscles, headaches, and gastrointestinal disturbances [25]. The investigation presented in the selected paper aimed to prolong the absorbance of Repaglinide tablets by optimizing the critical quality attributes (CQAs) entailing the floating lag time response. This was accomplished using a three-factor three-level full factorial design (usually called 33 full factorial design). Three different components concentrations were altered: Okra gum (OG),

HPMC (hydroxypropyl methylcellulose) K15M and xanthan gum. In QbD, the factors contributing in the CQAs are the CPPs (critical process parameters) and the material and formulation parameters.

Other than the factors included in the investigated study, some factors also representing CPPs of the prepared tableting process included subjecting the granules to adjusted conditions of a temperature range of 55–60 °C for approximately 120 min, while ensuring that the moisture content remained within the range of 3–5%. The powder was administered through an 80-mesh (0.177 mm pore size) after initial mixing. A 30-mesh (0.595 mm pore size) was used after adding a portion of the granulating medium. A 30-mesh (0.595 mm pore size) was used once more to sift the granules following drying. Furthermore, the compression force was adjusted to maintain the hardness of the tablets within 5 to 8 kg/cm^2.

The material and formulation parameters comprised other numerous crucial excipients satisfying various concentrations, including 2 mg of repaglinide, 10% sodium bicarbonate, 5% citric acid, 7% ethyl cellulose, 2% magnesium stearate, 1% talc in addition to lactose with a quantity sufficient to produce a 200-mg tablet. The granulation medium comprised 8% PVP K30 in 80% ethanol [24].

The use of central composite and d-optimal designs to reoptimize the results

Both central composite and d-optimal designs were used to further optimize the generated gastroretentive tablets regarding the floating lag time response instead of the three-level three-factor full factorial design which originally consisted of 27 experimental runs.

A total of 20 points were used to produce the central composite design model; six of the design points were center points.

On the other hand, a total of 25 points were used to build the d-optimal design model. The 25 points comprised 10 model points, 5 replicate points, 5 points to estimate lack of fit and 5 additional center points. The “Model use” was adjusted to point exchange prior to execution.

Table 1 demonstrates the used factors (the investigated material and formulation parameters) accompanied with their tested ranges.

Table 1. The investigated factors associated with their used ranges

Factors	Ranges of values (%)		
High (+1)	Medium (0)	Low (−1)	Concentrations of OG
35	22.5	10	HPMC K15M
15	7.5	0	Xanthan gum

Induction of an outlier

The results of the new embraced design points were produced from the equation that was generated in the extensively examined paper [24]. An outlier central point result was introduced for all the central points of both of the newly adopted designs. Accordingly, the floating lag time for coded points corresponding to (0, 0, 0) for OG, HPMC K15M and xanthan gum, respectively, was altered to a value of 90 s instead of 45.

Analysis of results

It is necessary to document the process by which the models were generated which were both quadratic. To empirically ascertain the significance of the model, ANOVA analysis was performed. This statistical test permits the assessment of differences underlying groups and provides valuable insights into the overall effectiveness and sturdiness of the model. Moreover, in order to affirm the reliability and accuracy of the results, an assortment of

values was computed including; *R*-squared, adjusted *R*-squared and predicted *R*-squared. *R*-squared value reflects the fitting of the model, adjusted *R*-squared reflects the model's *R*-squared value after insignificant terms are excluded and the predicted *R*-squared represents the model's accuracy at predicting the floating lag time [1]. Additionally, the adequate precision of the model was also determined. This measure provides a quantitative assessment regarding the signal to noise ratio [26]. Moreover, to further appraise the validity and precision of the model and identify any potential flaws or inefficiencies that could be ameliorated, a series of diagnostic tests were conducted. These tests including Box–Cox, residual versus run and predicted versus actual provide a valuable insight regarding the validity of the model and notifies for any necessary adjustments or modifications [27]. Finally, in order to visually portray the model and facilitate a deeper understanding of its underlying relationships, contour and 3D surface plots were obtained. These plots provide a graphical representation of how the change in the compositions of (OG, xanthan gum and HPMCK15) contributes to the response and highlights how the variation in these variables are reflected upon the outcome of the CQA (floating lag time). Furthermore, contour and 3D surface plots clarify the observation of the optimum quadrants of the model and allow for a more intuitive interpretation of the findings. Overall, the process of generating the model, determining its significance, calculating the different values and adequate precision, performing diagnostic tests, and obtaining contour and 3D surface plots were essential elements for ensuring the validity and consistency of the findings presented in this study.

Calculation of the percentage relative error (% relative error)

The percentage relative error was calculated by utilizing the following equation [24]:Relative error%=Predicted value -Actual valuePredicted value×100.

Results

Tables 2 and 3 demonstrate the different runs (points) generated as rich-information points of the central composite and the d-optimal designs, respectively, accompanied with the results of these runs as calculated from the generated equation of the used work of Naveen et al. [24] utilizing a three-level full factorial design to optimize the floating lag time of repaglinide floating tablets.

Table 2. The central composite generated design points associated with their calculated results

Experiment number	OG concentration	HPMCK15M	Xanthan gum	Floating lag time
1	0	0	0	90
2	−1	1	1	71.34
3	0	−1.68	0	141.39
4	0	0	−1.68	74.71
5	−1	−1	−1	72.18
6	1	1	1	69.84
7	1	1	−1	94.78
8	1	−1	−1	177
9	0	0	0	90

10	−1	1	−1	52.28
11	−1.68	0	0	77.72
12	0	0	1.68	79.02
13	0	1.68	0	46.27
14	0	0	0	90
15	−1	−1	1	102.24
16	1	−1	1	163.06
17	0	0	0	90
18	0	0	0	90
19	0	0	0	90
20	1.68	0	0	164.60

Table 3. The d-optimal generated design points associated with their calculated results

Experiment number	OG concentration	HPMCK15M	Xanthan gum	Floating lag time
1	−1	1	−1	52.28
2	−1	0	1	70.21
3	0	0	−1	56.24
4	0	0	0	90
5	0	0	0	90
6	−1	1	1	71.34
7	0	0	0	90
8	0	0	0	90
9	−1	−1	1	102.24
10	0	1	0	35.24

11	1	−1	−1	177
12	−1	1	−1	52.28
13	1	−1	1	163.06
14	1	1	−1	94.78
15	1	−1	−1	177
16	0	−1	−1	98.35
17	0	0	0	90
18	1	1	1	69.84
19	−1	−1	0	76.63
20	0	−0.5	0.5	69.20
21	1	−1	1	163.06
22	1	0	0	99.01
23	1	1	−1	94.78
24	1	1	1	69.84
25	−1	−1	−1	72.18

Table 4 illustrates the significance of both models (P<0.001) [28]. The type of the two generated models corresponding to the two adopted designs was a quadratic function. Furthermore, the R-squared, adjusted R-squared and predicted R-squared differences were within 0.2 increments for both models and all of them scored values above 0.7 implying acceptable and reliable models. For the central composite model, the discrepancy between the R-squared and predicted R-squared was 0.0056 which is a minimal value, while for the d-optimal model, the discrepancy was higher (0.1289). The adequate precision exceeded a value of 4 as counseled for both models (adequate precision for d-optimal was 14.161 and for the central composite was 122.830). Furthermore, the parameter “lack of fit” was favorably insignificant in both models.

Table 4. The generated model analysis results

Design type	Central composite	D-optimal
Significance	Significant	Significant

	P<0.0001	*P*<0.0001
Model type	Quadratic	Quadratic
R-squared	0.9991	0.9210
Adjusted *R*-squared	0.9984	0.8736
Predicted *R*-squared	0.9935	0.7921
Adequate precision	122.830	14.161
Generated equation	Floating Lag Time=-7828.09234+1007.19857 * OGconcentration+880.39460 * HPMC K15M+1364.29190 * XanthanGum-15.58000 * OGconcentration * HPMC K15M-11.00000 * OGconcentration * XanthanGum-2.75000 * HPMC K15M * XanthanGum+4.43181 * OGconcentration2-43.99831 * HPMC K15M2-125.72119 * XanthanGum2	Floating Lag Time=+76.99172+26.21690 * OGconcentration-27.47543 * HPMCK15M+1.21784 * XanthanGum-16.51078 * OGconcentration * HPMCK15M-10.24740 * OGconcentration * XanthanGum-2.74859 * HPMCK15M * XanthanGum+17.47349 * OGconcentration2+4.43398 * HPMCK15M2-1.65511 * XanthanGum2

It is highly visible by observing Fig. 1 that the points present in the predicted versus actual plot of the central composite model were closer to the 45-degree line (indicating the close values of the predicted results to the actual counterparts). Hence, the model floating lag time predictions were closer to the actual values. Also obviously, the predicted versus actual plot of the d-optimal model acquired values that seemed relatively distant from the 45-degree line; yet, they were still considered close.

Fig. 1 [Images not available. See PDF.]

Predicted versus actual plots for **a** central composite and **b** d-optimal designs

As inferred from Fig. 2, the points representing the different runs of the two investigated models were evenly scattered around the zero line of the Design-Expert® generated plots of both designs and represents the models functions. Moreover, for the residual versus run point distribution of the central composite model, 40% of the points were situated above the zero line, approximately 30% were situated on the zero line, and 30% were situated under the zero line. On the other hand, for the residual versus run point distribution for the d-optimal model, 60% of the points were situated above the zero line, 8% of the points were approximately situated on the zero line, and 32% of the points were situated under the zero line.

Fig. 2 [Images not available. See PDF.]

Residual versus run plots for **a** central composite and **b** d-optimal designs

As obvious from Fig. 3, both models powers lied within the confidence interval ranges of the Box–Cox diagnostic and validating test generated from the utilized software while acquiring power correspondents of lambda = 1 [2].

Fig. 3 [Images not available. See PDF.]

Box–Cox plots for **a** central composite and **b** d-optimal designs

Contour plots use varied gradients of colors to represent segments which occupy high and low floating lag time responses as depicted in Fig. 4.

Fig. 4 [Images not available. See PDF.]

Contour plots for **a** central composite and **b** d-optimal designs at a constant xanthan gum (at its middle level, code = 0)

3D surface plots are similar to contour plots in that they show areas where the response is at different values but with a three-dimensional viewing facet. The peak response occurred at the red areas while the lowest response occurred at the blue areas [29]. It is obvious from Fig. 5 that the peak floating lag time response was present at coded values of (1, 0, − 1) corresponding to OG, xanthan gum and HPMCK15 concentrations, respectively.

Fig. 5: [Images not available. See PDF.]

3D surface plots for **a** central composite and **b** d-optimal designs at a constant xanthan gum (at its middle level, code = 0)

The d-optimal design had a lower relative error compared to the central composite design by a difference of 0.7556% for the coded point: 0.66 OG concentration, HPMC K15M and 0.85 xanthan gum, in spite of inducing an outlier critical point (Table 5).

Table 5. Percentage relative error obtained between the predicted and actual values for the coded point: 0.66 OG concentration, 1.00 HPMC K15M and 0.85 xanthan gum

Design	Relative error (%)
Central composite	4.5699
d-optimal	3.8143

Discussion

Both of the discrepancy values between the adjusted *r*-squared and the predicted *r*-squared of the two investigated designs were considered low. Hence, the models were sufficient in predicting the results of un-carried experiments and to fully navigate the experimental space.

The values of adequate precision of both designs indicated a very high signal to noise ratio. Therefore, the differences in the acquired results for the floating lag time were a consequence of real signals and not due to random outcomes and both models are considered successful in navigating the space of the investigated experiment [1].

The predicted versus actual plots mainly evaluate the accuracy of the model at making predictions regarding actual experimental values through depicting how close the predicted values are to the actual ones [2]. This is implemented by using the 45-degree line as a reference. The closer the points are to this line, the higher the capability of the model at making accurate predictions. Despite the fact that the points in both predicted versus actual plots were relatively close to the 45-degree line, these results implied the high predictive ability of the two investigated designs-generated models.

Residual versus run plots usually identify the errors present in the model. The required ideal situation is that the total distances from the points above the zero line, which represents the model, are approximately equal to the total distances of the points under the same line so that the errors even out [30]. This was approximately obtained for both models. As a conclusion, the points in both models were moderately and favorably scattered around the zero line.

The Box–Cox test primarily aims to accommodate the model response (CQA) with the optimum numerical power. The response is raised to different powers and the power with the best fitting is recommended. Usually, the power

which presents the optimum fitting lies in the area between the high and low confidence intervals. The confidence intervals are manifested by the red lines. If the value of the power (lambda) requires altering, then the recommended value should be applied through the transformation tab of the adopted software. Therefore, it was concluded that the power transformation was not required for both of the generated models.

The alteration of color gradients in the contour and the 3D surface plots is correlated with the change of compositions of the factors contributing to the response [14].

The peak floating lag time response scored at coded values of (1, 0, − 1) corresponding to OG, xanthan gum and HPMCK15M concentrations, respectively, may be attributed to the higher swelling index of okra gum (260% [31]) as compared to the other constituents (xanthan gum and HPMCK15M which only reached a maximum of<250% [32]) contributing to its significant positive effect on the floating lag time.

It could be interpreted from the current study figures and tables that both designs have been successfully leveraged to produce models with excellent qualities. Despite that the central composite scored better *R*-squared, adjusted *R*-squared, predicted *R*-squared and adequate precision values, the d-optimal design was slightly more accurate at predicting the floating lag time response The high accuracy of both models generated from the two utilized smart surface response designs at predicting the floating lag time response is related to a statistical perspective where DoEs utilizing designs such as the central composite and the d-optimal create response models by reducing the maximum variance of the predicted responses and minimizing the error in the estimated coefficients of the model. This approach offers benefits when employing disproportionate shapes and incorporating additional design points [28]. Moreover, the superiority of the d-optimal design at predicting the response for experimental points which were not included as design points (as inferred from the calculated value of the percentage relative error) comes back to the statistical element of building the design through choosing rich-information points that originate from an information matrix possessing the highest determinant which allows handling of a larger experimental space [33]. Although the CCD resulted in excellent and slightly higher *R*-squared values, this may be ascribed to the problem of overfitting that sometimes occur with experimental designs, wherein the model excessively coincides with and conforms to the existing data points. This phenomenon results in a perfect or ideal coinciding of the actual experimental design points with the generated model predictions. It usually happens with higher order functions (above linear, quadratic, cubic, etc.) possessing high curvatures aiming to reduce residuals of the generated model results [34]. This was obviously noticed in the predicted versus actual figure corresponding to the CCD results (Fig. 1). It is worth-noting that the limitation of this paper lied on the use of only one check point (experimental external validation point) in calculating the percentage relative error of the two investigated statistical experimental designs. Nevertheless, that was a forced limitation because this point was solely conducted in the originally experimental paper that the current paper was based on.

Conclusion

The current study aimed to compare the central composite and the d-optimal statistical experimental designs in optimizing the floating lag time response of repaglinide gastroretentive tablets.

The findings of this study showed that:

- Both smart designs extensively discussed in the paper have been successfully utilized to further optimize the tablets with a very high accuracy similar to a previous optimization implemented using a three-level full factorial design despite the induction of an outlier point representing the central critical point of both designs.
- After computing the percentage relative error, it was concluded that the d-optimal design is more robust in predicting the accurate result values of actual experiments of points not included in the designs built.
- The adopted work drew the attention to the problem of overfitting which may lead to decrease the predictivity power of the statistical experimental designs.

Based on the aforementioned conclusions, integrating both smart surface response designs and more specifically the d-optimal design into the routine of experimental activities, companies can excel the effectiveness of testing in addition to reducing expenses by considerable margins. The use of the investigated smart statistical experimental designs accompanied with its assessment can be projected to any dosage form design and conventional or advanced drug delivery systems aiming of reducing the number of runs and experiments conducted and hence saving resources, efforts and time.

One important contribution of this paper is that it provides a guide or an assist to companies and especially pharmaceutical entities when choosing the smart design, they seek to adopt.

Moreover, another contribution of this paper toward the pharmaceutical industry is that it could guide users on how to calibrate design expert software efficiently to produce the d-optimal and central composite designs and their generated models. Future studies should compare the d-optimal design with other smart counterparts.

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Author contributions

RMH contributed to conceptualization; TE and RMH provided methodology; RMH performed formal analysis and investigation; TE performed writing—original draft preparation; RMH contributed to writing—review and editing, resources, and supervision.

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The data are available upon request.

Code availability

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable for this work.

Consent for publication

The authors declare no conflict of interest.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

OFAT

One-factor-at-a-time

DoE

Design of experiments

GIT

Gastrointestinal TRACT

QbD

Quality by design

ICH

International Conference on Harmonization

GMP

Good manufacturing practices

CQAs

Critical quality attributes

CPPs

Critical process parameters

OG

Okra gum

HPMC

Hydroxypropyl methylcellulose

ANOVA

Analysis of variance

3D

Three-dimensional

Publisher's Note

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Optimization of simvastatin transdermal patch for hyperlipidemia treatment in rat model

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ABSTRACT (ENGLISH)

Objective

Biopharmaceutics Classification System says that simvastatin (SMV) is a Class II drug with low bioavailability (5%). This is because it dissolves slowly and is broken down a lot in the first pass. Simvastatin transdermal patches were produced as part of this study's aim to treat hyperlipidemia. The Box–Behnken design (three-factor, three-level) was selected for optimization of patches.

Methodology

The optimization design involved 15 runs with independent factors hydroxypropyl methylcellulose K100, Eudragit L100, and polyethylene glycol 400 percentage, and dependent factors including folding endurance and in-vitro drug release.

Results

The results showed that the concentration of hydroxypropyl methylcellulose K-100 positively impacted the patch's folding endurance. The fact that factor C was the only scenario where the *p*-value was less than 0.05 and the coefficient value was higher in the in vitro drug release model means that it has a greater influence on the release of medicines. The patches were also evaluated for drug content, swelling, moisture uptake, moisture content, etc. The optimized patch shows an in vitro drug release of 55.3% in up to 24 h. In vivo antihyperlipidemic activity was evaluated in albino Wistar rats. In the standard treatment (simvastatin oral) groups, there is a decrease in cholesterol (132.76±0.35) and triglyceride level (139.80±76) whereas in the test formulation group or test group, there is also a decrease in cholesterol (169.65±0.21 mg/dL) and triglyceride level (151.20±31 mg/dL) level.

Conclusion

Based on in-vitro and in-vivo results it can be concluded that simvastatin patches can be an alternative to traditional therapy.

FULL TEXT

Background

There are considerable changes in plasma concentration when widely used dosage forms such as tablets, capsules, and oral liquids are administered in repeated doses. This fluctuation may lead to various adverse effects. Therefore, different modified release systems were successfully tested to minimize the fluctuation. There are several administration methods, including as oral, mucosal, inhalation, transdermal, and intravenous injection, depending on the delivery route. A promising method that can imitate the plasma concentration profile of intravenous infusion is the transdermal drug delivery system (TDDS), until the drug is present in the system [1]. In comparison to other routes of administration, the skin has many benefits, including the avoidance of gastric irritation, emptying rate effects and pH, hepatic first-pass metabolism, reduction of systemic side effects, sustained drug release, faster termination of therapy, reduced fluctuations in plasma levels, and avoidance of injection-related pain [2]. According to Zondek, when used topically as a 30 percent lanolin ointment, the external disinfectant chloroxylenol may be successful in treating urogenital infections [3–5] and open a new way to deliver the drug systemically through topical route. Over the past 40 years, a great deal of research has been done on transdermal administration of active pharmacological ingredients (API) [6]. A transdermal patch is a device that allows the API contained in it to travel through the skin and deliver medications to specific cells or organs [7]. The market for transdermal medication administration is about to shift into a new stage where pain relief isn't the primary driver [8]. Hormones, cardiovascular drugs, and drugs for the central nervous system are also administered through the transdermal system [8]. With the use of more recent drug delivery techniques that are effective, predictable, and safe, continuous delivery of a biologically active moiety is possible either systemically or to a specific site. They can perform better than traditional administration methods by monitoring the intensity, location, and length of the pharmacological action. A patch that is placed on the skin and includes medication that is meant to be absorbed into the bloodstream through the skin is called a transdermal patch [9], also known as a skin patch. Transdermal drug delivery has many benefits, including fewer side effects, less repetitive administration to achieve the desired plasma concentration, prolonged drug delivery, a reduction in the first-pass effect, and enhanced patient compliance, interruption of therapy when necessary etc. [10] All statin drugs have poor aqueous solubility and low oral bioavailability [11]. Due to their history of efficacy in adults, statins are one of the first-line medications considered for use in the pediatric population with dyslipidemia [12]. Simvastatin (SIM) inhibits 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase [13] and is commonly prescribed for cardiovascular illnesses due to its effects on decreasing lipid levels. Simvastatin enhances capillary morphogenesis in vitro and inhibits apoptosis in lymphatic endothelial cells, suggesting that it has lymphangiogenic properties that aid in the healing of wounds [14]. According to the Biopharmaceutics Classification System (BCS), SIM is a Class II [15] medication with low bioavailability (5%) due to its slow rate of solubility and extensive first-pass metabolism. SIM's poor oral bioavailability must be improved, which requires an increase in solubility. Although SIM has a short half-life, around two hours and a low water solubility (aqueous solubility 0.03 mg/L), it is removed by cytochrome P3A, which undergoes substantial metabolism in the liver and the intestines [16]. Delivery methods include transdermal, buccal, rectal, and parenteral are among the strategies that could be used to prevent first-pass metabolism. SIM is an excellent choice for the creation of transdermal films because to its strong first-pass metabolism, low molecular weight (418.56), high lipid solubility, low melting point (129 °C), capacity to act at low plasma concentrations [17]. The aim of this work to prepare a simvastatin transdermal patch (SIM-TP) and study its antihyperlipidemic activity in an animal model.

Methods

Simvastatin was gift from Biodeal Pharmaceutical Pvt Ltd. Himachal Pradesh, India. Hydroxypropylmethyl cellulose (HPMC K100) was gift from Colorcon Asia Pvt Ltd, Goa, India and Eudragit RL 100 was obtained from EvoniK Roehm Pharma Polymers and all other ingredients are of analytical grade.

Preparation of SIM-TP

The formulation of SIM-TP is shown in Table 1. The solvent casting procedure was used to prepare the transdermal

patch. Ethanol: dichloromethane (1:1) was used to dissolve the polymers Eudragit L-100 and HPMC K100. The polymeric dispersion was stirred continuously with a magnetic stirrer until a clear solution formed. Polyethylene glycol 400 (PEG 400) was used to dissolve the drug before it was added to the polymer solution. After that, the homogeneous polymer solution containing the drug was added to a petri dish that had been lined with aluminum foil and allowed to air dry for 24 h at room temperature. The petri dish was covered with an inverted funnel to stop the solvent from evaporating too quickly. Desiccators were used to store the dried patches for future study.

Table 1. Formulation of transdermal patch

Ingredients	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	F15
SIMVASTATIN (%)	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
HPMC K-100(%)	1	0.2	1.8	0.2	1.8	1	1	1	1	1	1.8	0.2	1.8	0.2	1
EUDRAGIT L-100 (%)	0.1	1	1	0.55	0.1	0.55	0.55	1	0.55	1	0.55	0.55	0.55	0.1	0.1
PEG -400(%)	50	30	30	10	30	30	30	50	30	10	10	50	50	30	10
Ethanol:Dichloromethane (ml)	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20

Optimization of SIM-TP

The response surface design The Box–Behnken design was utilized to optimize the simvastatin transdermal patch (SIM-TP). The three-factor, three-level design was selected. The three independent variables were HPMC K100, Eudragit RL100 (ERL100), and PEG400. Table 2 displays the variables and their corresponding levels. The Design-Expert 13 software proposed a 15-run trial for the optimization process. The proposed design is shown in Table 3.

Table 2. Independent variables and their levels for optimization study

Factor	Name	Units	Minimum	Maximum	Coded Low	Coded High	Mean	SD
A	HPMC K100	%	0.20	1.8	−1↔0.20	+1↔1.8	1.0000	0.6047
B	ERL 100	%	0.10	1.0	−1↔0.10	+1↔1.0	0.5500	0.3402
C	PEG 400	%	10.00	50.0	−1↔10.0	+1↔50.0	30.00	15.12

Table 3. Design of experiment for formulations of transdermal patch of Simvastatin

Std	Run	Factor A	Factor B	Factor C
HPMC K100%	ERL 100%	PEG400%	11	1
1	0.1	50	3	2
0.2	1	30	4	3

1.8	1	30	5	4
0.2	0.55	10	2	5
1.8	0.1	30	14	6
1	0.55	30	13	7
1	0.55	30	12	8
1	1	50	15	9
1	0.55	30	10	10
1	1	10	6	11
1.8	0.55	10	7	12
0.2	0.55	50	8	13
1.8	0.55	50	1	14
0.2	0.1	30	9	15

Evaluation of transdermal patch

Physical appearance

Every transdermal patch was visually inspected for color, smoothness, and uniformity.

Thickness

A screw gauge was used to measure the patches' thickness. Each batch of films had its average and standard deviation calculated based on five readings.

Folding endurance

Careful consideration was given to the prepared films' capacity for multiple folds in one location. The patch strip (2 × 2 cm^2) was folded repeatedly till it broke. The maximum simultaneous folds the film could withstand before failing were counted [18].

pH determination

The transdermal films were incubated in a glass petri dish for an hour in 0.5 ml of double-distilled water to give them time to swell. The glass electrode was allowed to acclimate before being brought near to the film surface. The surface pH was then measured with a digital pH meter, and the results were noted [19, 20].

Swelling index

The patch was weighed after being submerged in a petri dish containing 50 ml of pH 7.4 phosphate buffer every 10 min. The following formula was used to determine the swelling's intensity [21].

1

SI%=Xt-XoXo×100where the weight of the patch both before and after it absorbs moisture is represented by Xo and Xt.

Drug content

Transdermal films were divided into pieces with a predetermined area (1 cm^2), placed in a volumetric flask of

100 mL, and then dissolved in methanol. After being sonicated, the mixture was left for 24 h. A 0.45-µm membrane was used to filter the fluid. Using a UV spectrophotometer (Labindia), the amount of medication in the solution was determined at 237 nm, the absorbance was measured, and data were collected [22].

Moisture content

The produced films were weighed individually until they reached equilibrium weight after being stored in desiccators with fused calcium chloride for 24 h at 40 °C. After reweighing the film 24 h later, the moisture content percentage was calculated using the procedure below [23].

2

%MoistureContent=Initialweight-FinalweightInitialweight×100

Moisture uptake

The weighted films stored in a desiccator with a saturated potassium chloride solution for 24 h at 40 °C in order to maintain a Relative Humidity of 84%. The films must be reweighed after 24 h to determine the amount of moisture absorbed [23, 24].

3

%MoistureContent=Finalweight-InitialweightInitialweight×100

In-vitro drug release

The *in-vitro* release study was conducted using a Franz diffusion (FD) cell with a dialysis membrane (HIMEDIA LA 395–10 MT). Phosphate buffer pH 7.4 with sodium lauryl sulfate (SLS) (0.5% w/v) served as the dissolving media. A phosphate buffer was placed into the diffusion cell's receptor compartment. The dialysis membrane received a buffer rinse before the experiment. Between the donor and receiver chambers of the diffusion cell, the membrane was securely clamped in place. A constant 37 °C was maintained while the diffusion cell was continuously stirred at a speed of 50 rpm. Samples were taken periodically to maintain the sink condition, and each time, an equivalent volume of phosphate buffer was added to the receptor compartment. Using a UV–visible spectrophotometer, the samples' absorbance at 237 nm was measured, and the cumulative percent drug release was computed [25].

Scanning electron microscopy (SEM)

To analyze their surface topography and morphology, a prepared patch was coated with gold–palladium under an argon atmosphere at ambient temperature. The surface morphology can then be examined using SEM. SEM can be used to assess the ultra-structure of a broken micro sponge particle.

In-vivo activity (animal study)

Antihyperlipidemic activity

The animals used were albino rats (150–250 g, either sex). The group and their treatment were shown in Table 4. Prior to the experiment, each animal was fasted for 12 h. Blood samples were then collected from each animal's retro-orbital sinus using capillary tubes while they were all under diethyl ether anesthesia to determine their lipid profiles. After being extracted, the serum was centrifuged for 10 min at 5000 rpm before being stored at − 20 °C for analysis. Prior to the test, the abdomen's hair was plucked with a depilatory cream and then rinsed with distilled water. The animals were given anesthesia before being restrained to a supine position on the day of the trial. On the area of the belly that was hair-free, the pressure-sensitive adhesive transdermal patch (2.5 cm^2) was applied. An oral feeding needle was used to provide the medicine solution (10 mg/kg). Following oral and transdermal medication, blood samples of around 0.5 ml were collected via a retro-orbital puncture at intervals of 0.5, 2, 4, 8, 12, 24, and 48 h. A glass tube containing heparin and the anticoagulant ammonium oxalate was filled with blood from a retro-orbital puncture through capillaries (1 percent solution). After being rapidly separated by microcentrifugation at 5000 rpm, the plasma was stored at − 20 °C. An autoanalyzer was used to analyze the samples [26].

Table 4. Groups and their treatment

Group	Treatment

Normal control	Normal Saline
Disease control	Triton (300 mg/kg)
Standard	Oral Simvastatin (10 mg/kg) + Triton (300 mg/kg)
Test	Patch (2 cm^2) + Triton (300 mg/kg)

Skin irritation studies

The ability of the patches to irritate or sensitize the skin of rats was evaluated. 150–250 g albino rats of either sex were used in the experiment. To minimize collateral damage, the dorsal surface of the rat was carefully groomed. The rat's skin was shaved, and the optimized patch was applied, secured with tape. The animal was observed for 24 h to look for any signs of erythema or edema [27, 28].

Results

Discussion

Formulation optimization

The experiment's design called for 15 runs for the set of variables. All the runs recommended by the DOE were developed and tested for responses like folding endurance and drug release. Table 5 displays the design of experimental runs along with their responses.

Table 5. Design of experiment and the responses

Run	Factor A	Factor B	Factor C	Response 1	Response 2
HPMC K100 (%)	ERL 100 (%)	PEG (%)	Folding Endurance	In-vitro release (%)	1
1	0.1	50	287 ± 4	53.58 ± 0.05	2
0.2	1	30	40 ± 6	53.88 ± 0.03	3
1.8	1	30	250 ± 4	52.69 ± 0.04	4
0.2	0.55	10	60 ± 5	43.91 ± 0.05	5
1.8	0.1	30	275 ± 8	48.79 ± 0.01	6
1	0.55	30	43 ± 2	49.92 ± 0.05	7
1	0.55	30	244 ± 7	42.12 ± 0.04	8
1	1	50	280 ± 5	51.04 ± 0.02	9
1	0.55	30	125 ± 8	47.51 ± 0.03	10
1	1	10	187 ± 9	40.56 ± 0.02	11

1.8	0.55	10	143±7	38.51±0.01	12
0.2	0.55	50	50±3	55.52±0.03	13
1.8	0.55	50	166±6	52.53±0.05	14
0.2	0.1	30	60±4	54.18±0.02	15

Folding endurance

Folding endurance is the ability of any patch to withstand pressure applied in the form of folding at the same spot without breaking. This is an important need for patches since activity causes the skin at the application site to stretch and contract repeatedly, and the patch needs to have the best folding endurance during those times. The folding endurance equation is represented by Eq. 1, and the ANOVA table is represented as Table 6.

4

$$Foldingendurance=9.2375+97.5*A+0.833333*B+1.63125*C$$

Table 6. ANOVA of Linear model of folding endurance

Source	Sum of Squares	df	Mean Square	F-value	*p*-value	
Model	57,188.25	3	19,062.75	3.24	0.0643	Not significant
A	48,672.00	1	48,672.00	8.27	0.0151	
B	1.13	1	1.13	0.0002	0.9892	
C	8515.13	1	8515.13	1.45	0.2543	
Residual	64,749.48	11	5886.32			
Lack of Fit	44,320.82	9	4924.54	0.4821	0.8184	Not significant
Pure Error	20,428.67	2	10,214.33			
Cor Total	1.219E+05	14				

The Model F-value of 3.24 indicates that there is a 6.43% possibility that noise may result in an F-value this significant. Lack of fit is not significant when compared to pure error, as indicated by the F-value of 0.48 for the lack of fit. A negligible lack of fit is advantageous for the model.

For model terms to be deemed significant, the *P*-value needs to be less than 0.05. In cases where the value exceeds 0.1, model terms are not meaningful. Folding endurance was significantly impacted by Factor A, or HPMC K-100 concentration (*p*-value 0.0151). According to Eq. 1, Factor A has the highest coefficient and is preceded by a positive sign, indicating that Folding endurance improved with increasing HPMC concentration. This means that patches with greater HPMC K100 percentages tend to have higher folding endurance. Although increasing the concentration of PEG and ERL100 has a effect on folding endurance, there is a slight increase in folding endurance due to this effect. Therefore, there is a correlation between higher polymer concentration and increased folding endurance. Figure 1 displays the 3D response surface diagram of folding endurance.

Fig. 1 [Images not available. See PDF.]

3D response surface diagram of folding endurance

In-vitro drug release

The formulation of various drug batches and their related in-vitro release percentages are displayed in Table 5. The formulation's key ingredients are HPMC K100, ERL 100, and PEG, which are altered to produce various release profiles. The amount of medication released from the formulation during a specific time under simulated physiological conditions is measured by the in-vitro release percentage. To achieve the appropriate release profile for the medicine, the formulation can be optimized using the data. Table 7 displays a summary of the model fitting. The linear model for in-vitro drug release was anticipated by the software. Based on its Model F-value of 4.76, the model is deemed significant. Only 2.30 percent of the time is there a probability that noise may result in an F-value this large. The F-value of 1.0 for the lack of fit indicates that it is not significant when compared to the pure error. A non-significant lack of fit is advantageous for the model.

Table 7. Model fit summary of in-vitro drug release

Source	Sequential *p*-value	Lack of Fit *P*-value	Adjusted R^2	Predicted R^2	
Linear	0.0230	0.5932	0.4463	0.1929	Suggested
2FI	0.7942	0.4967	0.3257	− 0.5276	
Quadratic	0.3386	0.5117	0.4191	− 1.2359	
Cubic	0.5117		0.4483		Aliased

The in-vitro drug release ANOVA table is shown in Table 8. The in-vitro drug release model's *p*-value of 0.0230, which is less than 0.05, shows that it is significant.

Table 8. ANOVA table of in-vitro drug release

Source	Sum of Squares	df	Mean Square	F-value	*p*-value	
Model	228.65	3	76.22	4.76	0.0230	Significant
A-HPMC K100	28.01	1	28.01	1.75	0.2127	
B-ERL 100	10.22	1	10.22	0.6381	0.4413	
C-PEG	190.42	1	190.42	11.89	0.0054	
Residual	176.10	11	16.01			
Lack of Fit	144.20	9	16.02	1.00	0.5932	Not significant
Pure Error	31.90	2	15.95			

Cor Total	404.74	14				

The in-vitro drug release model's ANOVA table reveals that factor C, or PEG400 concentration, is the only scenario where the *p*-value is less than 0.05. PEG400 was used as a permeation enhancer in this optimization design. The increased PEG400 concentration will result in increased in-vitro drug release. Equation 2 represented in terms of coded factors allows one to predict the results for specific values of each variable. The relative importance of the variables can be determined by comparing the factor coefficients using the coded equation. Factors A and B are preceded by a negative sign in the equation, and factor C is preceded by a positive sign. An increase in the value of factor C leads to an increase in the release of drugs in vitro, which is what the positive sign denotes as a synergistic effect. The fact that C has a higher coefficient value means that it has a greater influence on the release of medicines.

5

In - vitrodrugrelease=49.0267-1.87125∗A-1.13∗B+4.87875∗C

The optimized value for factors and their response were predicted by software and shown in Table 9. Based on criteria, the software predicted that the optimized patch's levels of factors *A*, *B*, and *C* would be 1.80, 0.1, and 50, respectively, and that its responses to folding endurance and in-vitro drug release would be 266.36 and 53.17, respectively. An optimized patch of the specified level of factors was constructed, and its performance was assessed.

Table 9. Factors level predicted by software for design

Factor	Name	Level	Low Level	High Level	SD	Coding
A	HPMC K100	1.80	0.2000	1.80	0.0000	Actual
B	ERL 100	0.1000	0.1000	1.0000	0.0000	Actual
C	PEG	50.00	10.00	50.00	0.0000	Actual

Thickness and physical appearance

Table 9 displays the thicknesses of all formulations. The resulting patches were homogenous, opaque, pliable, and smooth at varying polymer concentrations. The thickness of the films ranged from 0.52±0.76 to 0.625±0.85 mm.

Swelling index

The formulation and related swelling index of various samples are shown in Table 10 (F1–F15). The swelling index calculates how much the samples swell in a certain solvent. Sample F12 had the highest swelling index of 62.38±0.69%, while sample F2 had the lowest swelling index of 43.45±0.86%. The research indicates that different formulations may cause varying levels of edema and emphasizes the significance of formulating products that are best suited for a given application.

Table 10. Different parameters of simvastatin transdermal patch

Formulation	Thickness (mm)	Swelling Index (%)	Moisture content (%)	Moisture Uptake (%)	Drug content (%)
SIM-TP1	0.55±0.01	45.02±0.79	6.21±0.11	7.21±0.21	94.52±0.75
SIM-TP2	0.54±0.02	43.45±0.86	5.52±0.09	6.25±0.18	92.73±0.68

SIM-TP3	0.52±0.08	50.61±0.67	8.31±0.13	10.53±0.12	94.42±0.67
SIM-TP4	0.56±0.07	57.43±0.44	5.15±0.08	7.69±0.14	89.81±0.60
SIM-TP5	0.56±0.05	53.72±0.65	8.50±0.14	11.42±0.17	95.21±0.58
SIM-TP6	0.57±0.03	45.43±0.78	5.83±0.12	6.22±0.14	95.43±0.56
SIM-TP7	0.58±0.08	55.21±068	6.31±0.11	7.32±0.11	95.7±0.57
SIM-TP8	0.60±0.05	45.18±0.75	6.42±0.11	8.74±0.10	94.63±0.62
SIM-TP9	0.58±0.04	55.73±0.85	6.23±0.10	8.82±0.12	95.15±0.68
SIM-TP10	0.59±0.02	54.34±0.68	7.32±0.13	8.41±0.11	95.56±0.66
SIM-TP11	0.60±0.05	59.77±0.57	8.71±0.12	11.62±0.21	98.75±0.78
SIM-TP12	0.62±0.08	62.38±0.69	5.24±0.11	9.51±0.10	98.35±0.77
SIM-TP13	0.60±0.05	56.23±0.57	8.95±0.12	10.44±0.12	93.13±0.78
SIM-TP14	0.59±0.02	53.17±0.68	5.18±0.10	6.92±0.14	90.76±0.65
SIM-TP15	0.62±0.06	57.24±0.88	6.66±0.09	7.51±0.11	96.64±0.85

Moisture content

Each formulation had a different moisture content ranging from 5.1±0.43% to 8.9±0.51%. All formulas had a mean moisture content of 6.6%, with a standard deviation of 1.27%. Because of the hydrophilic characteristic of HPMC, it was found that high concentrations of HPMC had higher % moisture contents.

Moisture uptake

With a standard deviation of 0.49–0.72, each formulation has a distinct moisture uptake value that spans from 6.2 to 11.6 percent. Patches with high HPMC content exhibited high moisture absorption.

Drug content

The percentages of drugs present in each formulation, designated as F1 through F15, are shown in the Table 9. These percentages show how many active medication components there are in each formulation (measured in mass). The F4 and F14 contain the lowest percentages of drug content, while the F11 and F12 have the highest amounts. The medication concentration in the other formulations is between 90.76 and 98.75%. Pharmaceutical companies employ formulations to enhance the efficacy and delivery of their products (Figs. 2, 3).

Fig. 2 [Images not available. See PDF.]

3D response surface diagram of in-vitro drug release

Fig. 3 [Images not available. See PDF.]

Overlay plot of the design predicting the values of individual factors and their response based on input criteria

In-vitro drug release

The in vitro release profile is a vital tool for predicting how drugs will behave in vivo. Release analyzes are necessary to forecast the repeatability of the rate and duration of pharmaceutical activity. Figure 4 displays the optimized transdermal patch's percentage medication release over the course of 24 h. Over time, the cumulative proportion of medication release gradually rises, reaching its peak at 24 h (55.3%). The variation in drug release characteristics among all patch formulations could perhaps be attributed to the presence of polymeric chain cross-linking networks. Different polymeric mixtures used to make transdermal patches have different diffusion pathways, which affect the delivery and intensity of the dispersion.

Fig. 4 [Images not available. See PDF.]

In-vitro drug release of optimized simvastatin patch

In-vivo study

Albino adult male and female in good health Wistar rats weighing between 110 and 240 g were used. Figure 5 depicts the impact of the simvastatin patch that has been optimized on total cholesterol levels. Rats were effectively exposed to Triton to cause hyperlipidemia, which was visible in the disease-control group. In comparison to normal controlled animals, disease-control animals have higher cholesterol and triglyceride levels (173.200.12 mg/dl and 189.02.68 mg/dl, respectively). The cholesterol level was seen to slightly decline in the test formulation. The levels of cholesterol and triglycerides are shown in the Fig. 5. The NC group has the lowest levels of both cholesterol and triglycerides, with average levels of 89.03 ± 0.89 mg/dL and 79.80 ± 0.28 mg/dL, respectively. The DC group rats treated with triton has the highest levels, with average levels of 173.20 ± 0.12 mg/dL for cholesterol and 189.02 ± 0.68 mg/dL for triglycerides, indicating the induction of hyperlipidemia and a higher risk for cardiovascular disease. In the standard treatment (simvastatin oral) groups, there is a decrease in the cholesterol (132.76 ± 0.35) and triglyceride level (139.80 ± 76) whereas, in the test formulation group or test group, there was also a decrease in cholesterol (169.65 ± 0.21 mg/dL) and triglyceride level of (151.20 ± 31 mg/dL) level. The triglyceride level in the standard treatment group and test group were comparable.

Fig. 5 [Images not available. See PDF.]

Effect of simvastatin patch on **A** Triglyceride **B** Cholesterol in triton-induced hyperlipidemia in rats

Scanning electron microscopy study

Any material's morphological or surface changes can be evaluated using scanning electron microscopy. Various magnifications of the SEM images of the optimized patches before and after drug release were displayed in Fig. 6. Prior to medication release, the optimized simvastatin patch may seem uniformly smooth in SEM pictures. At 10 µm magnification, the patch's surface exhibits a matrix structure with uniformly dispersed drug particles; nevertheless, at 50 µm magnification, the patch surface seems to be nearly smooth. To regulate the medication's release rate, thin film coatings might also be present on top of the drug matrix.

Fig. 6 [Images not available. See PDF.]

SEM images of optimized simvastatin patch before drug release (**A**, **B**) and after drug release (**C**, **D**)

The improved simvastatin patch's SEM pictures after drug release might reveal a rougher surface with fewer drug particles. Contrary to the previous photograph, the drug particles that were remaining on the surface were distributed unevenly. Drug elution may cause wear and tear on the patches, and drug release is confirmed by some porous forms on the surface of the patches, which demonstrate that the medication was gone out of the patches.

Conclusion

The formulation of SIM-TP was prepared by the solution casting method using the polymers Eudragit L-100 and HPMC K100. The Design-Expert 13 software proposed a 15-run trial for the optimization process. The three independent variables were HPMC K100, Eudragit RL100 (ERL100), and polyethylene glycol 400 (PEG400). Physical Appearance, Thickness, Folding Endurance, and pH Determination were evaluated. The results showed that With a *p*-value of 0.0151, factor A—the concentration of HPMC K-100—had a noteworthy effect on folding

endurance. A higher concentration of polymers was associated with greater folding endurance. The linear model for in-vitro drug release is significant, as evidenced by the model's *p*-value of 0.0230, which is less than 0.05. Factor C, or PEG400 concentration, is the only scenario where the *p*-value is less than 0.05. The factor coefficients can be compared using the coded equation to get the relative significance of the variables. The design expert 13 program was used to predict the values of factors A, B, and C and their responses to folding endurance and in-vitro drug release. The optimized patch's levels of factors A, B, and C were 1.80, 0.1, and 50, respectively, and its responses to folding endurance and in-vitro drug release were 266.36 and 53.17%, respectively. In-vitro drug release, in-vivo antihyperlipidemic action, and scanning electron microscopy were used to assess the improved patch. The improved patch released 55.30 percent of the medication during a 24-h period, which corresponded to the projected value. Transdermal patches were evaluated for thickness, physical appearance, swelling index, moisture content, and moisture uptake. All formulations had a mean moisture content of 6.6 percent, with a standard deviation of 1.27. The in-vivo activity of the optimized simvastatin patch was observed in albino adult male and female in good health Wistar rats, with the NC group having the lowest levels of cholesterol and triglyceride levels. The DC group rats treated with triton had the highest cholesterol and triglyceride levels, while the standard treatment (simvastatin oral) groups had a decrease in cholesterol and triglyceride levels. Scanning electron microscopy showed that the optimized simvastatin patch had a rougher surface with fewer drug particles after drug release. Drug elution may cause wear and tear on the patches, and drug release is confirmed by some porous forms on the surface. Based on the results, it can be concluded that the tailored simvastatin patch can be an alternative to treat hyperlipidemia.

Author contributions

All authors contributed to this work. N: Research Scholar, Collection of data, manuscript writing. AAand: Concept and designing, manuscript writing. DP: in-vivo activity and manuscript writing. Thank you very much for considering our manuscript for publication.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Ethics approval and consent to participate

Animal study was endorsed by the Institutional Animal Ethics Committee (IAEC) Hygia Institute of Pharmaceutical Education &Research, Lucknow, Uttar Pradesh under IAEC Approved no. HIPER/IAEC/95/02/2022.

Consent for publication

The authors declare no conflict of interest.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

SIM

Simvastatin

SIM-TP

Simvastatin transdermal patch

SEM

Scanning electron microscopy

HPMC

Hydroxy methyl propyl cellulose K100

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Cordycepin alleviates osteoarthritis by inhibiting chondrocyte ferroptosis via Keap1/Nrf2 axis

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ABSTRACT (ENGLISH)

Background

Intervertebral disc degeneration (IVDD) is a prevalent condition known to contribute to lower back pain and various spinal disorders. The progression of IVDD is closely associated with cell ferroptosis. This study aimed to explore the therapeutic potential of a reactive oxygen species (ROS)-responsive hydrogel loaded with garlic extract for the inhibition of cell ferroptosis and the treatment of IVDD.

Results

The study encapsulated garlic extract within the hydrogel using physical entrapment and controlled the release of the extract through the ROS-responsive degradation of the hydrogel. Our findings revealed that the hydrogel effectively inhibited the ferroptosis of nucleus pulposus cells induced by hydrogen peroxide. Furthermore, the hydrogel, when loaded with garlic extract, notably downregulated the expression of pro- ferroptosis genes and upregulated the expression of anti- ferroptosis genes.

Conclusions

This study demonstrated that the hydrogel loaded with garlic extract significantly mitigated IVDD. These results highlight the promising potential of ROS-responsive hydrogel loaded with garlic extract as a viable treatment option for addressing IVDD.

FULL TEXT

Background

Osteoarthritis (OA) is a prevalent chronic joint disorder characterized by the progressive degeneration of articular cartilage, remodeling of subchondral bone, and inflammation of the synovium [1]. With a prevalence of approximately 10% worldwide, it ranks among the most common musculoskeletal conditions [2]. OA is a complex ailment influenced by a combination of genetic, mechanical, and biochemical factors. Advanced age, obesity, and joint injuries are recognized as risk factors that contribute to the onset and progression of OA [3, 4]. Given the projected substantial increase in the global burden of OA in the near future, it has become a significant public health concern.

Ferroptosis is a regulated form of cell death characterized by the accumulation of lipid peroxides and reactive oxygen species (ROS), which ultimately leads to cell membrane damage and subsequent cell death. This distinct process stands apart from other known forms of cell death, including apoptosis and necrosis. It has gained recognition as a significant contributor to various human diseases, such as neurodegeneration, cancer, and cardiovascular diseases [5–7].

The Kelch-like ECH-associated protein 1 (Keap1)/nuclear factor erythroid 2-related factor 2 (Nrf2) pathway plays a crucial role in regulating oxidative stress and maintaining cellular redox homeostasis [8]. Keap1, located in the cytoplasm, normally interacts with the transcription factor Nrf2 and promotes its degradation through the ubiquitin–proteasome system under normal conditions [9]. Upon exposure to oxidative stress or electrophilic agents, Keap1 undergoes conformational changes, leading to the stabilization and activation of Nrf2. Subsequently, Nrf2 translocates to the nucleus where it binds to the antioxidant response element (ARE), resulting in the transcriptional upregulation of various cytoprotective genes, including heme oxygenase-1 (HO-1), glutathione peroxidase (GPx), and solute carrier family 7 member 11 (SLC7A11) [10, 11].

Recent studies have demonstrated a close association between the Keap1/Nrf2 pathway and ferroptosis, highlighting its dysregulation as a potential contributor to the pathogenesis of diverse diseases [12]. For instance, in cancer, the constitutive activation of the Keap1/Nrf2 pathway has been demonstrated to enhance tumor growth and confer resistance to chemotherapy through the inhibition of ferroptosis [13]. Conversely, the activation of the Keap1/Nrf2 pathway has been demonstrated to confer protection against ferroptosis-induced cell death across diverse cell types, including neurons, cardiomyocytes, and chondrocytes [14–16]. The involvement of ferroptosis and the Keap1/Nrf2 pathway in osteoarthritis remains poorly understood, necessitating further research to elucidate their respective contributions to the progression of the disease. Nonetheless, emerging studies propose the involvement of ferroptosis in articular cartilage degeneration and indicate the therapeutic potential of activating the Keap1/Nrf2 pathway in averting cartilage destruction in osteoarthritis.

Cordycepin, known scientifically as 3’-deoxyadenosine, is a notable compound isolated from the caterpillar fungus Cordyceps militaris [17]. Historically utilized in traditional Chinese medicine, cordycepin has witnessed a resurgence in scientific interest due to its diverse pharmacological properties, including anti-inflammatory, antioxidant, and anticancer effects [17–19]. This resurgence is supported by a systematic approach to reviewing its biological activities, reflecting a growing recognition of its potential as a therapeutic agent. Notably, cordycepin's structural similarity to adenosine—a key signaling molecule—underpins its broad physiological functions, ranging from immune system activation to potential anticancer activities [20]. Its mechanism of action includes modulation of cell survival, proliferation, migration, and inflammation, positioning cordycepin as a promising candidate for addressing complex diseases like osteoarthritis [21]. Research indicates that cordycepin may treat OA by regulating autophagy and oxidative stress levels, thereby inhibiting the expression of cartilage inflammation [22–24]. Nevertheless, the potential therapeutic effects of cordycepin on OA and its underlying mechanism of action remain incompletely understood. Ziwen Wang et al., suggest cordycepin modulates the Keap1/Nrf2 pathway, inhibiting cellular senescence in rodents [25]. This leads to the hypothesis that cordycepin could regulate the Keap1/Nrf2 pathway to inhibit chondrocyte ferroptosis, potentially improving OA conditions.

In this study, we aimed to investigate the impact of cordycepin on OA pathogenesis and chondrocyte ferroptosis. Our findings demonstrate that cordycepin can alleviate symptoms of OA by inhibiting ferroptosis in chondrocytes through the modulation of the Keap1/Nrf2 pathway. These findings offer novel insights into the molecular mechanisms driving OA pathogenesis and propose cordycepin as a promising therapeutic agent for this condition.

Methods

In vitro experiments

Isolation and culture of chondrocytes

One-week-old C57BL/6 mice were euthanized and disinfected by immersion in 75% alcohol. Using aseptic techniques, an incision was made in the skin and soft tissue surrounding the knee joint of the hind leg to expose the knee joint. The femoral condylar cartilage was excised, minced, and immersed in a Phosphate-Buffered Saline (PBS) solution supplemented with 1% penicillin and streptomycin for 15 min. Subsequently, the tissue was transferred to Dulbecco’s modified eagle's Medium (DMEM) culture medium supplemented with 0.2% collagenase and 5% Fetal Bovine Serum (FBS) and subjected to digestion for 6 h at 37 °C. Collagenase is employed to digest the extracellular matrix for the efficient extraction of chondrocytes and FBS is incorporated into the culture medium to provide vital nutrients, growth factors, and hormones necessary for the optimal growth and maintenance of the

cultured chondrocytes. After digestion, the resulting solution was centrifuged at 1200 rpm for 10 min, and the resulting precipitate was collected. The cells were then suspended in DMEM culture medium supplemented with 5% FBS and cultured at 37 °C under 5% carbon dioxide conditions for subsequent passages.

Cytotoxicity evaluation of cordycepin using the cell counting kit-8 (CCK-8) assay

In the cytotoxicity evaluation of cordycepin using the CCK-8 assay, chondrocytes were seeded at an initial density of 5,000 cells per well in 12-well plates. This seeding density was chosen to achieve logarithmic growth phase within 72 h of culture. The chondrocytes at passage number 3 were treated with DMEM/FBS medium supplemented with cordycepin at concentrations of 0, 10, 20, 40, 80, and 160 μg/mL for a duration of 72 h. In the treatment phase, the DMEM/FBS medium used for chondrocyte culture was composed of DMEM supplemented with 10% FBS, 1% penicillin–streptomycin. Subsequently, for the cytotoxicity evaluation, the CCK-8 solution (MCE, No. HY-K0301)was used at a final concentration of 10 μL per well and the cells were incubated for 2 h. The CCK-8 assay employs a water-soluble tetrazolium salt to produce a colorimetric change in response to cellular metabolic activity, allowing for the quantification of cell viability. The absorbance at 490 nm of each well was measured using an enzyme-linked instrument. The relative cell viability of each group was determined relative to the control group treated with 0 μg/mL cordycepin.

Measurement of Fe^{2+} content in chondrocytes

The Fe^{2+} content in chondrocytes was quantified using an Fe^{2+} assay kit (Elabscience, No. E-BC-F101). This assay kit is specifically designed to quantitatively determine Fe^{2+} levels in biological samples through a colorimetric assay, leveraging a specific ligand that forms a colored complex with Fe^{2+}. Chondrocytes from each group were collected, washed three times with PBS, and subsequently homogenized in an iron detection buffer. Subsequently, 5 μL of iron reductant and 5 μL of assay buffer were added to the standard well, and the resulting mixture was incubated for 30 min. The iron detection buffer provided with the Fe^{2+} Assay Kit, is formulated to optimize the conditions for Fe^{2+} ion interaction with the colorimetric probe, enhancing the specificity and sensitivity of the assay. Its composition ensures the stabilization of Fe^{2+} ions and prevents oxidation, thereby contributing to the precise quantification of Fe^{2+} levels. Following that, 100 μL of iron probe was added, mixed thoroughly, and incubated for 1 h. Subsequently, the absorbance of each well was measured at 593 nm using an enzyme-linked immunosorbent assay (ELISA) reader, and the relative Fe^{2+} content in each group of chondrocytes was calculated. Each experimental group was performed in triplicate.

Quantification of malondialdehyde (MDA) content in chondrocytes

Chondrocytes from each group were collected and washed three times with PBS following the protocol provided by the MDA kit (Elabscience, No. E-BC-K028-M). MDA, a product of lipid peroxidation, reacts with thiobarbituric acid under high temperature and acidic conditions to form a pink product, thiobarbituric acid reactive substances. Subsequently, the cells were lysed using a cell lysis solution, and the resulting supernatant was collected following centrifugation. A total of 0.2 mL of the supernatant was combined with 0.6 mL of the working solution, followed by heating in a 95 °C-water bath for 30 min, cooling in an ice bath, and subsequent centrifugation at room temperature for 10 min at 10,000 g. The resulting supernatant was collected, and the absorbance at 532 nm and 600 nm of each well was measured using an ELISA reader. The MDA content of each group was then calculated. The formula for calculating MDA content in cells is: $\text{MDA (nmol/mg protein)} = (\Delta A1/\Delta A2) \times C \div Cpr$, where ΔA1 is the optical density (OD) of the test tube minus the OD of the blank tube, ΔA2 is the OD of the standard tube minus the OD of the blank tube, C is the concentration of the standard (10 nmol/mL), and Cpr is the protein concentration of the sample (mg protein/mL). Each experimental group was performed in triplicate.

Detection of intracellular ROS levels in chondrocytes using fluorescent probes

Chondrocytes from various groups were gently washed three times with PBS and then incubated with 10 μM of either DCFH-DA fluorescent probes in the dark at room temperature for 30 min. Subsequently, chondrocytes were washed with PBS, and the fluorescence intensity of intracellular ROS was observed using a fluorescence microscope (Leica, No.DM2500). Each experimental group was performed in triplicate.

Quantitative polymerase chain reaction (qPCR) detection

RNA was isolated using the TRIzol reagent provided by Aidlab (Catalog No. 252250AX), following a protocol optimized for chondrocyte samples. For every 5×10^6 cells, 1 mL of TRIzol was added, and the mixture was homogenized thoroughly before transferring to an Eppendorf tube. Chloroform was added in a ratio of 200 µL per 1 mL of TRIzol, followed by vigorous shaking for 15 s and standing for 3 min. The mixture was then centrifuged at 12,000 g for 15 min. The aqueous phase was transferred to a new tube, mixed with an equal volume of isopropanol, and centrifuged at 12,000 g for 10 min at 4 °C. The RNA pellet was washed with an equal volume of 75% ethanol, centrifuged at 6000 g for 5 min, and the supernatant was discarded. The RNA pellet was air-dried and dissolved in 20 µL of DEPC-treated water for further analysis. β-actin was selected as our reference gene for normalization. In the assessment of RNA quality and quantity, we utilized a NanoDrop spectrophotometer. The NanoDrop provided measurements of RNA concentration and purity, with absorbance ratios at 260/280 nm used to assess protein contamination and ratios at 260/230 nm to evaluate organic compound or buffer contamination. Subsequently, the extracted total RNA was reverse transcribed into complementary DNA using a reverse transcription kit (Vazyme, No. R101-01/02). The DNA amplification step was carried out using the Applied Biosystems 7500 Real-Time PCR system, and the obtained results were analyzed using the 2-ΔΔCq method. The primer sequences are listed in Table 1. Each experimental group was performed in triplicate.

Table 1. The primer sequences

Name	Primer	Sequence
β-actin	Forward	5’- TGTCCACCTTCCAGCAGATGT-3’
Reverse	5’- AGCTCAGTAACAGTCCGCCTAGA-3’	Keap1
Forward	5’- TGGACTTTCGTAGCCTCCAT-3’	Reverse
5’-GCATTCCACACTGTCCAGAA-3’	Nrf2	Forward
5’-CAGCATAGAGCAGGACATGGAG-3’	Reverse	5’- GAACAGCGGTAGTATCAGCCAG-3’
GPX4	Forward	5’- CCTCTGCTGCAAGAGCCTCCC-3’
Reverse	5’- CTTATCCAGGCAGACCATGTGC-3’	SLC7A11
Forward	5’- CTTTGTTGCCCTCTCCTGCTTC-3’	Reverse
5’-CAGAGGAGTGTGCTTGTGGACA-3’	MMP13	Forward

5’-GCATTGGCTGAGTGAAAGAGAC-3’	Reverse	5’- ATGATGAACGATGGACAGATGA-3’
ADAMTS-5	Forward	5’- ATGATTCGCCTCGGGGCTC-3’
Reverse	5’- GCACTCTCCGAAGGGGATCT-3’	Col2a1
Forward	5’- GTGGAGGTGGACGCTACACTCA-3’	Reverse
5’-AGCCAGGTTGCCATCGCCATA-3’	aggrecan	Forward
5’-ACCAGACTGTCAGATACCCC-3’	Reverse	5’- CATAAAAGACCTCACCCTCC-3’

Western blot (WB) analysis

Chondrocyte cells were washed gently with PBS, and then lysed using Radioimmunoprecipitation assay buffer containing 1% phenylmethylsulfonyl fluoride and 1% phosphatase inhibitor on ice. The lysate was collected and centrifuged at 12,000 g at 4 °C for 20 min, and the supernatant was collected. Protein concentration of each sample was determined using a bicinchoninic acid assay kit (Beyotime, No. P0012). For the standard curve, a series of bovine serum albumin standards ranging from 0 to 2000 μg/mL were prepared. The assay was performed according to the manufacturer's instructions, with modifications to include a blank correction to account for background absorbance. Samples were diluted as necessary with PBS to fall within the dynamic range of the standard curve. Protein was separated by electrophoresis on a polyacrylamide gel electrophoresis (PAGE) gel and transferred to a polyvinylidene fluoride membrane using a wet transfer method. The PVDF membrane was blocked with 5% non-fat milk for 1 h at room temperature and then incubated with the primary antibody overnight at 4. After washing with Tris-Buffered Saline with Tween for three times, the membrane was incubated with the secondary antibody for 1 h at room temperature. The protein bands were visualized and imaged using an imaging system (ChemiDoc™ XRS+ System, Bio-Rad), and the intensity of each protein band was quantified (BandScan). The antibodies for WB are listed in Table 2.

Table 2. The antibodies for western blotting

Classification	Name	Manufacturer	Catalog number	Dilution ratio
Primary antibodies	β-actin	Abcam	ab227387	1:1000
Keap1	Abcam	ab119403	1:1000	Nrf2

Affinity	AF7006	1:1000	GPX4	Sigma-Aldrich
SAB5700944	1:1000	SLC7A11	KALANG	kl410Ra21
1:1000	MMP13	Abcam	ab219620	1:1000
ADAMTS-5	Abcam	ab41037	1:1000	Col2a1
Affinity	AF5456	1:1000	aggrecan	Abcam
ab313636	1:1000	Secondary antibodies	HRP Conjugated AffiniPure Goat Anti-rabbit IgG (H+L)	Boster

In vivo experiments

Grouping and modeling of mice

All mice were housed in a clean-level animal facility. The facility maintained a constant temperature of 22±2 °C and a relative humidity of 50±10%. The light/dark cycle was set to 12 h light/12 h dark. The bedding material used was autoclaved aspen wood shavings. Additionally, the mice had ad libitum access to food and water. Twenty 12-week-old C57BL/6 mice were randomly divided into four groups using a random number generator: the sham group, the OA group, the cordycepin group, and the cordycepin+brusatol group, with five mice in each group. All groups, except the sham, were used to create an OA model. The mice were carefully anesthetized with an intraperitoneal injection of pentobarbital sodium (30 mg/kg). During the surgeries, we utilized sterile surgical drapes to cover the operating table and wore sterile gloves throughout the procedure. All surgical instruments were sterilized using high-temperature autoclaving before procedure. For the surgical procedure, the knee joint of the right hind leg was prepared by shaving the surrounding hair to ensure a clean site. The knee joint area of each mouse was thoroughly disinfected with povidone-iodine prior to making any incisions. A precise 5 mm longitudinal incision was made along the medial side of the knee joint for optimal access to the joint cavity while minimizing damage to the surrounding tissues. After making the medial incision in the knee joint, we directly visualized the anterior cruciate ligament by flexing the mouse's knee joint. This approach allowed us clear access to the ligament. In the OA, cordycepin, and cordycepin+brusatol groups, the anterior cruciate ligament was gently transected to induce osteoarthritis-like conditions. The incision was then meticulously sutured for proper healing. In the sham surgery group, we followed the same surgical procedure as in the experimental groups up to the point of ligament manipulation, involving opening the joint cavity and suturing it closed, but without transecting the anterior cruciate ligament, serving as a control.

One week post-surgery, the mice in the sham and OA groups received an intra-articular injection of 10 μL PBS in the right knee joint. The cordycepin group received an injection of 10 μL cordycepin solution (8 mg/kg concentration), and the cordycepin+brusatol group received 10 μL of a combined solution of cordycepin (8 mg/kg) and brusatol (5 mg/kg). Injections were administered weekly for a total of four weeks. Five weeks after surgery, all mice were euthanized, and their right knee joints were harvested. The tissues were then embedded in paraffin, sectioned, and stained with Safranin O, hematoxylin and eosin (HE), and subjected to immunohistochemistry and immunofluorescence experiments.

Evaluation of osteoarthritis degree using the osteoarthritis research society international (OARSI) score

The degree of osteoarthritis was evaluated using the OARSI score, which is determined by multiplying the OARSI grade by the OARSI stage. The OARSI grade is determined based on the following criteria:
Grade 0 indicates an intact joint surface.
Grade 1 represents mild fibrillation or swelling of the joint surface.
Grade 2 indicates moderate fibrillation with superficial cartilage loss, typically affecting approximately one-third of the stained cartilage surface.
Grade 3 signifies deep fibrillation or fissuring with approximately two-thirds of the stained cartilage surface lost.
Grade 4 denotes erosion with exposed subchondral bone.
Grade 5 signifies destruction of a significant portion of the joint surface with extensive subchondral bone exposure.
Grade 6 represents total destruction of the joint surface with bone remodeling and osteophyte formation.
The OARSI stage is determined based on the extent of joint involvement and is categorized as follows:
Stage 0 indicates no involvement.
Stage 1 represents involvement of less than 10% of the joint surface.
Stage 2 signifies involvement of 10–24% of the joint surface.
Stage 3 represents involvement of 25–49% of the joint surface.
Stage 4 denotes involvement of more than 50% of the joint surface.
This comprehensive scoring system allows for the evaluation and classification of osteoarthritis severity based on the OARSI grade and stage, providing valuable insights into the disease progression.

Statistical analysis

Quantitative data were presented as mean ± standard deviation, providing a comprehensive summary of the central tendency and variability. To determine the significance of differences in OARSI scores among the various groups, a rigorous statistical analysis was conducted. This involved utilizing the Kruskal–Wallis analysis, a non-parametric test, followed by the Mann–Whitney U test for pairwise comparisons. Furthermore, for analyzing other statistical data, a one-way ANOVA was employed, followed by Tukey's post-hoc test to identify specific pairwise differences. Notably, a significance level of $P<0.05$ was considered statistically significant (*), while a more stringent level of $P<0.01$ was indicated by (**), ensuring robustness and reliability in our findings across all experiments.

Results

Establishment and verification of the ferroptosis model

Initially, a ferroptosis model was established in chondrocytes by stimulating them with interleukin-1 beta (IL-1β) at a concentration of 10 ng/mL, and the model was subsequently validated. IL-1β stimulation was found to upregulate Fe2 + expression, whereas the IL-1β + Fer-1 (ferroptosis inhibitor) group exhibited a significant reduction in Fe2 + expression compared to the IL-1β group (Fig. 1a). Similar trends were observed in the MDA content among the experimental groups (Fig. 1b). Furthermore, ROS levels in chondrocytes were assessed (Fig. 1c, d). Following IL-1β stimulation, an elevation in ROS levels was observed in chondrocytes, while treatment with Fer-1 demonstrated the ability to mitigate the effects induced by IL-1β. Additionally, to further confirm the establishment of the ferroptosis model, the expression of ferroptosis-related genes was assessed through qPCR analysis (Fig. 1e). The findings revealed that IL-1β treatment upregulated Keap1 expression and downregulated Nrf2, GPX4, and SLC7A11 expression. Notably, Fer-1 treatment successfully mitigated the effects induced by IL-1β. These findings were further supported by Western blotting analysis (Fig. 1g, h). Collectively, our results strongly suggest that IL-1β stimulation at a concentration of 10 ng/mL can induce ferroptosis in chondrocytes.

Fig. 1 [Images not available. See PDF.]

Establishment and verification of ferroptosis model. **a**,**b** Detection of Fe2 + and MDA contents in chondrocytes. **c**,**d** Fluorescence images and intensity analysis of ROS in chondrocytes. bar = 20 μm. **e** qPCR analysis of Keap1, Nrf2, GPX4, and SLC7A11. **f** WB analysis of Keap1 and Nrf2. **g** Quantification of specific signal intensities. *$p<0.05$, **$p<0.01$, n = 3

Cordycepin inhibits chondrocyte ferroptosis

Cordycepin's toxicity on chondrocytes was assessed using a CCK-8 assay (Fig. 2a, b). Chondrocytes were cultured in DMEM media supplemented with cordycepin at concentrations of 0, 10, 20, 40, 80, and 160 μM for three days. The results indicated that chondrocyte viability significantly decreased with 80 μM cordycepin compared to the control group. Consequently, a concentration of 40 μM cordycepin was selected for subsequent experiments. Furthermore, chondrocytes were divided into three groups: IL-1β group, IL-1β + cordycepin group, and IL-1β + cordycepin + brusatol group, where brusatol was used as a unique inhibitor of the Nrf2 pathway. Fe2 + detection results showed a significant decrease in its expression in the IL-1β + cordycepin group compared to the IL-1β group, while the IL-1β + cordycepin + brusatol group exhibited higher Fe2 + expression than the IL-1β + cordycepin group (Fig. 2c). Similar trends were observed in MDA detection (Fig. 2d). Moreover, cordycepin exhibited inhibitory effects on IL-1β-stimulated ROS expression, whereas brusatol counteracted this effect (Fig. 2e, f). Additionally, the expression of mitochondria in chondrocytes was observed using TEM in each experimental group (Fig. 2g). Our observations revealed a reduction or disappearance of mitochondria cristae in the IL-1β group, which could be mitigated by cordycepin treatment. qPCR and Western blotting results further confirmed the inhibitory effect of cordycepin on chondrocyte ferroptosis (Fig. 2h-j). The findings demonstrated that cordycepin treatment led to the inhibition of Keap1 expression and the promotion of Nrf2, GPX4, and SLC7A11 expression. However, the inhibitory effect of cordycepin was counteracted by brusatol (Fig. 2h-j).

Fig. 2 [Images not available. See PDF.]

Cordycepin inhibits chondrocyte ferroptosis. **a** Molecular formula of cordycepin. **b** CCK-8 assay for cordycepin cytotoxicity in chondrocytes. **c**,**d** Detection of Fe2 + and MDA contents in chondrocytes. **e**,**f** Fluorescence images and intensity analysis of ROS in chondrocytes. bar = 20 μm. **g** TEM images of chondrocytes in different groups. **h** qPCR analysis of Keap1, Nrf2, GPX4, and SLC7A11. **i** WB analysis of Keap1, Nrf2, GPX4, and SLC7A11. **j** Quantification of specific signal intensities. *p<0.05, **p<0.01, n = 3

Cordycepin attenuates chondrocyte matrix degradation

The effect of cordycepin on the matrix of chondrocytes was investigated by examining the expression of matrix metalloproteinase 13 (MMP13), a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS-5), collagen type II alpha 1 chain(col2a1), and aggrecan genes using qPCR in the IL-1β group, IL-1β + cordycepin group, and IL-1β + cordycepin + brusatol group (Fig. 3a). Compared to the IL-1β group, the IL-1β + cordycepin group exhibited significantly reduced expression of matrix-degrading enzymes MMP13 and ADAMTS-5, along with increased expression of col2a1 and aggrecan (Fig. 3a). WB analysis demonstrated similar findings (Fig. 3b, c). These findings suggest that cordycepin inhibits matrix degradation in chondrocytes. Additionally, brusatol, an Nrf2 inhibitor, can counteract the effect of cordycepin.

Fig. 3 [Images not available. See PDF.]

Cordycepin inhibits chondrocyte matrix degradation. **a** PCR analysis of MMP13, ADAMTS-5, Col2a1, and Aggrecan. **b** WB analysis of MMP13, ADAMTS-5, Col2a1, and Aggrecan. **c** Quantification of specific signal intensities. *p<0.05, **p<0.01, n = 3

Cordycepin inhibits ferroptosis of chondrocytes in vivo

A mouse model of osteoarthritis was established, and the mice were divided into the sham group, OA group, cordycepin group, and cordycepin + brusatol group. Figure 4a is the experimental timeline for the animal study, outlining the key time points throughout the research, starting with mice grouping and surgery (Week 0), followed by weekly injections (Weeks 1 to 4), and culminating in euthanasia and sample collection at Week 5. Immunohistochemistry results demonstrated that the cordycepin group exhibited decreased Keap1 expression and increased Nrf2 expression compared to the OA group (Fig. 4b–e). In contrast, the cordycepin + brusatol group showed increased Keap1 expression and decreased Nrf2 expression compared to the cordycepin group. The results were further confirmed using immunofluorescence (Fig. 4f–i). These findings suggest that cordycepin can inhibit chondrocyte ferroptosis by modulating the Keap1/Nrf2 axis.

Fig. 4 [Images not available. See PDF.]

Cordycepin inhibits ferroptosis of chondrocytes in vivo.**a** The experimental timeline for the animal study. **b** Immunohistochemical staining for Keap1 in mouse joint cartilage sections. bar = 200 μm. **c** Quantification of Keap1-positive cells. **d** Immunohistochemical staining for Nrf2 in mouse joint cartilage sections. bar = 200 μm. **e** Quantification of Nrf2-positive cells. **f** Immunofluorescence staining for Keap1 in joint cartilage. bar = 100 μm. **g** Quantification of Keap1-positive cells. **h** Immunohistochemical staining for Nrf2 in joint cartilage. bar = 100 μm. **i** Quantification of Nrf2-positive cells. *p<0.05, **p<0.01, n = 3

Cordycepin inhibits osteoarthritis in vivo

Immunohistochemistry was conducted to assess the expression of col2a1 and aggrecan in the sham, OA, cordycepin, and cordycepin + brusatol groups (Fig. 5a–d). The results demonstrated that cordycepin upregulated the expression of col2a1 and aggrecan in joint cartilage, whereas brusatol attenuated the effect of cordycepin. Furthermore, Safranine-O and HE staining was performed on cartilage slices from each group (Fig. 5e). The results indicated that the cordycepin group exhibited better preservation of joint cartilage, as evidenced by a significantly lower OARSI score compared to the OA group (Fig. 5f). However, in the cordycepin + brusatol group, the OARSI score was higher than that observed in the cordycepin group.

Fig. 5 [Images not available. See PDF.]

Cordycepin inhibits osteoarthritis in vivo. **a** Immunohistochemical staining for Col2a1 in joint cartilage. bar = 200 μm. **b** Quantification of Col2a1-positive cells. **c** Immunohistochemical staining for Aggrecan in joint cartilage. bar = 200 μm. **d** Quantification of Aggrecan-positive cells. **e** Safranine-O and HE staining of joint cartilage. bar = 200 μm. **f** OARSI scores of joint cartilages. *p<0.05, **p<0.01, n = 3

Discussion

The present study aimed to investigate the potential therapeutic effects of cordycepin on OA by inhibiting chondrocyte ferroptosis via the Keap1/Nrf2 signaling pathway. Our findings provide valuable insights into the specific mechanisms underlying the protective effects of cordycepin in OA and contribute to the broader understanding of targeted therapies for this debilitating joint disease.

Ferroptosis is an iron-dependent form of regulated cell death characterized by the accumulation of lipid peroxides and reactive oxygen species ROS, ultimately leading to cellular damage and dysfunction [26]. Emerging evidence suggests that ferroptosis plays a critical role in the pathogenesis of OA, contributing to the progressive degradation of articular cartilage [27, 28]. Therefore, identifying compounds that can inhibit chondrocyte ferroptosis represents a promising therapeutic strategy for OA.

We focused on cordycepin, a natural compound derived from Cordyceps species, and its potential role in alleviating OA by modulating ferroptosis. However, its specific effects on chondrocyte ferroptosis and its underlying mechanisms in the context of OA have not been thoroughly investigated. Our results demonstrated that cordycepin treatment significantly reduced the expression of iron metabolism-related proteins and oxidative stress markers in chondrocytes, indicating its inhibitory effect on ferroptosis. This suggests that cordycepin may act as a potent regulator of iron homeostasis and oxidative stress in chondrocytes, thereby protecting them from ferroptosis cell death. Importantly, we observed that cordycepin administration attenuated cartilage degradation and improved joint function in OA mice, further supporting its potential therapeutic benefits in managing OA progression.

The activation of the Keap1/Nrf2 signaling pathway was identified as a key mechanism underlying the protective effects of cordycepin against chondrocyte ferroptosis. The Keap1/Nrf2 pathway is known for its crucial role in cellular antioxidant defense mechanisms, regulating the expression of antioxidant enzymes and promoting the cellular adaptive response to oxidative stress [29]. Crucially, the role of Keap1 as a negative regulator of Nrf2, through its interaction and ubiquitination leading to Nrf2's degradation, emphasizes the importance of Keap1 in the balance of cellular responses to oxidative stress. We highlight the intricate balance controlled by the Keap1/Nrf2 axis and how cordycepin's modulation of this pathway contributes to its protective effects. Specifically, our findings suggest that

cordycepin interferes with the Keap1-mediated degradation of Nrf2, thereby enhancing the cellular defense against oxidative stress and ferroptosis.

To further validate the specificity of cordycepin in inhibiting chondrocyte ferroptosis, future studies could explore whether other compounds with similar structures or mechanisms of action exhibit comparable effects. Comparing the efficacy of cordycepin with other potential ferroptosis inhibitors would provide valuable insights into its specificity and potential advantages in the context of OA treatment.

Moreover, a deeper exploration into Keap1's regulatory mechanisms, particularly its interaction with Nrf2 and subsequent effects on ferroptosis-related molecules, would enrich our understanding of cordycepin's mode of action. Exploring the cross-talk between the Keap1/Nrf2 pathway and other cellular signaling pathways involved in OA pathogenesis, such as inflammation and apoptosis, may reveal additional targets for therapeutic intervention.

It is important to acknowledge the limitations of the current investigation. Firstly, our experiments were conducted primarily using in vitro and in vivo models of OA, which may not fully capture the complexity and heterogeneity of human OA. Further studies using human-derived chondrocytes and animal models that better recapitulate the pathophysiology of human OA are warranted. Secondly, while our results provide insights into the potential therapeutic effects of cordycepin, additional preclinical and clinical studies are needed to validate its efficacy, safety, and long-term effects in OA patients.

In conclusion, this research sheds light on the potential of cordycepin as a therapeutic agent for OA by inhibiting chondrocyte ferroptosis through the Keap1/Nrf2 axis. We provide evidence that cordycepin effectively protects chondrocytes from iron-induced cell death, attenuates cartilage degradation, and improves joint function. These findings contribute to the growing body of research on targeted therapies for OA and highlight the importance of ferroptosis as a potential therapeutic target. Future investigations should focus on further elucidating the molecular mechanisms underlying cordycepin-mediated chondroprotection and translating these findings into clinically applicable strategies for OA management. Ultimately, the development of novel therapeutic interventions that can modulate ferroptosis may offer new hope for patients suffering from OA and improve their quality of life.

Conclusion

This research demonstrates the significant therapeutic potential of the ROS-responsive hydrogel loaded with garlic extract in mitigating IVDD. By effectively inhibiting the ferroptosis of nucleus pulposus cells and regulating the expression of key ferroptosis genes, the hydrogel loaded with garlic extract exhibited promising results both in vitro and in an animal model. These findings underscore the critical role of oxidative stress in the pathogenesis of IVDD and suggest that the targeted delivery of garlic extract using the ROS-responsive hydrogel could serve as a novel and effective therapeutic strategy for combating IVDD. The development of such targeted interventions holds considerable promise in the advancement of treatments for IVDD and related spinal disorders, providing new insights for future research in the field of spinal health.

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Author contributions

JL contributed to study conception and drafted the manuscript. JL and ZL conducted the literature review, performed analysis, and acquired the data.

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Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

This study was approved by permission from the Ethics Committee of Shaoxing People's Hospital (Approval No.

2021-034).

Consent for publication

Not applicable.

Competing interests

The authors declare that no conflict of interest exists.

Abbreviations

IVDD
Intervertebral disc degeneration
ROS
Reactive oxygen species
OA
Osteoarthritis
Keap1
Kelch-like ECH-associated protein 1
Nrf2
Nuclear factor erythroid 2-related factor 2
ARE
Antioxidant response element
HO-1
Heme oxygenase-1
GPx
Glutathione peroxidase
SLC7A11
Solute carrier family 7 member 11
PBS
Phosphate-Buffered Saline
DMEM
Dulbecco's Modified Eagle's Medium
FBS
Fetal Bovine Serum
MDA
Malondialdehyde
ELISA
Enzyme-linked immunosorbent assay
qPCR
Quantitative polymerase chain reaction
WB
Western blot
PAGE
Polyacrylamide gel electrophoresis
OARSI
Osteoarthritis research society international
IL-1β
Interleukin-1 beta
MMP13
Matrix metalloproteinase 13
ADAMTS-5

A disintegrin and metalloproteinase with thrombospondin motifs 5
col2a1
Collagen type II alpha 1 chain
CCK-8
Cell Counting Kit-8

Publisher's Note

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Efficacy evaluation of Berberis aristata and Silybum marianum fixed dose combination on glycaemic and insulin resistance parameters in adult population: a systematic review and meta-analysis of randomized controlled trials

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ABSTRACT (ENGLISH)

Background

Diabetes is one of the most prevalent metabolic diseases with high rate of morbidity and mortality. The increased level of blood glucose level and increased insulin resistance is the hallmark of diabetes. Currently, various non-pharmacological and pharmacological therapeutic options are used for lowering the glucose level and improving the insulin activity. The current systematic review and meta-analysis study was conducted to evaluate the efficacy of *Berberis aristata* and *Silybum marianum* fixed dose nutraceutical combination on serum glucose and glycated haemoglobin level and insulin resistance parameters.

Main Body

Randomized controlled trials, identified from three online databases, evaluating the efficacy of *Berberis aristata* and *Silybum marianum* fixed dose combination were identified and evaluated as per pre-defined protocol. Quality of studies was evaluated using PEDro scale, and risk of bias was assessed using Cochrane Risk of Bias Tool. Pooled effect was reported as mean difference (MD) and 95% confidence interval, while the complete study was conducted as per PRISMA and Cochrane guidelines. After complete literature screening and evaluation process, seven studies were included in the final analysis. Data of 825 participants (active group: 416 participants and control group: 409 participants) were utilized for the statistical analysis. All included studies (except one) were of good quality. Supplementation of fixed dose combination significantly reduced glucose level (MD: − 5.26 mg/dl; $p=0.02$) and glycated haemoglobin (HbA1c) level (MD: − 0.69%; $p<0.0001$) as compared to control therapy, while greater insulin resistance reduction was observed in active group and the difference approached significance (MD: − 0.64 HOMA-IR score; $p=0.08$). Risk of bias analysis revealed some concerns regarding biasness (mainly due to randomization, outcome measurement and selected reporting biasness). All included studies had moderate risk of biasness. Sensitivity analysis revealed effect of particular study on overall heterogeneity observed, while neither significant publication bias nor any missing study was observed.

Conclusion

The results of current study suggest that *B. aristata* and *S. marianum* fixed dose combination is effective in improving glycaemic and insulin parameters and can be effective in diabetic population. The observed sensitivity of certain studies on overall heterogeneity and the moderate risk of biasness warrants further well-designed clinical studies to strengthen the results of current study.

FULL TEXT

Background

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both. Diabetes is a major and the most prevalent metabolic disease affecting millions of people worldwide [1]. In 2021, around 537 million adult population is estimated to have diabetes and it is estimated to reach 783 million by 2045 [2]. If left untreated, diabetes can lead to serious complications, such as cardiovascular disease [3, 4], chronic kidney disease [5, 6], blindness [7, 8] and lower limb amputations [9, 10]. The consequences of diabetes are far-reaching and can have a significant negative impact on the quality of life of individual. Diabetes is one of the top 10 causes of death globally, with an estimated 4.2 million deaths attributed to the disease in 2019, equivalent to 11.3% deaths from all causes and accountable to eight deaths every minute [11, 12]. In addition to its physical and emotional toll on individuals and families, diabetes also imposes a significant economic burden on healthcare systems and societies. According to the International Diabetes Federation (IDF), the global healthcare expenditure for diabetes was estimated to be $966 billion in 2021 and is projected to reach $1,054

billion by 2045 [13].

Pharmacological and non-pharmacological treatments are available to manage diabetes and reduce the risk of complications. The American Diabetes Association (ADA) and European Association for the Study of Diabetes (EASD) recommend a patient-centred approach to diabetes care, which includes individualized treatment plans based on patient medical condition and preference [14]. Pharmacological treatments for diabetes include oral hypoglycaemic agents, injectable medications such as insulin and glucagon-like peptide-1 receptor agonists, and other medications that target specific complications of diabetes, such as hypertension and dyslipidaemia, while non-pharmacological interventions such as lifestyle modifications, including diet and exercise, are also recommended to manage diabetes [15, 16]. The use of nutraceutical and herbal supplements in treating DM has raised in recent years due to their better efficacy and less side effects [17]. Nutraceuticals is a broad term which includes botanicals, herbal supplements, probiotics, prebiotics, vitamins, minerals, dietary fibres, polyunsaturated fatty acids, protein and amino acids, and other related substances [17]. Various herbal supplements have been studied for their efficacy in DM, including *Acacia arabica*, *Aegle marmelos*, *Allium cepa*, *Allium sativum*, *Aloe vera*, *Annona squamosa*, *Artemisia pallens*, *Azadirachta indica*, *Andrographis paniculata*, *Biophytum sensitivum*, *Beta vulgaris*, *Brassica juncea*, *Cassia auriculata*, *Boerhavia diffusa*, *Caesalpinia bonducella*, *Citrullus colocynthis*, *Cajanus cajan*, *Coccinia indica*, *Casearia esculenta*, *Catharanthus roseus*, *Camellia sinensis*, *Enicostemma littorale*, *Eugenia jambolana*, *Helicteres isora*, *Ipomoea batatas*, *Morus alba*, *Scoparia dulcis*, *Murraya koenigii*, *Ocimum sanctum*, *Punica granatum*, and many others [17].

Berberis aristata (Berberidaceae family) is a shrub native to the Himalayas and widely used in traditional medicine for its various therapeutic properties [18]. Its roots and stem bark contain several bioactive compounds, including berberine, which has been shown to have antidiabetic effects [18]. The AMP-activated protein kinase pathway (AMPK), that have a key role in glucose and lipid metabolism, is one of the key pathway which is activated by berberine supplementation, leading to improved glucose uptake and insulin sensitivity [19, 20]. Several clinical studies have demonstrated the efficacy of berberine in improving glycaemic control in patients with type 2 diabetes [21].

Silybum marianum (Asteraceae family), also known as milk thistle, is a plant native to the Mediterranean region and widely used for its hepatoprotective effects. The active component of milk thistle is silymarin, a complex mixture of flavonolignans that has been shown to have antioxidant, anti-inflammatory and neuroprotective properties [22]. Silymarin has been found to improve insulin sensitivity of insulin receptors, ameliorates insulin resistance and reduces hepatic glucose production [23, 24]. Silymarin has also been shown to protect against diabetes-related complications, such as diabetic nephropathy, by reducing oxidative stress and inflammation [25]. Clinical studies in humans have demonstrated the potential of silymarin to improve glycaemic control and reduce markers of oxidative stress in patients with type 2 diabetes [26].

Berberol is an herbal fixed dose combination containing 588 mg hydro-ethanolic extract from cortex from *B. aristata* standardized to contain at least 85% berberine and 105 mg hydro-ethanolic extract from fruits of *S. marianum* standardized to contain 60–80% flavanol-lignans calculated as silybin. The efficacy of this herbal fixed dose combination supplement has been evaluated in various clinical studies and has been shown to have a range of positive effects on human health, including the management of lipid and glucose metabolism, but no meta-analysis study has been previously conducted to synthesize the results of published clinical studies evaluating the efficacy of the fixed dose combination on markers of diabetes. Hence, the current systematic review and meta-analysis study was conducted to evaluate the effectiveness of a fixed dose combination of *B. aristata* and *S. marianum* supplementation in diabetes condition.

Main text

Study conduct

The current systematic review and meta-analysis study was conducted in accordance with the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA), Cochrane Handbook for systematic reviews of intervention and the Cochrane statistical method guidelines [27, 28]. The study was conducted based on a pre-

designed protocol, and randomized controlled clinical trials were evaluated as per pre-defined inclusion and exclusion criteria. The current study was based on the questions framed as per the PICOS (population, interventions, comparator, outcomes, study design) criteria (Table 1). Based on the PICOS criteria, the study question was as follows: Is *Berberis aristata* and *Silybum marianum* fixed dose combination effective in improving glycaemic index and insulin resistance parameters in participants with impaired glucose level and/or insulin resistance?

Table 1. PICOS criteria for study determination

Parameter	Description
Population	Participants with increased blood glucose and/or insulin resistance level
Intervention	Fixed dose combination of *Berberis aristata* and *Silybum marianum* (either as single therapy or in combination with other therapies)
Comparator	Either placebo, standard therapy alone, or any other supplementation other than the fixed dose combination of *Berberis aristata* and *Silybum marianum*
Outcomes	1. Blood glucose level 2. Glycated haemoglobin (HbA1c) level 3. Insulin resistance level (HOMA-IR score)
Study design	Randomized, controlled clinical study

HOMA-IR homeostatic model assessment for insulin resistance

Search strategy

The Google scholar, PubMed, and Science Direct online databases were searched independently from the year 2000 until November 2023 by two review authors. The literature search was conducted by using combination of Medical Subject Heading (MeSH) terms along with free-text words related to *B. aristata* and *S. marianum* fixed dose combination supplementation and its effect on glycaemic and insulin parameters. The complete search strategy is as follows: '(Berberol) OR (((*Berberis aristata*) OR (*B.aristata*) OR (*B. aristata*)) AND ((*Silybum marianum*) OR (*S. marianum*) OR (*S.marianum*))) AND ((glucose level) OR (glucose) OR (sugar level) OR (sugar) OR (glycated haemoglobin) OR (glycated haemoglobin) OR (HbA1c) OR (insulin resistance) OR (insulin sensitivity) OR (HOMA)) AND ((clinical trial) OR (clinical study) OR (randomized study) OR (randomized trial) OR (controlled study) OR (controlled trial))'.

Eligibility criteria

The articles were evaluated and screened based on pre-defined inclusion and exclusion criteria. Articles of randomized controlled clinical studies, available as full-text articles in the English language, evaluating the efficacy of *B. aristata* and *S. marianum* fixed dose combination on the serum glucose level, serum glycated haemoglobin level and insulin resistance parameters were included in the current study. Articles of in vitro studies, pre-clinical animal model studies and clinical studies of study design other than randomized controlled design, evaluating the efficacy of different interventions other than the *B. aristata* and *S. marianum* fixed dose combination on parameters other than the serum glucose level, serum glycated haemoglobin level and insulin parameters and not available as full-text article or available in language other than the English language were excluded from the current study.

Study selection and data extraction

After retrieval of articles from online databases and after duplicate removal, two review authors independently

screened the title and abstract of the studies for eligibility. Studies deemed eligible after initial screening were evaluated using full-text article evaluation.

Data from included studies were extracted by one review author and independently validated by other review author. Using a pre-designed excel worksheet, the following study characteristics were extracted from the included studies: lead author, publication year, indication, sample size, age, interventions provided and duration of study. Additionally, the data regarding glucose level (in mg/dl), glycated haemoglobin (HbA1c) level (in percentage) and homeostatic model assessment for insulin resistance (HOMA-IR) score were extracted in a separate pre-designed excel worksheet.

Study quality and risk of bias assessment

The Physiotherapy Evidence Database tool (PEDro scale) was used to evaluate the biasness within studies, while the Cochrane Risk of Bias Tool (RoB2) was used to evaluate the biasness between studies as both these scales have demonstrated high validity and inter-rater reliability [29, 30]. The PEDro scale evaluation and the RoB2 assessment were conducted independently by two review authors, and the overall judgment of assessment was discussed among authors. Any discrepancy(s) between the result of assessment were discussed among the authors by joint consensus.

The PEDro scale evaluates the internal and external validity, statistical sufficiency and the overall study quality and categorises the studies into following: high quality (≥8 points), moderate quality (4 – 7 points) and low quality (≤3 points).The criteria assessed by the tool are as follows: eligibility criteria specified, subject randomisation, allocation concealment, the similarity of baseline prognosis between groups, blinding of subjects, therapists and assessors, a primary outcome measurement on≥85% of initial subjects, use of intention-to-treat analysis, use of variability measures and use of between-group comparison methods [31, 32].

RoB2 tool assesses overall biasness that might have influenced the results of study based on five domains, namely randomization process, deviations from intended interventions, missing outcome data, measurement of the outcome and selection of reported result [33]. As per the study details, the RoB2 tool pre-designed form is filled and based on the tool algorithm, an outcome of low, some concern, or high risk of bias is generated along with the overall judgement. The overall judgement of independent assessment was discussed among authors, and any disagreement was discussed between the authors along with other review authors by considering the full-text of article for final conclusion.

Statistical analysis

The RevMan statistical software (Desktop v5.4) provided by Cochrane collaboration network was used for conducting meta-analysis. Data for individual evaluation parameter were presented separately as mean difference (difference between baseline and final value) and standard deviation (change from baseline) (SD_{change}). The data were evaluated using continuous evaluation method, and the pooled analysis effect was presented as pooled mean difference (MD) with 95% confidence interval (95% CI). SD_{change} for individual parameters was adopted from respective articles, and in case SD_{change} was not provided; then, it was estimated using the following formula adopted as per Cochrane recommendations [34].SDchange=(SDB2+SDF2)-(2×r×SDB×SDF)where "SD_B" and "SD_F" denote standard deviation at baseline and final visit, respectively, while "r" denotes the correlation coefficient, either obtained from other studies or considered to be 0.7 to provide a conservative estimate as undertaken from previous studies [35]. The effect of interventions on individual evaluation parameter was visually presented as forest plots for individual evaluation parameter. The model of effect analysis was decided based on the heterogeneity significance (I^2 value). If the heterogeneity was found to be low ($I^2 \leq 50\%$), the fixed effect model was utilized for the analysis of final data outcome, and if the heterogeneity was found to be high ($I^2 > 50\%$) then random effect model was utilized for the analysis of final data outcome. Sensitivity analysis was conducted by using leave-one-study-out analysis approach using the OpenMeta [Analyst] software. By using the sensitivity analysis, the effect of individual included studies on overall pooled effect and observed heterogeneity was evaluated. The Meta-Essential (v1.5) software package was used for publication bias assessment. Publication bias was statistically assessed using egger regression test and Begg–Mazumdar test, while publication bias was visually assessed using forest plot of individual

evaluation parameter. Additionally, the trimming and filling analysis was conducted to identify any missing study(s) and its effect on overall effect size (Cohen's d value). The *p*-value of<0.05 was considered to determine significance.

Results and discussion

Study selection process, study characteristics and quality assessment

The initial literature search revealed 811 articles and after duplicate removal, 633 articles were initially screened for eligibility. Eight studies were evaluated completely using full-text screening out of which seven studies [36–42] were included in the study and one study [43] was excluded from the study after the eligibility screening. The complete study selection process is presented in Fig. 1. The data of 825 participants were included in the final analysis, from which 416 participants were allocated to active therapy group, while 409 participants were allocated to control therapy group. The detailed characteristics of individual studies are presented in Table 2.

Fig. 1 [Images not available. See PDF.]

PRISMA study selection flowchart

Table 2. Characteristics of included studies

Author	Year	Indication	Treatment group		Control group		Control intervention	Study duration
N	Age	*N*	Age	Derosa [36]	2013	Dyslipidaemia	51	52 ± 10.5
47	52 ± 10.5	Placebo	3 months	Derosa [37]	2013	Dyslipidaemia	52	51.4 ± 9.5
50	51.4 ± 9.5	Placebo	3 months	Pierro [38]	2013	Type 2 diabetes	32	67.85 ± 10.81
31	66.35 ± 9.8	*B. aristata* supplement	4 months	Derosa [39]	2015	Dyslipidaemia	66	57.8 ± 12.6
62	57.9 ± 12.9	Placebo	3 months	Guarino [40]	2015	Obese + type 2 diabetes	25	54 ± 5
25	56 ± 7	Placebo	6 months	Derosa [41]	2016	Type 1 Diabetes	41	30.7 ± 8.1
44	29.8 ± 7.2	Placebo	6 months	Guarino [42]	2017	Obese + type 2 diabetes	68	56 ± 8

Age presented as mean ± standard deviation

N number of participants

The quality of included studies was evaluated using PEDro scale, and the overall result is presented in Table 3. Out of seven included studies, one study was of moderate quality [38], while all other studies were of good quality. While all studies reported eligibility criteria, randomization allocation, groups similarity at baseline, subject blinding, low dropout rate (below 15%), point and variable measures, and statistical comparison between the intervention groups, none of the included studies provided details of assessor blinding. While the assessor blinding may have significant impact on the overall result of clinical study, the major outcome parameters of current study are laboratory-evaluated parameters, and the influence of assessor biasness might be negligible in the current study context. The overall detailed quality evaluation and criteria for judgement are provided as Additional file 1.

Table 3. Quality assessment of included studies as per PEDro scale

Parameters	Study						
Derosa [36]	Derosa [37]	Pierro [38]	Derosa [39]	Guarino [40]	Derosa [41]	Guarino [42]	Eligibility criteria specified
1	1	1	1	1	1	1	Random allocation
1	1	1	1	1	1	1	Concealed allocation
1	1	0	1	1	1	1	Groups similar at baseline
1	1	1	1	1	1	1	Subject blinding

1	1	1	1	1	1	1	Therapist blinding
1	1	0	1	1	1	1	Assessor blinding
0	0	0	0	0	0	0	Less than 15% drop outs
1	1	1	1	1	1	1	Intention-to-treat analysis
1	1	0	1	1	1	1	Between-group statistical comparison
1	1	1	1	1	1	1	Point measures and variability
1	1	1	1	1	1	1	Overall score

Risk of bias assessment

Among the seven included studies, all the studies were found to have moderate level of biasness. All included studies had risk of biasness regarding randomization process, while moderate biasness regarding measurement of

outcome parameter was observed in three studies [37, 38, 41], and biasness regarding reported result selection was observed in three studies [40–42]. The result of risk of biasness assessment of individual studies is presented in Fig. 2A, while the result of overall assessment is provided as Fig. 2B.

Fig. 2 [Images not available. See PDF.]

Risk of bias assessment of (**A**) individual included studies and (**B**) overall risk of bias assessment

Meta-analysis

Among the included studies, the efficacy of interventions on plasma glucose level was evaluated in five studies [36–39, 41], and data of 470 participants (237 participants in active group and 233 participants in control group) were used for final analysis. Supplementation of *B. aristata* and *S. marianum* combination showed significant reduction in plasma glucose level (MD: − 5.30 mg/dl; 95% CI − 9.91 to − 0.70; $p=0.02$; Fig. 3) as compared to control group. Significant heterogeneity was observed among included studies ($I^2=90\%$). Sensitivity analysis revealed no significant effect of any included studies on overall observed heterogeneity (Table 4), while removal of two studies [39, 41] individually made the observed pooled effect insignificant (Fig. 6A).

Fig. 3 [Images not available. See PDF.]

Efficacy of *B. aristata* and *S. marianum* fixed dose combination in improving blood glucose level: meta-analysis result

Table 4. Result of sensitivity analysis using leave-one-study-out method

Studies (removed from analysis)	I^2 value (%)	Estimate	95% CI	*p*-value
Glucose level				
Overall	90	− 5.302	− 9.905 to − 0.698	0.024
Derosa [36]	91	− 6.433	− 12.799 to − 0.067	0.048
Derosa [37]	85	− 6.980	− 12.078 to − 1.882	0.007
Pierro [38]	92	− 5.969	− 10.836 to − 1.102	0.016
Derosa [39]	83	− 4.498	− 9.906 to 0.909	0.103
Derosa [41]	89	− 3.158	− 7.518 to 1.203	0.156
HbA1c level				
Overall	90	− 0.693	− 1.017 to − 0.370	< 0.001
Pierro [38]	91	− 0.776	− 1.133 to − 0.420	< 0.001
Guarino [40]	93	− 0.686	− 1.156 to − 0.215	0.004
Derosa [41]	92	− 0.754	− 1.136 to − 0.372	< 0.001

Guarino [42]	39	− 0.563	− 0.732 to − 0.394	<0.001
HOMA-IR score				
Overall	99	− 0.644	− 1.358 to 0.07	0.077
Derosa [37]	99	− 0.765	− 1.693 to 0.163	0.106
Derosa [39]	99	− 0.763	− 1.701 to 0.175	0.111
Guarino [40]	99	− 0.724	− 1.614 to 0.166	0.111
Guarino [42]	0	− 0.303	− 0.394 to − 0.211	<0.001

The effect of interventions on HbA1c level was evaluated in four studies [38, 40–42], and data of 328 participants (161 participants in active group and 167 participants in control group) were included in final analysis. Supplementation of *B. aristata* and *S. marianum* showed significant reduction in HbA1c level (MD: − 0.69%; 95% CI − 1.02 to − 0.37; $p<0.0001$; Fig. 4) as compared to control therapy. Random effect model was used for pooled effect estimate due to significant heterogeneity among included studies ($I^2=90\%$). Sensitivity analysis revealed no significant effect of individual studies on overall pooled estimate (Fig. 6B), while removal of one particular study [42] reduced the observed heterogeneity to a non-significant range ($I^2=39\%$) as detailed presented in Table 4.

Fig. 4 [Images not available. See PDF.]

Efficacy of *B. aristata* and *S. marianum* fixed dose combination in improving HbA1c level: meta-analysis result

Among the included studies, the effect of interventions on reducing insulin resistance parameter (HOMA-IR score) was evaluated in four studies [37, 39, 40, 42]. Data of 416 participants (active group: 211 participants; control group: 205 participants) were analysed that showed that supplementation of *B. aristata* and *S. marianum* combination reduced HOMA-IR score compared to control group, while the difference approached significance (MD: − 0.64; 95% CI − 1.36 to 0.07; $p=0.08$; Fig. 5). Significant heterogeneity was observed among included studies ($I^2=99\%$); hence, random-effect model was used for pooled analysis. Sensitivity analysis revealed removal of one particular study [42] reduced the overall observed heterogeneity to non-significant range ($I^2=0\%$) and reduced the overall pooled estimate, with the difference compared to control therapy reaching significance ($p<0.001$) as presented in Table 4 and Fig. 6C.

Fig. 5 [Images not available. See PDF.]

Efficacy of *B. aristata* and *S. marianum* fixed dose combination in improving HOMA-IR score: meta-analysis result

Fig. 6 [Images not available. See PDF.]

Result of sensitivity analysis using leave-one-study-out analysis for parameters of (**A**) blood glucose level, (**B**) HbA1c level and (**C**) HOMA-IR score

Publication bias

Egger test and Begg test were used to identify any publication bias among the included studies. From the data presented in Table 5, it was concluded that no publication bias was found for glucose level (Egger test $p=0.570$; Begg–Mazumdar test $p=0.624$), HbA1c level (Egger test $p=0.795$; Begg–Mazumdar test $p=0.497$) and HOMA-IR score (Egger test $p=0.375$; Begg test $p=0.174$) parameters. The funnel plots evaluating publication bias for glucose level, HbA1c level and HOMA-IR score are presented as Fig. 7A–C, respectively. The trimming and filling method showed the absence of any missing study.

Table 5. Outcome indicators and publication bias of studies on effects of combination therapy supplementation

Outcome	I^2 (%)	I^2 *p*-value	Egger test *p*-value	Begg–Mazumdar test *p*-value
Glucose level	90	< 0.00001	0.570	0.624
HbA1c level	90	< 0.00001	0.795	0.497
HOMA-IR Score	99	< 0.00001	0.375	0.174

Fig. 7 [Images not available. See PDF.]

Funnel plot for publication bias assessment of (**A**) blood glucose level, (**B**) HbA1c level and (**C**) HOMA-IR score

Findings and interpretations

The current meta-analysis study aimed to evaluate the efficacy of a nutraceutical composition composed of fixed dose combination of *B. aristata* and *S. marianum* on glycaemic indices and marker of insulin resistance. The studies included in the analysis were conducted on subjects suffering from diabetes and dyslipidaemia. The result of the present study shows that the nutraceutical composition is effective in reducing the level of glucose and glycated haemoglobin, while effect on insulin resistance was not significant.

Previous research has demonstrated the mechanism of action of *B. aristata* and *S. marianum* in reducing glucose level and provides improvement in diabetic condition. Berberine, the active constituent of *B. aristata*, has demonstrated to reduce the insulin level in type 2 DM patients by improving insulin sensitivity, while in end-stage type 2 DM and in newly diagnosed type 1 DM patients berberine has shown to increase the insulin secretion by protecting the pancreatic ß-cells because of the antioxidant and anti-lipid peroxidation activity of berberine [44]. This dual effect of berberine is due to its various mechanism of action. Berberine is shown to directly activate the AMPK pathway (action similar to metformin) by increasing the phosphorylation of Thr-172 unit of AMPK-α sub-unit, the catalytic domain of AMPK, which results to the downstream signalling activation, and thereby increasing the insulin sensitivity and glucose consumption [44, 45]. Additionally, berberine is shown to indirectly activate the AMPK pathway by inhibiting the mitochondrial oxidative respiration, by inhibiting the monoamine oxidase enzyme and the electron transporter chain complex-I, which reduces the oxidative ATP production by mitochondria and increases the AMP/ATP level, which ultimately initiates the AMPK-signalling pathway [44, 45]. Because of this dual effect, certain studies have claimed berberine activity to be similar to metformin and rosiglitazone [44]. In liver, berberine reduces gluconeogenesis by inhibiting various gluconeogenic genes, namely phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6Pase), Forkhead transcription factor-1 (FoxO1), sterol regulatory element-binding protein 1c (SREBP1), and carbohydrate-responsive element-binding protein (ChREBP), while increases hepatic glycolysis by increasing the mRNA expression of hepatic nuclear factor-4α (HNF-4α) [44, 45]. Berberine also inhibits various gastrointestinal enzymes including α-glucosidase, disaccharidase, sucrase-isomaltase complex and ß-glucuronidase, thereby reducing the intestinal absorption of dietary carbohydrates, action similar to acarbose [44, 45]. Because of these myriad of activities, berberine supplementation has clinically proven hypoglycaemic and insulin sensitization activity [46]. Silymarin, the active constituent of *S. marianum*, includes four structurally similar isoforms, namely silybin, isosilybin, silychristin and silydianin [47]. Various studies have demonstrated that silymarin exerts potent antioxidant activity through various different mechanisms, including inhibition of reactive oxygen species (ROS)-producing enzymes, thereby preventing free radicals formation, improving mitochondrial membrane integrity in stressful conditions, reducing inflammatory responses by inhibiting nuclear factor kappa-B (NF-κB) signalling pathway, maintaining optimal redox balance in cell by activating range of antioxidant enzymes and activation of nuclear factor-erythroid two-related factor (Nrf-2), thereby causing increase in non-enzymatic antioxidant potential [47]. This activity of silymarin is largely due to the presence of β-catechol group, which is

capable of donating hydrogen ions and thereby stabilizing free radical species, and by the presence of 2,3-unsaturation along with 4-oxo functional group and other functional groups which are capable of forming bonds with metal ions and stabilize them [48]. Due to these antioxidant and anti-inflammatory mechanisms, silymarin is found to be effective in reducing the diabetic complications including diabetic nephropathy, diabetic neuropathy and diabetic retinopathy [47]. The effects of silymarin on glucose metabolism and insulin activity are largely unexplored with very few studies identifying the exact mechanistic role. In liver, silymarin is found to reduce the activity of pyruvate kinase enzyme which leads to reduction in dihydroxyacetone phosphorylation and decrease in glucose-6-phosphate hydrolysis, ultimately causing reduction in hepatic gluconeogenesis [48]. In conditions of reduced insulin-producing capacity, silymarin supplementation is associated with increased Pdx1 transcription leading to increase in insulin gene expression and ultimately production, while by increasing the expression of Nkx61, a key transcription factor for maintenance of pancreatic β-cells health, silymarin supplementation improves the overall health of pancreatic β-cells and thus improves insulin secretion [23]. Additionally, silymarin supplementation is associated with improved insulin sensitivity by reducing the tumour necrosis factor-alpha (TNF-α)-mediated insulin resistance [23]. In normal condition, the binding of insulin to insulin receptors causes activation of insulin receptor substrate-1 (IRS-1) complex, which in turn activates the phosphatidylinositol 3-kinase-protein kinase B (PI3K)/Akt pathway leading to increased expression of glucose transporter type-4 (GLUT-4) on the cellular surface and thereby increases glucose consumption. As diabetes is associated with chronic inflammation, high TNF-α level causes activation of c-Jun N-terminal kinase (JNK) and I-kappa B-kinase (IKK) complex, which directly inhibits the activation of IRS-1 complex, which thereby reduces insulin-mediated activity and leads to insulin resistance [23]. Silymarin supplementation inhibits the TNF-α, JNK and IKK phosphorylation and activation, which ultimately leads to improved insulin sensitivity and reduced insulin resistance [23]. Due to these myriad of mechanisms, various studies have demonstrated that silymarin supplementation is associated with improved glucose metabolism and reduced insulin resistance [48].

In the current study, the sensitivity analysis revealed that study conducted by Giuseppe Derosa [39] and Giuseppe Derosa [41] has potential effect on the overall effect estimate of glucose level. The former study included patients with dyslipidaemia who were intolerant to high doses of statins, while subjects with type-I diabetes were included in the latter study. As the analysis revealed no publication bias in the evaluated parameters, it can be postulated that the effect of this nutraceutical composition is greater in these patient population, and this hypothesis would need to be examined by more clinical trials including similar patient population. Similarly, observation was seen in HOMA-IR parameter, where study conducted by Guarino et al. [42] had significant impact on overall effect-estimate. But, since the pooled estimate had *p*-value of 0.08 (approaching significance), and the absence of any publication bias, the difference observed from the study may be negligible.

The results of the current study are novel as no previous studies have evaluated the efficacy of *B. aristata* and *S. marianum* fixed dose combination on glycaemic and insulin resistance parameters using a systematic review and meta-analysis approach. A previous meta-analysis study included data from four clinical trials involving 491 participants evaluated the effect of *B. aristata* and *S. marianum* combination on markers of dyslipidaemia [49]. The study included data of subjects with dyslipidaemia, and the effect of supplementation was evaluated on low-density lipoprotein level, high-density lipoprotein level, total cholesterol level, and triglycerides level. In the current study, data of patients with diabetes and dyslipidaemia were included, and the markers evaluated were related to blood glucose and insulin resistance.

Strengths and limitations of current study

The current study has various strengths. Firstly, the current study is novel, and the results of the current study are in line with the results observed in individual clinical studies evaluating the effect of *B. aristata* and *S. marianum* fixed dose combination on glycaemic and insulin resistance parameters. Secondly, the transparency of the study is one of the strengths of the current study which was maintained by following the guidelines provided by PRISMA. The current study has few limitations too. Firstly, the significant heterogeneity and moderate level of biasness observed among included studies. While the feasibility of conducting meta-analysis in such scenario is questionable, the current study was entirely conducted as per a pre-designed and finalized protocol that was developed before the

initiation of the first step of review process (i.e. literature mining). In order to identify the cause of significant heterogeneity, sensitivity analysis was conducted using the leave-one-study-out method, and the individual studies having significant effect on the overall heterogeneity were determined for few outcome parameters, but more statistical correlation research is required for further identifying the exact cause of heterogeneity, which was not covered in the current study due to limited statistical scope. Secondly, the absence of assessor blinding in all of the included studies is one of the major limitations of the included studies. While the evaluation parameters of current study are laboratory evaluated parameters, the influence of assessor might reduce the reliability the results of the current study. Hence more clinical studies with true blinding of the subjects, the therapist and the assessors are required to validate the results of current analysis. Thirdly, the current study included adult population with impaired glucose metabolism and insulin resistance with no limitations on any particular disease indications; the results of the current study needs to be considered with caution, as impaired glucose metabolism and insulin resistance are observed in various conditions like metabolic complications (including diabetes, obesity, dyslipidaemia and non-alcoholic fatty liver disease), cardiovascular complications including hypertension, and gynaecological complications like polycystic ovarian syndrome as well. Hence, the results of the current study do not justify the use of this fixed dose combination nutraceutical supplement in disease indications other than diabetes, dyslipidaemia and obesity in which the clinical studies have been conducted. Lastly, the limited number of studies with a smaller number of participants utilized in the current study warrants further clinical studies.

Conclusion

The results of the current study suggest that the supplementation of *Berberis aristata* and *Silybum marianum* fixed dose combination is effective in improving glycaemic indices by reducing insulin resistance. However, in light of the study limitations including the low number of available studies, the high heterogeneity observed and the moderate risk of biasness, further well-designed clinical studies are warranted.

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Author contributions

All authors have studied and permitted the final manuscript for communication. VD was responsible for study conceptualization and ideation, protocol development, complete literature search, formal analysis and investigation, statistical analysis and development of initial manuscript. JK, DK and VS worked together with VD for the screening of literature searches and selection of eligible studies. JK, DK and VS worked together with VD in organizing the manuscript in proper format. VD and JK worked together in proper formatting the references and updating them regularly. The authors have read and approved the final version of manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

DM

Diabetes mellitus

IDF

International Diabetes Federation

ADA

American Diabetes Association

EASD

European Association for the Study of Diabetes

AMPK

AMP-activated protein kinase pathway

PRISMA

Preferred Reporting Items for Systematic Reviews and Meta-analyses

PICOS

Population, interventions, comparator, outcomes, study design

MeSH

Medical subject heading

HbA1c

Glycated haemoglobin

HOMA-IR

Homeostatic model assessment for insulin resistance

PEDro

Physiotherapy evidence database tool

RoB2

Risk of bias tool

SD_{change}

Standard deviation (change from baseline)

MD

Mean difference

95% CI

95% Confidence interval

PEPCK

Phosphoenolpyruvate carboxykinase

G6Pase

Glucose-6-phosphatase

FoxO1

Forkhead box protein O1

SREBP1

Sterol regulatory element-binding protein-1

ChREBP

Carbohydrate response element-binding protein

HNF-4α

Hepatocyte nuclear factor 4 alpha

ROS

Reactive oxygen species

NF-κB

Nuclear factor kappa B

Nrf-2

Nuclear factor erythroid 2–related factor 2

TNF-α

Tumour necrosis factor-α

IRS-1
Insulin receptor substrate 1
PI3K/Akt
Phosphoinositide-3-kinase–protein kinase B
GLUT-4
Glucose transporter type 4
JNK
C-Jun N-terminal kinase
IKK
I-kappa B-kinase

Publisher's Note

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Multi-epitope-based vaccine designing against Junín virus glycoprotein: immunoinformatics approach

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ABSTRACT (ENGLISH)

Background

The Junín virus (JUNV) is well known for causing argentine haemorrhagic fever (AHF), a severe endemic disease in farming premises. The glycoprotein of JUNV is an important therapeutic target in vaccine design. Despite using drugs and neutralizing weakened antibodies being used in the medication, neither the severity reduced nor eradicated the infection. However, this constraint can be resolved by immunoinformatic approaches.

Results

The glycoprotein fasta sequence was retrieved from NCBI to anticipate the B cell and T cell epitopes through the Immune Epitope Database. Furthermore, each epitope underwent validation in Vaxijen 2.0, Aller Top, and Toxin

Pred to find antigenic, nonallergic, and non-toxic peptides. Moreover, the vaccine is designed with appropriate adjuvants and linkers. Subsequently, physicochemical properties were determined in ProtParam including solubility and disulphide bonds in the SCRATCH server. The vaccine 3D structure was built using I-TASSER and refined in ModRefine. Docking between JUNV glycoprotein (PDB ID:5NUZ) with a built vaccine revealed a balanced docked complex visualized in the Drug Discovery studio, identified 280 hydrogen bonds between them. The docking score of − 15.5 kcal/mol was determined in the MM/GBSA analysis in HawkDock. MD simulations employed using the GROMACS at 20 ns resulted in minimal deviation and fewer fluctuations, particularly with high hydrogen bond-forming residues.

Conclusion

However, these findings present a potential vaccine for developing against JUNV glycoprotein after validating the epitopes and 3D vaccine construct through *in silico* methods. Therefore, further investigation in the wet laboratory is necessary to confirm the potentiality of the predicted vaccine.

FULL TEXT

Background

The Junín virus (JUNV) is a member of the *Arenaviridae* family, renowned for causing Argentine haemorrhagic fever (AHF), which is a severe endemic infection prevalent in populations residing around agricultural areas in Argentina [1]. The first cases were reported in the 1950s during the unleashed propagation of *Calomys musculus*. Since then, hundreds of cases have been registered annually. Humans will become victims by the inhalation of rodent aerosols or excreta, generally in the harvest season, so it is a rodent-borne virus [2]. Common symptoms of AHF include flu-like signs, such as headache, malaise, and fever. Primary infection sites are the lungs and then circulate to parenchymal tissues [3]. The final stage of AHF shows neurologic and haemorrhagic complications. The World Health Organization (WHO) has classified AHF as an emerging disease, warranting immediate research to design antiviral agents and vaccine targeting the virus components.

JUNV's genome consists of two single-stranded RNA segments: a small (S) segment of 3.4 kb and a large (L) segment of 7.2 kb. The S segment translates in the cytoplasm giving rise to nucleoproteins and glycoprotein precursors that mature into a glycoprotein complex after cleavage by cellular proteases. The glycoprotein is crucial for viral attachment to host cells, initiating the viral components' entry into the host cell [4–6]. Glycoprotein formation occurs in the endoplasmic reticulum and then infects parenchymal cells through circulation [4]. Glycoprotein also plays a role in cell pathogenicity [7].

In the late 1990s, an effective live-attenuated vaccine was developed, significantly reducing AHF incidence. However, there are no effective remedies to counter viral infection among victims [8]. Later, the application of immune plasma therapy to neutralize the antigens decreased the 1% fatality rate if treated before eight days of the onset of symptoms [9–12]. The FDA-approved nucleoside analogue ribavirin inhibits JUNV polymerase and serves as an antiviral, but its use is complicated due to side effects and less efficacy [12, 13]. Numerous small molecule (drugs) antiviral compounds have been reported as antagonists to JUNV in both in vitro [14–17] and in animal models [18, 19]. In silico approaches, such as molecular docking, have been employed to identify the potent drugs from the FDA against the glycoprotein of the Junin virus, revealing MK-3207 and dihydro-ergotamine [17]. Additionally, the live-attenuated vaccine strain Candid #1 (Can) has shown promise in inhibiting glycoprotein spread and reducing infections [18].

Until now, the experiments have primarily focussed on neutralized live-attenuated vaccines, but in this study, we aim to design a new vaccine specifically targeting the glycoprotein. Firstly, we have chosen the mature glycoprotein sequence in identifying and designing a new vaccine through immunoinformatics approaches such as predicting the B cell and T cell epitopes. Next, validating the peptide sequences to ensure the accuracy and reliability of epitopes. Subsequently, designing the vaccine by incorporating suitable adjuvants and linkers and analysing the physicochemical properties. Furthermore, detailed evaluations of the secondary and tertiary structural features of the designed vaccine sequence followed by refining the 3D structure. Furthermore, we checked the binding affinity score and molecular dynamic simulation of the designed vaccine. We presented the detailed methodology followed during

this study in a pictorial format, making it visually accessible and facilitating a better understanding in Fig. 1.

Fig. 1 [Images not available. See PDF.]

The methodology followed in this study

Methods

Sequence retrieval

The sequence of Junín virus glycoprotein precursor was retrieved from the National Centre for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/nuccore/MZ408914.1) [19] of 485 length peptide sequence. NCBI provides various tools and a repository database to access the sequence deposited in it.

Prediction of B cell and T cell [MHC I (T_C/CD4+), MHC II (T_H/CD8+)] epitopes

To predict B cell linear epitopes, we used the Immune Epitope Database (IEDB) (http://tools.immuneepitope.org/bcell/) and employed Bepipred Linear Epitope prediction 2.0 with a threshold value of 0.50 [20]. This prediction method is based on the input fasta format of the query protein sequence, and the server utilizes the random forest algorithm to distinguish between epitopes and non-epitope sequences found in crystal structures.

For predicting cytotoxic T cells/Class 1/CD4+ epitopes, we used artificial neural network 4.0 [21] and specified human MHC alleles as the source or reference to get the IC50 values of each predicted sequence in the IEDB Analysis Resource (http://tools.iedb.org/mhci/), which determines the subsequence's binding ability with the specific MHC class I. Helper T cells/Class II/CD8+ epitopes predictions were carried out with the NN-align 2.3 (NetMHCII) method [22, 23] with the human HLA-DR data set and all MHC II allele sequences between 12 and 18 mers in length. The resulting output provided IC50 values for predicted epitopes in the IEDB Analysis Resource (http://tools.iedb.org/mhcii/).

Validation of epitopes

The predicted epitopes were subjected to validation for allergenicity, antigenicity, and toxicity. Allergenicity refers to the ability of an antigen to induce abnormal and hyperresponsive [23]. AllerTOP v. 2.0 (http://www.ddg-pharmfac.net/AllerTOP/) was utilized for allergenicity prediction, employing the k-nearest neighbour algorithm on a training set of 2427 allergens and 2427 allergens, based on ACC uniform length and QSAR with different lengths [24]. Antigenicity, on the other hand, triggers the immune response and describes the ability to bind paratopes [25]. VaxiJen 2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) server used to predict the antigens and non-antigens [26]. This server was developed based on independent alignment methods with auto cross-covariance (ACC) of amino acid sequences transformation into principal vector properties to predict antigens and non-antigens. To access the peptides' toxicity, the ToxinPred server (https://webs.iiitd.edu.in/raghava/toxinpred/multi_submit.php) [27] was used. It utilizes the support vector machine (SVM) method with a threshold of 0.5 and an E value of 0.01 to predict the toxic and non-toxic peptides. Furthermore, population coverage analysis (http://tools.iedb.org/population/) [27] was performed to select the world population for MHC class I and MHC class II, which covers 3245 alleles from 16 geographical areas, 21 various ethnicities, and 115 countries.

Designing and characterization of structural vaccine

A serial arrangement of the vaccine was designed with beta-defensin 114 as an adjuvant and EAAK, AAY, GPGPG, and KK as flexible linkers [28, 29]. Epitope inclusion linkers and adjuvants were aimed to enhance protein stability and immunogenicity. To validate the designed vaccine, various physicochemical properties were calculated using ProtParam (https://web.expasy.org/protparam/) [30]. Antigenicity was determined using VaxiJen v 2.0 [26], while solubility with SolPro [31]. The presence of disulphide bonds was analysed through DLpro [32] through the SCRATCH web server [33] (https://scratch.proteomics.ics.uci.edu/). Allergenicity prediction was performed through AllerTOP v. 2.0 [24].

Secondary and tertiary structure assessment of antibody

Psipred [34] (http://bioinf.cs.ucl.ac.uk/psipred/) was designed based on the two feed-forward neural networks using the output from Position-Specific Iterated (PSI) – Blast. The SOPMA Server [35] (https://npsa-prabi.ibcp.fr/cgi-

bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) uses amino acid sequences by the self-optimized prediction method to predict the secondary structure features of the protein.

For in silico studies requiring tertiary structure of the protein 3D protein models using were generated using the servers such as Iterative Threading ASSEmbly Refinement (I-TASSER) [36] (https://zhanggroup.org/I-TASSER/). The prediction is based on the multiple threading approaches from the protein data bank through Local Meta-Threading Server v 3.0. The complete model was built by iterative template-based fragment assembly. The generated models were refined using the ModRefiner [37] (https://zhanggroup.org/ModRefiner/) servers until they achieved Ramachandran-favoured regions greater than 90%.

Molecular docking and MM/GBSA evaluation

The glycoprotein (GP) complex with the Fab antibody of the Junin virus was obtained from the Protein Data Bank (PDB ID: 5NUZ) (https://www.rcsb.org/) and determined using the X-ray diffraction method. The raw protein structure was made by eliminating the extra bound ligands, water, and het atoms followed by saving them in.pdb format for docking. The GP and the designed antibody were docked in the ClusPro protein–protein docking server (https://cluspro.bu.edu/home.php) [38], which was different from the protein–ligand docking that we performed in our previous studies [39].

Subsequently, the docked complex underwent Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) analysis to determine various interaction energies such as binding affinity, Vander Waal, electrostatic, polar solvation-free energy, and solvation-free energy based on the empirical methodologies after minimizing the complex in the ff02 force field over the HawkDock server (http://cadd.zju.edu.cn/hawkdock/) [40].

Molecular dynamic (MD) simulations

The MD simulations were performed on the refined tertiary structure of the antibody in a water medium using GROMACS [41] (https://simlab.uams.edu/index.php) from the WebGRO sim lab UAMS. GROMOS96 43a1 force field [42] initially used the TIP4P solvation model, to build the triclinic box and maintained 0.15 molarity of NaCl at pH 7.4. Using the steepest descent type of energy minimization 800 kJ/mol at 10,000 steps with NVT and NPT equilibration at temperature 300 k and pressure at 1 atmosphere. The final MD was performed by a leap-frog integer method at 20 ns of time.

Results

Sequence retrieval

The NCBI Virus accession number for the glycoprotein sequences of the GenBank ID: MZ408914.1, represents the glycoprotein sequence of the S segment of 485 length peptide sequence linear RNA genome isolated from the Argentinian mammarenavirus on Feb 2022.

Prediction and validation of B cell and T cell epitopes

The B cell and T cell epitopes were predicted using different algorithms from the IEDB, and their respective antigenicity (A>0.5), allergenicity (+), and toxicity (N) were manually analysed and curated (eliminated the non-antigenic (NA), allergenic (−), and toxic (T) peptides). Initially, fourteen B cell epitopes were retrieved as outputs, and they were curated based on properties such as antigenicity, allergenicity, and toxicity. Finally, four peptide sequences were selected for the vaccine construction in Table 1, after removing the duplicates. Based on the threshold values, numerous MHC class I and class II epitopes were obtained. Furthermore, filtered based on IC50 values (≤20), were selected followed by antigenic, non-allergen, and non-toxic peptides used in designing the antibody sequence in Table 2. Population coverage analysis for MHC I and MHC II epitopes was employed to estimate the potential target population for the predicted epitopes used in designing the vaccine, resulting in the best coverage value of 97.28% world population (Additional file 2: Table S1 and Figure S1).

Table 1. Predicted and selected B cell epitopes

Sequence	Antigenicity	Allergenicity	Toxicity

FSNNPHDLP	0.6	A	+	N
QHPADMSWCSKSDDQ	1.1295	A	+	N
DWHLDPPFLCRNRAKTE	1.4710	A	+	N
NNSYLNISDFRNDWI	0.8108	A	+	N

A Antigenicity; + Allergenicity; *N* Non-toxin

Table 2. Selected MHC class I and class II epitopes with antigenicity, allergenicity, and toxicity

Sequence	Allele	Antigenicity		Allergenicity	Toxicity
Tc/MHC class I					
FQTVSFSMV	HLA-A*02:06	1.51	A	+	N
KMKCFGNTA	HLA-A*30:01	1.22	A	+	N
TVSFSMVGL	HLA-A*68:02	1.83	A	+	N
ALNIALVAV	HLA-A*02:03	1.46	A	+	N
FVFLALAGR	HLA-A*68:01	1.52	A	+	N
NSYLNISDFR	HLA-A*68:01	0.88	A	+	N
TLNKSHLYIK	HLA-A*03:01	0.82	A	+	N
HTEFQTVSF	HLA-A*32:01	1.40	A	+	N
CPLPHRLNSL	HLA-B*07:02	1.21	A	+	N
Th/MHC class II					
IGLHTEFQTVSFSMVGLF	HLA-DPA1*03:01/DPB1*04:02	1.24	A	+	N
NGKLCLMKAQPTSWPLQC	HLA-DPA1*02:01/DPB1*01:01	0.93	A	+	N

LDHVNTLHFLTRGKNIQL	HLA-DQA1*01:01/DQB1*05:01	1.12	A	+	N
GNASFQISFDDIAVLLPQ	HLA-DPA1*01:03/DPB1*02:01	0.93	A	+	N
NTLHFLTRGKNIQLPRRS	HLA-DRB1*15:01	0.89	A	+	N
FLQEALNIALVAVSLIAI	HLA-DRB1*01:01	0.88	A	+	N
SFSMVGLFSNNPHDLPLL	HLA-DRB1*07:01	0.96	A	+	N
QEALNIALVAVSLIAIIK	HLA-DRB1*13:02	0.90	A	+	N

A Antigenicity; + Allergenicity; *N* Non-toxin

Designing and characterization of antibody structure

Before designing the vaccine, a suitable adjuvant, beta-defensin was selected, which is widely used. Additionally, appropriate linkers were exploited to connect intra B cells with KK, MHC I with AAY, and MHC II epitopes with GPGPG linkers and among them during vaccine designing. An EAAAK rigid linker, capable of forming an alpha helix at the amine terminal used to link the B cell epitopes and the adjuvant. The GPGPG linker was used between the MHC I epitopes and B cell epitopes. An AAY linker was employed to join the MHC I and MHC II epitopes followed by six histidine tags (6H) at the end in Fig. 2.

Fig. 2 [Images not available. See PDF.]

The sequential arrangement of epitopes in designing the vaccine

The designed antibody underwent physicochemical analysis of the nine descriptor values determined through ExPASY ProtParam. The vaccine comprises 431 residues with 47,835.77 Daltons molecular weight (**M.wt.**). The isoelectric constant point (**pI**) of the designed antibody was found to be 9.65. The negatively (Asp + Glu) [**−R**] and positively (Arg + Lys) [**+R**] charged residues were 29 and 57, respectively. The extinction coefficient (**EC**) value at 280 nm was 36,258 M^{-1} cm^{-1}. The instability index (**II**) was 34.87, a value greater than 40 refers to unstable, and a value less than 40 stability. The aliphatic index (**AI**) was 81.25 and the grand average of hydropathicity (GRAVY) of − 0.254 in Table 3.

Table 3. Physicochemical properties of the designed vaccine sequence

Protein	AA	M.wt	pI	(−)R	(+)R	EC[a]	II	AI	GRAVY
Antibody	431	47,835.77	9.65	29	57	36,258	34.87	81.25	−0.254

Ab Antibody; *AA* Total number of amino acids; *M. wt.* molecular weight; *pI* isoelectric constant; *(−)R* total number of negatively charged residues (Asp + Glu); *(+)R* total number of positively charged residues (Arg + Lys); *EC* extinction coefficient ([a] units of M^{-1} cm^{-1} at 280 nm measuring in the water); *II* Instability index; *AI* Aliphatic index; *GRAVY*

Grand average of hydropathicity

The descending order of the amino acid types in the antibody was: Polar>non-polar>basic>aromatic>acidic in Fig. 3. The constructed vaccine was validated as antigenic, non-allergenic, non-toxic, and had a solubility of 0.92. Moreover, it could form six disulphide bonds between the cysteine residues in Table 4 and Additional file 2: Table S2.

Fig. 3 [Images not available. See PDF.]

Different amino acid type content in the designed vaccine sequence

Table 4. Vaccine antigenicity, allergenicity, toxicity, solubility, and disulphide bonds forming the number

Descriptors	Antigenicity		Allergenicity	Toxicity	Solubility	No. of Di. S.B
Antibody	0.81	A	NA	N	0.92	6

A Antigen; *NA* Non-allergen; *N* Non-toxin

Secondary and tertiary structure assessment of antibody

The designed antibody fasta sequence was submitted to the SOPMA server for secondary structure prediction. The results indicated that the antibody comprises 26.68% of alpha helices, 41.07% of random coils, 25.99% of extended strands, and 6.26% of beta turns in Table 5. Figure 4, obtained from PSIPRED, exhibits the secondary structure confidence score and provides additional pictorial information about the secondary structure, as depicted in Additional file 2: Figure S2.

Table 5. Secondary features of the vaccine sequence

Feature type	No. of residues	Percentage (%)
Alpha helix	115	26.68
Extended strand	112	25.99
Bera turn	27	6.26
Random coil	177	41.07

Fig. 4 [Images not available. See PDF.]

The confidence level of prediction of the secondary structure via PSIPRED

The tertiary model obtained from the I-TASSER, though constructed upon validating in the Ramachandran plot, does not have enough amino acids in the allowed regions, leading to a low confidence score (C-score) of −2.17. The C-Score ranges from −5 to 2, where a higher value indicates a better model. Based on the confidence score, template modelling (TM-score) and root-mean-square deviation (RMSD) values were calculated to assess the structural similarity between the model and the actual protein. That resulted in a TM-Score of 0.46 ± 0.15 and 12.2 ± 4.4 [43]. The RMSD quantifies the similarity between two superimposed atomic coordinates [44]. The model was sent for structure refinement in the Galaxy refiner, and the results are shown in Additional file 2: Table S3. Based on the Global distance test (GDT) score, RMSD value, molprobity, clash score, rotamers value, and Ramachandran-favoured regions, model 1 was selected as a vaccine which was furthermore analysed. The GDT-HA represents the high-resolution value of the structure, and the MolProbity value is a log-weighted value determined from scores such

as clash score, Ramachandran-favoured percentage, and bad side-chain rotamers [45]. Ramachandran's favoured percentage regions have residues of 90.9% in the most favoured regions of the built 3D antibody. Overall, the selected model was refined and met the criteria for a suitable vaccine candidate, based on the various evaluation structural parameters and properties.

Molecular docking and MM/GBSA evaluation

The interactions between Junín glycoprotein and the designed antibody were analysed through the ClusPro server, and a balanced complex is retrieved in Fig. 5. The interactions between the residues were visualized in the drug discovery studio. Before this, the vaccine formed a total of nine hundred fourteen interactions with the glycoprotein. Including four S–S bonds, twenty-five electrostatic bonds, one-hundred eighty-three hydrophobic bonds, and seven hundred-two hydrogen bonds. Hydrogen bond-forming interactions play a prominent role in drug availability and enhancing the binding chances. Furthermore, the distance criteria of less than two angstroms (< 2 Å) interaction distance among the hydrogen bonds were 280 (Additional file 1).

Fig. 5 [Images not available. See PDF.]

The docked complex of JUNV glycoprotein (Chain C and L) with the designed vaccine (Chain A)

Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) was employed in determining the free energy between the glycoprotein and the designed vaccine (protein–protein interaction). Extensive methodologies in calculating the interaction energies include receptor's grid identification, pre-processing the input files, T leap function, scoring various energies, and scoring. Briefly van der Waals (VDW) energy was − 151.33 kcal/mol, electrostatic energy (ELE) was − 272.63 kcal/mol, polar solvation-free energy (GB) was 424.39 kcal/mol, and solvation-free (SA) energy was − 15.93 kcal/mol. The total binding free energy of the complex was − 15.5 kcal/mol represented in Table 6. Overall, the analysis indicates strong and favourable interactions between the Junin glycoprotein and the designed vaccine, with a significant binding free energy, suggesting the potent vaccine's effectiveness in combating the virus.

Table 6. MM/GBSA analysis representing various energies of the vaccine with glycoprotein

Energy types	Values (kcal/mol)
VDW	− 151.33
ELE	− 272.63
GB	424.39
SA	− 15.93
BA	− 15.5

VDW Vander Waal's energy, *ELE* Electrostatic energy, *GB* Polar solvation-free energy, *SA* Solvation-free energy, and *BA* Binding free energy affinity

Molecular dynamic simulations

Understanding the vaccine's conformational relative stability of the backbone atoms using the RMSD measure provides valuable insights since it measures the deviations of the extent atoms from the starting point. Less deviation (low RSMD) value signifies a better stable protein structure. For the constructed vaccine, the average lowest RMSD value was 0.24 nm in Fig. 6A. However, there was a slight increase in atomic deviations to 0.6 nm at 0.3 ns. This increase in RMSD values between 0.6 to 0.8 nm from 0.3 to 15 ns can be attributed to the modelled vaccine structural interactions optimization.

Fig. 6 [Images not available. See PDF.]

The molecular dynamic simulations of the 3D vaccine in water. **A** Root-mean-square deviation (RMSD) computes the average distance between the backbone atoms of starting structure (reference structure) with simulated structures (frame by frame) when superimposed. **B** Root-mean-square fluctuation (RMSF) computes fluctuations (standard deviation) of atomic positions of each amino acid (residues) in the trajectory [*X*-axis = Time; *Y*-axis = Residue number]. **C** Hydrogen interactions forming residues

Furthermore, the protein stability evaluated by root-mean-square fluctuations (RMSFs), which measures the protein residue's elasticity and binding site adaptation phenomena, was determined. The vaccine, RMSF contains evident residue cluster fluctuations. On average, there were two fluctuations for every hundred residues. Residues 200–320 showed high fluctuations with significant up and down levels. Maximum fluctuations were observed in the 390–400 residues range, while minimum fluctuations between 280 and 389 in Fig. 6B.

Hydrogen bond trajectories retained during the MD simulation were studied to evaluate the strength and binding affinity of the complex. The designed vaccine was found to form several hydrogen bonds with an average of 140 hydrogen bonds when interacting with the antigenic protein. The ability to form multiple hydrogen bonds was crucial in inhibiting the target protein, thereby strengthening the complex's free energy in Fig. 6C. Overall, analysis of RMSD, RMSF, and hydrogen bond trajectories provides valuable information about the stability and interactions of the designed vaccine, which were essential factors in determining its potential effectiveness as a vaccine.

Discussion

The general effective possibilities that are being examined are to control the dispense of Argentine haemorrhagic fever (AHF) by the design and development of suitable vaccines. The duration of the vaccine development from experimental studies to clinical trial studies takes an extensive period. In this modern era, due to the advancement of system applications in biology, numerous epitopes predicting epitopes are made available using various machine learning algorithms. Among them, multi-epitope-based vaccine designs employing predicting B cell and T cell epitopes have made a remarkable trend in bioinformatics. In addition, proper attention is required to develop a safe and viable vaccine.

To date, the primary approach to prevent the infection rate is neutralized monoclonal antibodies (MABs). Research on the MABs extracted from animal models (mouse) shows that the glycoprotein JUNV is involved in mimicking the human transferrin receptor 1 (hTfR1) during the binding process. This glycoprotein acts as a primary target and plays a predominant role in the prevention and treatment of infection [46]. The vaccine design according to the sequence-specific will act efficiently in inhibiting the antigen and will also be helpful in further mutational studies considering the evolution of the virus variants.

Three epitope types (B cell, $T_{C,}$ and T_{H}) predictions were performed using multiple servers including experimentally based HLA class I and HLA class II alleles [47]. All predicted epitopes were validated based on antigenicity/non-antigenicity, allergenicity/non-allergenicity, and toxicity/non-toxicity and shared maximum population coverage across the world. The number of B cell epitopes obtained after screening was less, indicating fewer interactions. Considering the T cell epitope number obtained, the cell-mediated immunity and immune response generated were high and long-lasting. A commonly used adjuvant (beta-defensin) was selected in designing and the linkers were added appropriately in Fig. 2. To ensure safety and efficacy, the designed vaccine over-checked their non-allergenicity, antigenicity, and non-toxicity. Furthermore, physicochemical, solubility and disulphide bond parameters were checked to determine the stability of the vaccine in Table 4 and Additional file 2: Table S2. This comprehensive validation paves potential applications in immunization against the target antigen.

The physicochemical properties in Table 3 and Fig. 3 include the pI as the pH at which no electrical charge is present on the molecule or the total number of negative and positive charges are equal [30]. The isoelectric focusing technique is performed based on the pI values for separating the molecule from the complex [48]. These values help to isolate the respective protein of interest in the wet lab experiments upon digestion. The glycoprotein's pI of 9.65 indicates that for wet-lab studies the experimental setup is to be maintained a basic environment during extraction.

EC is defined as the amount of light absorbed per mole of protein at a specific wavelength of light. The protein's EC value is calculated from the composition of tryptophan, tyrosine, and cysteine residues because these amino acids contribute significantly to measuring the protein's optical density in the 276–282 nm range [30]. Protein–protein and protein-ligands quantitative study can be understood through the EC values [49]. The EC value exhibited was high representing a good sequence for further studies. II indicates the protein stability under both in vivo and in vitro conditions. Proteins with II greater than 40 are considered unstable proteins, while II less than 40 are stable [50]. II of the designed vaccine was 34.87 inferring the stability. AI is another parameter that describes protein stability at temperatures. AI is defined as the relative volume occupied by aliphatic side chains like alanine (Ala), valine (Val), leucine (Leu), and isoleucine (Ile) [30, 48]. The high value of AI indicates the increase in the thermostability nature of the protein which is an additive factor for wet lab studies. The Aliphatic index (**AI**) of the vaccine sequence is 81.25 indicating the thermostability character. GRAVY value ranges from − 4 to + 4 indicating the hydrophilic and hydrophobic nature of the proteins [51]. The low GRAVY range indicates the possibility of being a globular (hydrophobic) protein rather than membranous (hydrophilic). Thus, the vaccine contains a globular protein residue with a value of − 0.254.

The tertiary structure of the vaccine has been generated from the I-TASSER and further processed in ModRefiner to achieve optimum descriptor values such as GDT-HA, RMSD, Molprobity, C-score, poor rotamers, and Ramachandran-favoured regions greater than or equal to 90% in Additional file 2: Table S3, aids to validate the designed structure all of which are crucial in validating the structural integrity [52, 53]. Subsequently, molecular docking of JUNV glycoprotein and the construct was performed to achieve a balanced complex model from the ClusPro server in Fig. 5. The post-docking analysis, specifically the MM/GBSA method in Table 6, of the docked complex resulted in favourable interaction energies within the docked complex. Moreover, MD simulations on the vaccine in water in Fig. 6 have indicated that the designed construct exhibited notable stability without higher deviations and fluctuations with maximum hydrogen bond forming ability that emphasizes the vaccine stability. These findings collectively underscore the designed vaccine's reliability and robustness for potential application in combating the JUNV.

Conclusion

In this study, we focussed on vaccine design based on the glycoprotein of the JUNV on employed immunoinformatics approaches to identify antigenic peptides because of the novelty of identifying the peptides using machine learning tools. The vaccine was constructed after selecting the antigenic, nonallergic, and non-toxic and deleting the duplicate and overlapping sequences. The vaccine was designed using 4 B cell, 9 MHC class I, and 8 MHC class II T cell epitopes with appropriate linkers and adjuvant. The tertiary model was generated and refined to meet suitable specific criteria especially Ramachandran-favoured regions greater than 90%. After the physicochemical properties' determination, molecular docking of JUNV glycoprotein with the designed vaccine performed a balanced complex model chosen for analysing the interactions among them showed 280 hydrogen bond forming residues with radii less than 2 angstroms. MM/GBSA methodology exhibited − 15.5 kcal/mol of binding free energy of the complex. The MD simulations of the vaccine that were performed exhibited fewer values of fluctuations and deviations that signify the vaccine's stability. However, the present work was based on in silico methodologies with promising results that will be evident in the wet lab works to justify the findings.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Antidiarrhoeal screening of Himalayan edible plant Begonia rubrovenia and its marker followed by its

validation using computational analysis

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ABSTRACT (ENGLISH)

Background

Diarrhoea has become one of the major areas of concern due to its high mortality rate contributing it to be the second largest cause of death in world. To explore the effectiveness of medicinal plant, the present investigation was undertaken to scientifically justify the traditional claim of the ethanolic root extract of the plant *Begonia rubrovenia* (EBV) against diarrhoea.

Results

EBV was standardized using HPLC with quercetin as marker and was further subjected to normal fecal excretion study at 100, 200 and 300 mg/kg, p.o. along with quercetin and loperamide. The study confirmed the effectiveness of EBV at 200 and 300 mg/kg followed by quercetin. In castor oil induced diarrhoea rat model, EBV at 200 and 300 mg/kg significantly delayed onset of diarrhoea, reduced the diarrhoeal faecal output which contributed in higher % protection. The effectiveness of EBV at 200 mg/kg was also confirmed through gastrointestinal motility, fluid accumulation and PGE_2 induced enteropooling tests. EBV and its marker quercetin also reduced the elevated level of NO and cytokines and restored the alterations in antioxidant enzymes, ions and enhanced Na^+/K^+–ATPase activity. Molecular docking, dynamics and network pharmacology study confirmed the role of quercetin in modulating the inflammatory mediators IL-1β, TNF-α and EP3 prostanoid receptor, where quercetin formed more stable complex with EP3 prostanoid receptor.

Conclusion

The study has scientifically justified the traditional use of the plants *B. rubrovenia* in treating diarrhoea, where quercetin played a critical role in the observed antidiarrhoeal potential of *B. rubrovenia* contributing in maintaining electrolyte balance, antioxidant status and inhibiting inflammatory mediators.

FULL TEXT

Background

Diarrhoea is an infection mainly associated with gastrointestinal (GI) tract, where defecation rate, volume and consistency of the stools get altered [1]. At present, diarrhoea is assessed to be the most prevalent situation hampering GI tract with 1–5% prevalence rate in adults in developed as well as developing countries like India [2, 3]. According to the WHO and UNICEF reports, about 1 billion diarrhoea cases are estimated worldwide, among which 3 million deaths occurs per year in children’s less than five years of age [4].

Different etiological factors attribute to the cause of diarrhoea like infectious agents (viruses, parasites and bacteria's), food allergies, intestinal dysfunction, alcohol consumption, decrease in uptake of bile salts and intake of some drugs, e.g., antineoplastic, antimicrobials, oral hypoglycaemic agents, antiretrovirals, β-blockers, proton pump inhibitors and non-steroidal anti-inflammatory drugs [3]. To handle this diarrhoeal condition, nontherapeutic approach, i.e. oral rehydration therapy is the primary step to maintain water and electrolyte loss along with zinc supplementation [5]. Therapeutic approach used to reduce the most consistent and clinically significant indications of diarrhoea have several side effects like dry mouth, severe constipation, nausea, vomiting, distension of abdomen and cramps [6] along with respiratory tract depression and paralytic ileus (mostly in children's due to loperamide drug) [7]. To minimize the health hazards of these agents, medicinal plants are most suitable approach to treat GI disorders like diarrhoea because they have lots of compounds, which help in efficacy enhancement and side effect neutralization [2]. Thus, to treat GI disorders like diarrhoea, natural entities are the most suitable therapeutic agent and also a good starter for the innovation of new drugs [3].

Begonia rubrovenia C.B. Clarke (Begoniaceae) is an ornamental plant majorly found in North Easton Himalayan regions especially in the states of Arunachal Pradesh, Meghalaya, Tripura and Manipur and is also found in Bangladesh [8]. In Meghalaya, the plant is locally named as Johusia by the Jaintia tribes where, the tender shoots and roots of the plant are commonly used as a vegetable and herbal tea prepared from the leaves, used as a best remedy to treat colic and dysentery like illness [8–10]. Traditionally, *B. rubrovenia* roots are used by the tribal peoples in Meghalaya to treat diarrhoea, liver disorders, stomach problems, peptic ulcers and skin related disorders [8, 10, 11]. Yet, there are very less reports available on the phytochemistry and pharmacological activities on this plant. However, the genus Begonia has been reported to have quercetin, rutin, luteolin, vitexin, isovitexin, orientin, isoorientin, friedelin and beta-sitosterol as some of the major phytoconstituents [12]. The plant under study is traditionally used to treat diarrhoea, however the traditional claim of the plant against diarrhoea is still not scientifically proven. Thus, the current study is an attempt to justify the antidiarrhoeal potential of *B. rubrovenia* using chemical-induced diarrhoea models following *in- vivo* and *in-silico* protocols.

Methods

Extraction and phytochemical evaluation

The plant *B. rubrovenia* was procured from West Khasi hills district of Meghalaya, India and was authenticated by Dr. Dongarwar, a botanist of our institute (Specimen number 10705). Next to authentication, the roots of the respective plant were dried under shade for at least 2–3 weeks. Then the dried roots were ground into coarse powder, further the coarse powder material (500 g) was extracted with ethanol as solvent (1.5 L) by Soxhlet assembly. After extraction, the volume of the collected extract was concentrated by rotary evaporator and the final extract (4.94% w/w) was kept under desiccator for further use. After complete extraction procedure, the obtained extract was screened for primary and secondary phytochemicals following standard test protocols [13]. Further, the screened phytoconstituents (total alkaloids, phenolics, tannins, saponins, and carbohydrates) were quantified as per the procedures described by Prasad et al. [12].

Standardization of extract

The occurrence of quercetin in ethanolic extract of *B. rubrovenia* (EBV) was confirmed by thin layer chromatography (TLC) screening and therefore quercetin ((Sigma-Aldrich, USA) was used as a standard marker for standardization of EBV using High Performance Liquid Chromatography (HPLC). For HPLC analysis, stock solution of EBV (1 mg/mL) and quercetin (0.1 mg/mL) was prepared in methanol. Further, a solution of methanol and water constituting 0.1% formic acid (80:20) was employed as a mobile phase with 10 µL of injection volume and 1.0 mL/min flow rate. The peaks of extract were matched with the standard quercetin peak with respect to retention time and presence of quercetin was confirmed [14].

Animals

Before selection of animals, the experimental protocol for antidiarrhoeal study was approved from the Institutional Animal Ethical Committee (IAEC/UDPS/2022/01 on 21/05/2022). After approval, healthy Wistar rats of either sex (150–200 g) were taken from the registered central animal house of our department (Reg. No.: 92/1999/CCSEA).

The animals were placed in their respective cages for at least 1–2 week to acclimatize in surrounding environment providing temperature, humidity and lightening facility along with feed and water as per standard protocol.

Acute oral toxicity study

Acute oral toxicity (AOT) study was carried out to determine the safety margin of EBV employing Organization for Economic Cooperation and Development (OECD)- 425 guidelines. Animals (overnight fasted) under the protocol were administered orally with EBV in increasing manner and different neurological and behavioural parameters were observed like sleep, palpitation, diarrhoea, drowsiness, sedation, tremors, lacrimation, writhing, salivation, gasping, convulsions and lowering respiratory rate for 48 h. Further, for any kind of mortality, these animals were monitored for another 14 days. In addition, weight of organs of the animals treated with EBV and control were measured and difference in their weights was recorded [15, 16].

Normal faeces excretion (NFE) rate

Previously fasted rats (3 h) were randomly categorized into six groups: First group (Normal control) was administered with suspension of 0.5% w/v carboxy methyl cellulose (CMC), second to fourth group was served with three different doses (100, 200 and 300 mg/kg) of EBV (dose selection was confirmed from AOT study), fifth group rats were treated with quercetin 50 mg/kg and the last group was served with standard loperamide drug (Torrent Pharmaceuticals India Ltd., India) at a dose of 2 mg/kg. All the test samples were prepared in 0.5% w/v CMC and were given orally using oral gavage. Weight of faeces in all the groups were noted in wet as well as in dry conditions (dried at 50 °C for 24 h) at 1st, 3rd, 5th and 7th hours after administration of EBV and finally wet to dry ratio was calculated [17].

Castor oil-induced diarrhoea

Castor oil induced (COI) diarrhoea rat model

In this model, castor oil was served as diarrhoea inducing agent, which was given after the gap of one hour of treatment to all the rats under study except the normal control rats. The fasted rats were randomly grouped into six groups, among them group 1 (Normal control) and group 2 (Diarrhoea control) rats received 0.5% CMC suspension. Rats from group 3–5 were administered with EBV at the dose of 100, 200 and 300 mg/kg p.o. respectively, and rats in groups 6 and 7 received quercetin and standard loperamide. All the rats after receiving castor oil (1 ml) were immediately placed in cages previously lined with plastic sheets and different diarrhoeagenic parameters were observed and determined for a period of 4 h after castor oil administration [18, 19].

COI gastrointestinal transit test model

This model was used to evaluate the antimotility effect of EBV and quercetin by employing charcoal meal. Approximately, 18 h fasted rats were divided into five groups, where 0.5% CMC suspension was prepared and given to Group 1 (Normal control) and Group 2 (Diarrhoea control) rats orally. Group 3 rats were administered with optimized dose of EBV, i.e. 200 mg/kg, which was confirmed from NFE study and COI diarrhoea rat model. Quercetin and atropine (0.1 mg/kg s.c.) (Sigma-Aldrich, USA) were administered to Group 4 and 5 rats, respectively. Further, 1 mL castor oil, was administered to rats, 30 min after the above treatment. This was followed by administration of 1 mL of suspension of 5% deactivated charcoal meal prepared in aqueous tragacanth (10%), 30 min after castor oil administration. Then, all the animals were sacrificed after 30 min of charcoal meal administration and intestinal part was isolated and distance travelled by the charcoal was measured with respect to total length of intestine. Finally, Peristaltic Index (PI) in percentage was calculated and was compared with diarrhoea control rats [18].

COI intestinal fluid accumulation test

Before commencing the protocol, rats were fasted for about 18 h and were categorized into 5 different groups with 6 animals in each group. Group 1 and 2 were served as normal control and diarrhoea control and received 0.5% CMC suspension by oral gavage. Groups 3–5 were treated with EBV (200 mg/kg), quercetin and loperamide, respectively. Diarrhoea was induced by giving castor oil (1 ml) to all rats except normal control rats just half an hour after the treatment. Immediately, 30 min after receiving castor oil, all the rats were sacrificed to remove the small intestine. Intestinal volume and weight of intestinal content of each rat were measured from each group [15].

PGE_2-induced enteropooling

Previously fasted rats were classified into four group (n = 6), where first (Normal control) and second (PGE_2 control) group animals received tragacanth suspension (2% w/v) orally and third and fourth group animals were treated with EBV extract (200 mg/kg) and quercetin (50 mg/kg) orally. PGE_2 (Astra Zeneca, India) was used as an inducing agent and was prepared in saline solution which contained 5% alcohol. PGE_2 was introduced via gavage in rats from groups 2 to 4. About 30 min later, all the rats were killed and small intestine was removed, intestinal volume was measured by using measuring cylinder [15].

Ions concentration and cytokines estimations

The colons isolated during the COI fluid accumulation test were first rinsed, homogenized in deionized water and finally centrifuged. The supernatant received after centrifugation was analysed for the determination of concentration of different ions like Na^{2+}, Ca^{2+}, Cl^- and K^+ with the help of Nulyte Electrolyte Analyzer [20]. Further, cytokines level (IL-1β and TNF- α) were also checked in previously isolated colonic tissues from different groups of rats using marketed Enzyme-linked Immunosorbent Assay (ELISA) kits (R&D Systems).

Na^+/K^+–ATPase assay

Na^+/K^+–ATPase assay was carried out to determine the efficacy of EBV and quercetin against Na^+/K^+–ATPase protein. The colonic tissues collected from the dissected rats from each group were first thoroughly cleaned. Then, they were homogenized according to the method by Gal-Garber et al. [21]. The homogenized solutions obtained were centrifuged and the supernatant collected were utilized for Na^+/K^+-ATPase assessment as per the method described by Parmar et al. [22].

Biochemical analysis and histopathology

The colonic tissues procured from the sacrificed rats were utilized for biochemical analysis. Initially, the colonic tissues arranged from COI fluid accumulation test were rinsed by tyrode solution, homogenized with 7.4 pH phosphate buffer and supernatants obtained after centrifugation of homogenized solutions were used for the estimations of different biochemical analytes. Nitric acid (NO) was estimated in the colonic segment by adapting the method given by Green et al. [23] and total carbohydrate content was evaluated by Yemm and Willis method [24]. Total DNA and protein concentration were determined by using methods described by Burton [25] and Lowry et al. [26] respectively. Further, the levels of antioxidants (SOD, CAT and LPO) were analysed in rat's colon with the help of standard protocol mentioned by Laloo et al. [27].

Histopathological studies were performed on colons collected from COI fluid accumulation testing. The initially blotted, dried and fixed (10% formalin) colons were dehydrated (with acetone) and finally embedding was carried out using paraffin wax. The colons fixed in paraffin wax were cut into small and thin sections with the help of microtome. Before observing the sections into the microscope (Leica DM-2000, Leica, Germany), they were stained with haematoxylin and eosin staining reagent for clear understanding of the histopathology.

In silico study

Preparation of the ligand

The ligand, quercetin, was prepared using the Marvin sketch tool in the Sanjeevani online program. The resulting 3D structure of the ligand was then imported into Biovia Discovery Studio 2022 (DS 2022) software. To optimize the ligand's energy, an energy minimization process was performed using the CharmM forcefield. Additionally, multiple conformations of the ligand were generated based on an *in-silico* pH of 7.4. Among these conformations, the one with the lowest energy was selected and subsequently docked into the active site of the enzyme protein.

Preparation of the proteins

The PDB structures of the proteins TNF-α (PDB ID: 1TNF), IL1-β (PDB ID: 1I1B), and EP3 prostanoid receptor (PDB ID: 6AK3) were obtained from the RCSB PDB website (https://www.rcsb.org/). The protein structures were prepared using the "Prepare Proteins" protocol incorporated in DS 2022. During the preparation, all water molecules were removed from the proteins and the protein structure was minimized using CharmM forcefield. The entire process was conducted with an *in-silico* pH of 7.4 to ensure consistency.

Molecular docking

The docking process was conducted using the CDOCKER protocol, which is known for its accuracy in predicting ligand–protein interactions. Following the docking simulations, the binding energy of ligand–protein complex was calculated. To refine the ligand conformation and to improve the accuracy of the results, an *in-situ* ligand minimization step was performed. The calculation of the binding energy took into account the non-bonded interactions, with a non-bond list radius of 14.0 Å employed for this purpose. Subsequently, an analysis of the binding poses and ligand orientation within the active site was conducted [28].

Molecular dynamic simulation

Among the three ligand–protein complexes obtained from molecular docking, the complex (Quercetin-6AK3) showing maximum binding energy was further subjected to molecular dynamics simulation using DS 2022. The simulation utilized CHARMm forcefield for both the small molecule and the protein. The system was set up with explicit periodic boundary conditions, maintaining an orthorhombic cell shape. The protein–ligand complex was solvated while ensuring a minimum distance of 7 Å from the boundary. A salt concentration of 0.145 was introduced, with sodium and chloride ions serving as the cation and anion, respectively. To initiate the simulation, an initial minimization step was performed using the steepest descent method. The minimization was carried out for a maximum of 5000 steps, with an RMS gradient of 0.1. Following the minimization, a standard dynamics cascade was executed, consisting of heating, equilibration, and production phases. The system was gradually heated from an initial temperature of 50–300 K over duration of 20 ps. Equilibration was then performed at 300 K without restraints for 50 ps. During the production phase, the system was simulated in the NPT ensemble at 300 K, using a temperature coupling decay time of 5 ps. The simulation was continued until the Root Mean Square Deviation (RMSD) reached a plateau. Snapshots of the system were saved every 20 ps during the production period. To assess conformational changes in the protein and ligand, several metrics were computed relative to the starting structure. These included the Root Mean Square Deviation (RMSD), Root Mean Square Fluctuation (RMSF), and Radius of Gyration (ROG). These metrics provided insights into the dynamics and stability of the protein–ligand complex throughout the simulation [29].

Network pharmacology

Novel bioactive targets based on docking results were selected to perform the network pharmacology. The suitability of the bioactive compound was evaluated using a cheminformatic tool and information regarding the bioactive target proteins was obtained from the UniProt database. The interactions between the bioactive target proteins and other molecules were analysed using STRING software ver 11.5 [30]. All the obtained data were then used to construct a network linking quercetin, target molecules, and protein–protein interaction data by Cytoscape ver 3.9.1 [31].

Statistical analysis

All the experimental data was analysed by one-way and two-way ANOVA using Tukey's multiple comparisons test, where results were represented as mean ± SEM (n = 6). The experimental data was statistically analysed with the help of GraphPad Prism having version 8 and the results were proven to be significant when p values were found to be <0.05.

Results

Phytochemical standardization

Approximately, 4.94% w/w yield of extract was obtained after extracting the roots from the plant *B. rubrovenia* using ethanol. The results from phytochemical screening revealed that EBV showed the presence of alkaloids, phenols, flavonoids, carbohydrates, proteins, amino acids and steroids. From phytochemical quantification, it was depicted that the extract was found to be rich in total phenolics (362.69 ± 17.44 mg/g GAE), total tannins (250.31 ± 14.47 mg/g TAE) and total flavonoid (197.36 ± 8.45 mg/g RE) contents. During HPLC standardization, well resolved and sharp peaks of quercetin was obtained which was matched with the peak of extract having retention time of 5.2 min and amount of quercetin was found to be 4.72% w/w in EBV (Fig. 1).

Fig. 1 [Images not available. See PDF.]

HPLC chromatogram of EBV representing the presence of quercetin. In figure, **A** HPLC chromatogram of standard

quercetin and **B** HPLC chromatogram of EBV showing presence of quercetin

AOT study

According to the AOT protocol, the rats under investigation did not depicted any abnormal type of behavioural and neurological symptoms up to the dose level of 2000 mg/kg, which confirmed that EBV up to a dose level of 2000 mg/kg was found to be safe (Details are available as Additional file 1).

Normal faecal excretion rate

Table 1 represents the effect of EBV on normal rate of faecal excretion. The results depicted that the rate of faecal excretion was significantly decreased at EBV 200, EBV 300 mg/kg and quercetin, respectively from 5th hr of treatment. But, in case of standard group, rate of faecal excretion was found to decline after 3rd hr of EBV administration itself. Wet to dry ratio was also found to be low in standard loperamide followed by EBV 200, EBV 300 mg/kg followed by quercetin.

Table 1. Effect of *B. rubrovenia* extract on normal faecal excretion rate

Treatment	Faecal wet weight (g) at various times (h) after treatment				Wet/dry weight of faeces
1	3	5	7	Normal Control	0.072 ± 0.003
0.328 ± 0.01	0.543 ± 0.02	0.905 ± 0.03	1.367 ± 0.05	EBV 100	0.379 ± 0.01[a]
0.380 ± 0.01	0.691 ± 0.03[a]	0.672 ± 0.03[a]	1.233 ± 0.07	EBV 200	0.516 ± 0.02[a]
0.542 ± 0.02[a]	0.463 ± 0.02	0.394 ± 0.01[a]	1.070 ± 0.04[a]	EBV 300	0.525 ± 0.02[a]
0.534 ± 0.02[a]	0.442 ± 0.02[a]	0.397 ± 0.01[a]	1.099 ± 0.02[a]	Quercetin	0.490 ± 0.02[a]
0.529 ± 0.02[a]	0.510 ± 0.02	0.470 ± 0.01[a]	1.108 ± 0.03[a]	Loperamide	0.451 ± 0.02[a]

Values are mean ± S.E.M. (n = 6)

In table *EBV* Ethanolic extract of *Begonia rubrovenia* at dose level of 100, 200 and 300 mg/kg, p.o.

[a] $p<0.05$ vs. Normal

[b] $p<0.05$ vs. Castor oil

Antidiarrhoeal evaluations

The results of COI diarrhoea model are demonstrated in Table 2, where, we observed that there was a significant delay in onset time in all the treated groups except in group administered with EBV at 100 mg/kg in comparison with the diarrhoeal rats. The other diarrhoeagenic features like total number of faeces, total number of wet faeces, total weight of faeces, total loss in body weight of rats, mean defecation rate, diarrhoea score and % protection also showed significant antidiarrhoeal effect in rats treated with EBV, quercetin and standard drug. From the overall observations, EBV at 200 and 300 mg/kg displayed similar response thus, confirming a ceiling effect of EBV from 200 mg/kg dose therefore, for further antidiarrhoeal studies, EBV 200 mg/kg was considered as optimized dose.

Table 2. Effect of *B. rubrovenia* extract on castor oil induced diarrhoea model

Groups	Onset time (min)	Total no of faeces	Total no of wet faeces	Loss in body weight	Total wt of faeces	Mean defecation in 4 h	Diarrhoea score	% Protection
Normal		4.50 ± 0.22		0.16 ± 0.005	0.27 ± 0.01	1.12 ± 0.05	–	100
Castor oil	52.33 ± 2.29	15.33 ± 0.76[a]	10.83 ± 0.74	1.03 ± 0.05[a]	2.07 ± 0.05[a]	3.83 ± 0.19[a]	28.33 ± 2.04	–
EBV 100	68.83 ± 3.04	11.33 ± 0.49[ab]	7.83 ± 0.30[b]	0.90 ± 0.04[a]	1.82 ± 0.08[ab]	2.83 ± 0.12[ab]	15.83 ± 0.79[b]	44.11
EBV 200	119.83 ± 5.54[b]	5.66 ± 0.33[b]	3.50 ± 0.22[b]	0.64 ± 0.03[ab]	0.93 ± 0.04[ab]	1.41 ± 0.08[b]	10.50 ± 0.61[b]	62.93
EBV 300	120.83 ± 6.20[b]	6.50 ± 0.42[b]	3.33 ± 0.21[b]	0.63 ± 0.03[ab]	0.94 ± 0.03[ab]	1.62 ± 0.10[b]	9.83 ± 0.47[b]	65.29
Quercetin	109.50 ± 5.85[b]	7.66 ± 0.33[ab]	3.33 ± 0.21[b]	0.72 ± 0.03[ab]	1.10 ± 0.06[ab]	1.91 ± 0.08[ab]	11.00 ± 0.44[b]	61.17
Loperamide	126.66 ± 6.95[b]	5.83 ± 0.40[b]	2.16 ± 0.47[b]	0.56 ± 0.02[ab]	0.66 ± 0.03[ab]	1.45 ± 0.10[b]	8.00 ± 0.73[b]	71.76

Values are mean ± S.E.M. (n = 6)

In table *EBV* Ethanolic extract of *Begonia rubrovenia* at dose level of 100, 200 and 300 mg/kg, p.o.

[a]*pP*<0.05 vs. Normal

The results of COI gastrointestinal transit test are represented in Fig. 2A. The peristaltic index was found to be maximum in case of diarrhoeal control rats; however, it was significantly decreased in rats treated with EBV 200 followed by quercetin treated group, which confirmed antimotility effect of extract and its marker quercetin.

Fig. 2 [Images not available. See PDF.]

Effect of EBV and its marker quercetin on COI gastrointestinal transit test (**A**), COI fluid accumulation test (**B**) and PGE_2 induced enteropooling test (**C**). Values are mean ± S.E.M. (*n* = 6), where **a***p*<0.05 vs. normal control/ethanol in saline and **b***p*<0.05 vs. diarrhoea control/PGE_2 + ethanol. In figure, EBV 200 corresponds to Ethanolic extract of *Begonia rubrovenia* at 200 mg/kg, p.o.

In COI intestinal fluid accumulation test, the intestinal weight and volume were found to be higher in castor oil control rats. Nevertheless, the rats treated with EBV and quercetin showed significant decline in both intestinal weight and volume. The maximum decline was observed in rats who received EBV followed by standard loperamide and quercetin (Fig. 2B). In PGE_2 induced enteropooling test, similar results were obtained where PGE_2 control rats showed maximum intestine volume, however rats receiving EBV and quercetin showed significant decrease in intestinal content (Fig. 2C).

Ions concentration and cytokines estimations

The results of ion concentration determination are given in Table 3, which demonstrated that there was a significant

decrease in the ion concentration of colonic tissue of castor oil control rats. However, in case of treatment groups, there was a significant recovery in the altered ions level. Figure 3A and B represents the cytokine profiling, where the results depicted that, groups receiving EBV, quercetin and standard showed significant decrease in the cytokine levels while castor oil control rats showed significant hike in the levels of cytokines as compared to normal rats.

Table 3. Effect of *B. rubrovenia* extract on castor oil induced intestinal fluid accumulation

Groups	Weight of intestinal content (g)	Volume of intestinal content (mL)	% Inhibition	Na^+ (mmol/l)	K^+ (mmol/l)	Ca^{2+} (mmol/l)	Cl^- (mmol/l)
Normal	1.47±0.05	1.80±0.05	100	75.44±3.59	28.86±1.35	17.20±0.88	70.06±3.49
Castor oil	4.04±0.28[a]	4.36±0.17[a]	–	57.33±2.62[a]	20.56±1.43[a]	18.90±0.80	52.08±2.13
EBV 200	2.48±0.10[ab]	2.06±0.14[b]	52.59	82.90±4.11[b]	27.40±1.30[b]	18.21±0.77	67.71±3.18[b]
Quercetin	3.09±0.07[ab]	2.41±0.09[ab]	44.57	76.33±3.82[b]	26.88±1.37[b]	18.60±0.92	65.21±2.92[b]
Loperamide	2.02±0.10[b]	1.95±0.07[b]	55.27	84.23±4.16[b]	28.06±1.43[b]	18.70±0.93	70.21±2.89[b]

Values are mean±S.E.M. (n=6)

In table *EBV* Ethanolic extract of *Begonia rubrovenia* at 200 mg/kg, p.o.

[a]$p<0.05$ vs. Normal

[b]$p<0.05$ vs. Castor oil

Fig. 3 [Images not available. See PDF.]

Effect of EBV and marker quercetin on IL-1β (**A**), TNF-α (**B**) and Na^+/ K^+–ATPase activity (**C**) in colonic tissue of COI diarrhoeal rat.Values are mean±S.E.M. ($n=6$). Where a $p<0.05$ vs. Normal control and b $p<0.05$ vs. Castor oil induced diarrhoea control. In Figure EBV 200 corresponds to Ethanolic extract of *Begonia rubrovenia* at 200 mg/kg, p.o.

Na^+/ K^+–ATPase assay

Figure 3C represents the results of effect of EBV and its marker quercetin on Na^+/ K^+–ATPase. From the overall results, it was observed that the groups treated with EBV and quercetin showed profound increase in Na^+/K^+ –ATPase activity in comparison with the diarrhoeal control rats. The results demonstrated by EBV were quite comparable to that of standard loperamide.

Biochemical estimations and histopathological examination

The results of biochemical evaluations are illustrated in Table 4, where we observed that the NO content was observed to significantly decline, whereas cellular proliferative factors like DNA, carbohydrates and proteins were found to significantly escalate in rats receiving EBV 200, quercetin and standard when compared with rats from diarrhoea control group. The results of antioxidant profiling revealed that, the amount of CAT and SOD went on to significantly decline, while LPO level significantly enhanced in rats from diarrhoea control group. Nevertheless, in treatment group's rats, both the antiperoxidative enzymes showed marked enrichment in their levels followed by decrease in peroxidative enzyme LPO.

Table 4. Effect of *B. rubrovenia* extract on biochemical parameters

Groups	NO (Units in mole/mg of protein)	Total carbohydrates (Conc mg/g of tissue)	Total protein (Units in mg/100 mg of tissue)	Total DNA (Units in mg/100 mg of tissue)	MDA (Units in Mole/mg of protein)	CAT (µMol H_2O_2 consumed/min/mg of protein)	SOD (Units/mg of protein)
Normal	3.59 ± 0.14	1.07 ± 0.05	0.98 ± 0.04	0.15 ± 0.007	7.35 ± 0.38	67.64 ± 2.65	1.75 ± 0.04
Castor oil	10.26 ± 0.51[a]	0.47 ± 0.02[a]	0.37 ± 0.01[a]	0.11 ± 0.005[a]	20.72 ± 1.79[a]	48.77 ± 2.88[a]	1.25 ± 0.02[a]
EBV 200	6.33 ± 0.39[ab]	0.94 ± 0.04[b]	0.73 ± 0.03[ab]	0.14 ± 0.007[b]	13.37 ± 0.55[ab]	108.60 ± 4.36[ab]	2.25 ± 0.09[ab]
Quercetin	8.79 ± 0.39[ab]	0.83 ± 0.04[ab]	0.56 ± 0.03[ab]	0.12 ± 0.005[b]	16.15 ± 0.82[ab]	103.08 ± 5.07[ab]	2.02 ± 0.16[b]
Loperamide	4.68 ± 0.29[b]	1.06 ± 0.05[b]	0.78 ± 0.03[ab]	0.16 ± 0.007[b]	11.16 ± 0.58[b]	120.45 ± 2.97[ab]	2.80 ± 0.14[ab]

Values are mean ± S.E.M. (n = 6)

In table *EBV* Ethanolic extract of *Begonia rubrovenia* at 200 mg/kg, p.o.

[a]p < 0.05 vs. Normal

[b]p < 0.05 vs. Castor oil

Figure 4 represents the histopathological changes observed in all the normal, treated and diarrhoea control rat colons. The histopathology of the rat colons in normal groups exhibited well intact and distinct microvilli with normalized glands, whereas the colonic tissues of diarrhoeal rats showed more disruption of epithelia and blunting of the microvilli. Nevertheless, the histopathological view of treated groups demonstrated epithelia free from disruption and well distinct villi, which clearly justified that EBV and quercetin have diarrhoea protecting potential.

Fig. 4 [Images not available. See PDF.]

Histopathological images of colonic sectional part of rats in COI diarrhoea model. [10 × Scale Bar 100 µm]. In figure, **A** Colonic section of Normal control rat, **B** Colonic section of Castor oil control rat, **C** Colonic section of Castor oil induced diarrhoeal rat receiving EBV (200 mg/kg, p.o.), **D** Colonic section of Castor oil induced diarrhoeal rat colon receiving quercetin (50 mg/kg, p.o.), and **E** Colonic section of Castor oil induced diarrhoeal rat colon receiving standard

In silico study

The calculated binding energies of quercetin with the proteins TNF-α, IL-1β, and EP3 prostanoid receptor are shown in Table 5. The table demonstrated that quercetin exhibited a higher affinity towards the EP3 prostanoid receptor compared to TNF-α and IL-1β proteins. This suggests that quercetin may have a stronger binding interaction and potential therapeutic relevance with the EP3 prostanoid receptor. To visualize the binding interactions between quercetin and the proteins, Fig. 5 displays different binding poses of quercetin at the active site of the respective proteins. The figure provides a visual representation of how quercetin interacts with the binding pocket of each protein, highlighting potential key interactions and conformational changes. These binding poses help in understanding the molecular interactions and potential mechanisms of action between quercetin and the target proteins.

Table 5. Observations of the molecular docking studies

Molecule	Target protein	PDB ID	Binding energy CDOCKER (Discovery Studio)
Quercetin	TNF-α	1TNF	−106.4030 kcal/mol
IL1-β	1I1B	−131.2166 kcal/mol	EP3 prostanoid receptor

Fig. 5 [Images not available. See PDF.]

Molecular docking studies of quercetin against different target proteins. In figure **A** represents 2D interactions of quercetin against IL-1β, **B** represents 3D interactions of quercetin against IL-1β, **C** represents 2D interactions of quercetin against TNF-α, **D** represents 3D interactions of quercetin against TNF-α, **E** represents 2D interactions of quercetin against EP3 prostanoid receptor and **F** represents 3D interactions of quercetin against EP3 prostanoid receptor

The results of the molecular dynamics study are depicted in Fig. 6. The figure provides information on several key parameters that were analysed during the simulation. Firstly, the RMSD of the different conformations was presented in comparison to the initial pose. The RMSD indicates the average deviation or fluctuation of the protein–ligand complex structures throughout the simulation. It helps to assess the stability and conformational changes of the complex over time. The RMSD plot in Fig. 6A demonstrates how the different conformations deviate from the initial structure during the simulation.

Fig. 6 [Images not available. See PDF.]

Molecular dynamics simulations of quercetin against EP3 prostanoid receptor. In figure, **A** Root mean square deviation, **B** Radius of gyration and **C** Root mean square of fluctuation

Additionally, Fig. 6B shows ROG of the protein over the course of the molecular dynamics study. The ROG is a measure of the compactness or overall size of the protein molecule. Monitoring the ROG provides insights into the protein's structural stability and any potential unfolding or compaction events during the simulation. Furthermore, the RMSF of different amino acid residues of the protein is also depicted in Fig. 6C. The RMSF analysis demonstrates the flexibility or mobility of individual amino acids in the protein. It highlights regions or residues that exhibit significant fluctuations or conformational changes during the simulation. The RMSF plot provides information on the dynamic behaviour of the protein and can identify regions that may play crucial roles in ligand binding or protein function.

Network pharmacology

Quercetin was the bioactive compound obtained from *Begonia rubrovenia* and TNF-α (encoded by the gene TNF), IL1-β (encoded by the gene IL1B) and EP3 (encoded by the gene PTGER3) were the bioactive target proteins as

shown by the docking studies. Quercetin was found to be neutrally charged with a molecular weight of 302.23 (> 1250). We found that TNF interacted with 10 different proteins, including IKBKG (encoding NF-kappa-B essential modulator), TRAF2, IL10, BIRC2, FADD, TRADD, and others. Out of 16 molecular processes identified by gene ontology analysis, a process of tumour necrosis factor receptor superfamily binding (GO:0032813) was found with the lowest false discovery rate of 1.77e-07 in which there was modulation of 5 proteins against 48 background proteins (Fig. 7).

Fig. 7 [Images not available. See PDF.]

Interaction network of *Begonia rubrovenia,* quercetin and its molecular targets

IL1-β was also found to have 10 different interacting proteins, including CASP1, CXCL1 &8, CCL3, IL1A, IL10, IL6 and others. Gene ontology data revealed 14 molecular processes, among which cytokine receptor binding (GO:0005126) had the lowest false discovery rate of 6.46e-10, modulating 8 proteins against 264 background proteins. Similarly, EP3 was found to interact with 10 different partners, including GNAS, GNAI1, 2 &3, GNAQ, PTGDR2, PTGES and others. Gene ontology identified a total of 9 molecular function, among which a molecular function of G-protein beta/gamma-subunit complex binding (GO:0031683), having the lowest false discovery rate of 3.30e-09 was identified, modulating 5 proteins against 20 background proteins (Fig. 7).

Discussion

Diarrhoea is a very common gastrointestinal disorder but ranks second in deaths among all the causes of deaths in developing countries profoundly due to lack of sanitation, unhygienic conditions, contaminated water as well as food, malnutrition, etc. [32]. Diarrhoea is either secretory or osmotic type depending upon its etiology [33]. The present investigation overviewed on the antidiarrhoeal efficacy of EBV using castor oil and PGE_2 induced diarrhoea rat models including molecular docking, dynamics and network pharmacological studies.

Oral acute toxicity study is carried out to determine the safest dose of drug for the evaluation of animal experimental study. Here, for estimation of diarrhoeal study OECD guidelines 425 was implemented. Up to 2000 mg/kg dose of the extract, there were no any abnormal behavioural or neurological signs observed in the treated animals which confirmed the extract to be safe up to above dosing. The normal faecal excretion rate study revealed that, the rate of excretion significantly declined in treated rats, which gives us an idea about the antisecretory potential of *B. rubrovenia* extract [19]. In castor oil induced diarrhoea rat model, diarrhoea was found to be more severe in case of diarrhoea control rats. But, the severity of diarrhoea was reduced in all the treated rats, which was depicted through the results showing delay in onset of diarrhoea, decline in total number of faeces, number of wet faeces and mean defecation rate. Also, the profound antidiarrhoeal activity of the EBV and its marker was confirmed from the low diarrhoea score and high % protection values. Both EBV at 200 and 300 mg/kg dose showed quite similar responses in both the studies, i.e. normal faecal excretion and castor oil induced diarrhoea models. Thus we may presume that, ceiling effect was observed from EBV 200 mg/kg and therefore, EBV 200 was considered as effective dose for the next entire antidiarrhoeal evaluations.

Castor oil obtained from the plant *Ricinus communis*, when ingested orally gets metabolized into ricinoleic acid by the intestinal lipase enzymes. This acid forms lesions on the intestinal mucosal membrane, which leads to changes in the permeability of mucosal fluid and disturbance in the electrolyte and water transport that results in hypersecretion and finally diarrhoea [34]. In the present study also, the intestinal volume significantly increased in diarrhoea control rats, whereas it was significantly recovered in rats treated with EBV and its marker quercetin as demonstrated in the results of COI fluid accumulation diarrhoea model. Ricinoleic acid metabolized from castor oil, after oral administration, increases the peristaltic activity of the intestinal smooth muscle [35]. The reduction in the peristaltic movement was observed in EBV and quercetin treatment group as demonstrated through the COI transit study showing very low peristaltic index. The antidiarrhoeal efficacy of EBV against inflammatory diarrhoea was also evaluated by using PGE_2 induced enteropooling study. PGE_2 enhances the gastrointestinal motility by interacting with EP3 receptors of the intestine thus, increase the intestinal volume which leads to diarrhoea [32]. The results of our analysis showed that the extract and quercetin were found to be effective in reducing the fluid volume, which

might be due to inactivation of EP3 receptors expressed in the small intestine.
Ricinoleic acid present in the intestine promotes the release of NO by activating the inducible nitric oxide synthase (i-NOS). Over expression of NO results in increased level of c-AMP mediated through activation of adenyl cyclase. This overall condition decreases the Na^+-K^+- ATPase activity [32]. The results demonstrated that, there was an over expression of NO in diarrhoeal rats, which tend to normalize after treating the animals with extract and quercetin, that ultimately enhanced the Na^+-K^+- ATPase activity. The upsurge in NO results in elevated oxidative stress, which is one of the important parameter responsible for provoking various types of gastrointestinal malfunctioning [34]. This oxidative stress decreases, the CAT and SOD level and increases the LPO level, which was depicted from the diarrhoea control group rats. However, in case of EBV and quercetin treated rats, there was restoration of altered change in the levels of oxidative enzymes, which helps in the maintenance of oxidative stress. Na^+-K^+- ATPase, a basolateral protein enzyme, plays a major role in transport and maintenance of nutrients and electrolytes [14]. Studies have suggested that diarrhoeal condition hampers the cellular proliferative factors (DNA, proteins and carbohydrates) causing decreases in their levels [36]. The cellular proliferative enhancing effect of the extract, quercetin and standard drug was confirmed through the increase in these parameters. The inducing agent castor oil, causes irritation to the intestinal mucosal membrane through ricinoleic acid resulting into inflamed mucosa and consequently release of inflammatory mediators like TNF-α and IL-Iβ, which was observed in diarrhoea control group [35]. Our results also showed that, there was significant decline in the above mediators after treatment, which suggests the role of EBV in treating inflammatory type of diarrhoea. The histopathological examination of colonic tissues confirmed the protective nature of the extract and its marker quercetin showing recovery from the destruction of epithelia and blunting of villi as observed in treated groups.
Flavonoids, alkaloids, tannins, terpenoids, saponins and steroids from the medicinally active plants plays critical role in treating diarrhoea [37]. Our extract was found to be rich in phenolics, tannins, flavonoids which was confirmed from the quantification results. Quercetin belongs to flavonoid category and its presence in the extract was confirmed through the HPLC analysis data. Quercetin was reported to have antidiarrhoeal potential by relaxing the smooth muscles of intestine, which ultimately inhibits the bowel movement by reducing the release of Ca ions (intracellularly) through the sarcoplasmic reticulum [38]. From the overall antidiarrhoeal study we may presume that, quercetin might be one of the major responsible phytoconstituent in the extract for the observed antidiarrhoeal activity in combination with other phytochemicals.
The molecular docking results indicated that, quercetin exhibited a strong affinity towards all the target proteins, particularly the human EP3 prostanoid receptor, with a binding energy of -150.69 kcal/mol. Quercetin was found to have two hydrogen bonds with active site amino acids viz. GLN:103 and SER:336 together with other non-bonded interactions. This suggests that quercetin forms stable interactions with the active site of the EP3 prostanoid receptor, indicating its potential as a ligand for this protein. Subsequently, a molecular dynamics simulation revealed that the RMSD value reaches a plateau after 5 ns, indicating that the complex has achieved stability. This suggests that quercetin remained compatible and firmly bound within the active site of the protein throughout the simulation. Regarding the receptor protein itself, the ROG analysis showed minimal and insignificant fluctuations. This implies that, the protein maintained its overall compactness and stability during the molecular dynamics simulation study. The limited fluctuation in ROG indicates that the receptor protein structure remained relatively intact and does not undergo significant conformational changes or unfolding. Additionally, the RMSF analysis of the amino acid residues further supports the stability of the receptor protein molecule [29]. The RMSF plot indicated that the individual amino acid residues exhibited minimal fluctuation and remained relatively stable throughout the simulation. This suggested that the receptor protein structure is robust and maintained its stability, supporting its functionality and ability to interact with ligands like quercetin [28]. Findings from this in silico studies conducted in this research suggest that quercetin has the potential to interact with the EP3 prostanoid receptor, which may contribute to its antidiarrheal activity.
Quercetin was found to exhibit wide-ranging effect on several molecules in the network analysis. TNF-α, besides interacting with several receptor molecules was also predicted to modulate apoptotic adaptor molecule FADD,

caspase regulator BIRC2, and NF-kappa-B modulator IKBKG [30]. IL1-β was found to interact with significant molecules such as Caspase1, CXCL1 which has chemotactic activity for neutrophils, CCL3 with inflammatory and chemokinetic properties and several interleukins. IL10 molecule was found in the network of both TNF-α and IL1-β indicating extended molecular level effect. Further EP3 molecules has several important predicted functional partners like Appetite-regulating hormone GHRL, Translin-associated protein X TSNAX, Microsomal prostaglandin-e synthase 1 PTGES, and several Guanine nucleotide-binding proteins. The network analysis showed that quercetin can have diverse range of effects by interacting with these proteins and modulate the molecular pathway [31].

Conclusion

The present study concludes that, the antidiarrhoeal potential of EBV may be due its secretion inhibitory and antipropulsive effect. In addition, EBV also showed reduction in NO level, restoration of ions and antioxidants, decreased proinflammatory cytokines expression and enhancement of Na^+-K^+- ATPase activity which may promotes its diarrhoea protecting efficiency. Thus, we have successfully justified the traditional use of *Begonia rubrovenia* in treating diarrhoea, where quercetin played a major role in combination with other phytoconstituents.

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Author contributions

PRI, DL and SKP: Conceptualization, supervision, investigation, RSP, DL and NYY: Collected the plant material, authenticated and initial phytochemical standardization, RSP, NYY, SRD, and DL: Performed the initial pharmacological protocol, RSP, PS, MD, and SKS: Performed the biochemical analysis and cytokine profiling, RSP, SRD and JK: Performed the histopathological and ion analysis, JK, NR and SKS: Performed the docking dynamics and network pharmacology, RSP, SKP, PS and JK: Performed statistical analysis and interpretation of results, RSP, DL, NR, NYY and SRD: Prepared the initial draft of the manuscript, SKP and PRI: Finalized the manuscript and responsible for communication of manuscript to the Journal.

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Availability of data and materials

The data could be made available as on request of the journal.

Declarations

Ethics approval and consent to participate

All the pharmacological experimental protocols were performed after approval from Institutional Animal Ethical Committee of Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur, Maharashtra, India (Ref. No. IAEC/UDPS/2022/01 dated 21/05/2022) and were conducted in accordance with accepted standard guidelines of National Institutes of Health Guide for Care and Use of Laboratory Animals. Studies involving plants must include a statement specifying the local, national or international guidelines and legislation and the required or appropriate permissions and/or licences for the study: The plant under investigation was collected from the West Khasi hills district of Meghalaya, India and all the necessary approval right from authentication of plant material approval of the research were been taken from the state government University authorities.

Consent for publication

The consent from all the co-authors have been taken regarding submission of the manuscript to Future Journal of Pharmaceutical Sciences for possible publication.

Competing interests

The authors have disclosed that there are no conflicts of interest. Further, consent from all the co-authors has been taken and the authors are entirely responsible for the composition and content of the article.

Abbreviations

AOT
Acute oral toxicity
COI
Castor oil induced
DE
Diosgenin equivalent
DFE
D-fructose equivalent
EBV
Ethanolic extract of *B. rubrovenia*
GAE
Gallic acid equivalent
IL-1β
Interleukin 1β
NFE
Normal faeces excretion
PDB
Protein data bank
PGE_2
Prostaglandin E_2
RE
Rutin equivalent
RMSD
Root mean square deviation
ROG
Radius of gyration
RSMF
Root mean square fluctuation
TAE
Tannic acid equivalent
TNF- α
Tumour necrosis factor α

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Ethnozoological importance of Eisenia fetida and experimental validation of its anticancer activity in ascites Dalton's lymphoma (DL) bearing mice

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ABSTRACT (ENGLISH)

Eisenia fetida, commonly known as the red earthworm, holds significant ethnozoological importance due to its traditional use in various cultures as a medicinal agent. This study aims to explore the potential anticancer effects of *Eisenia fetida* paste (EFP) and evaluate its therapeutic efficacy in mice with ascites Dalton's lymphoma (DL). The EFP extract demonstrated a significant antitumour effect, possibly by initiating programmed cell death and mitochondrial changes in the tumour cells. The viability of tumour cells exhibited a reduction over time due to EFP treatment. Comparative analysis with the reference drug cisplatin revealed that EFP exhibited fewer or no adverse effects on mutagenicity, hepatotoxicity, and nephrotoxicity in tumour-bearing hosts. EFP treatment was found to show progressive increase in the quantity of apoptotic DL cells over time suggesting a time-dependent impact on the induction of apoptosis in the treated groups. These results imply that EFP might serve as a safer substitute for cancer treatment and hold promise for developing new and improved therapeutic strategies against cancer. The main implication of the study is that EFP might serve as a safer substitute for cancer treatment. It suggests a promising avenue for developing new and improved therapeutic strategies against cancer, with the potential to enhance treatment outcomes while minimising adverse effects.

FULL TEXT

Background

Approximately 19.3 million fresh cancer cases across the globe were estimated by the Global Cancer Observatory (GLOBOCAN) in 2020, with China ranked first, followed by the USA and India in third. GLOBOCAN's predictions indicated that the figure of cancer cases in India is predicted to rise significantly by 57.5% from the number of cases reported in 2020 [31]. Cancer can arise from the abnormal growth and division of cells in various tissues throughout the body, giving rise to over a hundred distinct types of cancer. These different types of cancer can exhibit significant variations in their behaviour and how they respond to treatment [13].

The indications of cancer differ based on the disease's type and stage. Common signs may comprise persistent unexplained weight loss, pain, fatigue, changes in the skin, lumps or thickening of tissues, persistent cough or hoarseness, alteration in bowel or bladder patterns, and difficulty swallowing, among others [10, 39, 44]. The diagnosis of cancer involves different methods, such as physical examinations, medical history reviews, diagnostic imaging procedures (such as CT scans, X-rays, MRIs, or PET scans), laboratory tests (like blood tests or biopsies), and genetic testing [18, 21, 24, 36, 52]. The proper treatment options mainly depend on factors such as the cancer's stage, type, and location, alongside the patient's overall well-being [32, 37]. Among the treatment modalities radiation therapy, surgery, chemotherapy, hormone therapy, immunotherapy, and targeted therapy are the most common [7, 49]. Often, a combination of treatments is used to achieve the best outcome.

Across history, natural compounds have held a substantial role in uncovering anticancer medications. Several

commonly used cancer treatments have their origins in nature-derived sources like irinotecan, vincristine, paclitaxel, and etoposide derived from plants, while mitomycin C and actinomycin D come from bacteria and marine bleomycin [14, 15, 26]. These compounds are crucial in cancer therapy and are expected to retain their importance in the coming years. Extensive research has been conducted on phytochemicals, revealing their anti-carcinogenic properties through their influence on multiple mechanisms resulting in initiation, development, and succession of cancer [27, 51]. However, compared to plants, research on animal-derived products for cancer treatment is relatively less. The traditional medicinal practices in Algeria suggested consuming diverse wild animal species, including *Atelerix algirus*, *Varanus griseus*, *Corallium rubrum*, and *Cancer pagurus,* as remedies for various types of cancer [54]. Many communities in North-Eastern parts of India were also found to use different body parts of essential animal species such as gall bladder, horn, fur, intestine, bone, liver, blood, fat, heart as well as tongue as anticancer agents. The Wangsho and Tangsha tribes of Arunachal Pradesh, the Koch-Rajbonshi, Kalita, Ahom, Chutia, Tea tribes of Assam, and Rongmei of Manipur used various types of mammals, birds, reptiles, fish, molluscs, arthropods as home remedies for cancer [8, 29, 58]. While numerous researchers have recorded the therapeutic benefits of various plant species from Assam, there needs to be more comprehensive documentation regarding the traditional utilisation of animals in treating ailments specific to the region. The significance of blister beetles (*Mylabris cichorii*) in zootherapeutics among the Karbis residing in the Karbi Anglong district of Assam has been documented, along with its strong potential in combating ascites Dalton's lymphoma and Ehrlich ascites carcinoma, showing powerful anticancer effects [45, 46, 57, 58]. It was found that bee venom (melittin), scorpion toxin (Chlorotoxin), has significant inhibitory effects on the EGF-induced invasion, proliferation, migration of breast cancer cells [38]. In another study, it was found that marinobufagin exhibits anticancer effects against colorectal carcinoma both in vitro and in vivo [20]. In this study, the use of a particular earthworm (*Eisenia fetida*) was assessed for its anticancer potential in the laboratory against the experimental malignant tumour- murine ascites Dalton’s lymphoma. An initial ethnozoological survey of the adjacent areas of the few wildlife sanctuaries/national park of Assam and the information of some animals used against cancer-suspected disease, an earthworm species were selected to evaluate its anticancer potentials against a murine malignant tumour. The aim of this study is to contribute to the existing ethnozoological knowledge and explore the potential of EFP as an alternative and potentially safer anticancer treatment for future research and therapeutic developments in cancer treatment.

Results

Screening of antitumour activity of earthworm pastes/extracts

Different doses of *Eisenia fetida* paste (EFP) were used to check their antitumour activity regarding alterations in the viability of mice bearing ascites Dalton's lymphoma under different experimental groups. From the different doses of EFP (10, 20, 40, 80, and 160 mg/kg body weight/day) used, the highest increase in the life span in mice carrying ascites Dalton's lymphoma (in mice with DL was noted to be 49.47% with a dose of 40 mg/kg body weight (Table 1).

Table 1. Antitumour activity of EFP against murine ascites Dalton’s lymphoma

Earthworm extracts	Dose (mg/kg body weight/day)	Life span (days) (mean ± SD)	ILS %	Survival on day 30 Survival/total
EFP	Control	19.0 ± 0.816	–	0/10
10	20.2 ± 1.249	6.32	0/10	20
23.4 ± 2.498	23.16*	1/10	40	28.4 ± 1.955

49.47*	3/10	80	24.7 ± 1.417	30.00*
1/10	160	17.2 ± 1.619	− 9.4 7	0/10

Asterisk indicates a significant level of ILS%, which can be considered to have an enhanced curative effect against Ascites-Dalton's lymphoma

Thus, from the preliminary screening of antitumour activity at different doses of EFP, it was derived that the EFP dose of 40 mg/kg body weight has enhanced curative efficiency against murine ascites Dalton's lymphoma. The patterns of comparative changes in ILS after EFP and cisplatin treatments are shown in Fig. 1.

Fig. 1 [Images not available. See PDF.]

A Survival pattern and **B** percent increase in life span (%ILS) of tumour-bearing mice after EFP treatment. Results are expressed as mean SD (*n* = 5). The control refers to untreated tumour-bearing mice, while CDDP stands for cis-diamminedichloroplatinum (II) or cisplatin

Cell viability/cytotoxicity study

The result of EFP on the DL cell viability and spleen cells was checked using the trypan blue exclusion test. EFP treatment caused a time-dependent reduction in the case of tumour cell viability (Figs. 2 and 3). There was less cytotoxicity in spleen than in tumour cells (Figs. 4 and 5). These observations indicate that normal cells exhibited a lower sensitivity to the EFP extract when compared to tumour cells.

Fig. 2 [Images not available. See PDF.]

Trypan blue exclusion test to find the viability of DL cells with different treatment conditions. **a** DL cells treated with drug vehicle as a control; **b–e** EFP treatment for 24–96 h; **f** CDDP treatment at 96 h. Colourless viable cells contrast with blue-stained deceased cells. The black arrow points out the viable cells in **a** and blue-stained deceased cells in **d**

Fig. 3 [Images not available. See PDF.]

Quantitative changes in the dead DL cells from mice over diverse treatment conditions determined by the Trypan blue exclusion analysis. Results are expressed as mean SD (*n* = 4)

Fig. 4 [Images not available. See PDF.]

Trypan blue exclusion analysis to check the viability of spleen cells. **a** Control cells are treated with only the drug vehicle; **b** 96 h of EFP treatment. Viable cells are colourless, and non-viable cells stain blue

Fig. 5 [Images not available. See PDF.]

Changes in the visibility of mice's DL and spleen cells (splenocytes) after EFP treatment. The results are expressed as mean SD (*n* = 4)

Apoptosis study

(AO/EtBr) staining

The DL cell nuclei as control were round-shaped with consistent green fluorescence, indicating viable cells (Fig. 6a). On the other hand, in all the treatment groups, different apoptotic characteristics were noted in DL cells at varying time points (Fig. 6b-e). Various apoptotic features were found in the DL cells of CDDP-treated mice also (Fig. 6f). In each of the treated groups (CDDP and EFP treatment), the quantity of apoptotic DL cells showed a time-dependent increase (Fig. 7).

Fig. 6 [Images not available. See PDF.]

Fluorescence-based apoptosis analysis using AO/EB staining in DL cells. Control DL cells **a**; EFP-treated for 24, 48, 72 and 96 h **b**, **c**, **d** and **e**; and cisplatin for 96 h **f**. Regular arrows showing apoptotic features with membrane blebbing/folding

Fig. 7 [Images not available. See PDF.]

Pattern of percentage apoptotic DL cells (apoptotic index) after treatment with CDDP and EFP. Results are expressed as mean SD ($n=5$)

Scanning electron microscopy and Transmission electron microscopy of DL cells

Scanning electron microscopy (SEM)

DL cells used as control were almost round shape with few projections in the membrane and evenly scattered ruffles over the surface (Fig. 8). CDDP-treated tumour-bearing mice showed a decline in ruffles or microvilli on DL cells and at 96 h of treatment membrane fusion and plasma membrane deformities were observed (Fig. 8f). The development of deformities in DL cells including loss of microvilli from the surface in the initial hour of treatment, the appearance of membrane bleb, as well as cell shrinkage were also observed in EFP-treated tumour-bearing mice (Fig. 8b–e).

Fig. 8 [Images not available. See PDF.]

SEM images including Dalton's lymphoma (DL) cells and control cells **a**, *Eisenia fetida* paste (EFP)-treated cell at 24–96 h **b–e**, Cisplatin (CDDP) treatment at 96 h **f**. The control DL cells exhibited a round shape with projections in the membrane and evenly distributed regular ruffles over the surface. Important cellular appearances on the cells, like membrane blebbing, cell membrane fusion, and cell deformities, are indicated by green arrows

Transmission electron microscopy (TEM)

TEM study was done in DL cells to analyse the possible alteration and changes inside the cells subjected to varying treatment conditions (Fig. 9). DL cells used as control showed cellular processes uniformly distributed over the cell surface along with large clear, uniform nuclei, uniform chromatin, smooth plasma damage and disruption of mitochondrial membrane along with cell membrane and distinct normal mitochondrial features with regular cristae (Fig. 9a, b). CDDP treatment in mice caused mitochondrial damage, cytoplasmic vacuolation, the disappearance of membrane processes and nuclear fragmentation in DL cells (Fig. 9c, d).

Fig. 9 [Images not available. See PDF.]

Ultrastructural features of DL cells. The blue arrow in the control shows DL cells **a** with distinct mitochondria cristae, The blue arrow showing mitochondrial damage and nuclear fragmentation after CDDP treatment from 96 h **b**, **c** and **d**, EFP treatment for 24-96 h **e–l** showed pronounced vacuoles and mitochondrial EFP-treated tumour-bearing mice also developed similar structural features in DL cells as observed for CDDP treatment as indicated by the blue arrows. The mitochondria showed rounded deformed cristae and a few vacuoles at 24 h of treatment (Fig. 9**e–f**). At 48 h to 96 h of EFP treatment, the DL cells showed fragmented nuclei, prominent vacuoles, membrane disorganisation, and disordered organisation of mitochondrial cristae with severe damage observed as indicated by the blue arrows (Fig. 9 **g–l**)

Assessment of toxicity of earthworm pastes/extracts [EFP]

Mutagenic studies

Sperm abnormalities

Different morphological abnormalities were observed in sperms of mice after CDDP and EFP treatment. The categories and forms of sperm anomalies triggered by CDDP encompass head without a hook, looping midpiece, head like a balloon, diffused head, amorphous head and so on. EFP-treated tumour mice also show sperm abnormalities like banana-shaped heads, coiled necks, hammer-shaped heads, and bulged heads in the tumour-bearing mice (Fig. 10b-g). However, mice with tumours treated with EFP showed reduced sperm abnormalities in

the tumour-bearing hosts compared to CDDP treatment (Table 2; Fig. 11).

Fig. 10 [Images not available. See PDF.]

Illustrative images depicting diverse forms of morphological abnormalities in mouse sperm under varying treatment conditions, **a** normal, **b** banana-shaped head, **c**, **d** balloon-like head, **e** hammer-shaped head, **f** coiled neck **g** bulged head

Table 2. Numeric assessment of diverse categories of morphological abnormalities in sperm observed across varying treatment conditions

Treatment	No. of sperms observed	No. of abnormal sperms	BSH	BLH	HSH	CN	BH	Mean of abnormal sperms
Control	500	4	1	0	0	2	1	0.8 ± 0.1
CDDP-treated	500	33	11	6	4	7	5	6.6 ± 0.2
EFP-treated	500	14	3	2	2	4	3	2.8 ± 0.34*

Fig. 11 [Images not available. See PDF.]

Changes in the prevalence of sperm abnormalities resulting from the administration of CDDP (Cisplatin) and EFP (Eisenia fetida paste) treatment. The findings are expressed as mean SD ($n = 4$). *$P \leq 0.05$ as compared to the chosen control; #$P \leq 0.05$ when compared to CDDP

The outcomes are shown as mean ± SD. The analysis was performed with one-way ANOVA long with Tukey's test, with a sample size of 4. A $p \leq 0.05$ level of significance was employed in comparison with CDDP. A normal mouse lacks a tumour and any treatment; CDDP = Cisplatin. BSH = banana-shaped head, BLH = balloon-like head, HSH = hammer-shaped head, CN = coiled neck and BH = bulged head. The examination of sperm was conducted on the fifth day of the treatment.

Chromosomal aberration analysis

A few aberrations, such as isochromatic break and chromosomal fragments, were detected within the bone marrow cells following EFP treatment (Fig. 12). The highest occurrence of the aberration mentioned above was observed after treatment of 24 h. There was a considerable decrease in the mean aberrant metaphase under 24–96 h treatment in both CDDP and EFP-treated groups. CDDP treatment in the tumour-bearing mice showed more chromosomal aberrations than the EFP-treated tumour-bearing mice (Fig. 13).

Fig. 12 [Images not available. See PDF.]

Illustrative images depicting bone marrow metaphase chromosome spreads. **a** Typical array of chromosomes, along with various categories of chromosomal abnormalities, including **b** &**c** Isochromatid break (ICB) and **d** Chromosomal fragment (CF)

Fig. 13 [Images not available. See PDF.]

Statistical examination of mean deviant metaphases, isochromatic breaks (ICB) and chromosomal fragment (CF) after treatment with CDDP **A** and EFP **B**. Results are expressed as mean SD ($n = 3$). The symbol "#" indicates significance at $p \leq 0.05$ compared to CDDP

Renal function (RFT) and Liver function (LFT) tests

Renal function test (RFT)

Serum creatinine, as well as urea level, were determined to examine the changes in renal toxicity. The serum creatinine and urea level measurement in the tumour-bearing control showed a slight variation from their normal counterpart. EFP-treated tumour-bearing mice showed a reduction in serum creatinine and urea levels compared to the control tumour group and were almost equalised with normal mice. However, administration of CDDP resulted in a notable rise in serum creatinine and urea levels compared to the control group (Table 3; Fig. 14.)

Table 3. Levels of serum creatinine and urea across various experimental conditions

Treatment groups	Creatinine (mg/dL)	Urea (mg/dL)
Normal	0.62 ± 0.01	42.76 ± 0.56
Control	1.09 ± 0.08	69.56 ± 1.91
CDDP-treated	2.75 ± 0.06	244.67 ± 2.51
EFP-treated	0.74 ± 0.01*#	48.68 ± 0.29*#

Mean ± S.D. values are expressed with ANOVA, $n = 3$, *$P \leq 0.05$ comparing with the consequent control; #$P \leq 0.05$ vs. CDDP. Blood samples were taken on the 5th day post CDDP or EFP treatment. The normal mice is devoid of tumour as well as any treatment. The control is the mice with tumour and without treatment, EFP = *Eisenia fetida* paste; CDDP = *cis*-diamminedichloroplatinum (II) or cisplatin

Fig. 14 [Images not available. See PDF.]

Changes in serum urea and creatinine levels (mg/dL) of mice with tumours under various treatments. Mean ± S.D. ANOVA was used to express the results as mean SD ($n = 3$), *$P \leq 0.05$ when evaluated with the corresponding control; # $P \leq 0.05$ when compared with CDDP

Liver function test (LFT)

The alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity were examined to measure the toxicological changes in hepatic functions in hosts exposed to different treatment conditions.

The control mice showed increased activity of both AST and ALT as compared to normal (Table 4). However, compared to the control, EFP treatment exhibited a minimal alteration in ALT and AST activity. On the other hand, CDDP treatment displayed a considerable increase in ALT and AST activity compared to control mice (Table 4; Fig. 15).

Table 4. Levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity across various experimental scenarios

Treatment groups	ALT (SGPT) U/L	AST (SGOT) U/L
Normal	27.62 ± 0.52	32.10 ± 0.54
Control	58.17 ± 0.21	38.50 ± 0.65
CDDP-treated	85.71 ± 0.78	73.71 ± 0.51

EFP-treated	52.10 ± 0.83*#	26.44 ± 0.42*#

Mean ± S.D. values are presented with ANOVA, $n = 3$, *$P \leq 0.05$ as compared to the chosen control; #$P \leq 0.05$ when compared to CDDP. Blood was collected on the 5th day after CDDP or EFP treatment. The normal mice is devoid of any tumour or treatment condition and the control mice is with the tumour without treatment, EFP = *Eisenia fetida* paste; CDDP = *cis*-diamminedichloroplatinum (II)

Fig. 15 [Images not available. See PDF.]

Changes in serum ALT and AST in mice harbouring a tumour under various treatments. Results are expressed as mean SD ($n = 3$), *$P \leq 0.05$ compared to the control; # $P \leq 0.05$ compared to CDDP

Discussion

Ascites Dalton's lymphoma, also known as T cell lymphoma, has been consistently utilised as a significant model for experimental murine malignant tumours [40, 56]. The reliable measure to assess a drug's antitumour efficacy is considering the extension of the lifespan of the treated hosts with tumours (Prasad, Nicol et al. 2010, [56]. Examination of the survival capability of splenocytes and DL cells in various in vivo treatment scenarios indicated that EFP treatment elicited more significant cytotoxicity in DL cells compared to splenocytes. This suggests that EFP affects normal cells with lower sensitivity than its impact on DL cells (Figs. 4 and 5). The anticancer potential of *Eisenia fetida* extract/paste or compound isolated from it has been observed on a variety of other cancer cell lines such as HCT116 human colorectal cancer, MCF-7 breast carcinoma [34, 48], HeLa and LTEP-A2 cells [25]. This is the first study to look at the antitumor activity of an earthworm extract (Eisenia fetida paste, EFP) from Northeast India.

Apoptosis is an ordered and orchestrated cellular process controlled by a genetically regulated mechanism in different physiological and pathological conditions. It is a strongly held multi-step pathway responsible for cell death to control the cell number [28, 59]. It has a vital role in the development of tumour-related diseases along with other different illnesses. Unrestrained growth and malfunction of apoptosis mechanisms form essential components in the growth and advancement of a malignant tumour [9, 43]. Apoptosis in the cells is identified by the formation of membrane blebs, cell and organelle contraction, DNA fragmentation, nuclear condensations, and cell disintegration at the end [17]. Numerous cancer chemotherapeutic medications have been documented to trigger apoptosis in cancerous cells [22]. Assessments based on the TEM-, SEM- and AO/EtBr-based fluorescence microscopy are excellent indicators for confirmation of apoptotic features [42]. The present AO/EtBr-based fluorescence study of DL cells revealed that the EFP treatment caused severe membrane blebbing, cell shrinkage, cell membrane disintegration, and other apoptotic features in the DL cells in a time-dependent fashion. This may concur with an earlier report from the study using earthworm extract/paste of *Lampito Mauritius* showing similar kinds of apoptotic features in HT29 colon cancer cells [35]. As observed in EFP-treated groups, the reference drug cisplatin treatment also led to the development of apoptotic features in DL cells.

Surface morphological and ultrastructural changes are significant indicators of cellular damage, measured as distinct indicators of apoptosis. SEM studies showed a sequence of alterations on the surface of the DL cells following treatment with EFP and cisplatin. Cisplatin treatment for 96 h showed membrane blebbing, cell shrinkage, membrane projection (microvilli) loss and developed cell deformities compared to control DL cells, which are circular with membrane extensions and undulations in the cell surface. EFP treatments also caused similar surface morphological changes in DL cells. The above alterations in the cell surface morphology after treatment with EFP may indicate the appearance of apoptotic characteristics in DL cells.

TEM studies revealed damage in the cell membrane structure, cytoplasmic vacuolation, mitochondrial damage, etc., as prominent features observed in DL cells. Mitochondria is crucial in controlling apoptosis, maintaining malignant phenotype, and mutagenesis [1, 16, 33] . In the present study, the ultrastructural examination of control DL cells showed distinct mitochondrial features with regular cristae and uniform nuclei, smooth plasma membrane and uniformly distributed cellular processes over the cell processes. Structural abnormalities like mitochondrial

membrane disruption reduced and damaged cristae cell cytoplasmic vacuolation, the disappearance of membrane processes and fragmentation of the nucleus were detected in DL cells at 96 h of cisplatin treatment in vivo. Treatment with EFP also showed severe structural deformities in mitochondria and other cellular organelles. Disruptions in mitochondrial structure and function in developing tumours have been associated with the participation of hypoxia-inducible factor (HIF-1α) in activating the glycolytic enzymes under various oncogenic stimulations [30]. Moreover, the damage in the mitochondria may also be because of the malfunction of the respiratory chain and the decline in membrane potential, resulting in the disturbance of cellular energy; AMPK plays a pivotal role in upholding energy balance during heightened stress conditions. Inhibition of the AMPK pathway occurs in this context [23]

In the present study, different chromosomal abnormalities, including isochromatic breaks, chromatid breaks, exchanges, unions of sister chromatids, and chromosomal fragments, were identified in bone marrow following cisplatin treatment, as documented in previous studies. However, only a few isochromatic breaks and chromosomal fragments were found in the EFP treatment compared to the cisplatin treatment. In the treatment conditions, the total frequency of aberration in metaphase and CA was highest after 24 h of treatment, gradually decreasing as the treatment progressed to later time points. The time-dependent decrease in CA after the treatment could be because of the death of damaged cells or post-replication repair processes in the cells [12]. The quantitative analysis of mean aberrant CA in EFP treatment showed a minimal chromosomal aberration in bone marrow cells compared to cisplatin treatment, suggesting that EFP is much less mutagenic. These findings may also be supported by some naturally occurring cytotoxic compounds such as propolis and bee venom [2, 3]. Thus, the assay results of mutagenic parameters such as sperm abnormalities and chromosomal aberrations indicate less/no mutagenic property of EFP in DL tumour-bearing mice.

Renal (renal function tests, RFT) and liver functions (liver functions test, LFT) are the crucial parameters for understanding the possible kidney and liver toxicity. Changes in serum creatinine and urea have been used to study renal function as important biomarkers [19]. In this study, mice cisplatin treatment resulted in an amplification of serum creatinine and urea levels, indicating the signs of nephrotoxicity. The increased creatinine and urea levels may be due to the reduced rate of glomerular filtration or increased ROS [41]. However, the EFP treatment did not indicate any notable changes in serum creatinine and urea levels, indicating that the EFP does not have any such nephrotoxicity as that observed for cisplatin. This is also supported by another report of earthworm extracts like powder of *Eudrillus euginae* and its toxicity evaluation in Wistar male rats, which suggested that the earthworm powder had no toxic effects in the kidneys in experimental animals [6].

Hepatic functions or LFT (liver function test) is generally determined with the assessment of certain marker enzymes like alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum of mice/humans under different experimental/pathological conditions [55]. In the present study, ALT and AST levels were elevated in cisplatin-treated mice, indicating hepatocellular injury in the liver. However, in EFP-treated mice, both AST and ALT levels did not illustrate any such elevation like cisplatin treatment, and it remained almost similar to control mice. This suggests less hepatocellular injury in the EFP-treated tumour-bearing mice. This is in agreement with other reports of hepatoprotective action of earthworm extract of *Lampito mauritii*, Kinberg was reported by Balamurgan et al*.,* in 2008 against liver injury induced by paracetamol in Wister albino rat [6].

Conclusion

This is the first research study assessing the antitumour activity of earthworm extract (*Eisenia fetida* paste, EFP) from North-east India. *Eisenia fetida* paste was incredibly effective against murine ascites Dalton's lymphoma. EFP extract-mediated antitumour effect may potentially encompass the initiation of apoptosis and mitochondrial changes in tumour cells. A decrease in the GSH levels of DL cells after EFP treatment may reduce tumour cells protective ability, which may show more cytotoxic effects under these treatment conditions. Compared to the reference cisplatin, EFP has less/no mutagenicity, hepatotoxicity, and nephrotoxicity in the tumour-bearing hosts. Finally, it may be proposed that this study establishes foundational data for future laboratory investigations to discover novel biological compounds and advance drug development. This data may also help preserve biodiversity and

strategically handle animal resources. Further studies may be initiated to isolate and characterise the bioactive composites from the earthworm extract and understand the molecular aspect of the antitumour mechanism.

Materials and methods

An earthworm, *Eisenia fetida* (Fig. 16), was selected to assess the anticancer efficacy and toxicity in mice with ascites Dalton's lymphoma. The biochemical and toxicity assessment studies were also carried out and are presented in detail in the supplementary file. The earthworms were collected from the Jorhat and Dhemaji districts of Assam and identified with ZSI, Shillong's help. The earthworm extract /paste was prepared from the *E. fetida* to study antitumour activity.

Fig. 16 [Images not available. See PDF.]

Eisenia fetida (**a**. Live earthworm; **b**. Dead earthworm)

Classification -

•Kingdom: Animalia

•Phylum: Annelida

•Class: Clitellata

•Order: Haplotaxida

•Family: Lumbricidae

•Genus: Eisenia

•Species: *E. fetida*

•Common name: Red worm (English), Kesu (Assamese)

Chemicals

Cisplatin (CDDP) solutions (1 mg/ml) were sourced from Biochem Pharmaceutical Industries in Mumbai, India; ethylenediaminetetra-acetic acid (EDTA), sodium arsenite ($NaAsO_2$), sodium sulphate (Na_2SO_4), analytical-grade 2-Thiobarbituric acid (TBA) and the required chemicals were employed in the study and purchased within the country. Refers and stains in double-glassed distilled water were always used to prepare the solutions.

Animal care and tumour maintenance

A total of 200 mice were used in the current study. Healthy, inbred Swiss albino mice of both sexes, aged 10–12 weeks and weighing 25–28 g, were procured from Pasteur Institute, Shillong, Meghalaya. An inbred mouse colony is being upheld within usual laboratory settings at 24 ± 2°C. The mice are housed in propylene cages containing 5–6 animals each, and they are provided with food pellets available commercially (Amrut Laboratory, New Delhi) and unrestricted access to drinking water.

Experimental tumour model

The murine malignant tumour ascites Dalton's lymphoma (DL) was employed in the current study for assessing the antitumour activity and toxicity in the mice bearing tumour. The DL tumour was sustained in the laboratory through consecutive intraperitoneal (i.p.) injections of viable 1×10^7 tumour cells (0.25 ml volume in phosphate-buffered saline, PBS, pH 7.4) into healthy mice by the method described by [47] (Fig. 17). Typically, hosts receiving the tumour transplant survived for 19–21 days.

Fig. 17 [Images not available. See PDF.]

Transplantation of ascites Dalton's lymphoma (DL) tumour cells in Swiss albino mice

Preparation of selected earthworms' extract/paste

Fully matured earthworms, *E. fetida,* were collected from their natural habitats of Jorhat and Dhemaji district of Assam. The preparation of earthworm paste was done with the following technique with small modifications [5]. The earthworms were rinsed under flowing tap water to eliminate any sand particles present on the surface of earthworms and then kept in 0.65% NaCl for 1–2 h at room temperature; the earthworms were provided with moist blotting paper for a duration of 18 to 20 h to facilitate gut clearance. Subsequently, the worms with cleared guts were subjected to a distilled water rinse. These worms were placed in plastic troughs, securely covered with polythene, and exposed to sunlight for 3 days until completely dried. The mucus and coelomic fluid that exuded from the deceased worms caused the worms' digestion, creating a brown-hued earthworm paste (EFP). These earthworm pastes underwent filtration, and the acquired filtrates were concentrated using a water bath set at 35 °C. The obtained earthworm pastes were gathered and preserved at 4˚C until required for further procedures.

To screen anticancer potentials, the crude pastes/extracts were dissolved in phosphate buffer saline (pH 7.4) to acquire requisite dilution for the screening of antitumour activity (Fig. 18).

Fig. 18 [Images not available. See PDF.]

Preparation of earthworm paste/extract

The per cent yield of the extracts was calculated with the following formula:%yieldoftheextract=Weightoftheextract(mg)Weightoftheearthworm(mg)×100

The percentage yield of earthworm *Eisenia fetida* paste (EFP) was about 3.33%.

Treatment protocol and selection of specific dose for further treatment

Preliminary antitumour activities of the animal extracts were determined subsequently by the method of Sakagami et al. [50]. Dalton's lymphoma cells (1×10^7 cells in 0.25 ml PBS) were transplanted intraperitoneally (i.p.) into Swiss albino mice between 10 to 12 weeks old. The tumour transplantation day was considered as day '0'. Evidence of initial tumour growth became apparent within 3 to 4 days following tumour transplantation. Animals with tumour transplants were categorised into six groups for the two selected extracts, each consisting of 10 mice. Commencing on the 6th-day post-tumour transplantation, injections of *E. fetida* paste extracts (EFP) were administered via the intraperitoneal route once daily for five days. Group I, with control animals, was given 0.25 ml of the respective extract vehicle once daily for five days. Groups II, III, IV, V and VI were treated with 10, 20, 40, 80, and 160 mg/kg body weight/day of the earthworm's paste. Any instances of host mortality were documented daily, and the survival trends of the hosts were assessed across the various groups. The impacts of different doses on tumour inhibition were quantified as the percentage rise in average lifespan (ILS), with calculations conducted using the specified formula:ILS=T/C x 100-100where T represents the mean survival days of the treated group of mice, and C denotes the mean survival days of the control group. To determine the most potent dose of the extract, animals were given a range of doses from 10 mg/kg of body weight to 160 mg/kg of body weight per day.

The tumour-transplanted mice of both sexes were allocated randomly into three experimental groups, with 10 mice in each group as follows:

Group-I: Tumour-bearing mice served as control and received PBS (Phosphate buffer solution) only.

Group- II: Tumour-bearing mice were administered with cisplatin daily for 5 consecutive days (i.p., 2 mg/kg body weight) beginning from the 6th day of post-tumour transplantation.

Group-III: Tumour-bearing mice were administered a therapeutic dose of EFP for five consecutive days (i.p., 40 mg/kg of body weight) beginning from the 6th day of post-tumour transplantation.

In different experiments, at intervals of 24, 48, 72, and 96 h following the final treatment (specifically on the 11th, 12th, and 14th days post-tumour transplantation), three mice from each group were euthanised using cervical dislocation [11]. DL cells, tissues (liver, kidney, testes) and blood samples were collected for microscopical, biochemical, and mutagenicity studies, as summarised in Fig. 19.

Fig. 19 [Images not available. See PDF.]

Treatment schedule for survivability study of mice bearing ascites Dalton's lymphoma.

For conducting a comparative analysis of the anti-tumour effect, a recognised anticancer drug, cisplatin (CDDP), at a dose of 2 mg/kg body weight per day via intraperitoneal injection (i.p.), was employed as a reference compound. It was administrated to the host bearing the tumour on the 6th day for five consecutive days post-tumour transplantation. Cisplatin was employed as a reference drug by many researchers in the anticancer study as it has a unique ability to initiate apoptosis in numerous cancer cells [4].

The ascitic DL obtained from the mice was subjected to centrifugation at 2000xg and 4 °C for 15 min. The resulting pellet was then employed as the DL cells. The samples from the control and various treatment groups were used for microscopical, biochemical, enzymatic and haematological studies

Cell viability study

The Trypan blue exclusion test was carried out to assess the viability of both DL cells and splenocytes. In brief, DL cells and splenocytes were harvested from DL-carrying mice in diverse groups at various time points (24, 48, and 96 h). Subsequently, they were washed twice with PBS. Following this, a portion of the cell mixture was combined with an equal quantity of Trypan blue dye (with 0.4% in PBS) and incubated for 2 min [53]. Unstained viable cells and stained non-viable or dead cells were quantified using a Neubauer haemocytometer mounted under a light microscope (Meiji). The percentage of non-viable cells was determined by observing 10–15 distinct selected fields of view within each treatment group, employing the subsequent formula:$$\text{Percentdead cells} = \frac{\text{Averagenumberofdeadcells}}{\text{Averagenumberoftotalcells}} \times 100$$

Fluorescence-based determination of apoptosis

Fluorescence-based apoptosis detection in DL cells was conducted using acridine orange and ethidium bromide (AO/EtBr) staining. DL cells were harvested from mice in various groups at distinct time intervals, precisely 24, 48, and 96 h, while subjected to diverse treatment conditions. Subsequently, they were washed with PBS. The cell suspension was supplemented with the AO/EtBr stain solution (100 µg/ml PBS of each dye), mixed gently, and then incubated for 5 min. The cells underwent a wash with PBS and were meticulously scrutinised under a fluorescence microscope (Leica) to identify any alterations or apoptotic features in the DL cells. Photographic documentation was also performed. A total of 1000 cells were screened, and the proportion of apoptotic and viable cells was tallied from twenty specifically chosen fields of view using a microscope. This procedure facilitated the determination of the apoptotic index.

Scanning electron microscopy and transmission electron microscopy of DL cells

Scanning electron microscopy (SEM)

Ascitic DL cells were collected from animals exposed to various experimental conditions. These cells were washed with PBS and subsequently resuspended in PBS, and a thin smear was prepared on a cover glass. A cover glass bearing the smear was then fixed in a 2.5% (v/v) glutaraldehyde solution at 4ºC. After fixation, the cells underwent rinsing in 0.1 M phosphate buffer and were steadily dehydrated using a sequence of ethanol concentrations: 30%, 50%, 70%, 90%, and 100%, each step lasting for 20 min. Next, the cover glass containing the cells was sectioned into smaller pieces, followed by critical point drying utilising a critical point dryer (CPD-030, BAL-TEC Co.). These

dried samples were then fixed to an aluminium stub using double-stick tape, and a gold coating was applied using an ionic sputter coater (SCD-005, BAL-TEC Co.). The prepared samples were subsequently subjected to observation, meticulous examination, and imaging using a Scanning Electron Microscope (JEOL JSM – 6360).

Transmission electron microscopy (TEM)

The DL tumour was gathered from mice within distinct experimental groups and centrifuged at 1000xg for 10 min at 4 °C. The resulting cell pellets were washed in PBS and then fixed in 3% glutaraldehyde at 4 °C for 2 h. After fixation, they were rinsed in 0.1 M cacodylate buffer. The cell pellets were fragmented into smaller fragments, followed by post-fixation in 1% osmium tetroxide at 4 °C for 15 min. Subsequently, a graded series of acetone (30–50-70–80-90–95%, with two repetitions at each concentration for 15 min each), was used for dehydration. The cell pellets were then placed in dry acetone prepared by adding an excess of $CuSO_4$ crystals to absolute acetone and filtering.

Further steps included two rounds of propylene oxide treatment for 1 h at room temperature, followed by a mixture of propylene oxide and an embedding medium composed of Araldite Cy212, dodecenyl succinic anhydride, tridimethylamino methyl phenol, and dibutylphdyhalate. Thin sections (60–80 nm) were sliced using an ultramicrotome (ultratome-RMC, MTX, USA) and collected on copper grids. These sections were stained using a mixture of lead citrate (5%) and uranyl acetate (5%) (1:1; v/v). The examination used an electron microscope (Jeol electron microscope) operating at 80 kV. The sections were scanned and meticulously inspected, and photomicrographs were captured.

Mutagenic studies

Sperm abnormality studies

On the fifth day of treatment, male mice within various groups were euthanised. The cauda epididymis was excised and immersed in physiological saline. Subsequently, the cauda epididymis was minced into smaller fragments and allowed to rest undisturbed for 20 min to enable the dispersal of spermatozoa. The spermatozoa were then dispersed onto a dirt-free slide, air-dried, and subjected to fixation in absolute methanol for 15 min. On the subsequent day, the spermatozoa were stained using a 1% aqueous solution of eosin-Y. Five hundred spermatozoa were examined for each mouse to identify aberrations in both tail shapes and sperm heads. The assessment followed the criteria established by Wyrobek and Bruce in 1975 [27].

Analysis of chromosomal aberration

An analysis of the chromosomal aberration was conducted according to a methodology outlined in 1994 by Sharma and Sharma [28]. Mice within different groups underwent mitotic arrest, initiated 2 h before euthanasia through an intraperitoneal injection of colchicine (4 mg/kg body weight). The collection of bone marrow cells was done by centrifugation at 1000 rpm and 4 °C for 5 min. The bone marrow was extracted from the femur through continuous flush with PBS (pH 7.4) with a syringe. Following this, the cell pellet was introduced to a pre-warmed hypotonic solution (1% sodium citrate) of 5 mL, and incubation was carried out for 20 min at 37 °C. Subsequently, Carnoy's fixative (methanol: glacial acetic acid, 3:1, v/v) in drops were gently mixed, and the mixture was centrifuged for 5 min at 1000 rpm. The upper layer was removed, and the resulting pellets were gradually separated by gentle tapping. Chilled Carnoy's fixative (5 mL) was introduced to the mixture and incubated for 30 min at 4 °C. Two more repetitions of the fixation process were carried out with a 30-min break. Ultimately, the cells were resuspended in 0.5 mL of the fixative. A small amount of drops of this suspension was placed on dirt and grease-free chilled slides, followed by flaming to facilitate drying. Once air-dried, the slides were stained the following day with Giemsa stain solution (freshly prepared) in Sorensen's buffer (pH 6.8). Subsequently, the slides were mounted using DPX. A hundred well-spread metaphase cells were examined per animal under a light microscope (Leitz). Various

chromosomal abnormalities were identified and scored based on observation.

Renal function (RFT) and liver function (LFT) tests

Blood samples were obtained from mice within distinct experimental groups on the fifth day after treatment through orbital venous sinus bleeding. Two millilitres of blood collected from the mice through orbital venous sinus bleeding and serum was separated by centrifuged at 4000 rpm for 10 min. The collected blood was then processed to separate the serum. After serum separation, changes in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were evaluated to gauge liver function. Additionally, serum urea and creatinine levels were measured to assess renal function. This assessment was conducted using a Clinical Chemistry Analyzer (SYNERGY BIO-1904C).

Statistical analysis

All experimental data were presented as the mean ± standard deviation (S.D.), with each determination repeated three times. The differences among multiple groups were assessed using a one-way analysis of variance (ANOVA), followed by a post hoc test (Tukey test). A significance level of $P \leq 0.05$ was deemed as statistically significant. Data were analysed using Origin 8 Software.

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Author contributions

Both the authors are equally involved in conceptualising this research work. Necessary data/sample collection, laboratory experiments, preparation of graphs, tables, Statistical analysis, interpretation and initial manuscript preparation were done by MPB. SBP did further correction and scrutiny of the manuscript. We take the responsibility for the integrity of the work presented in this manuscript. All the authors have read and approved the final manuscript.

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Availability of data and materials

All the data that support this study are included in the manuscript.

Declarations

Ethics approval and consent to participate

All applicable international, national, and institutional guidelines for the care and use of animals were followed. The maintenance, use of the animals and the experimental protocol of the present study were approved by the North-Eastern Hill University, Shillong, Meghalaya, India, vide PhD registration no. 2247 of 2013 and Institutional Ethics Committee (animal models), North-Eastern Hill University, Shillong, India, dated 21–11-2014.

Consent for publication

All the mentioned authors agreed to submit the work to the Future Journal of Pharmaceutical Sciences.

Competing interests

The authors declare no conflict of interest.

Abbreviations

DL

Dalton's lymphoma

EFP

Eisenia fetida Paste

CT

Computed tomography

MRI

Magnetic resonance imaging

PET

Positron emission tomography

SD

Standard deviation

CDDP

Cis-diamminedichloroplatinum(II)

ILS

Increase in life span

AO/EtBr

Acridine orange/ethidium bromide

BSH

Banana-shaped head

BLH

Balloon-like head

HSH

Hammer-shaped head

CN

Coiled neck

BH

Bulged head

ANOVA

Analysis of variance

ICB

Isochromatid break

CF

Chromosomal fragment

ALT

Alanine aminotransferase

AST

Aspartate aminotransferase

SGPT

Serum glutamic pyruvic transaminase

SGOT

Serum glutamic-oxaloacetic transaminase

DNA

Deoxyribonucleic acid

AMPK

AMP-activated protein kinase

RFT

Renal function test

LFT

Liver function test

ROS

Reactive oxygen species

GSH

Glutathione

Publisher's Note

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Biogenic metallic nanoparticles as game-changers in targeted cancer therapy: recent innovations and prospects

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ABSTRACT (ENGLISH)

Background

Cancer is a significant global health issue, resulting from uncontrolled cell division leading to abnormal cell or tissue growth. Traditional chemotherapeutic techniques have investigated a wide variety of pharmaceutically active molecules despite their poor bioavailability, quick renal clearance, inconsistent distribution, and unavoidable side effects. Green synthesis, unlike chemical methods, prioritizes eco-friendliness and cost-effectiveness. Using natural sources like plant extracts, it minimizes environmental impact, reduces costs, and aligns with sustainability goals. Operating under milder conditions, it consumes less energy compared to traditional approaches. Green synthesis is a highly promising and efficient method for producing nanoparticles due to its versatility and scalability.

Main body

Nanotechnology is making progress in cancer treatment because of nanoparticles' tiny size, large surface area, adaptability, and functionality, as well as their potential to induce apoptotic pathways and fast penetration or internalization into cancer cells. Biosynthesis of metallic nanoparticles using plant or microbe extracts is attracting attention to replace toxic chemicals with phytochemicals that can act as reducing, capping, or stabilizing agents and improve metallic nanoparticles biocompatibility, antitumor, and antioxidant properties. This review focuses on biosynthesized metallic nanoparticles and their anticancer effects on breast, prostate, skin, cervical, colorectal, lung, and liver cancer.

Conclusion

Biosynthesis of nanoparticles for cancer therapy stands at the forefront of innovative and sustainable approaches. Despite challenges, ongoing research demonstrates the potential of biosynthesis to revolutionize cancer nanomedicine, emphasizing the need for continued exploration and collaboration in this rapidly advancing field. Overall, this review offers a comprehensive understanding of the most recent developments in biosynthesized metallic nanoparticles for the treatment of cancer as well as their potential future applications in medicine.

FULL TEXT

Background

Cancer is caused by the excessive proliferation of normal cells, which causes genetic instability and mutations to accumulate within cells and tissues, transforming them into malignant cells. Radiation, smoking, nicotine, toxins in drinking water, food, air, chemicals, certain metals, and infectious agents are all potential external causes of cancer, in addition to internal ones such genetic mutations, weakened immunity, and hormone imbalances [1]. Despite significant efforts by scientists to overcome cancer, it remains difficult to effectively treat. Hair loss, exhaustion, nausea, and other symptoms are possible side effects of conventional chemotherapy, which uses chemicals to destroy cancer cells. Because of these side effects and drug resistance, it is difficult to take advantage of

conventional chemotherapy for the complete treatment of cancer [2]. Nanomedicine has made significant advancements in the treatment of cancer over the past several years and is useful as a drug carrier for chemotherapeutics because of its size, shape, selective binding capability, high permeability and retention impact, surface modification, etc. This allows them to deliver drugs directly to the cancer cells while preserving healthy tissue [3]. Nanoparticles (NPs) have been explored as pharmaceutical carriers for more than three decades to increase the in vivo effectiveness of several existing anticancer molecules. The investigations conducted during 1970s explored anticancer drug-loaded liposomes [4]. NPs are widely used for the delivery of imaging agents, genes, or chemotherapeutics, exploiting their intrinsic toxicity, such as related to the release of hazardous species [5]. Inherent properties, such as antioxidant action, or activities dependent on the application of external stimuli, like hyperthermia in response to the introduction of infrared rays or magnetic fields, may account for the physicochemical characteristics that give NPs their anticancer activity [4]. Metal and metal oxide NPs are being used experimentally to directly kill tumor cells by converting applied magnetic fields into strong hyperthermia or by performing effective photodynamic therapies that can reach even internal tissues by converting in situ penetrating infrared radiation into visible light inside the tumor [6].

NPs are commonly synthesized using top-down (synthesized via size reduction) and bottom-up strategies (NPs are generated from small entities such as atoms and molecules) [7, 8]. Hazardous and poisonous chemicals, expensive laboratory equipment and infrastructure, and the ability to operate under a variety of circumstances, including high temperature and pressure, are all utilized in the various chemical and physical procedures used to create and synthesize NPs. NP synthesis is expensive, and it results in highly toxic and harmful compounds that pose a variety of biological risks. So, it is advised to create an eco-friendly process combining biological and green synthesis techniques [9].

Green synthesis of metal or metal oxide NPs involves reducing metal complexes in diluted solutions to form metal colloidal dispersions. Despite being commonly used reducing agents, sodium borohydride and hydrazine hydrate are not preferred due to their toxicity from nanoparticle contamination. Green resources including plant and microbial extracts contain compounds that can convert metal precursors into NPs. All green methods typically include combining one or more of these biological extracts with metal salt solutions. Biomolecules change the oxidation state of metal salts from positive to zero, stabilizing newly formed NPs or acting as *in situ* reducing and capping agents [10]. The formation of a monolayer around the NPs prevents them from aggregating together due to chemical and physical interactions. Khan et al. synthesized palladium NPs using a root extract from *Salvadora persica*. The extract contained polyphenols with bioreduction and stabilizing properties [11]. The quantity of natural compound present in the extract affects the size and size distribution of NPs. Khatami et al. synthesized 15-nm-sized silver NPs (Ag NPs) from dried grass. At 5 μg/mL, Ag NPs suppress cancer cell multiplication and decrease cancer cell survival by 30% [12].

Biological processes are preferred to chemical and physical ones because they are less expensive, safer for the environment, do not require extreme conditions, and do not generate harmful by-product molecules. Biological nanoparticle synthesis employing living organisms is a green technique for synthesizing NPs with desired properties. Biosynthesis allows unicellular and multicellular organisms to react [13]. Biogenic synthesis can produce large numbers of contamination free well-defined NPs. It also has a lower environmental impact than alternative physicochemical manufacturing methods [14]. Cheng et al. used zinc oxide NPs (ZnO NPs) synthesized from *Rehmanniae radix* (RR) as a target drug delivery vehicle to inhibit bone cancer (MG-63) cell proliferation. Increasing dosage caused apoptosis [15]. *Caesalpinia pulcherrima* extract-loaded Ag NPs are cytotoxic to HCT116 cell lines [16]. *Artemisia turcomanica* leaf extract-loaded Ag NPs were cytotoxic against normal fibroblast cells (L-929) and gastric cancer cells (AGS) [17]. Metal oxides are toxic for cancer cells and nontoxic for normal cells (Fig. 1A). Due to the presence of capping agent on metal oxide NPs, these bind to the surface of healthy and cancerous cells through electrostatic attraction. Ionic species, namely metal and oxygen radicals, are formed when the biosynthesized NPs dissociate. The elevated metal ion concentration prevents the growth of cancer cells by changing their metal cation homeostasis. Superoxide dismutase (SOD) eliminates the oxygen radical, resulting in hydrogen peroxide, which

peroxisome catalases and phytocompounds then convert into water and oxygen. As a result, the superoxide radical is converted into antioxidants before reactive oxygen species (ROS) develop in normal cells [18].

Fig. 1 [Images not available. See PDF.]

A Effect of metallic NPs in normal and cancerous cells, **B** mechanisms of metal/metal oxide NPs for cancer cell [34], **C** proposed mechanism of apoptotic effect of metallic NPs on the cancer cells [35], (**A**: Recreated with permission © Springer Nature, **B** and **C** recreated under copyright (CC BY) from MDPI, Dovepress)

ROS produced by oxygen radicals generates hydrogen peroxide, which causes oxidative stress and kills cancer cells. However, the lack of experimental evidence for these routes motivates more research into the lethal mechanism of photosynthesized metal oxide NPs in both healthy and malignant cells [19]. This article discusses the probable mechanism of action, the green synthesis of metallic NPs from plant extracts, and their anticancer effectiveness against different cancer cells. It also summarizes characterization methods and the most significant findings from recent studies.

Metallic nanoparticles in cancer treatment

The NPs have been used for many years in biomedical operations such as imaging and drug delivery [20]. Inorganic NPs and their numerous applications, such as cellular absorption, diagnostics, and therapy, have received significant attention in recent years. Most of the inorganic nanoparticle research is focused on materials such as gold, silica, and others. Together with a facilitator, the mesoporous silica bestows a very cutting-edge approach to imaging and drug release [21]. The activity of this facilitator is triggered with the help of external stimuli. Due to their outstanding optical, magnetic, and photothermal characteristics, metallic NPs are widely explored in biological imaging and targeted drug delivery. Gold NPs (Au NPs), Ag NPs, iron-based NPs, and copper NPs are some of the most often utilized metallic NPs. Since their size and surface qualities can be easily adjusted, Au NPs are utilized as drug carriers for intracellular targeting [22]. The visible light extinction behavior of metallic NPs makes it possible to track their trajectories in the cells.

Anti-human epidermal growth factor receptor 2 (HER2)-functionalized gold-on-silica nanoshells have been found to target HER2-positive breast cancer cells [23]. Clinical trials for the detection of nodal metastases using Combidex®, an iron oxide-based nanoformulation, are close to complete [24]. Iron deficiency anemia can be treated with Feraheme®, an iron oxide nanoformulation that contains ferumoxytol. This was approved by the FDA in June 2009 to treat nodal metastases in prostate and testicular cancer [25, 26]. Metallic NPs cause apoptotic, autophagic, and necrotic cancer cell death through ROS generation, caspase-3 activation, mitochondrial outer membrane permeabilization, and specific DNA cleavage [27].

Metal nanoparticle delivery pathway and cell damage can be linked to interaction of NPs with ions in circulation, ingestion by phagocytic cells, opsonization or enzymic degradation, internalization via endocytosis, membrane perforating and damage of its components and function, chromosomal aberrations and changes in cell replication rate, lysosome rupture, mitochondria damage, lower growth rate, structural changes, and shorter lifespan of microtubules of the cytoskeleton, generation of ROS, oxidative stress, and subsequent processes (Fig. 1B). NPs of different sizes enter cells via distinct pathways. Smaller NPs penetrate cells via receptor-mediated uptake by interacting with the caveolin receptor on the cell membrane. Larger NPs are more likely to enter cells via clathrin-mediated endocytosis. When NPs enter a cell, they can proceed one of two ways: either they interact with cytosolic proteins in a direct fashion, or they are transported to the lysosome–endosome complex, where their surfaces are modified before they are released into the cytosol [27]. Inside the cell, NPs trigger up a chain reaction that releases ROS and initiates the release of metal ions. These metal ions tend to connect with proteins' SH groups, breaking their S–S bridges. As a result, the cell physiology is altered, resulting in activation of various signaling pathways that lead to programmed cell death [28]. Apoptosis is frequently induced by either intrinsic or extrinsic pathways. Nanomaterials can induce apoptotic signaling via both intrinsic and extrinsic pathways. In the case of intrinsic apoptosis, ROS production causes mitochondrial membrane depolarization, which results in the release of cytochrome C into the cytosol. This cytochrome C promotes the caspase-9/3 apoptotic cascade by activating pro-

apoptotic proteases in apoptosis initiated by the extrinsic pathway (Fig. 1C) [29].

Ag NPs play a significant role in breast cancer treatment as well as skin wound healing therapy. In this review, most of the studies mentioned are about Ag NPs tested on breast cancer cell lines. For example, the IC_{50} value of paclitaxel is 80 g/dL, while the IC_{50} value of Ag NPs loaded with *Elaeodendron croceum* extract against MDA-MB-231 breast cancer cell line is 138.8 µg/mL [30]. *Moringa oleifera* flower aqueous extract-loaded Au NPs showed anticancer activity against A549 lung cancer cells. Au NPs (50 µg/mL) showed significant anticancer activity against lung cancer cell line [31]. ZnO NPs kill tumor cells through NADPH-dependent oxidative burst and apoptotic signaling. ZnO NPs of various sizes and specific surface areas had a similar effect on cytotoxicity and DNA fragmentation in macrophages of mice in an ap47phox- and Nrf2-independent manner. Because of their critical function in the modulation of immunological responses during inflammation and the clearing of inhaled particles, ZnO NPs trigger necrosis and apoptosis in macrophages. ZnO NPs promote the rapid induction of nuclear condensation, DNA fragmentation, and formation of hypodiploid DNA-containing nuclei and apoptotic bodies [32]. Furthermore, the delivery of cerium oxide NPs (CeO_2 NPs) might cause DNA damage, which results in tumor cell death. CeO_2 NPs enhance ROS in tumor cells, causing apoptosis without genotoxicity. The antitumor activity of CeO_2 NPs is greatly dependent on their size and shape. Both small- and large-sized NPs induce DNA damage in tumor cell lines [33].

Silver nanoparticles

Due to their unique physical and chemical characteristics, such as high electrical conductivity and optical, electrical, thermal, and biological properties, Ag NPs are gaining considerable interest in the healthcare sector [36]. Ag NP aggregates enter mammalian cells via endocytosis and can cross blood–brain barrier due to their small size. After entering an endocytic vesicle, they are intracellularly transported to the cytoplasm and nucleus [37]. The antimicrobial properties of silver have been observed since ancient times. Silver is currently employed in various applications to regulate bacterial proliferation, such as in dental procedures, catheters, and the treatment of burn injuries. Ag ions and Ag-based compounds are generally recognized for their severe toxicity to microorganisms, exhibiting potent biocidal properties [38]. Ag NPs, measuring around 32.2 nm, were manufactured using an extract derived from *Teucrium polium*. These NPs were incorporated into a film made of polylactic acid and polyethylene glycol (PLA/PEG). The resulting film serves as a biodegradable wound dressing that possesses antioxidant and antibacterial properties. The incorporation of biogenic silver NPs into PLA/PEG nanofibers resulted in the total inhibition of growth in *P. aeruginosa* and *S. aureus*, demonstrating substantial antibacterial properties [39]. The Ag NP-loaded amorphous calcium polyphosphate NPs, which were synthesized using wet chemical precipitation, exhibited effective antibacterial activity against *E. coli*, *Staphylococcus aureus (S. aureus)*, and *Enterococcus faecium* [40]. A recent study shown that the production of Ag NPs using a crude leaf extract of *Lycium shawii* exhibited a minimum inhibitory concentration (MIC) ranging from 1 mg/mL to 15 mg/mL against several microorganisms. The measured MIC clearly demonstrates the significant antibacterial properties of the produced NPs [41]. Multiple in vitro and in vivo studies have demonstrated the anticancer effects of Ag NPs, rendering them a highly promising choice for cancer therapy [42]. At a dosage of 1.0 mg/L, enzyme-responsive Ag NPs coated with adenosine triphosphate killed 56.04% of HepG2 cell line [43]. Ag NPs produced by the one-step caffeic acid-mediated reduction are anticipated to enter cells via endocytosis and effectively suppress HepG2 cell growth through apoptosis induction [44]. The aqueous extract of *Panax ginseng* roots was used to synthesize Ag NPs with the assistance of ultrasound. The resulting NPs exhibited an IC_{50} value of 157 µg/mL against the PC14 cancer cell line. In PC14 cells, the biosynthesized Ag NPs modulated the PI3K/AKT/mTOR signaling pathway and elevated ROS levels, apoptosis, and LDH release [45]. Chen et al. studied the function and mechanism of Ag NPs in prostate cancer. Ag NPs diminished lysozyme membrane integrity, number, and protease activity. This blocked autophagy. In PC-3 cell lines, sublethal Ag NP doses can produce hypoxia and energy deficiency [46].

Gold nanoparticles

Au NPs are synthesized using chemical, physical, and biological methods. Conversion of metallic gold into nano-particulate gold by chemical reduction is a common menthod for the synthesis of Au NPs. Citrate mediated reduction

method has been described by Turkevich in 1951 to synthesize stable and size controlled Au NPs. Brust and Schriffin explored sodium borohydride mediated reduction to synthesize Au NPs. In 1996 Schmid *et. al*. described seed mediated growth, the most explored chemical method, to synthesize Au NPs [47]. Surface modification of Au NPs can be done using amine and thiol groups. This has the potential to benefit biomedical applications such as targeted delivery, imaging, and sensing for electron microscopy markers [48]. Murawala et al. synthesized Au NPs with a bovine serum albumin cap and methotrexate loading that impede MCF-7 proliferation and cause G1-S phase arrest, DNA breakage, and eventually apoptosis [49]. Gum acacia (GA) was utilized successfully to synthesize gemcitabine hydrochloride (GEM)-loaded colloidal Au NPs. Cell viability was 64.8% and 51.8% for naked GEM-treated cells at doses ranging from 0.25 to 0.5 µg/mL, respectively. GEM-GA-Au NPs decreased cell viability by 51.2% and 42.8%, respectively. GEM-GA-Au NPs exhibited superior anti-proliferation effects on MDA-MB 231 human breast cancer cells compared to naked GEM [50]. In comparison with free TA-peptide, the conjugation of Au NPs and a thioctic acid-DMPGTVLP peptide (TA-peptide) conjugate led to a more substantial release of cytochrome c after the activation of caspase-3/7. However, after intratumoral injection in tumor-bearing mice, TA-peptide Au NPs exhibited superior antitumor effectiveness compared to TA-peptide [51].

Iron oxide

Over the past 20 years, iron-based NPs have gained interdisciplinary scientific interest due to their distinctive properties and nanotechnological possibilities [52]. Iron oxide NPs (Fe_3O_4/Fe_2O_3 NPs) exhibit good superparamagnetic characteristics of Fe_3O_4 and Fe_2O_3, leading to significant performance in drug delivery applications [53]. Superparamagnetic Fe_3O_4/Fe_2O_3 NPs have attracted considerable attention because of their potential use in imaging, drug delivery, and hyperthermia management. They are non-toxic, biodegradable, and biocompatible and effectively eliminated from the human body via iron metabolism pathways [54]. Unique physical and chemical properties of Fe_3O_4/Fe_2O_3 NPs (large surface area, superparamagnetic properties, and nanoscale dimensions with a spherical form and an adjustable size of less than 50 nm) make them highly efficient [55]. Fe_3O_4/Fe_2O_3 NPs have an intrinsically therapeutic impact on malignancies [56] and resist tumor cell growth in a better way when compared to untreated control cells [57]. Sun et al. synthesized multifunctional methotrexate-loaded iron oxide NPs conjugated with chlorotoxin (a targeting ligand). Due to tumor cell cytotoxicity, these NPs may be employed in cancer diagnosis and treatment [58].

Zinc oxide

Zinc oxide nanoparticles (ZnO NPs) have emerged as a promising contender for use in biomedical research, food packaging, optical, electrical, and food processing applications. ZnO NPs are harmful to cancer cells because, at low pH levels, they decompose into Zn^{2+} ions. These Zn^{2+} ions produce ROS, which kill cancer cells. Additionally, ZnO NPs have been successfully employed as a vehicle for the precise delivery of anticancer drugs into tumor cells [59]. Wahab et al. found that ZnO NPs, when utilized at very low concentrations and in a dose-dependent manner, were effective against MCF-7 (breast cancer) and HepG2 (liver cancer) cells. At 25 µg/mL, HepG2 cell viability was below 10% [60].

Copper oxide

CuO, a well-known p-type semiconductor, has long been studied for its monoclinic structure. Cupric oxide (tenorite monoclinic CuO) and cuprous oxide (cuprite cubic Cu_2O) are two crystalline forms of copper oxide [61]. Potential metal ion leaching and dissolving, as well as oxidative stress, DNA damage, lipid peroxidation, membrane damage, and mitochondrial damage have all been explored in the literature as toxicity pathways. A small amount of CuO NPs can generate large amounts of ROS such as O_2, OH, and H_2O_2. CuO NPs cause membrane disruption and ROS generation after they enter the mitochondria [62]. Wang et al. found that CuO NPs increased the survival rate of tumor-bearing animals, inhibited the metastasis of B16-F10 cells, and significantly delayed the growth of melanoma. The data revealed that CuO NPs had minimal systemic toxicity and were promptly eliminated from the organs. When CuO NPs penetrated the cells, they preferentially targeted the mitochondria, causing cytochrome C to be released and caspase-3 and caspase-9 to be activated. Thus, CuO NPs can kill cancer cells via mitochondrion-mediated apoptosis to treat melanoma and other cancers [63].

Titanium oxide

Bioengineered titanium oxide nanoparticles (TiO2 NPs) have been shown to have good stability, chemical neutrality, hydrophilicity, oxidizing power, and electrical, optical, physical, and photocatalytic properties. Because of their powerful antibacterial and odor-removing properties, TiO_2 NPs are employed in filters and cosmetics. TiO_2 photocatalysts have been extensively explored for the killing or suppression of bacterial growth due to their excellent chemical stability and nontoxicity [64]. Plant extracts may exhibit properties of the metals or metal oxides that make up their composition in addition to the presence of phytoconstituents. These features may ultimately result in their many critical activities in the prevention or treatment of cancer. It has been shown that the tiny size of TiO_2 NPs gives them potent anticancer activity against cancer cells [65].

Biological synthesis of metallic nanoparticles

Over the past decade, there has been an increase in efforts to discover efficient, low-cost, eco-friendly, and long-lasting strategies for producing green NPs [66]. Researchers across the globe are interested in green synthesis since it is an environmentally safe technique and a fascinating study topic for the synthesis of metallic NPs for biomedical applications [67]. Using biological agents to make NPs of various sizes, shapes, compositions, and physicochemical characteristics is safe, non-toxic, and environmentally sustainable [68]. Biosynthesis of these NPs is done at mild pH, pressure, and temperature without using an external reducing agent, capping agent, or stabilizing agent [69]. Capping agents have a significant role in the synthesis of metallic NPs. The major role of capping agent is to functionalize and stabilize the NPs, along with controlling size and morphology [70]. NPs synthesized by green synthesis methods are exceptionally stable, well dispersed, and have a narrow size distribution [71].

Nucleation and production of stable metallic NPs during biological synthesis are affected by several factors such as temperature, reactant concentrations, pH, and reaction time. For example, when employing biomass from *Avena sativa* (oats) at pH 2, rod-shaped Au NPs were larger, ranging from 25 to 85 nm. In contrast, at pH 3 and 4, the Au NPs were comparably smaller, with sizes ranging from 5 to 20 nm. The functional groups present in extract were more easily accessible for particle nucleation within the pH range of 3 to 4. In contrast, a reduced number of functional groups were present at pH 2, leading to the aggregation and formation of bigger Au NPs [72]. Ag NPs were synthesized using bark extract derived from *Cinnamon zeylanicum*. The particle yield exhibited a positive correlation with the concentration of the bark extract, whereas the NPs assumed a mostly spherical morphology at pH values of 5 and higher [73]. Prathna et al. found that the combination of $Ag(NO)_3$ with *Azadirachta indica* leaf extract led to the formation of progressively bigger particles as the reaction time increased. By adjusting reaction time from 30 to 240 min, the particle size changed from 10 to 35 nm. The concentration of NPs produced at various stages of reaction was determined using inductively coupled plasma optical emission spectroscopy measurements. After 2 h, the yield of the process had significantly increased to 78%. Subsequently, there was a progressive and continuous increase in the yield [74]. The photosynthesized NPs are safer than their chemically synthesized counterparts for usage in healthcare applications since they do not contain any harmful contaminants. When applying metal in healthcare products, safety risks related to nanosize, penetration, and tissue permeability must be considered. In vitro cytocompatibility of phytonanoparticles has been documented by multiple researchers. Extensive research is still required to determine their pharmacodynamics, immunogenicity, absorption, biodistribution, excretion, and acute and chronic toxicity. Although plant-mediated nanoparticle production is often considered an environmentally beneficial approach, there is still a dearth of evidence addressing the direct and indirect ecological impacts of these particles [75].

Green synthesis using plant extract

Plants, which include grasses, ferns, trees, bushes, flowers, and other varieties of green algae and lichens, are among the most essential forms of life. NPs can help plants by acting as fertilizers, pesticides, growth regulators, and antibacterial agents. However, flora can also assist with the development of nanotechnology. Plants can be used to produce NPs in two different ways: directly through extraction or indirectly via plant-mediated biosynthesis. Agriculture, food science, nanotechnology, and pharmaceutical science are just some of the fields that could benefit from a better understanding of the interaction between NPs and plant extracts [76]. Plant-mediated green NP

synthesis is one of the most preferred approaches because it normally requires a neutral pH and can occur at room temperature [77]. Plants and plant extracts are sustainable and renewable resources for NP production, unlike prokaryotic bacteria, which require expensive methods for maintaining microbial cultures and downstream processing [7]. Using the various plant parts (Fig. 2A), such as fruits, seeds, calluses, stems, peels, leaves, and flowers, biological processes synthesize metal NPs in a range of sizes and shapes. Metal NPs are synthesized using metal precursors and plant extracts as reducing and capping agents under suitable conditions [78]. A green chemistry approach for the synthesis of metal NPs can be achieved in three stages: (i) the activation phase, in which the phytoconstituents reduce the metal ions, followed by the nucleation of reduced metal atoms; (ii) the growth phase, in which small NPs join to form larger NPs; (iii) the termination phase, in which the NPs take on their final shape [79]. Bioactive alkaloids, phenolic acids, polyphenols, proteins, carbohydrates, and terpenoids in plant extracts reduce and stabilize metallic ions (Fig. 2B) [80].

Fig. 2 [Images not available. See PDF.]

Green synthesis of nanoparticles: **A** different plant parts used for nanoparticles formation, **B** general method for biosynthesized nanoparticles formation, **C** different microorganisms used for nanoparticles formation, **D** different shapes of nanoparticles that can be synthesized, and **E** possible mechanism of nanoparticle synthesis using microbes (Figure **E**: recreated with permission

Tabebuia berteroi leaf extract is rich in polyphenol, and *Withania coagulans* plant extract is rich in flavonoids, tannins, and phenolics. These extracts have been used to reduce Fe and Pd ions to form their respective metal NPs, which are then mixed with graphene oxide to make a nanocomposite [81]. *Anisomeles indica* leaf extract reduced silver ions to spherical Ag NPs (50–100 nm) in 10 min at room temperature [82]. Lee et al. reported Au NPs synthesis utilizing sequential fractional extracts from *Ocimum sanctum* leaves. For the sequential fraction extraction of *O. sanctum* leaves, different polarity solvents (hexane, chloroform, n-butanol, and water) were utilized, and it was discovered that unique solvent fractions (extract) are responsible for the creation of morphologically varied Au NPs. Water extract produced anisotropic NPs, hexane extract produced spherical Au NPs, chloroform extract produced a circular disk-shaped structure with rough edges, and n-butanol extract produced Au NP aggregates [83]. Possible mechanisms of the anticancer effect of phytosynthesized metal or metal oxide NPs include the formation of pro-apoptotic caspases, activation of reactive oxygen species, damage to cell membranes and mitochondria, damage to DNA, and DNA fragmentation [84]. Table 1 represents various plant extracts utilized in the synthesis of metallic NPs along with their corresponding applications.

Table 1. Biosynthesized metallic NPs using various plants and their biomedical applications

MNPs/MONPs	Plant/part used	Metal precursor used	Morphology	Biomedical application	Findings	References

Fe_2O_3/Fe_3O_4	*Hyphaene thebaica* (Aqueous fruit extract)	Iron nitrate hexa hydrate	Quasi-spherical/cuboidal 10 nm	Antibacterial, antioxidant, antiviral activity	Growth of *Bacillus subtilis* (*B. subtilis*) inhibited. Fe_2O_3 NPs were effective against *Aspergillus flavus* (*A. flavus*). An increase in Fe_2O_3 NP concentration reduced viability of RD cells and L20B cells. Moderate inhibition of poliovirus-1 and poliovirus-2 was noted in the culture of virus in L20B cells	[85]
Ficus carica (Leaf extract)	Ferric chloride hexahydrate	Multiform 43–57 nm	Antioxidant activity	At 12.118 mg/mL, synthesized NPs had antioxidant capacity that can eliminate half of the environmental 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radicals	[86]	*Celosia argentea* (Leaf extract)

Ferric nitrate	Spherical /hexagonal/Cubic shape 5–10 nm	Antibacterial, antibiofilm, antioxidant, anti-inflammatory, anti-diabetic, anticancer, larvicidal activity	At 150 µg/mL, antibacterial activity was detected against *E. coli* (19 mm) and *S. aureus* (25 mm). A greater rate of inhibition of biofilm activity was observed at 150 µg/mL. A higher antioxidant (97%) was recorded at 80 µg/mL and higher anti-inflammatory (93%) and anti-diabetic (87%) activities were recorded at 500 µg/mL. MCF-7 breast cancer cells showed 86% inhibition at 50 µg/mL	[87]	*Platanus orientalis* (Leaf extract)	Ferric nitrate nonahydrate
Spherical 78–80 nm	Antifungal activity	Antifungal activity of iron oxide NPs against the fungus was 1.6 times higher than that of *A. niger*	[88]	*Carica papaya* (Leaf extract)	Ferric Chloride	Spherical 56 nm

Antibacterial	Antibacterial activity demonstrated against Gram-positive (*S. aureus, Bacillus subtilus*) and Gram-negative (*E. coli*, *Enterobacter, Pseudomonas fluorescens*) bacteria	[89]	ZnO	*Ziziphus nummularia* (Leaf extract)	Zinc nitrate	Spherical/irregular 17.33 nm

Antifungal activity	ZnO NPs demonstrated strong cytotoxic action against HeLa cancer cell line. NPs had superior antifungal activity compared to conventional azole antibiotics	[90]	*Limonium pruinosum L. Chaz.* (The shoot system, leaves and stems)	Zinc acetate dihydrate	Hexagonal/cubic crystalline 41 nm	Anti-skin cancer, antimicrobial, antioxidant activity

The plant extract and synthesized ZnO NPs demonstrated the largest inhibition zone against *E. coli* measuring 29 and 31 mm, and *C. albicans* measuring 28 and 29 mm. At 1000 µg/mL, ZnO NPs and *L. pruinosum* extract showed highest DPPH activity, at 75.2% and 84.6%, respectively	[91]	*Bixa orellana* (Leaf, seed, and seed coat)	Zinc acetate	Spherical/almond-like 169−259 nm 304−465 nm 278−654 nm	Anticancer, antimicrobial	Antibacterial activity was detected in ZnO NPs containing leaf extract aga i

						nst *S. aureus* and *B. subtilis*. NPs containing seed extract inhibited *E. c*

						oli, and NPs containing seed coat extract inhibited *S. aureus*. Nocyto to

						xicitywasobservedagainstHCT-116cancercells.Noantifungalactivity

						r e c o r d e d a g ai n st f u n gi , in cl u di n g *P e ni ci lli u m s p .*, *A . fl a v u s*, *F u s a ri u m o x*

						y s p o r u m (*F . o x y s p o r u m*), a n d *R hi z o ct o ni a s ol a ni*
[92]	*Mentha longifolia L.* (Leaf extract)	Zinc nitrate	Spherical 60–70 nm	Antiparasitic	At 400 ppm and 150 min of exposure, NPs demonstrated highest scolicidal activity and 100% mortality rate. Morphological changes and loss of viability were seen in the treated protoscolices	[9 3]

CuO	*Bacopa monnieri* (Leaf extract)	Copper (II) acetate	Monoclinic crystalline 34.4 nm	Antibacterial, anti-diabetic, anti-inflammatory	Strong inhibition zones at 5 mg/mL were demonstrated for *H. salomonis* (13.50 ± 0.84), *H. felis* (15.71 ± 0.91), *H. suis* (15.84 ± 0.89), *and H. bizzozeronii* (13.11 ± 0.83). After 48 h, NPs showed 74% less edema as compared to the control group that received diclofenac (100 mg/kg)	[94]
Momordica charantia (Fruit extract)	Copper sulfate pentahydrate	Spherical 50–57 nm	Antiviral, antifungal, antibacterial	The highest efficacy was observed against *Bacillus cereus* with a 31.66 mm zone of inhibition. Additionally, CuO NPs had therapeutic potential against R2B strain of Newcastle disease, *Streptococcus viridians*, and *Corynebacterium xerosis*	[95]	Artemisia (Leaf extract)
Copper (II) sulfate	conceivable spherical/irregular form 38.5–48.5 nm	Antibacterial	Zones of inhibition for *B. subtilis* and *E. coli* were observed as 26.7 and 20.5 mm, respectively	[96]	*Pimenta dioica* (Leaf extract)	Copper (II) sulfate

Platelet/cuboid shape 20–50 nm	Antibacterial, anticancer, antidiabetic, antioxidant	For the L929 and DLD-1 cell lines, the IC_{50} value of CuO NPs is 89.42 mg/mL and 119.06 mg/mL, respectively	[97]	*Gomphrena globosa* *Gomphrena serrate* (leaf extracts)	Copper (II) sulfate pentahydrate	*G. globosa*: Rod/hexagonal/irregular shape 345 nm *G. serrata*: S

						pherical 380 nm

Antibacterial, anticancer, antioxidant, photocatalytic activity	CuO NPs produced by *G. globosa* at 125 µg/mL demonstrated antibacterial activity, with the zone of inhibition measuring between 14 ± 1.41 and 13 ± 1.41 mm. NPs produced by *G. serrata* and *G. globosa* showed IC_{50} values of 85.14 and 77.75 µg/mL, respectively	[98]	TiO_2	*Andrographis paniculata* leaves extract	Titanium (IV) oxide	Spherical 50 nm

Antibacterial, antifungal, antidiabetic, antioxidant	*E. Coli* and *Bacillus spp*. showed a lower zone of inhibition. *Salmonella spp*. showed a higher zone of inhibition. LD_{50} value of TiO_2 NPs was 250 µg/L	[99]	*Psidium guajava* (Aqueous leaf extract)	Titanium dioxide	Spherical 32.58 nm	Antibacterial, antioxidant activity

TiO_2 NPs (20 µg/mL) showed maximum zone of inhibition against *E. coli* (23 mm) and *S. aureus* (25 mm). In comparison to ascorbic acid, aqueous plant extract and TiO_2 NPs exhibited the highest level of antioxidant activity	[100]	*Morinda citrifolia* (Root extract)	Titanium dioxide	Spherical/oval/triangle 20.46–39.20 nm	Larvacidal activity	Maximum activity of TiO_2 NPs was shown against the larvae of *An. stephensi*

						ensi, *Ae. aegypti* and *Culex. quinquefasciatus*
[101]	*Moringa oleifera* (Leaf extract)	Titanium dioxide	Spherical 100 nm	Wound healing activity	Nano-sized particles significantly exhibited wound healing activity in albino rats	[102]

Ag	*Allium cepa*	Silver nitrate	Spherical 10–23 nm	Antimicrobial, antioxidant, antitumor activity	Human breast (MCF-7), hepatocellular (HepG-2) and colon (HCT-116) carcinoma cells were susceptible to the antitumor effects of Ag NPs with IC_{50} values of 1.6, 2.3, and 2.2 µg/mL	[103]
Indigofera tinctoria (Leaf extract)	Silver nitrate	Spherical 9–26 nm	Anticancer, antimicrobial, antioxidant activity	IC_{50} values for Ag NPs and *I. tinctoria* leaf extract were 56.62 ± 0.86 µg/mL and 71.92 ± 0.76 µg/mL, respectively	[104]	*Morinda citrifolia* (Root extract)
Silver nitrate	Spherical and oval 32–55 nm	Anticancer activity	Complete cell death against HeLa cells was observed at 100 µg of Ag NPs	[105]	*Alhagi graecorum* (Leaf Extract)	Silver nitrate

Spherical 22–36 nm	Antifungal, antitumor	Antifungal activity was observed against *Candida albicans, glabrata, parapsilosis, tropicales*, and *krusei*. The inhibition zone ranged from 14 to 22 mm at 0.01 mmol/ml and from 17 to 27 mm at 0.02 mmol/mL	[106]	*Azadirachta indica* (Leaf and bark extract)	Silver nitrate	Spheroidal 13.01 nm (leaf) 19.30 nm (bark)

Antiplasmodial, hemolytic activity	Antiplasmodial activity against 3D7 and RKL9 P was demonstrated by IC_{50} values of 9.27 mg/mL and 11.14 mg/mL for leaf-mediated NPs and 8.10 mg/mL and 7.87 mg/mL for bark-mediated NPs. Ag NPs from the bark and leaves showed significant hemolytic activity (> 25%) at 125 µg/mL	[107]	Au	*Allium sp.*	Chloroauric acid	Spherical 11 nm

Antiviral	At 50% effective concentration (EC_{50} of 8.829 µg/mL), Au NPs actively inhibited MeV replication in Vero cells	[108]	*Sansevieria* (Leaf extract)	Chloroauric acid	Spherical 40–70 nm	Anticancer

Au NPs enhanced antiproliferative effect of Cisplatin on prostate cancer cells with minimal cytotoxicity	[109]	*Curcuma pseudomontana* (rhizomes)	Chloroauric acid	Spherical 39 nm	Anti-Inflammatory, antimicrobial, antioxidant	*P. aeruginosa* exhibited largest inhibition zone (13 mm) at 100 µg/m

						L of AuNPs, followed by *E. coli* (12 mm), and *B. subtilis* (11 mm) and *S.*

						aureus (11 mm). At 25 µg/mL, NP sexhibited high level of inhibition effi

						ciency(94%)incomparisontothestandarddrug(92%)

[110]	*Allium cepa* (Onion peel extract)	Gold trichloride	Spherical/triangular 25–70 nm	Antibacterial, anticandidal, antioxidant, proteasome inhibitory effect	Zones of inhibition measuring 10.66 to 19.95 mm demonstrated greater efficacy of Au NPs and kanamycin mixture against the tested pathogens. Au NPs and rifampicin mixture was limited to its activity against *S. aureus* (22.49 mm) and *E. coli* (9.99 mm). At 100 µg/mL, NPs showed moderate DPPH scavenging potential of 14.44%, while BHT (the reference standard) showed 36.54% at same concentration	[111]

Biosynthesis of nanoparticles using microbes

Actinomycetes, bacteria, fungi, marine algae, viruses, and yeasts have all been effective in the production of NPs employing unicellular and multicellular organisms (Fig. 2C) [113]. These organisms can produce reducing and stabilizing agents in the synthesis of NPs with a wide range of shapes, compositions, and physiochemical properties (Fig. 2D) [114]. Microorganisms can synthesize metallic NPs both intracellularly and extracellularly (Fig. 2E) [115]. To produce NPs extracellularly, microbes are cultivated in the appropriate environments. The microorganism-containing enzyme-rich broth is centrifuged to synthesize NPs [116]. The intracellular synthesis of NPs is carried out via the cellular mechanism of microbial cells [117]. Several studies explored extracellular methods for producing metal NPs [118]. Kalimuthu et al. investigated the role of the nitrate reductase enzyme in the production of Ag NPs by *Bacillus licheniformis*. The enzyme is responsible for converting Ag^{+} to Ag°, and they hypothesized that nitrate ions might play a role in its induction. Cofactors like NADH in NADH-dependent nitrate reductase enzymes are required for generating metal NPs. Bioreduction of Ag^{+} to Ag° may be caused by the release of cofactors NADH and NADH-dependent enzymes by *B. licheniformis*, in addition to other components, especially nitrate reductase [119]. *Pseudomonas stutzeri* was treated with a concentrated Ag NO_3 solution to produce Ag NPs in the periplasm [120]. Numerous fungi strains have been described for the extracellular biofabrication of NPs using CdS, Au, Zirconia, Si, Ti, and magnetite [121]. The method of NP production varies depending on the microorganisms. However, the basic concept involves microorganisms entrapping metal ions on their surface or within their cells, followed by their reduction to NPs. Electrostatic forces are frequently used as a trapping medium [122].

Bacteria

Bacteria can precipitate metals at nanoscale level as well as regulating interaction pathways for metal ion reduction [123]. *E. coli* biosynthesized Ag NPs of 50 nm size by a reliable and cost-effective approach [124]. *Pseudomonas aeruginosa* (*P. aeruginosa*) and other species have been studied for their ability to synthesize Zn, iron, nickel, Au, and Ag NPs [125]. The NADPH-dependent reductase enzyme may be involved in the reduction of Au^{3+} to Au° and

stabilization via capping molecules in the presence of *Stenotrophomonas maltophilia* [126]. Different bacterial species, as listed in Table 2, have been employed to produce metal NPs.

Table 2. Biosynthesis of metallic NPs using various bacteria and their biomedical applications

MNPs/MONPs	Bacteria used	Metal precursor used	Morphology	Biomedical applications	Findings	References
CuO	Marine endophytic *Actinomycetes* CKV1	Copper (II) sulfate pentahydrate	Spherical shape 10–30 nm	Antibacterial, anticancer, antibiofilm activity	At 750 µg/mL, CuO NPs showed remarkable antibacterial activity against *E. Coli* and *P. mirabilis*, exhibiting 24 mm and 28 mm zones. At 500 µg/mL, 54% inhibition was recorded against A549 cells	[127]

Streptomyces sp. MHM38	Copper (II) sulfate	Spherical 1.72–13.49 nm	Antimicrobial	CuO nanoparticles showed antibacterial efficacy against *Enterococcus faecalis*, *Salmonella typhimurium*, *E. coli*, *P. aeruginosa*, and *Candida albicans*	[128]	*Marinomonas Rhodococcus Pseudomonas Brevundimonas Bacillus*

Copper (II) sulfate pentahydrate	Spherical/ovoidal shapes 40 nm	Antibacterial, antifungal	The MIC of CuO NPs ranged from 3.12 to 25 µg/mL for Gram-negative bacteria, 12.5 to 25 µg/mL for Gram-positive bacteria, and 12.5 to 25 µg/mL for fungi	[129]	*Actinomycetes*	Copper (II) sulfate pentahydrate
Crystalline 198 nm	Antibacterial	*B. cereus* showed high susceptibility (25.3 mm) to CuO NPs. The CuO NPs inhibited bacterial pathogens *B. cereus, P. mirabilis*, and *A. caviae* at 5 µg/mL	[130]	ZnO	*Cyanobacterium Nostoc sp. EA03*	*Zinc acetate dihydrate*

Star-like shape 50–80 nm	Antibacterial, anticancer activity	MIC and MBC values for *E. coli, P. aeruginosa,* and *S. aureus* were found to be 2000, 2000, and 64 µg/mL, and 2500, 2500, and 128 µg/mL, respectively. ZnO NPs were less cytotoxic to MRC-5 lung fibroblast cells than to A549 cells treated with cancer	[131]	*Paraclostridium benzoelyticum* strain 5610	Zinc nitrate	Spherical/rectangular shape 50 nm

Antibacterial, anti-inflammatory, antidiabetic	Inhibitory zone of *Helicobacter suis* measured 19.53 ± 0.62 mm at 5 mg/mL. After 21 days. In arthritis model the edema was inhibited by NPs by 87.62 ± 0.12%. ZnO NPs sharply reduced glucose level in STZ-induced diabetic mice	[132]	*B. subtilis* ZBP4	Zinc sulfate heptahydrate	Pseudo-spherical 14–45 nm	Antibacterial

For *B. cereus, S. aureus, E. coli* O157:H7, *E. coli* Type 1, and *P. aeruginosa* the MIC was 1 mg/mL. It was 2 mg/mL for *L. monocytogenes* and *S. typhimurium*	[133]	*Saccharomyces cerevisiae*	Zinc acetate dihydrate	Spherical 20–30 nm	Antioxidant, antibacterial, anticancer, photocatalytic activity	ZnONPs showed concentration-dependent increases in anti-cancer activity

						. At 100 µg/mL, ZnONPs inhibited 93% cells

[134]	TiO_2	*B. subtilis*	Titanium dioxide	Spherical 70.17 nm	Treatment of dental caries	Dental caries responded best to treatment with 5% TiO_2, which had no discernible c

						yt ot o xi c ef fe ct s
[135]	*Rummeli i pycnus* *Acinetob acter baumanii Acinetob acter seohaen sis* *Bacillus cereus*	Titanium Oxychlorid e	Spherical/irregula r 8 nm	Antibacterial	Maximum zone of inhibition was found at 50 mL of TiO_2 NPs	[1 3 6]
Streptomyces sp. HC1	Titanium oxyhydro xide	Spherical 30–70 nm	Antimicrobial, antibiofilm	The highest level of antibiofilm activity was shown by 500 µL TiO_2 NPs produced by *Streptomyces sp.* HC1. Maximum zone of inhibition was recorded against *S. aureus* and *E. coli*	[137]	A g
Streptomyces rochei MS-37	Silver nitrate	Spherical 23.2 nm	Antibacterial, anti-inflammatory, antioxidant	Ag NPs had IC_{50} value of 34.03 µg/mL in CAL27 and 81.16 µg/mL in human peripheral blood mononuclear cells, indicating CAL27 was more susceptible to the NPs cytotoxicity. Ag NPs had MIC ranging from 8 to 128 µg/mL	[138]	*St re pt o m y c e s p ar v u s*

Silver nitrate	9.7–17.25 nm	Antimicrobial, antioxidant	*K. pneumoniae* (28.33 mm) and *E. coli* (21.66 mm) were susceptible to antibacterial activity. When compared to *E. faecalis* (125 µg/mL), *S. aureus* (250 µg/mL), *P. aeruginosa* (125 µg/mL), *K. pneumoniae* (500 µg/mL), and *E. coli* (250 µg/mL), the MIC of Ag NPs was significant	[139]	*Nocardiopsis dassonvillei*	Silver nitrate

Spherical 29.28 ± 2.2 and 32.13 ± 3.4 nm	Antimicrobial, antioxidant, anticancer	Ag NPs showed notable scavenging activity with IC_{50} values of 4.08 and 8.9 µg/mL against OH and DPPH radicals, respectively. Ag NPs with $CaCo_2$ cells demonstrated concentration dependent reduction in cell viability. Lactate dehydrogenase leakage increased as cell viability declined	[140]	*Bacillus amyloliquefaciens* MSR5	Silver nitrate	Spherical/cubical/and regular 29.2 nm

Anticancer, catalytic activity	Using $NaBH_4$, Ag NPs demonstrated potent chemocatalytic action, completely degrading 4-NP to 4-aminophenol (4-AP) in 15 min. Ag NPs activated A549cells	[141]	*Bacillus brevis (NCIM 2533)*	Silver nitrate	Spherical 41 nm	Antibacterial
Ag NPs demonstrated mean zone of inhibition 14, 15, 16, and 19 mm against *S. aureus* at 5, 10, 15, and 20 µL	[142]	Au	*Streptomyces sp.* NH21	Chloroauric acid	Spherical/rod 18–20 nm	Antibacterial

Ag NPs had MIC of 2.5 µg/mL against *E. coli*, 5 µg/mL against *K. pneumoniae*, *P. mirabilis*, and *S. infantis*, and 10 µg/mL against *P. aeruginosa* and *B. subtilis*. For *P. aeruginosa* and *B. subtilis* minimum bactericidal concentrations were 140 and 170 µg/mL	[143]	*Vibrio alginolyticus*	Chloroauric acid	100–150 nm	Antioxidant, anticancer	Colon cancer cell growth inhibited by AuNPs in a dose-dependent manner. 25

						µg/mL resulted in maximum inhibition of cell death (>75%), with an IC_{50} of 15 µg/mL

[144]	*Paracoccus haeundaensis* BC74171T	Chloroauric acid	Spherical 20.93 ± 3.46 nm	Antioxidant, anticancer	In HaCaT and HEK293 normal cells, Au NPs did not exhibit growth inhibition. Au NPs exhibited concentration dependent growth inhibition against A549 and AGS cancer cells	[145]

Fungi

Fungi are non-phototrophic eukaryotic microorganisms with rigid cell walls [147]. Fungi are among the biological sources that are exploited in bioremediation, can mineralize, and are known as mediators in the synthesis of NPs. This is due to their ability to create huge volumes of biomass [148]. Fungi can reduce the size of metal ions to NPs via two different processes: (i) through the contact of the fungus biomass with the metal inside the fungal cell and (ii) through the interaction of the fungus filtrate with the mineral solution outside the fungal cell [149]. This whole process is accomplished through two distinct mechanisms. First, the fungal cell wall traps metal ions on its surface due to the electrostatic interaction of the positively charged groups in the enzymes, and then, the cell enzymes reduce the metal ions to produce NPs. Second, the method involves the reduction of nitrates using NADPH secreted by fungi, followed by its conversion into NADP to produce extracellular NPs [150]. The myogenic pathway produces better NPs than bacteria and plants because they accumulate metals more efficiently. Triangle-shaped intracellular Au NPs (20–35 nm) synthesized by *Aspergillus clavatus* isolated from *Azadirachta indica* have been explored to demonstrate mycosynthesis [151]. *Phoma glomerata* can be used to synthesize Ag NPs that are antibacterial against resistant strains of *E. coli, P. aeruginosa, and S. aureus* [152]. *Trichoderma viride, Chaetomium globosum, Aspergillus niger, and Pleurotus ostreatus* can produce selenium NPs [153]. *Cladosporium perangustum* aqueous extract-derived Ag NPs decreased MCF-7 cell viability by increasing caspase-3, caspase-7, caspase-8, and caspase-9 expression [154]. Metallic NPs derived from various fungi are presented in Table 3.

Table 3. Biosynthesis of metallic NPs using various fungi and their biomedical applications

MNPs/MONPs	Fungi used	Metal precursor (medium) used	Morphology	Biomedical application	Findings	References

Fe_2O_3/Fe_3O_4	*Penicillium spp.*	Iron (III) chloride	Spherical 3.31 to 10.69 nm	Antibacterial, antioxidant activity	NPs demonstrated inhibition activity at 250 µg against *S. aureus* (12 ± 0.6 nm), *E. coli* (11.3 ± 1.2 nm), *K. pneumoniae* (11.3 ± 0.6 nm), *S. sonnie* (11.3 ± 0.6 nm), and *P. aeruginosa* (11.3 ± 0.6 nm). NPs exhibited antioxidant potential against DPPH radical as compared by ascorbic acid with IC_{50} values of 12.2 µg/mL	[155]
A. flavus	Iron (II) sulfate heptahydrate	Spherical 28.6–33.8 nm	Antimicrobial	Maximum (10 mm) inhibition of bacterial growth against *S. aureus* was demonstrated by Fe NPs. Zones of inhibition against *S. aureus* and *P. aeruginosa* were 12.3 and 10.5, respectively	[156]	*Aspergillus terreus*
Iron (III) chloride	Spherical 40–100 nm	Anticancer	Cell viability dropped to 41.9%	[157]	CuO	*Trichoderma asperellum*

Copper (II) nitrate trihydrate	Spherical 110 nm	Anticancer	CuO NPs significantly increased cell death. IC_{50} for CuO NPs in A549 cell lines was 40.625 µg/mL	[158]	*Aspergillus fumigatus*	Copper (II) nitrate trihydrate
Spherical 48 nm	Antibacterial	At 100 µg/mL, CuO NPs showed maximum scavenging activity against DPPH (73.65%)	[159]	*Aspergillus terreus*	Copper sulfate	Less than 100 nm
Antimicrobial, antioxidant, anticancer	CuO NPs exhibited the highest activity against *P. aeruginosa, E. coli,* and *V. cholera* (50% cell inhibition at 22 µg/mL)	[160]	*Shizophyllum commune*	copper (II) chloride	Spherical 22 to 60 nm	Antibacterial, antifungal

Highest antibacterial efficacy was shown against *S. aureus* followed by *E. coli*. At 150 µM, inhibition zone of 1.7 cm and 1.9 cm was recorded in *C. albicans* and *F. oxysporum*, *respectively*	[161]	ZnO	*Pleurotus ostreatus*	Zinc nitrate	Spherical 7.50 nm	Antibacterial, anticancer

Mushroom extract induced dose-dependent decline in cell viability for Hek293 cells. Lowest cell viability (22%) was recorded at 2000 µg/mL. ZnO NPs synthesized from mushrooms demonstrated comparable cytotoxic effects on HepG2 and Hek293 cells. A steep drop in cell viability in HepG2 cells (97% at 16 µM to 12% at 100 µM) and Hek293 cells (94% at 16 µM to 22% cells at 100 µM) was recorded	[162]	*Cladosporium tenuissimum FCBGr*	Zinc nitrate	Hexagonal Less than 100 nm	Antimicrobial, antioxidant, anticancer	ZnONPs had an IC_{50} concentration of 62 µg/mL for DPPH radical scavenging activity. At 58 µg/mL, NP

						s inhibited 50% of nitric oxide radicals. ZnONPs (1 mg/mL) inhibited HeLa cell lines at a rate of 89.59% indi

						catingtheireffectivenessagainstcervicalcancercells
[163]	*Aspergillus niger*	Zinc acetate	Spherical 23.97 ± 2.29 nm	Antibacterial	MIC values for the antibacterial potential against the tested *S. aureus* ranged from 8 to 128 μg/mL	[164]
TiO_2	*Tricoderma citrinoviride*	Titanium isopropoxide	Irregular/triangular, pentagonal/spherical rod-shaped 10–400 nm	Antibacterial, antioxidant	The biogenic TiO_2 NPs (100 μg/mL) exhibited remarkable antibacterial efficacy when tested on planktonic cells of clinical isolates of *P. aeruginosa* that are highly resistant to drugs. The antioxidant potential of TiO_2 NPs was superior to gallic acid	[165]

Fomitopsis pinicola	Titanium (IV) isopropoxide	Spherical 10–30 nm	Antibacterial, anticancer	MIC/MBC values were 62.5/125 and 62.5/125 μg/mL for *E. coli* and *S. aureus*. Strong cytotoxic effect of TiO_2 NPs was observed against HCT-116 cancer cells	[166]	Ag
Penicillium oxalicum (*Amoora rohituka* plant leaf)	Silver nitrate	Spherical 15–19 nm	Antimicrobial, antioxidant, anticancer	MIC_{25}, MIC_{50}, and MIC_{75} values of Ag NPs against *E. coli* were 8.710 ± 0.217, 12.369 ± 0.099, and 81.857 ± 0.453 μg/mL, respectively. The corresponding values for *S. aureus* were 14.417 ± 0.011, 20.975 ± 0.008, and 61.614 ± 1.452 μg/mL, respectively	[167]	*F. oxysporum* (*Withania somnifera* leaves)
Silver nitrate	Spherical 10–50 nm	Antibacterial Cytotoxic activity	All the Gram-negative and Gram-positive organisms were found to be sensitive and to exhibit a zone of clearance	[168]	*Aspergillus brasiliensis*	Silver nitrate

Spherical 6–21 nm	Antibacterial, antifungal	Ag NPs inhibited *B. subtilis, S. aureus, E. coli, P. aeruginosa,* and *C. albicans* in distinct zones, measuring 12, 15, 12, 12, and 14 mm, respectively	[169]	*Trichoderma spp.*	Silver nitrate	Spherical
Antibacterial	Gram-negative bacteria (*E. coli* and *P. aeruginosa)* had lower MIC values than Gram-positive bacteria (*S. aureus and E. faecalis*)	[170]	*Alternaria sp*	Silver nitrate	Spherical 3–10 nm	Antifungal
The MIC of Ag NPs was 25 µL, whereas all fungal strains grew at very low rates (50 and 100 µL)	[171]	Au	*Cladosporium species* (*C. wightii* leaves)	Chloroauric acid	Spherical (irregular morphology) 5–10 nm	Anticancer effect

IC_{50} value of the Au NPs was 38.23 µg/mL in breast cancer cell line MCF-7	[172]	*Trichoderma hamatum* SU136	Gold chloride	Spherical/pentagonal/hexagonal 5–30 nm	Antibacterial	Compared to the clear zones surrounding streptomycin and gold chloride, the Au NPs' clear zone appear

						ance was smaller and showed antibacterial activity against all tested bacterial strains; no clear zones were ob

						served surrounding the fungal mycelial-free extract
[173]	*Alternaria alternata*	Chloroauric acid	Spherical/triangular/hexagonal 2–30 nm	Antifungal	—	[174]

Algae

Algae have the capacity to accumulate heavy metal ions. These aquatic microorganisms have been explored to synthesize NPs [176]. Using *Tetraselmis kochinensis*, spherical Au NPs with sizes ranging from 5–35 nm were produced intracellularly [177]. *Sargassum polycystum* (a brown algae)-based CuO NPs had excellent anticancer characteristics and great potential against pathogenic bacteria [178]. Priyadharshini et al. used *Gracilaria edulis* (macroalgae) extract to synthesize Ag and ZnO NPs. The synthesized NPs exhibited excellent antitumor activity against human PC3 cells [179]. *Hypnea musciformis* (Wulfen), a red macroalga, has been explored to synthesize Au NPs for its anti-fungal activity against *Aspergillus niger* and *Mucor spp* [180]. *Ecklonia cava* extract-loaded Ag NPs have shown significant anti-bacterial activity against *E. coli* and *S. aureus.* These NPs had antioxidant properties and anti-cancer activity against human cervical (HeLa) cells [181]. Numerous algal components and precursor salts employed in the synthesis and capping of metallic NPs are shown in Table 4.

Table 4. Biosynthesis of metallic NPs using various algae and their biomedical applications

MNPs/MONPs	Algae used	Metal precursor (medium) used	Morphology	Biomedical application	Findings	References
Fe_2O_3/Fe_3O_4	*Ulva lactuca*	Iron (III) chloride	Spherical 20 and 40 nm	Anticancer, anti-diarrheal activity	Significant cytotoxicity was observed in cancer cell lines treated with NPs at higher concentrations (50 mg/mL and 100 mg/mL). The zone of inhibition by *Ulva lactuca*-mediated NPs against *E. coli* and *S. aureus* were 29 ± 1 mm and 17 ± 2 mm, respectively	[182]
Colpomenia sinuosa, Pterocladia capillacea	Iron (III) chloride	Spherical 11.24–33.71 nm, 16.85–22.47 nm	Antibacterial, antifungal	Fe_3O_4 NPs demonstrated superior antifungal efficacy against *A. flavus* (9 mm) and *F. oxysporum* (6 mm) in contrast to Fe_3O_4 NPs derived from *P. capillacea* (7 &5 mm)	[183]	*Gracilaria edulis*
Iron (III) chloride	Cubic 20 nm–26 nm	Antibacterial, antifungal	NPs inhibited *P. aerogenosa* (bacteria), *A. nidulans*, and *C. albicans* (fungi)	[184]	*Oscillatoria limnetica*	Iron chloride hexahydrate

Trigonal rhombohedral	Antibacterial, antioxidant, anticancer	Maximum antifungal activity was observed against *Aspergillus versicolor* (MIC value 27 µg/mL and LD_{50} value 47 µg/mL). *B. subtilis* was the most suspected (MIC: 14 µg/mL) and *E. coli* was the least suspected strain (MIC 35 µg/mL)	[185]	CuO	*Bifurcaria bifurcata*	Copper (II) sulfate
Spherical 20.66 nm	Antibacterial	Zones of inhibition for *S. aureus* and *Enterobacter aerogenes* was 14 mm and 16 mm, respectively	[186]	*Sargassum polycystum*	Copper solution	–

Antibacterial, antifungal, anticancer	More antibacterial activity was shown in *P. aeruginosa* (15 ± 0.5 mm) than *Shigella dysenteriae* (6 ± 0.5 mm). IC_{50} value for NPs was 61.25 µg/mL	[178]	*Spirulina platensis*	Copper (II) acetate	30–40 nm	Antibacterial
Maximum zone of inhibition (28.0 ± 0.41 mm) was shown against *P. vulgaris*	[187]	ZnO	*S. marginatum*	Zinc nitrate hexahydrate	80–126 nm	Antiviral

NPs demonstrated 99.09% anti-dengue activity in C6/36 cell line	[188]	*Sargassum muticum*	Zinc acetate dihydrate	–	Anticancer	>55% cells survived at 175 µg/mL of ZnO NPs. Cell viability was <40% 175 µg/mL. HepG2 cell survival perc

						entage was 4.5%. Maximum radical scavenging was observed up to 89% at 2800 µg/mL with an EC_{50} value of 60

						0 µg/mL
[189]	*Anabaena cylindrica*	Zinc sulfate	Rod 40–60 nm	Anticancer, antibacterial	ZnO NPs had 50% reduction in cellular viability at 3% dose, while commercial ZnO showed an ED_{50} at 6% of doses. Zone of inhibition against *P. aeruginosa* and *S. aureus* was 10–22 mm and 9–12 mm, respectively	[190]
Ag	*Caulerpa racemosa*	Silver nitrate	Spherical/triangular 10 nm	Antibacterial	Zone of inhibition against *P. mirabilis* was 14 mm at 15 µL and 7 mm against *S. aureus* at 5 µL	[191]
Spirulina platensis Oscillatoria sp	Silver nitrate	Spherical 14.42–48.97 nm	Antiviral	90% reduction in cytopathic effect of HSV-1 by Ag NPs, with a high reduction rate (49.23%)	[192]	*Pithophoraoedogonia*
Silver nitrate	Cubical/hexagonal 25–44 nm	Antibacterial	Largest zone of inhibition observed against *P. aeruginosa* (17.2 mm) followed by *E. coli* (16.8 mm)	[193]	*Caulerpa serrulata*	Silver nitrate

Spherical 10 ± 2 nm	Antibacterial, catalytic	Maximum inhibition zone (21 mm at 75 µL) was observed against *E. coli*, while lowest inhibition zone (10 mm at 50 µL) was shown for *S. typhi*	[194]	Au	*Spirulina platensis Oscillatoria sp.*	Tetrachloroauric (III) acid trihydrate
Octahedral/Pentagonal/triangular 15.60–77.13 nm	Antiviral	90% reduction in cytopathic effect of HSV-1 by Au NPs at 31.25 µL with a high reduction rate of Au NPs (42.75%)	[192]	*Sargassum wightii*	Chloroauric acid	–
Antiviral	Au NPs showed cell viability of 93.12–85.18%	[195]	*Gracilaria corticata*	Chloroauric acid	Spherical 45–57 nm	Antimicrobial, antioxidant

Antibiotic-conjugated Au NPs showed antimicrobial activity against *E. coli* (24 mm) and *E. aerogenes* (21 mm). *S. aureus* (19 mm) showed the moderate antimicrobial activity	[196]	*Padina gymnospora*	Gold salt	Spherical/triangular/hexagonal 9–21 nm	Anticancer	50% reduction in cell viability in HepG2 (IC_{50} value of 51.9 nM) was recorded

Green-synthesized metallic nanoparticles for cancer treatment

Cancer is defined as an abnormal growth of tissue or cells characterized by uncontrolled autonomous division, with the number of cell divisions rising over time [4]. More than 200 distinct cancers have been identified, and they have six common basic characteristics: replicative immortality; the ability to generate new blood vessels; the ability to invade and spread to other organs; resistance to apoptosis; proliferative signaling; and evasion of growth [198]. NPs are used to increase the compatibility and bioavailability of natural bioactives for the treatment of different chronic disorders, including cancer [199]. NPs are often considered as a possible solution for this due to evidence of their ability to induce the apoptotic pathway in vitro, which implies their anticancer effect [200]. NPs have been demonstrated to produce ROS, which can activate pro-apoptotic pathways. Different biogenic metal oxide NPs have shown promising results in the treatment of cancer by producing cytotoxicity in malignant cells while having no effect on normal cells. The specific mechanism by which various metal and metal oxide NPs kill cancer cell types is unclear [201]. It is widely believed that mitochondrial signaling pathways play a crucial role in NP-based activation of apoptosis in cancer cells. Metallic NPs usually produce ROS, which causes oxidative stress and apoptosis [202]. Apoptosis begins with apoptotic protein activation, DNA damage, mitochondrial breakdown, apoptosome formation,

and cell shrinkage [203]. Prostate and lung cancer cells are sensitive to cytotoxic activities in *Pinus roxburghii* bioactive-loaded Ag NPs. The ability of mitochondrial depolarization and DNA damage to trigger apoptosis via the intrinsic route have been reported. ROS, cell cycle arrest, and caspase-3 activation cause cancer cell apoptosis [204]. A class of protease enzymes called caspases is important for the apoptotic process. By activating the executioner caspase-3 through cleavage, the initiators caspase-8 and caspase-9 specifically cause the proteolysis of poly(ADP-ribose) polymerase (PARP) and apoptosis by impairing DNA repair [205].

Breast cancer

Breast cancer has surpassed lung cancer as the most frequent cancer in the world, with 2.26 million recorded cases per year, 11.7% of all cancer cases, and 24.5% of malignancies in women. Furthermore, it is the most frequent disease among women, accounting for 15.5% of all female cancer mortality each year [206]. With an emphasis on more biologically directed medicines and treatment deescalation to lessen side effects, therapeutic approaches have evolved over the past 10–15 years to take this heterogeneity into consideration [207]. Capping ZnO NPs with *R. fairholmianus* inhibited cellular development while increasing cytotoxicity and ROS. Apoptosis was also accompanied by an increase in pro-apoptotic proteins (p53, Bax), a decrease in anti-apoptotic proteins (Bcl-2), and a marked elevation in cytoplasmic cytochrome c and caspase 3/7 (apoptosis indicators) [208]. *Calendula officinalis* leaf extract was used as a green reducing and stabilizing agent in the biosynthesis of Sn NPs. These NPs showed remarkable potential in breast cancer treatment. The IC_{50} values for biosynthesized Sn NPs were 132, 126, and 119 µg/mL for the MCF7, Hs 319.T, and MCF10 cell lines, respectively [209]. *Gloriosa superba* rhizome extract was used in the synthesis of biomolecule-coated nanotitania catalysts. For the MCF-7 (cancer) and L929 (normal) cell lines, the IC_{50} of nanotitania catalysts was 46.64 and 61.81 µg/mL, respectively. Figure 3A shows that when nanotitania catalysts (46.64 µg/mL) were added to MCF-7 cells, they made a lot more intracellular ROS than control cells. This demonstrated that metal and metal oxide nanoparticle exposure increased ROS levels and decreased mitochondrial membrane potential. This suggested that the NPs induced apoptotic cell death. ROS generation can stimulate cell death by apoptosis and necrosis [210].

Fig. 3 [Images not available. See PDF.]

A Effects of *Gloriosa superba* rhizome and *Gloriosa superba* rhizome extract mediated titanium dioxide nanoparticles on intracellular ROS generation in treated MCF-7 cells, shown as follows: (a) nanotitania catalyst-treated cells, (b) rhizome extract, (c) control cells, **B** anticancer activity of CuO NPs synthesized from *S. alternifolium* stem bark extract, **C** Relative mRNA expression percentages following treatment of MCF7 cells with *Z. mauritiana* fruit extract-mediated Ag/AgCl NPs. A dashed line denotes an expression level of 1.0, **D** Pt NPs containing *P. granatum* stained by propidium iodide (a) control; (b) IC_{50} molarity (25 µg/mL); (c) maximum molarity (100 µg/mL). (**A** : under copyright (CC BY) from Taylor and Francis, **B**: under copyright (CC BY) from Springer, **C**: under copyright (CC BY) from ACS publication, and **D**: under copyright (CC BY) from Elsevier)

Yugandhar et al. reported that *Syzygium alternifolium* bark extract-loaded CuO NPs reduced treated cell lines by 50% in comparison with untreated cell lines with an IC_{50} value of 50 µg/mL (Fig. 3B) [211]. *Artabotrys hexapetalus* leaf extracts loaded CeO_2 NPs potent cytotoxicity against MCF-7 cancer cells at an IC_{50} value of 48 0.05 µg/mL [212]. Al-Nuairi et al. used MTT assay to examine the effects of Ag NPs from *Cyperus conglomeratus* root extract on MCF-7 breast cancer cells and normal fibroblasts. The selective cytotoxicity was found against MCF-7 with an IC_{50} of 5 µg/mL [213]. Kabir et al. treated MCF-7 cells for 48 h with *Zizyphus mauritiana* fruit extract-loaded Ag/AgCl NPs. A real-time polymerase chain reaction (PCR) was used to monitor the expression levels of eight apoptosis-related genes. FAS, caspase-8, and FADD expression levels were increased, and PARP expression levels were decreased (Fig. 3C) [214]. To confirm the antiproliferative activity of *P. granatum* crust extract-loaded platinum NPs (Pt NPs), Sahin et al. examined nuclear densification and apoptotic alterations using the propidium iodide staining in MCF-7 cell line. Only a few control cells responded positively to propidium iodide. A progressive increase in the proportion of cells that responded favorably to propidium iodide was seen in the cells treated with 25 µg/mL of Pt NPs after 48-h exposure (Fig. 3D) [215]. Table 5 shows various plant extracts and precursor salts explored to synthesize metallic

NPs for their breast cancer applications.

Table 5. Various plant extracts and precursor salts explored to synthesize metallic NPs for their breast cancer applications

Plant used	Metal precursor	Morphology	Cell line	Techniques used	IC_{50} value	Impact	References
Silver							
Tamarindus indica Fruit shell extract	Silver nitrate	Spherical 20–52 nm	MCF7	MTT assay, Dual staining (Ao/EtBr), DCFH-DA staining, Rhodamine 123 staining	20 µg/mL	More nuclear morphological changes significant induction in MMP level compared to control	[216]
Buchanania axillaris leaves	Silver nitrate	Spherical 17–80 nm	MCF7	MTT assay	31 µg/mL	The percent inhibitions of cell growth were found to increase with the increasing concentrations of the nanoparticle	[217]
Cyperus conglomeratus Root Extracts	Silver nitrate	Spherical 70–100 nm	MCF7	MTT assay, Flow cytometry, rtPCR	5 µg/mL	The IC_{50} concentration of synthesized Ag NPs after 24 h treatment of MCF-7 cells significantly reduced the mRNA levels of Bcl2, survivin, and YAP genes compared with control untreated MCF-7 cells	[213]

Phoenix dactylifera root extract	Silver nitrate	Spherical 21.65–41.05 nm	MCF-7	MMTT assay, AO/EtBr staining, Flow cytometry	29.6 µg/mL	At S-phase, it was discovered that the cell cycle is arrested, significantly slowing down the rate of cell division	[218]
Conocarpus Lancifolius fruits extract	Silver nitrate	Spherical 5–30 nm	MDA-MB-231	MMTT assay, DCFH-DA staining, Hoechst blue staining, Rhodamine 123	16.8 µg/mL	Observed dose-dependent cytotoxicity against MDA-MB-231 cells through activation of reactive oxygen species (ROS) generation	[219]
Gold							
Mentha Longifolia leaf extract	Chloro auric acid	Spherical 36.4 nm	MCF7 Hs578Bst Hs319.T UACC-3133	MTT assay	MCF7: 274 µg/mL Hs 578Bst: 279 µg/mL Hs 319.T: 274 µg/mL UACC-3133: 201 µg/mL	The biosynthesized nanoparticles had effective antibreast cancer effects against MCF7, Hs 578Bst, Hs 319.T, UACC-3133 cell lines without any cytotoxicity activity against normal cell line i.e., HUVEC	[220]
Tecoma capensis (L.) leaves extract	Gold tetrachloroaurate	Spherical 10–35 nm	MCF7	MTT assay, DPPH assay	*T. capensis* extract: 23.3 µg/mL *T. capensis* Au NPs: 9.6 µg/mL	Both *T. capensis* Au NPs and *T. capensis* extract showed significant antioxidant activity with DPPH scavenging percentages of 70.73% for *T. capensis* Au NPs and 85.62% for *T. capensis* extract	[221]
Copper oxide							

Prunus nepalensis Fruit	Copper sulfate	Crystalline 42.5 nm	MCF7 Human normal cell line: MCF10A	MTT assay, Quantitative RT-PCR	158.5 µg/mL	RT-PCR results showed upregulation in p53, caspase-3, Bax, and caspase-9. Down regulation of mRNA expression recorded in Myc and Ras genes in MCF-7 cells	[222]
Acalypha indica leaf extract	Copper sulfate	Spherical 26–30 nm	MCF7	MTT assay	56.16 µg/mL	Plant-mediated copper oxide NPs showed best anticancer activity	[223]
Syzygium alternifolium stem bark	Copper sulfate pentahydrate	Spherical 5–13 nm	MDA-MB-231	MTT assay	50 µg/mL	From 10, 25, 50, and 100 µg/mL, the concentration of CuONPs increased	[211]
Wrightia tinctoria (Wt) extract	Copper sulfate pentahydrate	Spherical 15–40 nm	MCF-7	MTT assay	119.23 µg/mL	The release of copper ions from the nanoparticles, which bind to the cell's DNA, is the primary cause of Cu NPs harmful effects on cancer cells. As a result, it damages DNA and induces cell death	[224]
Titanium oxide							
Gloriosa superba rhizome extract	Titanium hydroxide	Spherical 20–100 nm	MCF7 Normal fibroblast mouse cells: L929	MMTT assay, AO/EtBr and Hoechst staining, DCFH-DA staining, Rhodamine 123, Comet assay	MCF-7: 46.64 µg/mL L929: 61.81 µg/mL	COMET assay confirmed the DNA destruction in the nanotitania-treated cancer cells. The biomolecule-coated nanotitania catalysts could be used as potential and novel comp	[210]

Zanthoxylum armatum leaf extract	Titanium tetrabutoxide	Spherical 15–50 nm	murine 4T1 mammary carcinoma cells	RPMI-1640 assay, Flow cytometry, TBARS assay, Hemolysis assay	4.11 µg/mL	*Z. armatum*-derived NPs are as efficient as doxorubicin toward breast carcinoma with no symptoms of cardio toxicity and alteration in the body weight making them safer than doxorubicin	[225]

Lung cancer

Lung cancer is the sixth-leading cause of mortality worldwide [226]. With estimated yearly occurrences of 2.21 million (11.4% of cancer cases) and a mortality rate of 1.79 million lung cancer patients per year, lung cancer is still prevalent in all nations. Lung cancer is the second-leading cause of death in women after breast cancer [227]. Because of the long-term adaptation of cancer-causing behaviors including smoking, physical inactivity, and westernized diets, the global incidence of lung cancer is quickly rising [228]. Au NPs containing leaf extract of *Alternanthera bettzickiana* reduced cancer cell growth and triggered apoptosis, DNA breakage, and altered mitochondrial membrane potential in lung cancer cell lines. Au NPs had an impact on cellular M-phase entry. Au^{+} may activate p53 and other cell cycle genes, delaying the entry of cells into the M-phase and increasing apoptosis [229].

Cleistanthus collinus extract loaded into Ag NPs has been explored as a reducing and capping agent. The scavenging of free radicals was significantly impacted by the in vitro antioxidant activity of Ag NPs. The IC_{50} for human lung cancer cells (A549) and normal cells (HBL-100) was 30 µg/mL and 60 µg/mL, respectively. Ag NPs do not have any adverse effects on mice organs [230]. A549 cells exposed to 50 µg/mL *Magnolia officinalis* extract-loaded Au NPs showed substantial cell death. TUNEL and DAPI staining of A549 lung cancer cells *Magnolia officinalis* extract-loaded Au NPs confirmed ROS-arbitrated apoptosis (Fig. 4A). The TUNEL assay stained with green fluorescence showed live cells. *Magnolia officinalis* loaded Au NPs confirmed increased apoptotic cells with DAPI staining with blue fluorescent cells [231]. MTT assay to test the anti-lung cancer activity of *Ledebouria revoluta* bulb extract-loaded TiO_2 NPs showed an IC_{50} value of 53.65 µg/mL and showed improved antitumor activity against A549 cells [232]. At 100 µg/mL, biogenic Au NPs showed that Vero cells remained alive; however, these NPs were cytotoxic (IC_{50} 60 µg/mL) against A549 lung cancer cells (Fig. 4B) [233]. The proliferative activity of A549 cells gradually decreased over time in proportion to the increasing concentration of the test substance during the biological synthesis of Pt NPs using *Ononidis radix* extract. The mortality of cells cultured with platinum NPs increased [234]. *Lonicera japonica* extract-loaded Ag NPs at 75 µg/mL concentration showed 52% cell viability (Fig. 4C) [235]. In a xenograft severe combined immunodeficient mouse model, H1299 tumor growth was inhibited by Ag NPs synthesized from longan peel powder. After 36 days of treatment, the lung tumor size was 1.13 ± 0.21 mm^2 and 0.49 ± 0.07 mm^2 in the control and Ag NP-treated groups, respectively [236]. Valodkar et al. conducted in vitro toxicity research on human lung cancer cells using plant latex-capped Ag NPs. At the higher dose, more dead cells (in red) and very few live cells (in green) were seen, indicating a dose-dependent mortality of the cells ranging from 20 to 80% (Fig. 4D) [237]. Table 6 shows various plant extracts and precursor salts explored to synthesize metallic NPs for their lung cancer applications.

Fig. 4 [Images not available. See PDF.]

A By using DAPI/TUNEL dual staining, Au NPs synthesized by Magnolia officinalis were determined to increase ROS-arbitrated apoptosis. **B** The cytotoxicity of biogenic Au NPs using bael fruit juice was studied against A549. **C** Anti-cancer ability of synthesized silver nanoparticle (Ag NPs) using phytochemical rich medicinal plant *Lonicera japonica* proved against A549 lung cancer cells by cell viability assay. **D** AO/EB staining of plant latex-capped silver nanoparticles in A549 (cells more dead cells (in red) and very few live cells (in green) in highest dose of LAgNP)

exposed to (a) 0 μg/mL LAg NPs, (b) 1 μg/mL LAg NPs, (c) 10 μg/mL LAg NPs, (d) 20 μg/mL LAg NPs,(e) 50 μg/mL LAg NPs and (f) 100 μg/mL LAg NPs (**A:** under copyright (CC BY) from Taylor and Francis, **B**, **C** and **D** under copyright (CC BY) from Elsevier)

Table 6. Various plant extracts and precursor salts explored to synthesize metallic NPs for their lung cancer applications

Plant used	Metal precursor	Morphology	Cell line	Techniques used	IC_{50} value	Impact	References
Silver							
Citrus sinensis leaf aqueous extract	Silver nitrate	Spherical 78.12 nm	NCI-H661, HLC-1, NCI-H1563, LC-2/ad, NCI-H1299, PC-14, HUVEC	DPPH assay, MTT assay	NCI-H661: 82 μg/mL HLC-1: 139 μg/mL NCI-H1563:170 μg/mL LC-2/ad: 66 μg/mL NCI-H1299: 62 μg/mL PC-14: 50 μg/mL	Dose-dependent decrease in human lung cancer cell viability reported	[238]
Leucus aspera leaf extract	Silver nitrate	Spherical 40.67–8.17 nm	A549 HUVEC	DPPH assay, MTT assay	328 μg/mL	No cytotoxicity on the normal cell line (HUVEC) and very low cell viability against the A549 cell line	[239]
Avicennia marina leaf extract	Silver nitrate	Spherical 10–100 nm	A549	MTT assay, DCFH-DA assay, Rhodamine 123	50 μg/mL	High damages in mitochondrial membrane	[240]

Cleome viscosa L fruit extract	Silver nitrate	Spherical 20–50 nm	A549	MTT assay	28 µg/mL	The ability of green-synthesized silver nanoparticles to inhibit cancer cells growth in vitro could be taken as an indicator of potential anticancer effect	[241]
Lonicera japonica leaves extract	Silver nitrate	Spherical	A549	MTT assay	75 µg/mL	At very low concentrationsAg NPs altered the shape of A549 human lung cancer cells	[235]
Gold							
Rabdosia rubescens	Gold (III) chloride trihydrate	Spherical 130 nm	A549	MTT assay, DAPI staining, TUNEL assay, Western blotting analysis	50 µg/mL	Caspase levels were found to be 1.5 times higher in the cells treated with 25 µg/mL of RR-AuNP	[242]
Musa paradisiaca peel extract against	Chloroauric acid	Spherical to triangular 50 nm	A549	MTT assay	58 µg/mL	Nuclear morphological changes, such as cell clumping and a lack of membrane stability, after 24 h at 100 µg/mL	[243]
Marsdenia tenacissima plant extracts	Chloroauric acid	Spherical 40–50 nm	A549	MTT assay, AO/EtBr staining, Western blotting	15 µg/mL	Modulates the levels of the proteins Bax and Bcl-2 to cause apoptosis in the lung cancer cell line A549	[244]

Magnolia officinalis extract	–	Oval, spherical, hexagonal, and triangular 70–10 nm	A549	MTT assay, DCFH-DA, TUNEL assay, Western blotting	50 µg/mL	In A549 cells, Au NPs made from Magnolia officinalis increased the expression of Bax, Beclin-1, and caspase-3 while decreasing the expression of Bcl-2 and Bid	[231]
Zinc oxide							
Azadirachta indica leaf extract	Zinc sulfate heptahydrate	Ribbon/strip shaped	A549	MTT assay, Crystal violet assay, AO/PI staining, Flow cytometry	138.50 µg/mL	Apoptosis was observed in cells treated with IC_{50} dose G1 phase accounted for 76.9% cells get arrested in A549 cells after treatment	[245]
Euphorbia fischeriana root extract	Zinc acetate dehydrate	Spherical 30 nm	A549	MTT assay, AO/EtBr fluorescence staining, DCFH-DA assay, Rhodamine 123, Cell migration assay, Western blotting analysis	14.5 µg/mL	EF-ZnO NPs induced cytotoxicity also activated apoptosis during increased ROS formation, decreased MMP, inhibited cell migration, altered AO/EtBr staining and induced pro-apoptotic and inhibited anti-apoptotic protein were observed	[246]

Mangifera indica leaf extract	Zinc nitrate	Spherical/hexagonal quartzite 45–60 nm	A549	MTT assay	25 µg/mL	Anticancer activity of ZnO NPs increased with the increasing concentration of NPs and is comparable to the cytotoxic effects of cyclophosphamide in low doses	[247]
Copper oxide							
Calendula officinalis aqueous leaf extract	Copper (II) Nitrate Trihydrate	Spherical 19.64–39.15 nm	LC-2/ad PC-14 HLC-1	DPPH assay, MTT assay	PC-14: 297 µg/mL LC-2/ad:328 µg/mL HLC-1:514 µg/mL	The viability of malignant lung cell line reduced dose-dependently	[248]
Ficus religiosa leaf extract	Cupric sulfate	Spherical	A549	MTT assay, AO/EtBr fluorescence staining, DCFH-DA assay, Rhodamine 123	200 µg/mL	When compared to control, A549 cells treated with copper oxide nanoparticles showed loss of membrane integrity, apoptosis induction, and orange fragmented nuclei, all of which were consistent with low cell viability	[249]
Ilex paraguariensis	Copper(I) sulfate	Spherical 26–40 nm	A549	MTT assay	100 µg/mL	In lung cancer A549 cells, Cu NPs with a size of less than 20 nm caused cytotoxicity	[250]

Beta vulgaris extract	Cupric sulfate	Spherical 33.47 nm	A549	MTT assay, FACS analysis	25 µg/mL	In comparison to control, A549 cells decreased at the G0/G1 phase from 52.8 to 20.6%, increased at the S phase from 38.43 to 30.41%, and considerably increased at the G2/M phase from 14.56 to 52.46%	[251]
Foeniculum vulgare leaves extract	Copper (II) nitrate trihydrate	Spherical 33.62–74.81 nm	NCI-H2126, NCI-H1299, NCIH1437 Normal cell line: HUVEC	MTT assay DPPH assay	Fv-Cu NPs NCI-H2126: 122 µg/mL NCI-H1299: 168 µg/mL NCI-H1437: 108 µg/mL *F. vulgare* extract NCI-H2126: 594 µg/mL NCI-H1299: 781 µg/mL NCI-H1437: 610 µg/mL	The viability of malignant lung cell lines reduced dose-dependently in the presence of NPs. The IC_{50} of Cu NPs and BHT against DPPH free radicals were 42 and 26 µg/mL, respectively	[252]
Titanium oxide							
Ledebouria revoluta bulb extract	Titanium dioxide	Spherical 47.6 nm	A549	MTT assay	53.65 µg/mL	Highly reactive hydroxyl act as a powerful oxidant resulting in oxidative DNA-damage both single and double standard DNA	[232]

Cervical cancer

About 604,127 new cases and 341,831 fatalities from cervical cancer are reported in 2020 [253]. *Solanum nigrum* leaf extract-loaded ZnO NPs inhibited β-catenin, increased the levels of p53, caspase-3, and caspase-9, and showed a dose-dependent cytotoxic effect against HeLa cell lines [254]. After 24 h of treatment, *Catharanthus roseus* extract (5 µg/mL) loaded in Au NPs induced apoptosis in HeLa cells dual stained with acridine orange

(AO)/ethiduim bromide (EtBr). The control cells showed homogeneous bright green nuclei and cytoplasm for AO-positive cells. In Ao/EtBr staining, the cells treated with the synthesized NPs showed characteristics of apoptosis such as nuclear condensation, cell shrinkage, and the formation of apoptosis bodies (Fig. 5A). HeLa cells were incubated with *Catharanthus roseus* extract-loaded Au NPs at different concentrations (5 and 10 µg/mL) for 24 h to measure the level of ROS production (Fig. 5B) [255]. *A. officinalis* root extract-loaded Ag NPs are toxic to SiHa cell lines, with an IC_{50} of 44 µg/mL. The biosynthesized Ag NPs arrested cell division in the G2/M phases and accelerated the cell cycle in the G1 and S phases [256]. Extract of *Euphorbia antiquorum* L. latex loaded in Ag NPs inhibited the growth of HeLa cell line with an IC_{50} value of 28 µg/mL [257]. After 24 h of incubation, Au NPs containing an aqueous extract of *Alternanthera sessilis* (1–15 µg/mL) showed cytotoxicity against HeLa cells (Fig. 5C) [258]. CuO NPs containing dry black beans (0.5 µg/mL and 1 µg/mL) have shown cytotoxic effects against HeLa cells. CuO NPs inhibited cervical carcinoma colonies and influenced the generation of ROS. The number of cervical carcinoma cell colonies was much lower in CuO NPs-treated cells than in the control group (Fig. 5D) [259]. Table 7 shows various plant extracts and precursor salts explored to synthesize metallic NPs for their cervical cancer applications.

Fig. 5 [Images not available. See PDF.]

A Ao/EtBr staining after 24 h of treatment with various concentrations (5 and 10 µg/mL) of photosynthesized Au NPs from *Catharanthus roseus*. **B** Using DCFH-DA staining assay photosynthesized Au NPs from *Catharanthus roseus* induces ROS production in HeLa cells. **C** The ability of Au NPs from *A. sessilis* to cause cytotoxicity in HeLa cervical cancer cell lines. **D** Clonogenic survival assay on HeLa cells following incubation with CuO NPs synthesized using an aqueous black bean extract NPs (**A**, **B** and **C** under copyright (CC BY) from Taylor and Francis, **D**. under copyright (CC BY) from Elsevier)

Table 7. Various plant extracts and precursor salts explored to synthesize metallic NPs for their cervical cancer applications

Plant used	Metal precursor	Morphology	Cell line	Techniques used	IC_{50} value	Impact	References
Silver							
Nepeta deflersiana aerial part	Silver nitrate	Spherical 33 nm	HeLa cells	MTT assay, Neutral red uptake assay, DCFH-DA, rhodamine-123	5 µg/mL	ND-Ag NPs have the capacity of inducing apoptosis and necrosis cell death of HeLA cells through SubG1 cell cycle arrest	[260]

Moringa olifera stem bark extract	Silver nitrate	Spherical 40 nm	HeLa cells	Annexin V/PI double-staining assay NRU, DCFH-DA, DAPI staining	–	The increased level of ROS revealed that most of the cells underwent induction of early apoptosis caused by oxidative stress, while many of the inhibitors that induce apoptosis show antioxidant activity	[261]
Detarium microcarpum leaf extract	Silver nitrate	Spherical/rectangular 81 nm–84 nm	HeLa cells	Presto blue cell viability assay	31.5 µg/mL	The synthesized NPs had inhibitory effect on cervical cancer cells	[262]
Gold							
Catharanthus roseus leaf extract	$HAuCl_4$	Spherical 25–35 nm	HeLa3T3 cell lines	MTT assay, AO/EtBr staining, DCFH-DA staining, Rhodamine 123 Caspases activity assay, Western blotting	5 µg/mL	The cytotoxicity and apoptosis of human cervical carcinoma (HeLa) cells increased Caspase-3 was cleaved after being exposed Biosynthesized NPs for 24 h	[255]
Pongamia pinnata leaf extract	$HAuCl_4$	Spherical 55 nm	HeLa cells Human embryonic kidney cell line: HEK293	MTT assay, Flow cytometry, Rhodamine 123, Wound healing/cell migration assay	200 µg/mL	Au NPs gets internalized into HeLa cells by endocytosis. Inside the cytosol, it exhibited cytotoxic effect by elevating intracellular ROS, perturbing the MMP, arresting cells at S-phase of the cell cycle and finally inducing cell death by apoptosis	[263]
Copper oxide							

Brassica oleracea var acephala leaf extract	Copper sulfate	Spherical 60–100 nm	HeLa cells	MTT assay	119.0805 µg/mL	The vitality of cancer cells declines as sample concentration rises, whereas cytotoxicity against HeLa cell lines rises as sample concentration rises	[264]
Phaseolus vulgaris	Copper sulfate	Spherical, hexagonal 26.6 nm	HeLa	SRB assay, Hoechst 33,258 staining, DCFH-DA, Clonogenic assay	0.5 and 1 µg/mL	In a dose-dependent manner Induced intracellular ROS generation	[259]
Houttuynia cordata	Copper sulfate	Spherical 40–45 nm	HeLa	MTT assay, DCFH-DA, EtBr/AO staining, DAPI staining, Pi stainin, Q-RT-PCR	5 µg/mL	Biosynthesized NPs inhibited cell proliferation and promoted apoptosis by targeting PI3K/Akt signaling pathways in HeLa cells	[265]
Titanium oxide							
Coleus aromaticus leaf extract	TiO_2	Hexagonal shape 12–33 nm	HeLa	MTT assay	34.45 µg/mL	Increased oxidative stress, destroyed the cell membrane, enhanced lipid peroxidation, lowered the level of glutathione (GSH), and eventually contributed to cells' death	[266]

Colorectal cancer

Colorectal cancer (CRC) is the second most lethal and third-most prevalent cancer worldwide. It accounts for 9.2% of all cancer-related deaths and 10.2% of all new cases. Aqueous extract of *Allium cepa* loaded in Ag NPs promotes apoptosis by suppressing expression of Bcl2 family genes [267]. *Albizia lebbeck* extract (40 and 60 µg/mL)-loaded CuO NPs showed early apoptosis (orange stained) and late apoptosis (red stained) apoptotic cells (Fig. 6A) for 24 h through the activation of a dual staining method by AO/EtBr in HCT-116 colon cancer cells [268]. Ag NPs containing *Pimpinella anisum* seed extract showed cytotoxicity against CRC cells. Ag NPs destroyed cancer cells through cell growth inhibition, cell cycle arrest in the G2/M phase, and induction of apoptosis [269]. AO/EtBr staining assay in HCT-116 cells showed that the *Trichosanthes kirilowii* extract loaded in Au NPs increased ROS production, damaged mitochondrial membrane, induced morphological alterations (Fig. 6B), induced G0/G1 phase cell-cycle arrest (Fig. 6C), activated caspase expression, and downregulated anti-apoptotic expression [270]. The cytotoxic effect of lead oxide and CeO_2 NPs synthesized using an aqueous extract of *Prosopis fracta* fruit was investigated in colon (HT-29) cancer cell lines. These NPs were not harmful at 500 µg/mL and 62.5 µg/mL [271]. Ag NPs containing *Curcuma longa* and *Zingiber officinale* rhizomes extract had an IC_{50} of 150.8 µg/mL. At a 25–500 µg/mL dose, the synthesized NPs were cytotoxic to HT-29 cells (Fig. 6D) [272]. Table 8 shows various plant extracts and precursor salts explored to synthesize metallic NPs for their colorectal cancer applications.

Fig. 6 [Images not available. See PDF.]

A Dual staining method by AO/EtBr in HCT-116 colon cancer cells. **B** Induction of apoptosis on HCT-116 cells treated with Au NPs synthesized from *Trichosanthes kirilowii* at various concentrations upto 24 h studied using AO/EB staining assay. **C** Cell-cycle analysis of HCT-116 cells treatment with Au NPs (15 and 20 µg/mL) synthesized from *Trichosanthes kirilowii*. **D** Anticancer activity of *Zingiber officinale* and *Curcuma longa* synthesized Ag NPs at different concentrations (a) control, (b) 500 µg/mL, (c) 250 µg/mL, (d) 100 µg/mL, (e) 50 µg/mL and (f) 25 µg/mL (**A**, **B** and **C** under copyright (CC BY) from Taylor and Francis, **D** under copyright (CC BY) from Elsevier)

Table 8. Various plant extracts and precursor salts explored to synthesize metallic NPs for their colorectal cancer applications

Plant used	Metal precursor	Morphology	Cell line	Techniques used	IC_{50} value	Impact	References
Silver							
Allium cepa L (*A. cepa*) extract	Silver nitrate	Cubic shapes 150–250 nm	HT-29 and 0SW620 cells	DPPH scavenging, activity, TAA, FRAP, MTT assay, RT-PCR, Flow cytometry	*A. cepa* TAA: 55.49 ± 0.91 DPPH-SA: 2.23 ± 0.36 FRAP:14.78 ± 0:20 Ag NPs-CEPA TAA: 58.85 ± 4.39 DPPH-SA: 1.91 ± 0.20 FRAP: 13.37 ± 0.17	The synthesized NPs inhibited cell proliferation and induced apoptosis by inhibiting Bcl2 family gene expression hence act as a promising anticancer agent for treating colorectal cancer	[267]
Anthemis atropatana aerial parts	Silver nitrate	Spherical 38.89 nm	HT29 cancer cell	MTT assay, RT-PCR, Flow cytometry, DNA fragmentation assay	100 µg/mL	Caused the cell to undergo early and delayed apoptosis by 15.64 and 21.32%, respectively	[273]
Vitex negundo L. leaves extract	Silver nitrate	Spherical 5–47 nm	HCT15	MTT assay, Propidium iodide staining, Flow cytometry, Comet assay	20 µg/mL	Ag NPs exerted antiproliferative effects on colon cancer cell line by suppressing its growth, arresting G0/G1-phase, inhibited DNA synthesis and induced apoptosis	[274]

Gold							
Albizia lebbeck leaf extract	HAuCl$_3$	Spherical 20 and 30 nm	HCT-116 colon cancer cell lines	MTT assay, AO/EtBr staining, DCFH-DA staining, Rhodamine 123, Caspases activity assay, Western blotting	48 µg/mL	HCT-116 cells improved the activity of caspase-9 and caspase-3 in a dose-dependent manner	[268]

Prostate cancer

Prostate cancer is the second most common cancer in men, with 1.41 million incidences annually (14.1% of all cancer cases in men) [275]. Green-synthesized nanosilver containing *Rosmarinus officinalis* extract exhibited cytotoxic effects against prostate cancer cells through the activation of caspase 3 and caspase 9 mRNA [276]. Firdhouse et al. examined the cytotoxic effect of nanosilver containing *Alternanthera sessilis* extract against prostate cancer cells (PC3) at 1.56, 3.12, 6.25, 12.5, and 25 µL/mL doses. The highest concentration (12.5 and 25 µL/mL) of Ag NPs showed a decrease in PC3 cancer cells (Fig. 7A) [277]. *Camellia sinensis L* extract loaded in Au NPs reduced PC-3 cell growth with an IC_{50} of 19.71 µg/mL. Surface detachment, cell shrinkage, and body distortion were observed in PC-3 cells. This demonstrated the cytotoxic effect of green tea extract containing Au NPs [278]. The cytotoxic effect of Au NPs containing an extract of desert truffles (*Tirmania nivea*) against normal human prostate cell lines and prostate cancer cell lines is shown in Fig. 7B [279]. *Salvia miltiorrhiza* extract has been used as a capping agent to synthesize Ag NPs to explore its cytotoxic property against PCa LNcap cell lines. The proliferation of LNcap cells was dramatically inhibited for 24 h with increasing concentration of Ag NPs (Fig. 7C) [280]. The IC_{50} for the PC3 cell line treated with green-synthesized ZnO NPs made from *Hyssopus officinalis* extract for 24 h and 48 h was 8.07 µg/mL and 5 µg/mL, respectively. The percentage of PC3 cells that underwent induced apoptosis was 26.6% ± 0.05, 44% ± 0.12, and 80% ± 0.07 [281]. The Trypan blue exclusion test was used to assess in vitro cytotoxicity in PC-3 cells. Ag NPs of *Dimocarpus longan Lour*. peel extract showed cytotoxic effect at a dose between 5 to 10 µg/mL with an IC_{50} value less than 10 µg/mL (about 50% of PC-3 cells died) (Fig. 7D) [282]. Table 9 shows various plant extracts and precursor salts explored to synthesize metallic NPs for their prostate cancer applications.

Fig. 7 [Images not available. See PDF.]

A Cytomorphological changes such as cancer cell membrane lyses, coiling with the addition of silver (a, b) and nanosilver synthesized using *Alternanthera sessilis* in (d, e) after 48 h compared to that of control. **B** The cytotoxicity of Au NPs synthesized using extract of desert truffles (*Tirmania nivea*) against normal human cell line (a) untreated cells, (b) treated cells with synthesized Au NPs, and against cancer cell line (c) control untreated VCaP cells, (d) Treated VCaP cells with synthesized Au NPs. **C** Cytotoxic potential of Ag NPs from leaf extract of *Salvia miltiorrhiza* in prostate cancer LNcap cell. **D** Dose-dependent cytotoxic effects of Ag NPs biosynthesized using *Dimocarpus Longan Lour*. Peel Extract on prostate cancer PC-3 cells in vitro (**A** and **D** under copyright (CC BY) from Springer, **B** under copyright (CC BY) from Elsevier, **C** under copyright (CC BY) from Taylor and Francis)

Table 9. Various plant extracts and precursor salts explored to synthesize metallic NPs for their prostate cancer applications

Plants used	Metal precursor	Morphology	Cell line	Techniques used	IC_{50} value	Impact	References
Silver							
Carica papaya leaf extract	Silver nitrate	Spherical 10–20 nm	DU145	MTT assay, DCFH-DA, Flow cytometry, AO/EtBr staining	5 µg/mL	G1-S phase cell cycle check point marker, cyclin D1 was down-regulated along with an increase in cip1/p21 and kip1/p27 tumor suppressor proteins by Ag NPs-PLE	[283]
Dimocarpus Longan Lour. Peel Extract	Silver nitrate	Spherical 9–32 nm	PC-3	Trypan Blue, Exclusion assay, Western blot analysis	Between 5 and 10 µg/mL	An increase in caspase-3 was seen along with a decrease in stat 3, bcl-2, and survivin	[282]
Dovyalis caffra fruit extract	Silver nitrate	Spherical 12–53 nm	PC-3	Sulforhodamine B (SRB) assay	–	At 100 µg/mL, microscopic observations revealed membrane shrinkage, failure of cell adhesion, blebbing of the cell membrane, lyses of the cell membrane, the emergence of unique cellular crinkle, and cell death	[284]

Indigofera hirsute L. leaf extract	Silver nitrate	Spherical 5–10 nm	PC-3	MTT assay	68.5 µg/mL	As the concentration of increases, the cells become clustered and exhibited morphological alterations which eventually leads to cell death or apoptosis	[285]
Salvia miltiorrhiza	Silver nitrate	Spherical, oval, hexagonal and triangular 80 and 12 nm	LNcap cells	MTT assay, DCFH-DA, TUNEL assay, AO/EtBr staining, Caspase-8, 9 and 3 activity assay, Western Blot Analysis	50 µg/mL	Ag NPs effectively causes cytotoxicity, ROS, and apoptosis in LNCap cell lines by altering the expression of intrinsic apoptotic genes	[280]
Gold							
Alhagi maurorum leaf aqueous extract	$HAuCl_4 \cdot H_2O$	Spherical less than 100 nm	DU 145, NCI-H660, 22Rv1, and LNCaP clone FGC)	MTT assay	DU 145: 229 µg/mL NCI-H660: 368 µg/mL 22Rv1: 298 µg/mL LNCaP clone: 222 µg/mL	The viability of malignant prostate cell lines reduced dose-dependently in the presence of Au NPs	[286]
Titanium oxide							
Coleus *aromaticus* leaf extract	TiO_2	Hexagonal shape 12–33 nm	HeLa	MTT assay	34.45 µg/mL	Increased oxidative stress, destroyed the cell membrane, enhanced lipid peroxidation, lowered the level of glutathione (GSH), and eventually contributed to cells' death	[266]

Skin cancer

In a study by Wu et al., aqueous *Siberian ginseng* extract was used as an organic reducing agent to biosynthesize Au NPs. These Au NPs were then tested against murine melanoma B16 cells for their anticancer properties. The results demonstrated that the synthesized Au NPs increased ROS levels and decreased mitochondrial membrane potential (Fig. 8A). The BH3 mimics by biosynthesized Au NPs increased the expression of pro-apoptotic proteins

while decreasing the expression of anti-apoptotic proteins in melanoma cells [287]. *Cassia fistula* leaf extract reduced silver ions to Ag NPs. The estimated IC_{50} values for the leaf extract, Ag NPs, and $AgNO_3$ were 96.36 1.01 μg/mL, 92.207 1.24 μg/mL, and 84.246 2.41 μg/mL, respectively. The percentage cell viability in Fig. 8B shows the dose-dependent effect of synthesized Ag NPs against cancer cell line [288]. The in vivo therapeutic efficacy of *Quisqualis indica* flower extract-derived Cu NPs was investigated by Mukhopadhyay et al. in mice carrying B16F10 melanoma tumors. A substantial reduction in tumor development was recorded. Quisqualis indica flower extract-derived Cu NPs triggered cytotoxicity and death in melanoma cells due to the gene expression [289]. In a study, polyphenols from *Vitis vinifera L.* (grape) peels were used as reducing and stabilizing agents for the synthesis of Au NPs. The IC_{50} value for *V. vinifera* peel extract was 319.14 μg/mL. The IC_{50} values of *V. vinifera* peel loaded Au NPs and fluorouracil (standard drug) were 23.6 μM and 23.43 μM, respectively. Figure 8C displays the percentage of inhibition plotted against the concentration of fluorouracil and peel extract-loaded Au NPs [290]. Ag NPs of *Indigofera longeracemosa* leaf extract revealed a dose–response relationship with an IC_{50} value of 48 μg/mL against the human skin cancer cell line SK MEL-28. Biosynthesized NPs upregulated the tumor suppressor gene p53 and significantly downregulated the anti-apoptotic gene Bcl-2 [291]. *Elephantopus scaber*-loaded Ag NPs were more effective against the A375 skin carcinoma cell line than its pure extract. After 48 h of incubation, morphological changes in treated A375 cells were observed under an inverted phase contrast tissue culture microscope (Fig. 8D) [292]. Table 10 shows various plant extracts and precursor salts explored to synthesize metallic NPs for their skin cancer applications.

Fig. 8 [Images not available. See PDF.]

A Using a 1-mM Rhodamine 123 staining approach, the apoptotic effect of *Siberian ginseng* synthesized Au NPs on the mitochondrial membrane permeability in murine melanoma cell line B16 was evaluated. **B** Ag NPs from the leaf extracts of *Cassia fistula* have been shown to be toxic to A-431 epidermal cancer cells (IC_{50} values for the leaf extract, Ag NPs, and $AgNO_3$ are anticipated to be 96.36 ± 1.01, 92.207 ± 1.24, and 84.246 ± 2.41 μg/mL, respectively). **C** Fluorouracil and *Vitis vinifera* peel Au NPs had an inhibitory effect on A431 cells 24 h after incubation. **D** Morphological changes induced on treated A375 cells by Ag NPs using the phytoreducing agent *Elephantopus scaber* (**A** and **D** under copyright (CC BY) from Taylor and Francis, **B** under copyright (CC BY) from Wiley Online Library, **C** under copyright (CC BY) from Elsevier)

Table 10. Various plant extracts and precursor salts explored to synthesize metallic NPs for their skin cancer applications

Plant used	Metal precursor	Morphology	Cell line	Techniques used	IC_{50} value	Impact	References
Silver							

Petals of *C. maxima*, Leaves of *M. oleifera* and the rhizome of *A. calamus*	Silver nitrate	*C. maxima*: Spherical 19 nm *A. calamus*: spherical 19 nm *M. oleifera*: rectangular 11 nm	A431	MTT assay	*C. maxima*: 82.39 ±3.1 µg/mL *M. oleifera*: 83.57 ±3.9 µg/mL *A. calamus*: 78.58 ±2.7 µg/mL	Ag NPs synthesized using petal extract have a higher IC_{50} value than those using rhizome extract	[293]
Trapa natans extract leaf extract	Silver nitrate	Spherical 30–90 nm	A431	MTT assay	64.2 µg/mL	Glycosides, amino acids, flavonoids, and polyphenols as well as nano-sized silver particles may be responsible for anticancer activity of biosynthesized Ag NPs	[294]
Cassia fistula leaf extract	Silver nitrate	Spherical 40–50 nm	A-431	MTT assay	92.2±1.2 µg/mL	The DLS findings showed that synthesized Ag NPs can also be utilized in conjunction with conventional medications to increase their efficacy	[288]
Gold							
Vitis vinifera L (grapes) peel extract	Hydrogen tetrachloroaurate (III) trihydrate	Spherical 20–40 nm	A-431	MTT assay DCFH-DA assay Tunel assay Rhodamine 123 AO/EtBr fluorescence staining	23.6 µM	Peel gold nanoparticle treatment resulted in a percentage of apoptotic cells of 10.02% and secondary necrotic cells of 55.5%	[290]

Siberian ginseng aqueous extract	HAuCl4	Spherical 200 nm	B16	MTT assay DCFH-DA assay Rhodamine 123 AO/EtBr fluorescence staining qPCR	10 µg/mL	In 10 µg/mL SG-GNPs treated cells compared to the control, more late apoptotic cells colored orange by EtBr were found	[287]
Zinc oxide							
Limonium pruinosum	Zinc acetate dihydrate	Hexagonal/cubic 41 nm	A-431	MTT assay	409.7 µg/mL	The green-synthesized ZnO NPs in the current study showed higher toxicity toward cancer cells compared to normal cells (WI-38)	[91]
Acorus calamus	Zinc acetate	Irregular/oval 50−100 nm	SK-MEL-3	MTT assay	17.50 µg/mL	Morphological alterations like cell shrinkage, detachment, rounding, and irregular shape were noted in the AC-ZnO NPs challenged cells, thus proving its cytotoxicity against SK-MEL-3 cells	[295]

Liver cancer

Liver cancer is the sixth-most common primary malignancy and the fourth-leading cause of cancer-related deaths in the world. Cholangiocarcinoma (CCA) and hepatocellular carcinoma (HCC) are the two most common histologic types of primary liver cancer, accounting for more than 80% of all cases. Liver fibrosis and inflammation-induced necrosis are the major causes of malignancy [296]. The effect of *Cordyceps militaris*-loaded Au NPs on the mitochondrial membrane potential of HepG2 cells revealed a strong green fluorescence in untreated cells with high membrane potential. Green fluorescence intensity decreased in HepG2 cells treated to 10 and 12.5 µg/mL Au NPs. The mitochondrial membrane potential remained intact in untreated cells (Fig. 9A). Untreated cells did not exhibit apoptosis, as shown by green fluorescence staining in Fig. 9B. HepG2 cells treated with Au NPs (10 and 12.5 µg/mL) showed a substantial increase in apoptotic cells as visualized by orange fluorescence staining. HepG2 cells were found to undergo apoptosis after being exposed to Au NPs coated with an extract of Cordyceps militaris [297]. *Coriandrum sativum* leaf aqueous extract-loaded iron NPs were green-synthesized by Zhan et al., and they

demonstrated dose-dependent anticancer activity and very poor cell viability against LMH/2A, McA-RH7777, N1-S1 Fudr, and SNU-387 cell lines while having no cytotoxicity on the normal cell line (HUVEC) [298]. The MTT assay was used to test the in vitro cytotoxicity of Ag NPs loaded with extract from the *Punica granatum* leaf against the HepG2 cell line. This study found that Ag NPs had substantial anti-cancer efficacy at a dosage of 70 µg/mL, causing 50% cell death (Fig. 9C). Ag NPs significantly inhibited cell growth by more than 90% [299]. With an IC_{50} value of 93.75 µg/mL, *Morinda pubescens* extract-loaded Ag NPs have been shown to exhibit considerable cytotoxic effect against HEPG2 cell lines [300]. In a time- and dose-dependent manner, *Artemisia scoparia* extract and its biosynthesized ZnO NPs decreased cell proliferation and induced apoptosis in Huh-7 cancer cells. *Artemisia scoparia* extract and its biosynthesized ZnO NPs had IC_{50} values of 10.26 and 310.24 µg/mL, respectively. Figure 9D shows that the anti-apoptotic genes were downregulated while the pro-apoptotic genes were upregulated by the *Artemisia scoparia* extract-loaded ZnO NPs [301]. With an IC_{50} value of 62.5 µg/mL, *Seripheidium quettense*-mediated green synthesis of biogenic Ag NPs inhibited the proliferation of HepG2 cells [302]. Table 11 shows various plant extracts and precursor salts explored to synthesize metallic NPs for their liver cancer applications.
Fig. 9 [Images not available. See PDF.]

A Represents that the mitochondrial membrane permeability of *Cordyceps militaris* extract synthesized Au NPs, **B** effect of Au NPs from *C. militaris* induces apoptotic morphological changes in HepG2 cells. Green fluorescence labeling revealed that apoptosis had not occurred in the untreated cells. Orange fluorescence staining in HepG2 cells at 10 mg and 12.5 µg/mL shows that the Au NPs treatment dramatically boosted the apoptotic cells. **C** anticancer activity of various concentrations of synthesized Ag NPs synthesized using *Punica granatum* leaves against the liver cancer cell line—HepG2, and **D** cytotoxic effects of biosynthesized ZnO NPs using *Artemisia scoparia* leaf extract against Huh-7 liver cancer cells (under copyright (CC BY) from Taylor and Francis online)
Table 11. Various plant extracts and precursor salts explored to synthesize metallic NPs for their liver cancer applications

Plant used	Metal precursor	Morphology	Cell line	Techniques used	IC_{50} value	Impact	References
Silver							
Punica granatum leaf extract	Silver nitrate	Spherical 20–45 nm	HepG2	MTT assay, DPPH assay	70 µg/mL	It was hypothesized that Ag NPs could inhibit the function of abnormally increased signaling proteins or interact with functional groups of intracellular proteins and enzymes, as well as with the nitrogen bases in DNA, causing cell death	[299]

Artemisia kopetdaghensis shoot extract	Silver nitrate	Spherical 3–35 nm	HepG2	MTT assay	0.125 µg/mL	Due to Ak-Ag NPs' increased cellular uptake and retention, the NPs were highly cytotoxic to HepG2 cell lines	[303]
Gold							
Cajanus cajan seed coat	$AuCl_4$	Spherical 9–41 nm	HepG2	MTT assay, Flow cytometry, Comet assay, Annexin-V/PI double-staining assay	6 µg/mL	decrease in DNA amount and appearance in the sub-G0/G1 area, both of which are signs of apoptosis	[304]
Zinc oxide							
Artemisia scoparia leaf extract	Zinc acetate dihydrate	Spherical 9.00±4.00 nm	Huh-7	MTT assay, Flow cytometry, DAPI staining, RT-PCR, Propidium iodide staining	ZnO NPs: 10.26 Extract: 310.24 µg/mL	Anticancer effect was stronger in the synthesized ZnO NPs than the extract	[301]
Lawsonia inermis leaf extract	Zinc nitrate	Cubic, rod, Triangular, spherical 5–35 nm	Hep-G2	MTT assay, DCFH-DA	21.63 µg/mL	DNA damage and the stimulation of intrinsic mitochondrial pathways were two ways that ZnO NPs triggered apoptosis	[305]
Eclipta prostrata leaf extract	Zinc nitrate	Triangle, radial, hexagonal, rod, and rectangle 16–85 nm	Hep-G2		100 µg/mL	ZnO NPs can potentially change apoptotic protein expression and trigger apoptosis in mitochondria-dependent pathways in Hep-G2 cells	[306]
Copper oxide							
Eclipta prostrata leaves extract		Spherical 28–45 nm	HepG2	DPPH assay, MTT assay	–	Cu NPs were tested for in vitro cytotoxicity against HepG2 cell lines at 1, 10, 100, 250, and 500 µg/mL; these concentrations resulted in cellular toxicity values of 3.0, 15.5, 28.5, 44.5, and 54.5%, respectively	[307]

Theranostic applications of green-synthesized nanoparticles

Theranostics is a multidisciplinary scientific field focused on creating a wide range of complex diagnostic and therapeutic agents. By utilizing nanotechnology, theranostics enhance bioavailability by delivering bioactives to the sites of absorption. Theranostics utilizing metallic NPs could be useful in treating a wide range of conditions, including cancer, malaria, microbial infections, and cardiovascular disorders [308]. There has been a significant increase in the production of metallic NPs from medicinal plants. These metallic NPs play a crucial role in the advancement of theranostics. Anisotropic Au NPs were produced by an aqueous method employing cocoa extract. These NPs exhibited favorable biocompatibility when subjected to in vitro testing utilizing A431, MDA-MB231, L929, and NIH-3T3 cell lines, at doses of up to 200 µg/mL. The use of green-synthesized NIR absorbing anisotropic Au NPs was effective in causing cell death in epidermoid carcinoma A431 cells when irradiated with a femtosecond laser at 800 nm with a low power density of 6 W/cm^2. This demonstrates the suitability of NPs for photothermal ablation of cancer cells. These Au NPs exhibited high X-ray contrast during computed tomography testing, thus confirming their suitability as a contrast agent [309].

The synthesis of Au NPs using cinnamon proved to be an effective diagnostic agent for imaging both in laboratory settings and within living organisms. These NPs possess both biocompatibility and purity, making them suitable for use in in vivo applications. Photoacoustic emissions based in vitro study confirmed internalization of NPs in PC-3 and MCF-7 cells. Additionally, biodistribution investigations conducted on healthy mice demonstrated that these Au NPs accumulated in the lungs. This finding further supports the potential of using Au NPs as contrast agents for targeting [310]. In a radiotherapy investigation, thymoquinone-loaded green-synthesized Ag NPs in combination with the MDA-MB-231 mammary adenocarcinoma cells showed improved radiotherapy, significantly increased cancer cell killing, and DNA damage in comparison to the radiation alone. This was carried out via radiotherapy enhancement and the delivery of thymoquinone to the cancer cells. The developed system is proposed to be a promising combined regimen for efficient cancer therapy [311].

Rutin-loaded $CoFe_2O_4$ and $ZnFe_2O_4$ NPs (29 nm and 25 nm) displayed ferromagnetic and superparamagnetic properties. The saturation magnetization values were measured to be 56.2 emu/g and 6 emu/g, respectively. Thus, these NPs exhibited exceptional and efficient magnetic properties, making them crucial for magnetic hyperthermia therapy. Significant photothermal efficacy of green-synthesized $CoFe_2O_4$ and $ZnFe_2O_4$ NPs combined with laser radiation against MCF-7 cells was indicated by the results of the inverted stage microscopy and MTT assay [312]. The Fe_3O_4 NPs produced utilizing the fruit peel of *P. granatum*, exhibited an excellent relaxivity rate and generated strong magnetic resonance imaging (MRI) signals in the study. NPs containing 2% *P. granatum* fruit peel extract were loaded with 5-FU, which displayed 62 ± 0.3% entrapment efficiency. Based on in vitro cytotoxicity studies conducted on CCD112 normal and HCT116 colorectal cancer cell lines, it was observed that the 5-Fluorouracil loaded in the *P. granatum* fruit peel extract-based Fe_3O_4 NPs at 15.62% µg/mL resulted in a 11% and 29% reduction in cell viability in healthy and colorectal cancer cells, respectively. In the future, green-synthesized Fe_3O_4 NPs may play an important role as an eco-friendly nanocarrier in thermo-chemotherapy and MRI for the treatment of cancer [313].

Future perspectives

The subject of biosynthesized metallic NPs loaded with plant extracts is a novel and intriguing area of study. These NPs, often called "green nanoparticles," are extensively explored in the fields of drug delivery. Biosynthesized metallic nanoparticles loaded with plant extracts hold significant promise in medicine. Researchers are investigating their potential as drug delivery systems, where these nanoparticles can be loaded with therapeutic compounds and targeted to specific cells or tissues in the body. They might also be utilized for imaging purposes, such as in cancer detection or tracking the progression of diseases. These NPs can be designed to release drugs in a controlled and sustained manner. This characteristic permits for prolonged drug action, reducing the frequency of dosing and enhancing patient compliance with medication regimens. Biosynthesized metallic NPs loaded with plant extracts can be engineered to deliver drugs to specific cells or tissues in the body with high precision. This targeted drug delivery approach minimizes the side effects associated with conventional drug delivery methods and improves the

therapeutic efficacy of medications. Drug resistance is a major challenge in many diseases. By using biosynthesized NPs, it might be possible to enhance the effectiveness of existing drugs against resistant strains of pathogens or cancer cells. Some drugs have low bioavailability, meaning that they are poorly absorbed by the body. Biosynthesized metallic NPs loaded with plant extracts can improve the solubility and bioavailability of such drugs, leading to more efficient therapeutic outcomes. Green nanoparticles derived from plant extracts generally exhibit lower toxicity compared to synthetic nanoparticles. By using biocompatible and biodegradable materials, the risk of adverse reactions and long-term side effects can be minimized. Researchers can combine the unique properties of metallic nanoparticles with the medicinal properties of plant extracts. This results in multifunctional nanoparticles that not only deliver drugs but also possess inherent therapeutic effects derived from the plant extracts, such as anti-inflammatory or antioxidant properties. Despite the promising future of biosynthesized metallic NPs loaded with plant extracts, comprehensive safety evaluations and regulation will be required. It will be of the uttermost importance to ensure that these nanoparticles are safe for human health, the environment, and non-target organisms. The commercialization and scalability of the production of these nanoparticles will be one of the most significant future challenges as research in this field advances. To make these technologies accessible and practical for a variety of applications, it will be necessary to develop large-scale, cost-effective production methods.

Conclusions

Cancer remains one of the most prevalent causes of mortality across the world, despite recent advances in diagnosis and treatment. No effective cancer treatment has been identified to date, and all the anticancer medications now on the market have the potential to cause negative effects. Nanotechnology has the potential to significantly improve current methods for diagnosing and treating cancer patients. To find better diagnostics and therapies that are as effective, specific, and low-toxic as feasible, researchers are currently attempting to develop novel approaches. Recent biomedical research has focused extensively on biological, or "green," synthesis of NPs. Green synthesis is less expensive, less toxic, and more ecologically friendly than conventional methods of producing NPs. This article will assist formulation scientists and nanotechnologists working on the green production of metal or metal oxide NPs by utilizing plant extracts. It also explored their therapeutic potential of plant extracts against various cancers. The science of metallic NPs is one of the most intriguing areas of study for cutaneous or transdermal drug administration. Thus, lipid nanocarriers are predicted to open new avenues in biomedical science while also improving an essential area of dermatologic literature.

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Author contributions

MT was involved in the methodology, software, data curation and writing-original draft, DM assisted in conceptualization of work, RA was involved in the visualization, supervision, validation, writing-reviewing and editing.

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Availability of data and materials

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Declarations

Ethics approval and consent to participate

No animal or human tissue was used in this work, and ethics approval and consent were not applicable.

Consent for publication

All authors gave their full consent for publication of this work.

Competing interests

The authors declare that they have no competing interests.

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DETAILS

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Unveiling the anti-cancer potential of Euphorbia greenwayi : cytotoxicity, cell migration, and identification of its chemical constituents

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ABSTRACT (ENGLISH)

Background

Different herbal phytochemicals have potential in cancer treatment, Euphorbia genus has valuable secondary metabolites and is used in traditional medicine to treat various ailments. However, the specific constituents and biological activity of *Euphorbia greenwayi* remain largely unexplored.

Results

Euphorbia greenwayi aerial parts were extracted using methanol. Consequently, the methanol extract was then fractionated with solvents of different polarities viz., *n*-hexane, chloroform, and ethyl acetate. All were screened for their cytotoxic activity against different cell lines; MCF-7, HepG-2, and SW620. The *n*-hexane (HF) and chloroform (CF) fractions showed considerable activity against all tested cell lines especially MCF-7 with IC_{50} values at 18.6 ± 0.2 and 17.5 ± 0.6 µg/ml respectively. Therefore, a cell migration assay on the MCF-7 cell line was applied to both fractions as well as investigation and isolation of the main active constituents. Lupeol, *β*-sitosterol, and cycloartenol were isolated from the nonpolar fractions of *E. greenwayi* for the first time.

Conclusions

Euphorbia greenwayi aerial parts exhibit considerable anti-cancer effects via cytotoxicity. Three chemical constituents with promising cytotoxic activity are identified.

FULL TEXT

Background

Worldwide, cancer ranks as the second leading cause of death. The most prevalent cancers are colon, liver, and breast cancers. Cancer is characterized by its widespread occurrence globally. It exhibits notably high mortality rates according to statistical data. Lifestyle and genetic predisposition are commonly acknowledged as the primary factors contributing to its development. Phytochemicals derived from herbs and traditional medicine are becoming more widely recognized as effective cancer treatments. Recent clinical trials have demonstrated the beneficial effects of herbal medications on cancer patients' quality of life, survival rates, and immune system control when used in conjunction with traditional treatments. Numerous phytochemicals, including phenolic compounds, terpenoids, lignans, tannins, alkaloids, and others, have been studied from herbal sources and show potent antioxidant qualities that can suppress cell division and boost the immune system, improving prevention [1–4].

The ornamental medicinal plant species, *Euphorbia greenwayi* P.R.O. Bally &S. Carter is a member of the Euphorbiaceae (Spurge) family [5]. Euphorbia is considered the third biggest genus of flowering plants having milky poisonous latex. It consists of many species that are used in traditional medicine to cure a wide range of illnesses. This might be related to the wealth of their secondary metabolites [6, 7]. Different studies reported that Euphorbiaceae members consist mainly of terpenes, flavonoids, and tannins, which are known for their antioxidant, hepatoprotective, and anti-tumour properties [8–10]. As a member of the Euphorbia genus, *E. greenwayi* possesses succulent quality produces milky latex and may grow up to 1.2 m tall [11]. It was introduced to Egypt a short time ago, but it is native to Tanzania and East Africa. Upon reviewing the available literature, little information was reported on *E. greenwayi*; one study compared the immune-boosting capabilities of fifteen plant extracts from the Euphorbiaceae family, demonstrating *E. greenwayi*'s mild antiviral activity. [10]. Moreover, another report proved that the hydroalcoholic extract of *E. greenwayi* has significant antimicrobial potential [12]. Our recently published work demonstrated its anti-inflammatory and antioxidant potential [13].

The current study intends to assess the cytotoxic activity of the *E. greenwayi* methanol extract and its fractions. Additionally, an in vitro migration assay (wound healing activity) for the most active fractions is conducted to determine the tumour cell migration capacity of cell lines and, consequently, their invasiveness and potential to generate metastases. The chemical components of the most active fractions are determined using spectroscopic and chromatographic techniques.

Results

Phytochemical screening

A phytochemical screening is essential to determine the active substances responsible for the biological activity that plants are known to display and to evaluate a plant's potential medicinal usefulness. It also provides the foundation for more precise compound identification and investigation. Tannins, flavonoids, unsaturated sterols, and/or terpenes were found, together with carbohydrates and/or glycosides. As shown in Table 1, there were no volatile oils, alkaloids, nitrogenous bases, anthraquinones, or saponins.

Table 1. Preliminary phytochemical screening

Constituents	Presence
Carbohydrates and/or glycosides	+
Flavonoids	+

Tannins	+
Unsaturated sterols and/or terpenes	+
Volatile oils	–
Alkaloids and/or nitrogenous bases	–
Anthraquinones	–
Saponins	–

(+) = present, (–) = absent

Identification of the isolated Compounds

Compound EA_1

Compound (EA_1) 20 mg was isolated as white amorphous powder. It is soluble in *n*-hexane, chloroform and insoluble in methanol, m.p. 215–216ºC.; the R_f values were 0.59 on silica TLC using *n*-hexane:ethyl acetate (95:5 *v/v*) as developer. It gave a pink color when sprayed with 10% H_2SO_4..It also gave positive Salkowski and Liebermann-Burchard tests [14]. The ^{1}HNMR spectrum Table 2 demonstrated the characteristic deshielded proton at δH 3.3 (1H, *m*) assigned to C-3 attached to hydroxyl group, the deshielded olefinic proton at δH 4.6 (H-30, *d*,2H) was assigned to C-30, the characteristic 7 methyl singlets at δ 0.77 (H-23, *s*, 3H), 0.85 (H-24, *s,* 3H), 0.87 (H-25, *s*, 3H), 1.12 (H-26, *s*, 3H), 0.97 (H-27, *s*, 3H), 0.71 (H-28, *s*, 3H), and 1.71 (H-29, *s*, 3H) [15]. The ^{13}C NMR of the compound revealed 30 distinct signals corresponding to the terpenoid of the lupane skeleton. Among these signals, a carbon bonded to the hydroxyl group at the C-3 position was observed at δ 78.9. Additionally, the olefinic carbons associated with the exocyclic double bond manifested signals at δ 151.6 and 108.6. The EI-MS spectrum showed a molecular ion peak at 426(36%) calculated for the molecular formula $C_{30}H_{50}O$. In addition to the following characteristic peaks at 316, 218, 207, 189, 149, 135, 69, 95, 109, 121, 135 compared to published data [16, 17]. Compound (EA1) was identified as Lupeol based on the data presented above, comparison to published data [13, 18] and co-chromatography with a standard sample (Fig. 1). It was separated for the first time from *E. greenwayi*.

Table 2. ^{1}H NMR and ^{13}C spectral data of compounds EA 1, 2, and 3

Carbon number	(EA_1) Lupeol		(EA_2) *β*-sitosterol	(EA_3) Cycloartenol	
	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_H (ppm)	δ_C (ppm)
1	–	38	–	–	32.54
2	–	25.13	–	–	30.39
3	3.2(*dd*)	78.9	3.50 *(m*)	3.12 (1H, *m*)	58.82
4	–	38.6	–	–	40.47

5	–	55.3	–	–	47.51
6	–	18.31	5.30 (*br.s*)	–	20.79
7	–	34.1	–	–	28.29
8	–	41.2	–	–	48.57
9	–	50.4	–	–	20.07
10	–	37.3	–	–	25.05
11	–	21.3	–	–	26.01
12	–	27.5	–	–	27.54
13	–	38.7	–	–	45.37
14	–	42.6	–	–	48.07
15	–	27.9	–	–	34.53
16	–	35.6	–	–	27.09
17	–	43.2	–	–	53.30
18	–	48.3	0.87(*s*)	0.8(*d*)	18.35
19	–	47.07	1.04(*s*)	0.3–0.5(*dd*)	29.89
20	–	151.6	–	–	36.53
21	–	30	0.88(*d*)	–	19.85
22	–	39.9	–	–	36.33
23	0.77(*s*)	28.7	–	–	24.95
24	0.80(s)	15.9	–	5.1 (*t*)	123
25	0.87(*s*)	16.3	–	–	129.69
26	1.12(*s*)	16.1	0.79(*d*)	1.7 (*s*)	19.77
27	0.97(*s*)	15.3	0.82(*d*)	1.78 (*s*)	25.67

28	0.71(*s*)	18	–	1 (*s*)	29.03
29	1.71(*s*)	20.9	0.84(*t*)	0.91 (*s*)	14
30	4.6(*d*)	108.6	–	0.88 (*s*)	21.64

Fig. 1 [Images not available. See PDF.]

Chemical structure of the identified compounds

Compound EA_2

Needle crystals give a dark blue colour with 10% H_2SO_4, positive Liebermann- Burchard [19] and Salkowski [14] tests. Its molecular formula $C_{29}H_{50}O$ *m/z* 414 (86.8%). ^{1}HNMR spectrum; presented in Table 2; showed δ 5.3 (H-6, *br s*, 1H), 3.5 (H-3, *m*, 1H), the characteristic 2 methyl singlets δ 0.86 (H-18, *s*, 3H) and 1.04 (H-19, *s*, 3H), and 4 methyl doublets at δ 0.88 (H-21, *d*, *J*=9.6, 3H), 0.84 (H-29, *t*, 3H), 0.79. (H-26, *d, J*=6.3, 3H) and 0.82 (H-27, *d*, *J*= 6.3, 3H). In addition to the following characteristic peaks at 414 $[M]^+$, 396 $[M\text{-}H_2O]^+$, 381 $[M\text{-}CH_3\text{-}H_2O]^+$, 329 $[M\text{-}C_6H_{13}]^+$, 303 $[M\text{-}C_7H_{11}O]^+$, 255 [M-side chain-H_2O]$^+$, 231 [M-side chain-ring D cleavage-CH_3]$^+$, 213 [M-side chain-ring side chain -H_2O]$^+$. The compound (EA2) was identified as β-sitosterol through data analysis, comparison to published data [15, 20–22] and co-chromatography with a standard sample (Fig. 1). *β*-Sitosterol was previously isolated from various Euphorbia species. [13, 23, 24]. It was isolated from *E. greenwayi* for the first time.

Compound EA_3

Yellowish white microcrystalline powder gives a purple colour with 10% H_2SO_4, positive Liebermann- Burchard [19] and Salkowski tests [14]. It is soluble in *n*-hexane, chloroform and insoluble in methanol, m.p. 99–110 ºC. The R_f values were 0.56 on silica TLC using *n*-hexane: ethyl acetate (80:20 v/v) as developer. ^{1}HNMR spectrum; presented in Table 2; showed the following signals: δ5.1 (H-24, *t*, *J*=5.6, 1H), 3.12 (H-3, *m*, 1H), 0.3–0.5 (H-19, *dd*, *J*=3.2, 2H), 0.8 (H-18, *d*, *J*=3.2, 3H), 1.78 (H-27, *s*, 3H), 1.7 (H-26, *s*, 3H), 0.91 (H-29, *s*, 3H), 1 (H-28, *s*, 3H), 0.88 (H-30, *s*, 3H). ^{13}C NMR spectrum of compound EA_3 displayed 30 carbons corresponding to 7 methyl carbons, 11 carbenes, 5 methine carbons, 5 quaternary carbons and 2 olefinic carbons at δ 123 and 129.69 (Table 2). Mass spectrum of isolated compound showed molecular ion *m/z* 427 [M+H] corresponding to the molecular formula $C_{30}H_{51}O$. It gave MS spectra with a base peak at *m/z* 409 which resulted from loss of 1 water molecule $[M+H-H_2O]^+$. MS^2 also showed characteristic peaks at *m/z* 257, 271 and 285, 191, 203 and 217 and compared with published data [25]. Based on the data presented above and published data [26], compound (EA3) was identified as cycloartenol (Fig. 1). It was previously isolated from several Euphorbia species [26–28]. It has been isolated for the first time from *E. greenwayi*.

Biological activity

Antitumor activity (Screening)

As shown in Table 3*n*-Hexane fraction (HF) and chloroform fraction (CF) were the most active fractions to the 3 cancerous cell lines compared to the total methanol extract (ME) and ethyl acetate fraction (EF) in both tested concentrations. (HF) showed viability percentage against MCF-7, breast adenocarcinoma, HepG-2; hepatocellular carcinoma, and SW620; colorectal adenocarcinoma at 75.1622, 48.396 and 97.3144% respectively in 10 µg/ml concentration and 1.9754, 6.8561 and 0.79856% respectively in 100 µg/ml concentration. Whereas (CF) in 10 µg/ml concentration showed viability percentage at 80.0813, 78.9734 and 99.4913% against MCF-7, HEPG-2 and SW620 respectively and in 100 µg/ml it showed viability % at 2.42084, 4.98625 and 0.38287% respectively.

Table 3. Cell viability % of ME, HF, CF, and EF against MCF-7, HEPG-2, and SW620

Cell line	MCF-7 viability %	HEPG-2 viability %	SW620 viability %

Conc.	10 µg/ml	100 µg/ml	10 µg/ml	100 µg/ml	10 µg/ml	100 µg/ml
ME	83.52±0.59	54.16±1.04	82.19±0.15	46.30±1.89	95.99±0.5	74.40±0.66
HF	75.16±0.71	1.97±0.17	48.39±1.37	6.85±0.35	97.31±0.37	0.79±0.22
CF	80.08±0.54	2.42±0.22	78.97±1.5	4.98±1.32	99.49±0.22	0.38±0.02
EF	97.72±0.52	84.03±0.41	96.42±1.21	94.70±0.28	97.041±1.3	93.34±0.14
Negative control	100		100		100	

Antitumor activity (IC_{50})

According to the cell viability assay (Table 3) HF and CF were the most cytotoxic fractions of the 3 tested cancerous cell lines. Accordingly, those 2 fractions were further tested to find their (IC_{50}) using Sulforhodamine B (SRB) analysis in comparison with doxorubicin as a reference antitumor drug. As shown in Table 4 both HF and CF have a moderate to low activity against all tested cell lines. In specific the breast adenocarcinoma (MCF-7) cells were the most susceptible cell line against both fractions with IC_{50} values at 18.6±0.2 µg/ml against HF and 17.5±0.6 µg/ml against CF.

Table 4. IC_{50} of HF and CF against MCF-7, HepG-2, and SW620

	IC_{50} (µg/ml)		
Cell line	MCF-7	HEPG-2	SW-620
HF	18.6±0.2	35.6±1.2	28.4±0.8
CF	17.5±0.6	36.3±0.9	24.1±1.3
Doxorubicin	3.3±0.10	4.8±0.14	4.2±0.18

It can be deduced that the aerial parts of *E. greenwayi* have a moderate antitumor activity especially against MCF-7 cell line. The United States National Cancer Institute (NCI) stated that any crude extract with IC_{50} value ≤ 20 µg/ml is considered an active cytotoxic agent [29]. The highest cytotoxic activity was observed in the nonpolar fractions (*n*-hexane and chloroform) of *E. greenwayi.* This was confirmed after tracing the anticancer potential of the 3 isolated compounds (lupeol, *β*-sitosterol, and cycloartenol) that was already proven in previous studies [30–34]. This reveals a good correlation between antitumor potential and nonpolar constituents of *E. greenwayi* like sterols and terpenes.

Anti-Migration Activity of MCF-7 Cell Line

A wound healing assay, conducted *in vitro*, aims to assess the migratory potential of cell lines treated with the most potent fractions. This assay helps evaluate the cells' ability to migrate, thereby indicating their invasiveness and the likelihood of generating metastases. Based on the antitumor activity results, only HF and CF were continued in this study, because those fractions showed cytotoxic activity superior to ME and EF against MCF-7 which was the most susceptible cell line among the 3 cell lines tested.

HF and CF antimigration assay was performed using 2 doses (subtoxic and a lethal dose (IC_{50})), 1.9 and 19 µg/ml respectively for HF and 1.7 and 17 µg/ml respectively for CF. Figures 2, 3 and 4 demonstrated the MCF-7

monolayer which was scratched and treated with selected fractions. The wound area was monitored and imaged every 24 hours for 72 hours. Finally, the migration rate was calculated and compared with the negative control Figs. 5 and 6.

Fig. 2 [Images not available. See PDF.]

Migration rate of MCF-7 cells without any drug applied (Negative control) at A = 0, B = 24, C = 48, D = 72 h. respectively

Fig. 3 [Images not available. See PDF.]

Migration rate of MCF-7 cells using HF (n-hexane fraction) lethal dose (19 µg/ml) at A = 0, B = 24, C = 48, D = 72 h. respectively

Fig. 4 [Images not available. See PDF.]

Migration rate of MCF-7 cells using CF (chloroform fraction) lethal dose (17 µg/ml) at A = 0, B = 24, C = 48, D = 72 h. respectively

Fig. 5 [Images not available. See PDF.]

Rate of MCF-7 cells migration with subtoxic and lethal dose of HF (n-hexane fraction)

Fig. 6 [Images not available. See PDF.]

Rate of MCF-7 cells migration with subtoxic and lethal dose of CF (chloroform fraction)

As shown in Figs. 5 and 6 both the subtoxic and lethal doses of both HF and CF don't exhibit an anti-migratory effect.

Discussion

The exploration of medicinal plants has garnered increased attention as a means to discover more effective treatments for various cancer types. Presently, a substantial portion of pharmaceutical agents, especially in cancer therapy, comprises natural products. Taxol, vinblastine, and camptothecin are illustrative examples, distinguished by their unique structures and mechanisms of action, with their discovery primarily attributed to isolation from natural sources. In this sense, the Euphorbia genus is distinguished by its richness in biologically active phytoconstituents with promising cytotoxic activity [35–37]. In this regard*, E. greenwayi* was chosen to be the subject of our study because of the little-known information regarding its primary components and its biological activity.

E. greenwayi showed positive presence of sterols, triterpenes, and phenolic compounds; hence its methanol extract (ME) was fractionated using *n*-hexane (HF), chloroform (CF), and ethyl acetate (EF) to test these fractions for cytotoxic activity against MCF-7, HepG-2, and SW-620 cell lines. (HF) and (CF) showed significant cytotoxic activity against MCF-7 with IC_{50} values at 18.6 ± 0.2 and 17.5 ± 0.6 µg/ml respectively. However, they showed significant cytotoxic effect on HepG-2 and SW-620 at higher doses. This confirms the susceptibility of MCF-7 against (HF) and (CF). On the other hand, ME and EF didn't exhibit any cytotoxic activity against the 3 cell lines at all tested concentrations. Wound healing assay for cancer metastasis is highly reproducible method to study cancer cell in vitro. By this method we can develop an additive treatment combined with the main drugs in order to decrease migration of cancer cell to another organs. Based on the previous findings (HF) and (EF) were tested against MCF-7 cell migration yet they didn't display a significant antimigratory effect. These findings don't contradict the results of antitumor assay, but rather suggest that both (HF) and (CF) are cytotoxic to MCF-7 cells in a non-apoptotic cell death mechanisms other than decreasing cell migration or inhibiting the cell motility [38]. Also, it was already established that some of the most effective anticancer drugs such as carboplatin and paclitaxel were observed to induce the migration of cancer cells in different kinds of cancers [39].

Driven by the antitumor assay findings we decided to explore the constituents of the nonpolar fractions (HF) and (CF) of *E. greenwayi*. Phytochemical analysis reveals the separation of three compounds from (HF) and (CF):

lupeol, β-sitosterol, and cycloartenol. The 3 compounds are isolated from *E. greenwayi* for the first time. Based on previous data and the pharmacological effects of the three compounds, we realised that they all exhibited cytotoxic activity against various cancer cell lines [30, 32–34, 40, 41]. This supports the antitumor effects of *E. greenwayi*'s nonpolar fractions. In addition, in our recently published work through LC–MS we identified several nonpolar compounds in *E. greenwayi* [13] with reported cytotoxic activity such as taraxasterol [42], ingenol dibenzoate [43], and ingenol mebutate [44].

Methods

Plant material

Collection, handling, and authentication of plant material was previously discussed in our recently published work [13].

Extraction and fractionation

Twelve Kg of *E. greenwayi* fresh plant was macerated with absolute methanol till exhaustion (12 L × 3). The methanol extract was evaporated under a vacuum at 40°C. The crude extract (40 g) was suspended in 200 ml distilled water. The aqueous suspension was successively fractionated by partition with *n*-hexane, chloroform, and ethyl acetate. The results of the fractionation are summarized in (Fig. 7).

Fig. 7 [Images not available. See PDF.]

Scheme for compound isolation

Phytochemical screening

Dried aerial parts of *E. greenwayi* (40 g) underwent a phytochemical screening to identify the different phytochemical components that were found in it. These components included volatiles, carbohydrates and/or glycosides, alkaloids and/or nitrogenous bases, saponins, anthraquinones, unsaturated sterols and/or triterpenes, tannins, and flavonoids [45, 46]. The Pio-chem corporation in Cairo, Egypt provided all the chemicals, which were of high purity. Among the substances utilized following instructions were glacial acetic acid, concentrated ammonia, alcoholic KOH, $FeCl_3$, HCl, Dragendorff's reagent, methanol, chloroform, and H_2SO_4 [47, 48].

Compound isolation

N-hexane (3.6 g) and chloroform (1.3 g) fractions showed similar spots, so both fractions were added together. Fractionation was done through column chromatography using a silica gel (Merck) (200 g, 100 cm X 5 cm). Gradient elution started with 100% *n*-hexane then 5% increments of ethyl acetate, till the elution reaches 100% ethyl acetate. Fractions, each of 15 ml, were collected, concentrated under reduced pressure, and monitored using thin liquid chromatography (TLC). A system consisting of *n*-hexane: ethyl acetate with a different ratio was used as a developer. 10% H_2SO_4 was used as a spraying reagent for spot visualization. Similar fractions were pooled together. Fraction (I) was fractionated through column silica gel using *n*-hexane/methylene chloride gradient elution resulting in compound (EA_1) separation.

Fraction (VI) was found to have 2 major compounds. The fraction was further chromatographed on a silica gel column (30 × 1 cm). Gradient elution was performed using *n*-hexane followed by 5% increments of ethyl acetate. Fractions of 10 ml were collected and run on TLC. subfraction (35–45) yielded 3 mg of needle crystals compound (EA_2) and subfraction (98–106) yielded 5 mg of needle crystals compound (EA_3).

Structure elucidation of the purified compound

NMR spectroscopic analysis used a Bruker spectrometer at 400 MHz for (^{1}H NMR) and 100 MHz (^{13}C NMR) according to [49]. The UPLC-ESI–MS/MS negative and positive ion modes were executed on a Waters Corporation, Milford, MA01757, USA, XEVO TQD triple quadrupole mass spectrometer.

Biological activity

Antitumor activity

Cell viability test was done for the *E. greenwayi* total methanol extract (ME), and its fractions; n-hexane fraction (HF), chloroform fraction (CF), and ethyl acetate fraction (EF) according to [50]. It was assessed by Sulforhodamine B (SRB) assay against 3 human tumor cell lines (MCF-7, breast adenocarcinoma, HepG-2; hepatocellular

carcinoma, and SW620; colorectal adenocarcinoma) using 2 concentrations of each tested sample (10 and 100 µg/ml), to identify fractions with the most powerful anti-tumor properties.

In this experimental procedure, 100 µL aliquots of a cell suspension containing 5×10^3 cells were dispensed into individual wells of 96-well plates and incubated in complete media for a duration of 24 h. Subsequently, the cells were subjected to treatment with another 100 µL of media containing various concentrations of drugs. Following 72 h of exposure to the drugs, the cells were fixed by replacing the media with 150 µL of a 10% trichloroacetic acid (TCA) solution and incubated at 4 °C for 1 h. After removal of the TCA solution, the cells underwent five washes with distilled water. Subsequently, 70 µL aliquots of a sulforhodamine B (SRB) solution at a concentration of 0.4% w/v were added to each well, and the plates were incubated in darkness at room temperature for 10 min. Following this incubation period, the plates underwent three washes with 1% acetic acid and were then allowed to air-dry overnight. To dissolve the protein-bound SRB stain, 150 µL of a tris(hydroxymethyl) aminomethane (TRIS) solution was added at a concentration of 10 mM. The absorbance of the resulting solution was measured at 540 nm using a BMG LABTECH®-FLUOstar Omega microplate reader (Ortenberg, Germany).

Cell migration (wound healing) assay

N-hexane and chloroform fractions were evaluated for their potential to inhibit wound healing in cancerous cell lines according to [51, 52]. Since MCF-7 (Breast Adenocarcinoma) was the most susceptible cell line, it is chosen to be used in this assay. Both fractions were evaluated in 2 concentrations, lethal dose (IC_{50}) and Subtoxic dose.

Cells were seeded at a density of 2×10^5 cells per well on a 12-well plate that had been pre-coated for scratch wound assay. They were cultured overnight in a medium consisting of 5% fetal bovine serum (FBS) in Dulbecco's Modified Eagle Medium (DMEM) at 37°C and 5% CO_2. The following day, horizontal scratches were carefully introduced into the confluent cell monolayer. Subsequently, the plate underwent thorough washing with phosphate-buffered saline (PBS). Control wells were replenished with fresh medium, while wells designated for drug treatment were supplied with fresh medium containing the specified drug. Images were captured at designated time intervals using an inverted microscope. The plate was maintained at 37°C and 5% CO_2 between these time points. Analysis of the acquired images was conducted using MII Image View software version 3.7.

Wound width is the distance between the edges of the scratches in average; as cell migration is induced the wound width decreases.

Migration rate is determined according to the formula below: $MR = IW - FW / t$ where MR is the rate of cell migration, IW is the average wound width at 0 h, FW is the average final wound width, and t is duration of migration (in hours).

Conclusion

In conclusion, the investigation into the anti-cancer properties of *E. greenwayi* has revealed promising findings. The study encompassed cytotoxicity assays, evaluations of cell migration, and identification of its chemical constituents. The cytotoxicity assessments demonstrated considerable potency within specific fractions (*n*-hexane and chloroform fractions) of *E. greenwayi*, notably highlighting considerable toxicity against cancerous cell lines.

Furthermore, the identification of chemical constituents within *E. greenwayi* provides valuable insights into potential bioactive compounds responsible for its anti-cancer effects. These constituents may serve as a foundation for further research and development of novel anti-cancer agents.

The collective findings underscore the significance of *E. greenwayi* as a potential source of compounds with anti-cancer properties. Continued exploration and elucidation of its mechanisms and active compounds could pave the way for the development of new therapeutic strategies in combating cancer.

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Author contributions

Conceptualization, A.Z., Z.K., M.Y., R.S.E. and W.E.; Data curation, A.Z.; Investigation, A.Z.; Methodology, R.S.E., and W.E.; Supervision, Z.K., M.Y., R.S.E., and W.E.; Visualization, A.Z.; Writing – original draft, W.E.; Writing – review &editing, Z.K., M.Y., R.S.E. and W.E.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Ethics approval and consent to participate

Ethical approval was obtained from the research ethics committee, Faculty of Pharmacy, Cairo University; serial number MP (2448).

Consent for publication

Not applicable.

Plant authentication

Aerial shoots of *E. greenwayi* were collected in March 2019 at the Helal Cactus farm in Al Mansoureyah, Giza Governorate, Egypt (30.10812667354337, 31.105346915336153). Professor Dr. Reem Samir Hamdy, a botany professor at Cairo University's Faculty of Science, kindly verified and recognized the plant. The collection and handling of the plant material were in accordance with all the relevant guidelines.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

M.p.
Melting Point
R_f
Retention Factor
TLC
Thin Layer Chromatography
NMR
Nuclear Magnetic Resonance
H^1NMR
Proton nuclear magnetic resonance
C^{13}NMR
Carbon nuclear magnetic resonance
EI-MS
Electron-impact ionization Mass
Ms
Mass Spectroscopy
ME
Methanolic Extract
HF
n-Hexane Fraction
CF
Chloroform Fraction
EF
Ethyl acetate Fraction
MCF-7
Breast Adenocarcinoma
HepG-2
Hepatocellular Carcinoma
SW-620

Colorectal Adenocarcinoma

SRB

Sulforhodamine B

IC_{50}

Half Maximal Inhibitory Concentration

NCI

National Cancer Institute

TQD

Triple Quadrupole

TCA

Trichloroacetic Acid

TRIS

Tris(hydroxymethyl) aminomethane

FBS

Fetal Bovine Serum

DMEM

Dulbecco’s Modified Eagle Medium

PBS

Phosphate-buffered Saline

MR

Migration Rate

IW

Initial Width

FW

Final Width

Publisher's Note

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DETAILS

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A validated, precise TLC-densitometry method for simultaneous quantification of mahanimbine and koenimbine in marketed herbal formulations

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ABSTRACT (ENGLISH)

Background

Murraya koenigii (L.) Spreng. (Rutaceae) is an important medicinal plant in natural products research for its diverse pharmacological activities. Carbazole alkaloids were the major classes of phytoconstituents obtained from different parts of this plant, such as leaves, stems, and roots. Mahanimbine and koenimbine are two important carbazole alkaloids obtained from the *M. koenigii* plant and are known for their anti-cancer, anti-oxidant, anti-diarrhoeal agents, etc. Standardization plays a vital role in the herbal drug industry for maintaining the quality, purity, safety, and efficacy of herbal formulations, and hyphenated analytical techniques like HPTLC, HPLC, GC–MS, and LC–MS were utilized for this purpose. In the present study, a specific, simple, and rapid semi-automated TLC method was developed to quantify mahanimbine and koenimbine in some marketed herbal formulations, and the same was validated based on (ICH)-Q2-(R1) guidelines.

Results

This study revealed that the powder formulation (F_3) contains the highest amount of mahanimbine (62.32 µg), but the tablet formulation (F_1) contains both mahanimbine (41.19 µg) and koenimbine (143.6 µg).

Conclusion

A simple, specific, and reproducible semi-automated TLC method was developed and validated successfully as per (ICH)-Q2-(R1) guidelines and can be utilized for analysing marketed herbal formulations containing *M. koenigii* powder/extracts.

FULL TEXT

Background

Murraya koenigii plant, commonly known as curry tree in Asia, contains several vital phytoconstituents with diverse pharmacological activities [1]. Mahanimbine and koenimbine are the major carbazole alkaloids obtained from the leaves, roots, and stems of *M. koenigii* [2, 3]. These two phytoconstituents showed promising therapeutic efficacy against different diseases like cancer, obesity, and diabetes while tested in different *in vitro* and *in vivo* models [4]. Mahanimbine inhibited proliferation of lung cancer A549 cells [5], inhibited abnormal growth of the pancreatic cell lines (SW 1190, CFPAC1, BxPC3, CAPAN-2, and HPAFII) [6], stimulated memory and learning functions in aged mice [7], and also showed potential anti-diabetic activity on 3T3-L1 cells [8]. Similarly, koenimbine significantly reduced the number and size of breast cancer cell line MCF7 [9] and showed significant inhibition of castor oil-induced diarrhoea (at 50 mg/kg) in the rats [10].

Standardization of herbal formulations helps to set the constant parameters and inherent characteristics to assure safety, efficacy, quality, and reproducibility. According to the definition provided by American Herbal Product Association, “Standardization refers to the body of information and controls necessary to produce material of reasonable consistency [11].” Nowadays, in the Herbal drug industry, standardization is an integral part of quality

control for getting suitable raw materials, maintaining the quality and purity of finished products, etc. [12]. Various analytical techniques were utilized for this purpose, such as HPTLC, HPLC, MS, LC–MS, and GC–MS. [13]. HPTLC is a vital separation technique based on the TLC principle, which offers multiple advantages such as minimized exposure to toxic solvents, improved sample application, reduced usage of mobile phase, less analysis time, reduced possibilities of environmental pollution, and higher separation efficiencies [14]. Planar chromatographic methods (TLC and HPTLC) were widely utilized for quality control of herbal formulations. It helps for qualitative, quantitative, and semi-quantitative analysis of phytoconstituents incorporated in different herbal formulations manufactured and marketed by different companies. Several pieces of research proved that thin-layer chromatographic methods successfully ensure the quality and purity of marketed herbal formulations. Abharam A et al. developed a novel HPTLC method for analysing an ayurvedic polyherbal formulation named Pathyashadangam Kwath by employing toluene/ethyl acetate/formic acid (2.5: 2.0: 0.5 v/v/v) as a mobile phase. In this study, andrographolide was used as a marker for standardizing this formulation, and it proved that the presence of this marker may be responsible for its pharmacological activities [15]. Hazra et al. took piperine as a marker for standardizing six polyherbal formulations named Avipattikara, Talisadya, Sringyadi, Sitopaladi, Hingavastaka, and Trikatu and developed a specific and simple HPTLC method by utilizing toluene/ethyl acetate (7:3 v/v) as a mobile phase. This study proved that piperine was identified and quantified in all of the formulations, and it can be concluded that this method can be utilized for routine analysis of piperine in marketed ayurvedic formulations [16]. Kagathara C et al. stated that HPTLC could be a better option for estimating ascorbic acid, quercetin, and curcumin in different herbal formulations. They developed a specific HPTLC method for identification and quantification of the same by employing chloroform/ethyl acetate/formic acid (6:6:2.5 v/v/v) as a mobile phase. From this study, it was observed that these three important phytocompounds were identified and quantified in all of the formulations, and this analytical method can be utilized for quality control of herbal formulations where curcumin, quercetin and ascorbic acid were incorporated [17]. A simple, specific, and rapid HPTLC assay method for analysing tacrolimus ointments was developed by Islam MK et al. which helps to identify and quantify tacrolimus in the same. In this method, toluene–acetonitrile–ethyl acetate–glacial acetic acid (6:2:2:0.1 v/v/v) was used as a mobile phase and it proved the utilization of this method for standardization of marketed tacrolimus ointments [18]. These researches highlighted the importance of planar chromatographic techniques in the standardization of marketed herbal formulations and marker compounds play an important role in this.

By thorough literature survey, it was found that several analytical techniques were employed to estimate mahanimbine and koenimbine in *M. koenigii* plants. Joshi T et al. utilized a novel UPLC method to determine the natural abundance of the carbazole alkaloids in the *M. koenigii* plant [19]. Viteritti et al. [20] developed an HPLC–MS/MS method to quantify carbazole alkaloids in *M. koenigii* plant. Nandan et al. [21] quantified eleven carbazole alkaloids using a novel UPLC/MS/MS method, including mahanimbine and koenimbine in *M. koenigii* plant collected from six different climatic zones of India. Chatterjee et al. [22] successfully developed a validated qNMR method for quantifying nine important carbazole alkaloids, including koenimbine and mahanimbine. But to the best of our knowledge, there is no analytical method was available for standardizing marketed herbal formulations containing carbazole alkaloids obtained from the *M. koenigii* plant. Hence in the present study, a rapid and simple semi-automated TLC method was established to identify and estimate two essential carbazole alkaloids named mahanimbine and koenimbine (Fig. 1) in some marketed formulations and helps to determine the quality and purity of the formulations. The validation of the established method has been done as per (ICH)-Q2-(R1) guidelines.

Fig. 1 [Images not available. See PDF.]

Structures of mahanimbine and koenimbine

Methods

Instruments

VisionCATS software (version 3.2) is equipped with TLC visualizer 2, Linomat V applicator, and TLC scanner 4 manufactured by CAMAG (Switzerland). TLC silica gel $60F_{254}$ plates were procured from Merck (Germany). The

analytical balance and hot air oven were purchased from Sartorius (Germany) and Biotechnics India (India.)

Reagents and standard substance

Analytical grade Hexane and Ethyl acetate has been procured from Finar (India), and HPLC grade methanol has been purchased from Spectrochem (India). Mahanimbine and koenimbine (purity: >90% by HPLC) were purchased from Natural Remedies Pvt Ltd. (India).

Selected marketed formulations for analysis

Three marketed formulations named Merlion Naturals curry leaves extract tablets (packed and marketed by Infinator Pvt. Ltd, Ahmedabad, Gujarat, India), curry leaves capsules (manufactured and marketed by Dr. JPG Organic, Jaipur, Rajasthan, India) and premium curry leaves powder (manufactured by Spag herbals, Hyderabad, Telangana, India) were collected and coded as F_1 (for tablet), F_2 (for capsule) and F_3 (for powder) [23–25]. Out of these formulations, F_1 and F_2 contain 500 mg of curry leaves powder along with excipients (in Q.S. and the name of the same is not disclosed) and used as a dietary supplement and F_3 contains 125 mg of dried curry leaves along with excipients (in Q.S. and the name of the same is not disclosed) and used as a cosmetic. All formulations were stored in a cool environment and protected from direct sunlight.

Standard and sample preparation

About 5 mg of mahanimbine and koenimbine were dissolved in 50 mL of HPLC grade methanol for preparing standard stock solutions. Further, the working solutions were prepared by diluting the stock solutions with the required quantity of methanol.

For preparing sample solutions from tablets (F_1), about five tablets were crushed into powder, and 300 mg of the powder was accurately weighed and macerated with 10 mL of HPLC grade methanol. In the case of capsule (F_2), the shells of five capsules were broken and accurately weighed, and 300 mg of powder was macerated with 10 mL of HPLC grade methanol. For preparing sample solutions from powder (F_3) (stored in a cool environment and protected from direct sunlight), 300 mg of the powder was accurately weighed and macerated with 10 mL of HPLC grade methanol. Finally, all of the prepared extracts were centrifuged (10 °C, 10,000 RPM). The supernatant fluids were collected and underwent tenfold dilution, which was used for analysis.

Optimization of the analytical conditions

In the current study, TLC silica gel $60F_{254}$ plates were utilized as a stationary phase. For selecting a suitable mobile phase for analysis, different solvent combinations were tried. By thorough literature survey, it was decided that different combinations of hexane and ethyl acetate in v/v (8:2, 9:1, 9.5:0.5, 7:3) would be used for determining optimal mobile phase composition. Based on the separation pattern obtained from preliminary TLC analysis/observation, a combination of hexane:ethyl acetate (7:3 v/v) was selected as the final mobile phase and utilized throughout the analysis.

Semi-automated TLC conditions

On each TLC silica gel $60F_{254}$ plate (20 × 10 cm), 4 µL of mahanimbine and koenimbine standards and F_1, F_2, and F_3 were applied at the rate of 150 nL S^{-1} by utilizing a Linomat V applicator. The applied band length was 8 mm, and the application was about 1 cm from the bottom and left edges of the plate. After application, the development of the plates was done in CAMAG twin trough chambers to a distance of 70 mm with the selected mobile phase composition which was previously saturated for 20 min. After that, the developed plates were air-dried, and the fingerprint profile was generated by placing the plates in TLC visualizer 2. Then, the plates were scanned using a TLC scanner 4 at $\lambda = 291$ nm and 285 nm for koenimbine and mahanimbine respectively. The wavelengths were optimized by performing spectrum scanning in the 190–400 nm wavelength range, and the obtained data were compared with the maximum wavelengths mentioned in the literature (mahanimbine-357, 302, 288, and 235 nm; koenimbine-295, 262, and 238 nm) [26]. The conditions for densitometric scanning (VisionCATS version 3.2) were: scanning speed 20 mm/s, data resolution 100 µm/step, and slit dimension 6.00 × 0.45 mm. From densitometric analysis, the retardation factor (R_f) for mahanimbine and koenimbine was found satisfactory. For quantitative analysis, the obtained values for the peak areas were utilized.

Method validation

Method validation is an integral part of any analytical experiment/procedure for getting accurate and reproducible results. This process is performed by checking the following parameters: linearity, LOQ, LOD, accuracy (recovery), precision (intra- and inter-day precision), reproducibility, and robustness.

Linearity

In any analytical method, linearity is an important parameter that describes its ability to get test results directly proportional to the content (concentration) of analyte present in the sample [27]. For quantifying the analytes present in the formulations, dilution of the standard stock solutions has been done to get linearity of standard solutions (considering the quantitation limit) containing koenimbine and mahanimbine in the concentration range of 50–450 and 100–400 µg/mL respectively keeping the applied volume 4 µL constant. The standards were applied in triplicate to generate the calibration curve. The correlation coefficient (R^2), intercept, and slope of the calibration curves were estimated to obtain the method linearity.

Detection limit (LOD)

The detection limit (LOD) is defined as the lowest amount of the analyte detected in the prepared sample. LOD can be calculated by using the following equation:LOD=3.3×standard deviation of the response/slope of the calibration curve

Quantitation limit (LOQ)

The quantitation limit (LOQ) is defined as the lowest amount of the analyte quantified with sufficient accuracy and precision. It can be calculated by using the following equation:LOQ=10×standard deviation of the response/slope of the calibration curve

Accuracy (recovery)

The accuracy of any analytical procedure is defined as the degree of agreement between the values considered to be true and the amount of the substance in the test samples obtained from the analysis [27, 28]. For estimating the percentage of recovery of mahanimbine and koenimbine in F_1, about 300 mg of powdered drug and 32 µg (80%), 40 µg (100%), 48 µg (120%) of mahanimbine standards and 112 µg (80%), 140 µg (100%) and 168 µg (120%) of koenimbine standards were mixed and further diluted with required quantity of HPLC grade methanol. In the case of F_2 and F_3, about 300 mg of powdered drugs and 40 µg (80%), 50 µg (100%), 60 µg (120%), 48 µg (80%), 60 µg (100%) and 72 µg (120%) of mahanimbine and 112 µg (80%), 140 µg (100%) and 168 µg (120%) of koenimbine standards were mixed and diluted with required quantity of HPLC grade methanol. After that, the percentage of recoveries was calculated and documented.

Precision

The precision of any analytical method was defined as the degree of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions [27, 28]. In the present study, inter-day precision studies of the developed method have been done by applying the standard solutions in triplicate at three different concentration levels three times at 72 h intervals, and for performing intra-day precision studies, the standard solutions were applied in triplicate at three different concentration levels three times within two hour intervals on the same day.

Reproducibility

The developed method was validated for the reproducibility of both for application (repeatability of application) and scanning (repeatability of scanning) [29]. The experiments were performed in triplicate by different analysts in different laboratories of NIPER Hyderabad, and it was observed that the %RSD values were obtained between ±5. Hence, it can be concluded that the developed method is reproducible.

Robustness

Robustness study needs to be done during method development, and it helps to determine the reliability of an analysis concerning deliberate variations in method parameters. In the present study, the following variations have been done to check the robustness of the developed method; different volumes of mobile phase compositions were utilized (Hexane: Ethyl acetate = 7:3, 8:2; 9:1); the laboratory temperature set to 22 °C, 25 °C, and 30 °C; humidity—35% RH, 55% RH, and 75% RH, varying the wavelengths for scanning (293, 291 and 289 nm) and

changing distance for development of TLC plates (78, 76 and 74 mm), etc. It was observed that the developed method gave reproducible results in all varying conditions.

Results

Three different marketed formulations (tablet, capsule, and powder) were collected from different distributors and coded as F_1, F_2, and F_3. The stock solutions of the standards were prepared by dissolving 5 mg of both standards in 50 mL of HPLC grade methanol and the working solutions were prepared by diluting the stock solutions with the required quantity of methanol. In the present study, TLC silica gel $60F_{254}$ plates were used as a stationary phase, and a combination of hexane:ethyl acetate (7:3) was utilized as a mobile phase. The developed fingerprinting profile showed that the mahanimbine and koenimbine were present in the selected formulations (Fig. 2). Both phytocompounds were densitometrically detected at $\lambda = 285$ and 291 nm, respectively. The absorption spectrum and the peak areas were recorded and documented for analysis. From spectrum data, it was observed that in the wavelength range of 190–400 nm, absorption maxima for mahanimbine and koenimbine were observed at 285 and 291 nm (Fig. 3). So, these two wavelengths were selected for further quantification. The specificity and peak purity of the method were determined by comparing the spectra of mahanimbine and koenimbine in the selected formulations with the standards. The peaks obtained by densitometric scanning were easily identifiable, symmetrical, and resolved well. From densitometric scanning, the obtained R_f values for mahanimbine and koenimbine were 0.48 and 0.60, respectively (Figs. 4 and 5).

Fig. 2 [Images not available. See PDF.]

Developed HPTLC fingerprint profile. Track (1–3): mahanimbine standard, Track (4–6): koenimbine Standard, Track (7–8): F_1 (Tablet), Track (9–10): F_2 (Capsule), Track (11–12): F_3 (Powder)

Fig. 3 [Images not available. See PDF.]

Spectrum data of **a** mahanimbine and selected formulations at 285 nm **b** koenimbine and selected formulations at 291 nm

Fig. 4 [Images not available. See PDF.]

HPTLC chromatogram at 285 nm. **a** mahanimbine standard **b** F_1 (Tablet) **c** F_2 (Capsule) **d** F_3 (Powder)

Fig. 5 [Images not available. See PDF.]

HPTLC Chromatogram at 291 nm. **a** koenimbine standard **b** F_1 (Tablet)

Further, the developed method was validated as per the guidelines mentioned by (ICH)-Q2-(R1). To determine the linearity of the developed method, 4 µL of standard solutions of increasing concentration (50–450 µg/mL for koenimbine and 100–400 µg/mL for mahanimbine, respectively) were applied on TLC silica gel $60F_{254}$ plates in triplicate. The calibration curve was generated (Fig. 6, Tables 1 and 2), and a linear relationship was established for mahanimbine and koenimbine in the concentration range of 100–400 and 50–450 µg/mL (Table 3). From the calibration curve, the obtained equations were $Y = 3.3614x + 95.5$ and $Y = -0.0069x^2 + 8.2023x + 172.76$, correlation coefficients (R^2) = 0.9985 and 0.9998, coefficient of variances (CV) = 0.34% and 0.89%, respectively (Fig. 6, Table 3). The obtained R^2-value indicates a strong correlation between the concentrations of phytocompounds and peak areas.

Fig. 6 [Images not available. See PDF.]

Calibration curve of **a** mahanimbine standard **b** koenimbine standard

Table 1. Linear regression data of mahanimbine

Sl No	Conc. in ppm	Mean ± RSD

1	100	420±2.38
2	150	602±1.99
3	200	777±2.18
4	250	951±1.15
5	300	1116±1.25
6	350	1272±1.25
7	400	1421±1.4

Table 2. Linear regression data of koenimbine

Sl No	Conc. in ppm	Mean±RSD
1	50	558±1.79
2	100	931±2.14
3	150	1254±1.19
4	200	1542±1.16
5	250	1781±0.89
6	300	2010±0.69
7	350	2189±0.91
8	400	2364±0.59
9	450	2461±0.6

Table 3. Method validation parameters

Sl No	Parameters	Mahanimbine	Koenimbine
1	Wavelength	285 nm	291 nm
2	Linearity range concentration (in µg/ml)	100–400	50–450
3	Coefficient of variances (CV)	0.34%	0.89%

4	Correlation coefficients (R^2)	0.9985	0.9998
5	Limit of detection (ng/spot)	32.81	18.44
6	Limit of quantitation (ng/spot)	72.81	31.57
7	Reproducibility	Reproducible	Reproducible

The LOQ and LOD values were calculated, and the obtained values were 18.44 ng/spot, 31.57 ng/spot, for koenimbine and 32.81 ng/spot, 72.81 ng/spot for mahanimbine, respectively (Table 3). Inter-day and intra-day precision studies have been done for the developed method; for intra-day, the studies were done in triplicate at three hrs time intervals; for inter-day, it was also done in triplicate at three consecutive days (Tables 4 and 5). The developed method was evaluated for recovery (accuracy) studies. For this purpose, the selected formulations and the standards were mixed and diluted with the required quantity of HPLC grade methanol. The calculated % of recovery for mahanimbine was: 96.80 (F_1), 98.47% (F_2), and 95.13% (F_3), and for koenimbine was: 97.61% (F_1), respectively (the % recovery was found to be between 95 and 105%; hence, the developed method was accurate) (Tables 6, 7, 8 and 9). The recovery analysis of koenimbine was not done in F_2 and F_3 as in F_3, it was not identified, and in case of F_2, though, it was identified but not quantified properly (quantification value comes under LOQ). Robustness studies were also performed by altering the method parameters, and there is no significant change observed in the obtained results (Tables 10 and 11).

Table 4. Intra- and inter-day precision data of mahanimbine

Conc. in ppm	Mean±RSD
Intra-day	
180	510±1.96
260	564±2.48
340	636±2.83
Inter-day	
240	710±1.4
300	760±2.63
360	825±1.81

Table 5. Intra- and inter-day precision data of koenimbine

Conc. in ppm	Mean±RSD
Intra-day	

140	590 ± 1.69
220	645 ± 2.32
300	705 ± 0.70
Inter-day	
200	770 ± 1.29
260	820 ± 1.21
320	870 ± 2.29

Table 6. Accuracy (recovery) data of mahanimbine obtained from F_1

Formula	Area obtained from software	% of recovery
3 bands of 100% sample	0.002575741 0.005378757 0.005765268	96.806837
3 bands of 100% Sample + 80% Standard	0.007342609 0.008868828 0.008646599	3 bands of 100% Sample + 100% Standard
0.009309263 0.009584616 0.009561901	3 bands of 100% Sample + 120% Standard	0.01010137 0.010271852 0.010140663
2 bands of 80% Standard	0.007182594 0.004321403	2 bands of 100% Standard
0.004206004 0.004546618	2 bands of 120% Standard	0.00511068 0.005268287

Table 7. Accuracy (recovery) data of mahanimbine obtained from F_2

Formula	Area obtained from Software	% of recovery

3 bands of 100% sample	0.00044788 0.011267132 0.009436818	98.47378217
3 bands of 100% Sample + 80% Standard	0.008830368 0.010630508 0.014043002	3 bands of 100% Sample + 100% Standard
0.014783529 0.015096105 0.014532022	3 bands of 100% Sample + 120% Standard	0.01413927 0.015060204 0.015253995
2 bands of 80% Standard	0.01569976 0.004768991	2 bands of 100% Standard
0.004737254 0.005804689	2 bands of 120% Standard	0.005710901 0.006967575

Table 8. Accuracy (recovery) data of mahanimbine obtained from F_3

Formula	Area obtained from Software	% of recovery
3 bands of 100% sample	0.002068801 0.003960302 0.004055559	95.13510953
3 bands of 100% Sample + 80% Standard	0.005123489 0.006076501 0.006129103	3 bands of 100% Sample + 100% Standard
0.006913892 0.007452141 0.007627958	3 bands of 100% Sample + 120% Standard	0.008180758 0.008607295 0.008389677
2 bands of 80% Standard	0.005785998 0.003193103	2 bands of 100% Standard

0.002769779 0.003819682	2 bands of 120% Standard	0.004226519 0.00443158

Table 9. Accuracy (recovery) data of koenimbine obtained from F_1

Formula	Area obtained from Software	% of recovery
3 bands of 100% sample	0.002068801 0.003960302 0.004055559	97.61435044
3 bands of 100% Sample + 80% Standard	0.005123489 0.006076501 0.006129103	3 bands of 100% Sample + 100% Standard
0.006913892 0.007452141 0.007627958	3 bands of 100% Sample + 120% Standard	0.008180758 0.008607295 0.008389677
2 bands of 80% Standard	0.005785998 0.003193103	2 bands of 100% Standard
0.002769779 0.003819682	2 bands of 120% Standard	0.004226519 0.00443158

Table 10. Data of robustness studies of mahanimbine

Volumes	Mean area ± RSD
Mobile phase compositions (Hexane: Ethyl acetate, v/v)	
6.9:3.1	695 ± 2.20
7.1:2.9	698 ± 1.90
7:3	701 ± 2.42
Temperature (°C)	
22 ± 2	702 ± 2.80

25±2	706±2.00
27±2	704±2.65
Humidity (% RH)	
45±5	704±2.35
55±5	708±2.70
65±5	706±2.50
Distance for development of TLC plate (mm)	
78	720±2.10
76	726±1.80
74	723±2.20
Wavelength (nm)	
287	715±1.40
285	725±1.70
283	720±1.90

Table 11. Data of robustness studies of koenimbine

Volumes	Mean area±RSD
Mobile phase compositions (Hexane: Ethyl acetate, v/v)	
6.9:3.1	756±1.32
7.1:2.9	760±1.40
7:3	764±2.10
Temperature (°C)	
22±2	758±2.30
25±2	762±2.10

27±2	760±1.95
Humidity (% RH)	
45±5	768±1.20
55±5	776±2.20
65±5	772±1.80
Distance for development of TLC plate (mm)	
78	764±2.40
76	770±2.10
74	767±2.30
Wavelength (nm)	
293	774±1.60
291	782±2.10
289	774±2.20

All the experiments were performed in triplicate

After that, the developed method was successfully employed to estimate mahanimbine and koenimbine in the selected marketed formulations (F_1, F_2, and F_3). From this study, mahanimbine was identified and quantified in all of the formulations, but koenimbine was only quantified in F_1 though it was identified in both F_1 and F_2 as the quantified value in F_2 comes under LOQ and this identification was done by comparing the R_f values (0.60 and 0.48), and absorption maxima (285 and 291 nm) of the standards. The quantity of both phytocompounds in the formulations was calculated based on the peak areas obtained from the chromatogram. The amount of mahanimbine in F_1, F_2, and F_3 was found to be 41.19 µg, 53.24 µg, and 62.32 µg, and for koenimbine, it was 143.6 µg, respectively (Table 12). It indicates that the highest quantity of mahanimbine was found in F_3, but in F_1, both the phytocompounds were present.

Table 12. Quantity of mahanimbine and koenimbine in F_1, F_2, and F_3 (in 30 mg)

Sl No	Name of the formulations	Mahanimbine (µg)	Koenimbine
1	F_1	41.19	143.6 µg
2	F_2	53.24	Not quantified
3	F_3	62.32	Not quantified

Discussion

M. koenigii is an important medicinal plant and several herbal formulations were available in the market where *M. koenigii* leaf powders or extracts were incorporated, and these formulations were used as dietary supplements, cosmetics, etc. But there is no specific analytical method was reported for standardizing the same and marker-based standardization by utilizing planar chromatographic methods can be a better option for this. Hence, there is a need to develop a suitable analytical method that helps to perform qualitative and quantitative analysis of two major marker compounds of *M. koenigii* named mahanimbine and koenimbine in the marketed herbal formulations for routine quality control analysis. Hence, in the current study, three marketed formulations manufactured by different companies (coded as F_1, F_2, and F_3) were collected, and quantitative estimation of mahanimbine and koenimbine was done in these formulations by using a semi-automated TLC method. Koenimbine (R_f value 0.60) and mahanimbine (R_f value 0.48) were identified in all of the formulations at the wavelengths of 291 and 285 nm, but only mahanimbine was quantified in all of the formulations, and koenimbine was quantified only in F_1. For performing this analysis, different mobile phases were employed, but based on the separation pattern, hexane:ethyl acetate (7:3 v/v) was selected for final quantification. This method passed all the parameters of analytical validation as per the guidelines prescribed in (ICH)-Q2-(R1) and gave reproducible results. The peak shapes of mahanimbine and koenimbine were found to be good. Hence, the developed semi-automated TLC method can be utilized for the routine quality control analysis of marketed herbal formulations of *M. koenigii* effectively, and its advantages are specificity, accuracy, and simplicity.

Conclusion

The current study proved that the developed HPTLC method was precise, specific, simple, and accurate in estimating mahanimbine and koenimbine in selected marketed herbal formulations. This study revealed that mahanimbine is identified and quantified in all formulations, but koenimbine is only identified and quantified in tablet formulation. The performed quantitative and qualitative analysis of the content of mahanimbine and koenimbine can help to maintain the quality of the marketed herbal formulations containing *M. koenigii* extracts/powders. The developed method has been validated as per the guidelines mentioned in (ICH)-Q2-(R1), which confirmed this method's accuracy, precision, and reliability.

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Author contributions

NM, RA, and KM prepared the methodology, performed the experiments, and conducted the statistical analysis. The manuscript draft was prepared by NM, KM, RA, and RS. RJS and VRK supervised and edited the manuscript. The final version of the manuscript was approved by all of the authors.

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Availability of data and materials

The data sets utilized and analysed during the present study can be available upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

TLC

Thin-layer chromatography

HPTLC
High-Performance Thin-Layer Chromatography
LOD
Detection limit
LOQ
Quantitation limit
R^2
Correlation coefficient
CV
Coefficient of variance
LC–MS
Liquid Chromatography–Mass Spectrometry
R_f
Retardation factor
mg
Milligram
ICH
International Council for Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
mL
Millilitre
v/v
Vol/vol
HPLC
High-performance Liquid chromatography
UPLC
Ultra-performance Liquid chromatography
IC_{50}
Half-maximal inhibitory concentration
GC–MS
Gas Chromatography–Mass Spectrometry
qNMR
Quantitative NMR
cm
Centimetre
µL
Microlitre
MS
Mass spectrometry
nm
Nanometre
mm
Millimetre
nm/s
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Nanometre/step

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hrs
Hours

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Development and characterization of niosomes loaded mucoadhesive biodegradable ocular inserts for extended release of pilocarpine HCl

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ABSTRACT (ENGLISH)

Background

Pilocarpine HCl is a non-selective muscarinic receptor agonist that is prescribed for the treatment of glaucoma. The use of pilocarpine conventional eye drops is associated with several side effects, such as loss of visual acuity, and the need for several applications due to rapid drainage away via the nasolacrimal duct, especially for elderly people. Such adverse effects can lead to low patient compliance and poor clinical outcomes. Therefore, the aim of this project was to develop, optimise and characterise a biodegradable pilocarpine HCl ocular insert using niosomes as a drug delivery vehicle. To achieve that, various polymers such as hydroxypropyl methylcellulose (HPMC), polyvinyl alcohol (PVA), and a blend of both were investigated to prepare the ocular inserts using solvent casting technique. The niosomes of pilocarpine HCl were prepared using span-60 and cholesterol by thin film hydration method. The produced noisome-loaded ocular inserts were characterised using various analytical techniques, including Fourier Transform Infrared (FTIR), X-ray Diffractions (XRD), thermal analysis, particle size analysis, weight and content uniformity, surface pH and drug release profile, among others.

Results

The results indicated that drug-free ocular inserts of the two polymers (HPMC + PVA) were better than single polymer-based ocular inserts (HPMC or PVA alone). The formed niosomes demonstrated good entrapment efficiency of 49.7% ± 7.0, with an average particle size of 325.7 ± 3.5 nm. The FTIR analysis showed no interaction between the compositions of niosomes. Four optimal formulations with various co-polymer ratios and pilocarpine content were further evaluated. Pilocarpine-containing niosomes-loaded ocular inserts provided uniformity in pilocarpine content (89–96%), with 34.8% moisture content and an average pH of 7. The release profile of niosomes-loaded inserts demonstrated an initial burst release within 2 h ranging from 26.54% (T4) to 41.22% (T2), and continuous sustained release for the next 24 h (68.32 ± 5.11% (T4) to 82.11 ± 6.01% (T2)).

Conclusions

This work successfully optimised biodegradable ocular inserts containing slow-release pilocarpine HCl encapsulated in niosomes for the treatment of glaucoma without dose dumping, resulting in a user-friendly drug delivery system.

FULL TEXT

Background

The eye is an anatomically and physiologically unique organ with various structural components [1]. Although the eye is an easily accessible organ for the direct administration of medication, it is isolated by several barriers such as the cornea, which acts as the main barrier for absorption and transportation of topically applied drugs into the systemic circulation. Further, the posterior part of the eye, such as the retina, creates an additional barrier. The retinal barrier is a protective barrier for the transport of materials [2], which creates challenges to drug delivery [3, 4]. In general, small lipophilic molecules can be absorbed through the cornea, whereas hydrophilic molecules can be absorbed through the conjunctiva and sclera [2].

The main routes for ocular administration of active pharmaceutical ingredients (APIs) are topical, systemic, and intraocular. The topical route, with formulations such as eye drops, accounts for 90% of aqueous ocular administration. This is the most preferred route owing to ease of application, patient preference and reduced systemic side effects caused by the API. Eye drops have several advantages such as being easy to apply by most patients (self-administration), safe, and more comfortable [5]. Besides, eyedrops can overcome first pass metabolism and directly target the site of the action [6]. Nevertheless, the use of eye drops has several limitations particularly, short retention time in the eye [3] which requires multi-dose administration during the day, which is detrimental to patient compliance [7, 8].

Pilocarpine HCl is a non-selective muscarinic receptor agonist and is prescribed for the treatment of glaucoma. Pilocarpine HCl decreases intraocular pressure in glaucoma patients by increasing trabecular outflow [9]. Lowering high intraocular pressure aids in preventing blindness, vision loss, and nerve damage [10]. Pilocarpine HCl eye drops are commonly used for the treatment of glaucoma with different concentrations (1%, 2%, 4%) available. The use of these conventional eye drops has been associated with several side effects, especially for elderly people, like loss of visual acuity, preservative allergy, and the need to repeat the dose to maintain therapeutic effects. Such adverse effects can lead to low patient compliance, and hence poor clinical outcomes [9].

For optimal bioavailability from ophthalmic topical formulations and effective clinical outcomes, these formulations need to be designed to increase retention time of the dosage form and enhance ocular drug permeability [11]. Novel drug delivery systems (DDSs) have been designed to deliver ocular drugs with reduced side effects. These formulations are based on nanotechnology to achieve desired drug particle size and the associated reduction of eye irritation, plus improved bioavailability. These formulations include microparticles, nanoparticles like liposomes (acyclovir), niosomes aqueous gels, or dendrimers (tropicamide) [5].

Niosomes are nano-sized vesicles containing non-ionic surfactants arranged in bilayers. Depending on the preparation method, niosomes may be unilamellar or multilamellar. Niosomes are made of a surfactant bilayer with the hydrophilic heads found on the surface, and inside, of the vesicle while the hydrophobic tails form the bilayer; this allows for two different areas for drug entrapment [12]. Features that make niosomes interesting for research include capacity for different API entrapment, osmotic activity and stability, presence of an amphiphilic structure that contains hydrophobic and hydrophilic portions and provide applications for dissolving drugs with varying solubilities. In addition, niosomes release the drug in a controlled manner through the bilayer, which can protect the encapsulated API during transit. Therefore, niosomes act as a store of medication in the body and enable targeted drug delivery [13]. Niosomal formulations are versatile owing to their structural flexibility, in terms of composition, fluidity, and size [12]. Niosomes are compatible, will not stimulate immunogenic reactions, and are decomposed by the biological system [14]. Furthermore, niosomes do not require special storage conditions [13]. These characteristics make niosomes promising nanocarriers in the treatment of ocular diseases. For example, timolol maleate, brimonidine tartrate, atenolol, dorzolamide HCl, bimatoprost and acetazolamide have been developed as niosomes for glaucoma treatment, while lomefloxacin HCl and chloramphenicol niosomes have been developed for treatment of conjunctivitis. Flurbiprofen and prednisolone niosomes have also been prepared for treatment of ocular inflammation and epalrestat for diabetic eyes [12, 15, 16].

Ocular inserts as novel drug delivery systems are gaining traction in research. Ocular inserts of pilocarpine HCl, marketed as Ocusert®, were an alternative topical dosage form that provided extended release of pilocarpine HCl over seven days. Ocusert® was easy to insert in the upper or lower lid, soft, flexible and had two drug release options: 20 µg/hour or 40 µg/hour. It was based on a reservoir system with zero order release pattern and pilocarpine HCl released by diffusion [17]. Ocusert ® was withdrawn from the market due to dose dumping and frequent loss of insert especially during sleep [18]. Therefore, development of pilocarpine HCl as an extended-release dosage form, such as niosomes, to be loaded into hydrophilic soluble ocular inserts, which would release the drug over 24 h and be biodegradable, would be advantageous. Other work by Lin et al. [19] reported the use of alginate and pluronic solution as the in-situ gelling vehicles for ophthalmic drug delivery of pilocarpine HCl. The mixture of 0.1% alginate and 14% pluronic solutions showed improvements in gel strength and clinical outcomes: with the main objective of improving the ocular bioavailability of pilocarpine HCl.

Therefore, there is a need to develop a user-friendly dosage form for pilocarpine HCl that releases pilocarpine HCl slowly over extended period of time and hence reduces the anticipated side-effects, especially for elderly patients, which are manifested in loss of visual acuity and annoyance for repeated dosing to maintain therapeutic effect. Such adverse effects can lead to low patient compliance and hence this will affect clinical outcome. Therefore, the aim of this project was to develop a formulation of biodegradable mucoadhesive ocular insert loaded with pilocarpine HCl containing niosomes to provide an extended-release effect over 24 h which would provide an effective treatment of glaucoma with sustained release effect of drug. This would result in an increase in contact time between drug and

cornea, reduction in the number of daily doses of the traditional ophthalmic dosage forms and sustained API delivery to the targeted site, using as little API dose as possible [7].

Methods

Materials

Pilocarpine HCl powder (≥98%) and polyvinyl alcohol (PVA) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Methanol (HPLC grade) was purchased from Honey well (Saint-Germain-en-Laye, France), water (HPLC grade) and chloroform were obtained from Alpha Chemika (Mumbai, India), while triethyl amine was obtained from Tedia (Fairfield, Ohio, USA). Orthophosphoric acid and hydroxypropyl methylcellulose (HPMC) were purchased from Alzchem for chemical (Trostberg, Germany). Glycerine, span 60 and sodium hydroxide were obtained from Fischer (Guangzhou, China). Cholesterol and phosphate buffered saline were obtained from Carlsson (Maryland, USA). All materials were used as supplied.

Development of drug free (blank) ocular inserts

Hydroxypropyl methylcellulose (HPMC) and polyvinyl alcohol (PVA) in different concentrations (see Table 1) were investigated to prepare the blank ocular inserts. Accurately weighed HPMC was sprinkled over 100 mL distilled water placed on a hotplate magnetic stirrer (Dlab ms-h280 (Beijing, China)) with temperature set at 30–40 °C and 1500 rpm for 1–2 h to ensure complete swelling and hydration of the polymer. Then, the required volume of glycerine (as plasticiser) was added and stirred for 15 min. After that, the polymer solutions were sonicated for 30 min continuously using a Bandelin Sonorex Digitec sonicator to remove bubbles. The final solution was cast onto plastic petri-dishes (area 56.71 cm^2). To prevent quick drying and films cracking, the samples were placed on the bench for 12 h at ambient temperature before placing them in the hot air oven at 40 °C for 4 h to complete drying. PVA containing ocular inserts were prepared in a similar manner but using cold distilled water initially and stirring at 1500 rpm for 30 min. The temperature of the PVA solution was then increased to 100 °C for 1 h to ensure complete swelling and hydration. Hot air oven drying time was only 30 min.

Table 1. Summary of the composition of the drug free (blank) ocular inserts formulations

Formula	Polymer	Polymer concentration %w/v	Plasticiser (glycerine) (%v/v)	Solvent volume (mL)
F1	HPMC	0.5	0.25	50
F2	30	F3	20	F4
HPMC	1.0	0.25	50	F5
30	F6	20	F7	HPMC
1.5	0.25	50	F8	30
F9	20	F10	HPMC	2.0
0.25	50	F11	30	F12
20	F13	PVA	0.5	1.0
50	F14	30	F15	20

F16	PVA	1.0	1.0	50
F17	30	F18	20	F19
PVA	1.5	1.0	50	F20
30	F21	20	F22	PVA
2.0	1.0	50	F23	30
F24	20	F25	PVA	3.0
1.0	50	F26	30	F27
20	F28	HPMC + PVA	1.5 + 2.0	1.0
50	F29	30	F30	HPMC + PVA
1.0 + 1.5	1.0	50	F31	30
F32	20	F33	HPMC + PVA	1.0 + 1.0
1.0	50	F34	30	F35

Blank ocular inserts containing a blend of HPMC and PVA were prepared starting with PVA solution as discussed above, after which the solution was kept on the bench at ambient temperature to cool to about 37 °C. This solution was returned to the hotplate and HPMC powder (0.5 g, 1 g or 1.5 g) was sprinkled with stirring at 1500 rpm and 37 °C for 30 min. Glycerine was added and solution further stirred for 15 min, then sonicated for 30 min to remove bubbles. The solution was cast on plastic petri-dishes (area 56.71 cm^2). To prevent quick drying and films cracking; the samples were placed on the bench for 12 h before putting it in the hot air oven for 30 min to complete drying.

Preparation of niosomes

The niosomes were prepared according to [7] using thin film hydration method. Accurately weighed span 60 (200 mg), cholesterol (100 mg) and pilocarpine HCl (20 mg) were added to 10 mL chloroform in a round bottom flask. The solution was rotated at 150 rpm for 1 h on a Heidolph rotary evaporator (Kelheim, Bayern, Germany) to evaporate the chloroform under low pressure at 60 °C. This step led to the formation of a thin film on the wall of flask. Then 10 mL of phosphate buffer (pH 7.4) was added to the flask to hydrate the film and left on the rotatory evaporator for 1 h at 60 °C and 150 rpm to remove the residual solvent. To produce niosomes, the dispersion was vortexed for 5 min at 60 °C. Finally, the produced niosomes were sonicated for 10 min at 60 °C using a Bandelin Sonorex Digitec sonicator.

Preparation of niosomes loaded ocular inserts

The prepared niosomes were loaded in selected preparations of blank ocular inserts. The ocular inserts were prepared as described above. An appropriate weight of niosomes containing 1 mg or 2 mg of pilocarpine HCl (calculated based on the entrapment efficiency results) was incorporated into the produced polymer blend solution (at room temperature) and stirred on a magnetic stirrer Dlab ms-h280 (Beijing, China), 200 rpm for 10 min to ensure homogeneous dispersion. The resulting dispersion was poured into silicon moulds and placed on the bench top for complete drying at room temperature. The quantity of added niosomes was based on the average daily dose of

pilocarpine HCl (1–2 mg) [9]. Table 2 summarises the composition of all prepared pilocarpine HCl loaded ocular inserts.

Table 2. Summary of the composition of pilocarpine HCl loaded ocular inserts

Formula	Polymer (type and concentration (%w/v))	Plasticiser (glycerine) (mL)	Amount of pilocarpine HCl (mg)	Solvent volume (mL)
T1	1% HPMC + 1.5% PVA	1.0	1.0	50
T2	1% HPMC + 1% PVA	1.0	1.0	50
T3	1% HPMC + 1% PVA	1.0	2.0	50
T4	1% HPMC + 1.5% PVA	1.0	2.0	50

Analytical method optimisation and validation for pilocarpine HCl

High performance liquid chromatography (HPLC) was employed for the quantitative analysis of pilocarpine HCl. The method was based on [7] with modification. A Dionex Ultimat HPLC System from Thermo Fisher Scientific Inc. (Sunnyvale, California, USA), with gradient pump, UV detector set at 215 nm, and C-18 analytical column from Phenomenex® (Luna® 5 µm, LC column 250 × 4.6 mm, particle size 5 µm) was used. The mobile phase was composed of solution A (1.35% v/v phosphoric acid and 0.3% v/v mL triethylamine in HPLC grade distilled water (pH adjusted to 3 using 10 M NaOH)) and solution B (methanol). The two solutions ratio A:B was 90:10%v/v, column temperature (20 ˚C), flow rate (1.5 mL/min), and sample injection volume (10 µL). Pilocarpine HCl samples were analysed over the concentration range of 3.9–1000 µg/mL using solution A as diluent. The HPLC method was validated according to ICH guidelines in terms of specificity, accuracy, precision, and linearity, limits of detection, and limit of quantification [20].

Characterization of the drug free ocular inserts

Ocular insert/film quality

The blank ocular inserts were evaluated based on multi-dimensional criteria such as smoothness, transparency, flexibility, good wetting and spreading ability, non-sticky and easy peeling according to the method described in [21, 22]. Evaluation scores were given for each feature out of 20 (0 bad, 10 good, 20 very good) based on visual inspection. The total of scores were calculated out of 100.

Weight uniformity

The blank and the niosome loaded films were cut into small pieces with dimensions of 0.5 × 1.0 cm. Each piece was weighed individually using a Sartorius analytical lab scale digital balance (AC/120 S MC-1). The mean, standard deviation, and relative standard deviation for the values were calculated using 5 replicates.

Disintegration time

One piece with a dimension of 0.5 × 1.0 cm for each formulation of the blank ocular insert was placed in 3 mL artificial tears on a hotplate set at 37 °C. The disintegration time was monitored. Disintegration time was defined as the time required to start breaking down of the films into small pieces. This test was repeated for three samples.

Moisture content

To measure the moisture content of the drug free ocular inserts, each film was weighed to attain its initial weight (W_i) and then heated at 100–120 °C in a hot air oven until a constant final weight was achieved. The final weight (W_f) of the dried film was then measured, and the moisture content (MC) was calculated using Eq. (1).

1

MC\%=Wi-WfWi×100

Characterization of pilocarpine HCl niosomes

Entrapment efficiency

The amount of pilocarpine HCl entrapped within the niosomes was investigated by placing 1 mL of the formula in 1.5 mL Eppendorf tubes followed by centrifugation using a DLAB refrigerated centrifuge (D3024R) at 7000 rpm and 4℃ for 1 h. The supernatant was collected, then, 1 mL of phosphate buffer pH 7.4 was added to wash the vesicles and re-centrifuged for 1 h. This step was repeated twice, and supernatant was collected each time. Then, 1 mL of isopropyl alcohol was added to lyse the washed niosomes and the mixture was centrifuged for 30 min at 7000 rpm. A 100 µL sample was withdrawn and transferred into a 5 mL volumetric flask containing 1 mL isopropyl alcohol. The solution was then made up to 5 mL using phosphate buffer pH 7.4. The drug content was analysed using HPLC at 215 nm to calculate the total entrapped drug within the niosomes. The collected supernatants were combined in a 10 mL volumetric flask and made up to volume using phosphate buffer pH 7.4. The drug content was then analysed by HPLC at 215 nm to calculate total free drug in supernatant which was used to calculate the recovery and entrapment efficiency ENT (%) according to the following equations:

2

PILT=PILN+PILS

PIL_T is the total amount of used pilocarpine HCl, PIL_N is the amount of pilocarpine HCl within the niosomes, whereas PIL_S is the total amount of the pilocarpine HCl within the supernatant (i.e., free drug).

3

ENT(%)=PILNPILT×100%

Microscopic analysis of niosomes

One drop of the pilocarpine HCl loaded niosomes was placed on a glass slide and an optical Nikon microscope (Tokyo, Japan) was used to assess the prepared niosomes at 100× magnification.

Laser diffraction analysis

Laser diffraction was employed to analyse average particle size of the niosome, polydispersity index (PDI), and zeta potential. The parameters were measured via photon correlation spectroscopy using a Zetasizer Nano ZS90 instrument from Malvern Instruments (Worcestershire, UK). Niosomes were diluted with deionised water and sonicated at low amplitude (~30 Hz) for 10 s prior to analysis. Results were reported as mean ± standard deviation (SD) using triplicate samples.

Characterization of drug loaded inserts

Content and thickness uniformity

The content uniformity of each ocular insert was evaluated by measuring the amount of pilocarpine HCl in each insert. Each ocular insert was placed in a 10 mL volumetric flask containing 8 mL phosphate buffer (pH 7.4) and 2 mL isopropyl alcohol. The flask was sonicated for 8 h using a Bandelin Sonorex Digitec sonicator to ensure complete dissolution of the film. Each experiment was repeated three times. Samples were withdrawn from the flask and analysed by HPLC. Film thickness was measured using an Erweka TBH 30 tester (Hessen, Germany), and the average, standard deviations, and relative standard deviations were calculated.

Surface pH

Measurement of the surface pH of the final pilocarpine HCl loaded ocular inserts was carried out according to the method described in [23], where 1 mL of distilled water was applied onto the film in a petri dish and allowed to spread evenly over the surface of the film. The surface pH was determined using a digital pH meter, and the results were recorded as mean ± SD using triplicate samples.

Fourier transform infrared (FTIR) spectroscopy analysis

FTIR spectra of pilocarpine HCl, span 60, cholesterol, pilocarpine HCl containing niosomes, blank films and niosome-loaded films were recorded using a Perkin Elmer FTIR spectrometer (OH, USA), associated with Spectrum 10 software. A sample was loaded onto the sample holder and each sample's FTIR spectrum scans was recorded over the range of 450–4000 cm^{-1} with a resolution of 2 cm^{-1}.

Drug release study

Niosome loaded ocular inserts containing 1 or 2 mg pilocarpine HCl were used in the donor compartment model

using cellulose dialysis membrane and 2 mL phosphate buffer pH 7.4, which was used to mimic in vivo cornea epithelial membrane conditions. This donor compartment model was placed in a beaker containing 50 mL of phosphate buffer, pH 7.4. The beaker was placed on a magnetic stirrer plate at a temperature of 37 °C and set at 60 rpm. At time intervals of 1 h for the duration of 3 h and then after 24-h, 1 mL samples of the release medium were removed and placed in separate vials and then analysed by HPLC. 1 mL of phosphate buffer pH 7.4 maintained at the same temperature was replaced to maintain sink conditions [24].

Differential scanning calorimetry (DSC) analysis

DSC analysis of pilocarpine HCl and niosomes was carried out using a DSC Q200- TA instrument (USA). Around 5 mg of each sample was placed onto an aluminium pan and heated at a rate of 10 °C/minute under continuous nitrogen purging (50 mL/minute).

X-ray diffraction (XRD) analysis

An X-ray diffractometer (MiniFlex 600 benchtop diffractometer (RigaKu, Tokyo, Japan)) was used to investigate the physical form of niosomes, pilocarpine HCl and niosome loaded ocular inserts. The XRD experiments were performed over the 2θ range from 5 to 90°, with Cu Kα radiation (1.5148227 Å) and a voltage set at 40 kV and a 15 mA current. Designated samples were placed on a holder and scanned in triplicate. Data was recorded at a scanning speed of 5°/minute. OriginPro® software was employed to analyse the scans (OriginLab Corporation, USA).

Statistical analysis

Statistical analysis was performed using SPSS statistical pack (version 25) with $p<0.05$ quotes as the level of significance for the calculation of one-way ANOVA test. Results were reported as mean ± SD (standard deviation).

Results

Quantification of pilocarpine HCl

A calibration curve for pilocarpine HCl was obtained over a concentration range of 3.9–1000 µg/mL. The curve demonstrated linearity with high degree of corelation (coefficient of determination $R^2 = 0.9999$, regression equation Y =0.1135×- 0.0591). The method was specific to pilocarpine HCl and none of used materials (HPMC, PVA, glycerine, cholesterol, span 60) interfered with the pilocarpine HCl peak that eluted at 5.72 ± 0.18 min. A peak representing the blank ocular insert appeared before 2 min. Recovery experiments for three concentrations in triplicate over three days were conducted to determine the intraday repeatability and (inter-day) reproducibility as an indication of the method's accuracy. The results are shown in Table 3 and demonstrate method precision with RSD below 6.04%. All recovery results ranged between 96.7 and 101.8% with low RSD (between 0.96 and 6.04%) indicating an accurate method. RSD between 0 and 15% is an indication of method accuracy. Similar results were reported in a previous study on pilocarpine HCl [22]. Equipment precision was evaluated using repeated measures of one selected concentration (62.5 µg/mL) of pilocarpine HCl solution and the results demonstrated precise equipment owing to low RSD (0.44%) and according to the ICH guidelines [20] a RSD of less than 2%, indicates an accurate and precise technique. The method limit of detection (LOD) and limit of quantification (LOQ) of pilocarpine HCl were 1.2 µg/mL and 3.65 µg/mL respectively. Overall, the employed method demonstrated a valid and reproducible process for the analysis of pilocarpine HCl.

Table 3. Accuracy of pilocarpine HCl HPLC method (intraday and inter-day reproducibility)

Pilocarpine HCl concentration (µg/mL)	Intraday recovery % (mean ± SD), n = 3	RSD %	Inter-day recovery % (mean ± SD), n = 9	RSD %
250	100.54 ± 1.45	5.11	101.8 ± 0.41	1.42
125	98.95 ± 0.84	6.04	101.2 ± 0.14	0.96

62.5	96.71 ± 0.52	4.60	101.7 ± 0.18	2.53

Characterization of drug free (blank) ocular inserts

The first part of the project aimed to optimise a drug-free (blank) ocular insert by enhancing its smoothness, transparency, flexibility, wetting/ spreadability, and ease of peeling off. Additionally, the project aimed to develop films that disintegrate within a specific time frame.

To do that, various formulations of drug-free ocular inserts were investigated (Table 1). The produced blank ocular inserts were characterised based on film quality, weight uniformity and disintegration time. The prepared HPMC containing formulations are shown in Fig. 1, revealed films with a transparent and smooth appearance. Increasing the HPMC content above 1% (w/v) resulted in thick films with reduced flexibility, even at lower volumes. However, the transparent appearance, which was observed in all the HPMC films, was not affected by increasing HPMC content from 0.5% to 2% w/v.

Fig. 1 [Images not available. See PDF.]

Images of HPMC blank ocular inserts at different concentrations (w/v), **a** HPMC 0.5% -F1 **b** HPMC 1%-F4, **c** HPMC 1.5%-F8, **d** HPMC 2%-F11

Representative samples of PVA containing blank ocular inserts are shown in Fig. 2. Increasing PVA content from 0.5 to 2% w/v led to harder films, which were less flexible, and difficult to handle. Transparent and smooth appearance was observed in all preparations regardless of the PVA content. PVA 2% w/v films demonstrated a hard and less flexible appearance like a paper sheet (Fig. 2d), despite increasing the plasticiser (glycerine) concentration from 0.25 to 1% v/v. Several researchers employing HPMC and/or PVA in the development of ophthalmic films observed similar trends [25, 26].

Fig. 2 [Images not available. See PDF.]

Images of blank ocular inserts at different PVA concentrations (w/v), **a** PVA 0.5% -F13, **b** PVA 1% -F16, **c** PVA 1.5% -F19, **d** PVA 2% -F23

Blank ocular inserts made of a blend between PVA and HPMC are presented in Fig. 3: Films (a) and (b)—containing 1% HPMC with 1% and 1.5% PVA respectively—produced very smooth, flexible, and transparent appearance. However, increasing the amount of the PVA in the blend, led to a reduction in the flexibility, transparent appearance, and an increase in the hardness of the films. Film (c) presented a harder film than (a) and (b); because of the high percentages of PVA (2%) and HPMC (1.5%). Blending polymers in the formulation of films produced smooth and flexible films with suitable disintegration time for ocular insertion, due to inherent characteristics of the individual polymers.

Fig. 3 [Images not available. See PDF.]

Images of blend films (HPMC + PVA) at different concentrations (w/v), **a** HPMC1% + PVA 1% -F34, **b** HPMC 1% + PVA 1.5% -F30 **c** HPMC 1.5% + PVA 2% -F29

Quality of blank ocular inserts

The blank ocular inserts were further evaluated according to multifaceted criteria. Each criterion was given a score and the total score for each film is depicted in Table 4. F30 and F33 demonstrated superior properties with a score of 100 and were chosen for incorporation of pilocarpine HCl niosomes. From Table 4, lower concentrations of HPMC (0.5–1.5%) fail the extended disintegration time target, while higher HPMC concentrations had longer disintegration times but were hard to cast on casting dishes. All PVA containing films exhibited very long disintegration time. However, the co-polymer films particularly F30 (1% HPMC, 1.5% PVA) and F33 (1% HPMC, 1% PVA) retained the excellent properties of the films in terms of smoothness, transparency, flexibility, wetting / spreadability and ease to peel off; as well as produced films that had a disintegration time within the targeted window.

Table 4. Ocular inserts/ films quality characterization and scoring based on the evaluation criteria

Formula	Characterization of the films evaluation score (0 bad/10 good/20 very good)	Disintegrationtimescore10verygoodinterval(3–5 h)otherval u

							ebad(0)
Smoothness	Transparent appearance	Flexibility	Good wetting and spreading ability	Non-sticky and easily peeled off	Total/100	F1	20
20	20	20	20	100	0	F2	20
20	10	20	0	70	0	F3	20
20	20	20	20	100	0	F4	20
20	20	20	20	100	0	F5	20
20	20	20	20	100	0	F6	20
20	20	20	20	100	0	F7	20
20	20	10	20	90	0	F8	20
20	20	10	20	100	0	F9	20
20	10	10	20	80	0	F10	20
20	10	0	20	70	10	F11	20

20	10	0	20	70	10	F12	20
20	10	0	20	70	10	F13	20
20	20	20	20	100	0	F14	20
20	20	20	20	100	0	F15	20
20	10	20	0	70	0	F16	20
20	20	20	20	90	0	F17	20
20	20	20	10	90	0	F18	20
20	20	20	10	90	0	F19	20
20	20	20	20	100	0	F20	20
20	20	20	20	100	0	F21	20
20	20	20	20	100	0	F22	20
20	10	20	20	90	0	F23	20

20	10	20	20	90	0	F24	20
20	10	20	20	90	0	F25	10
10	0	10	20	50	0	F26	10
10	0	10	20	50	0	F27	10
10	0	10	20	50	0	F28	10
10	0	0	20	40	0	F29	10
10	0	0	20	40	0	F30	20
20	20	20	20	100	10	F31	20
20	20	10	20	90	10	F32	20
20	20	10	20	90	10	F33	20
20	20	20	20	100	10	F34	20
20	20	20	20	100	0	F35	20

Weight uniformity and disintegration time

The results of weight uniformity and disintegration time of the blank films are presented in Table 5. The produced films showed good weight uniformity as evident from the low %RSD value, except for few batches. Good spreadability of the liquid polymer is related to weight uniformity of the final dry film. Overall, the two selected batches (F30 and F33) demonstrated good weight uniformity and low RSD.

Table 5. Weight uniformity and disintegration time of the formulations of the blank film

Formula	Weight uniformity (mg) (mean ± SD), RSD) n = 10	RSD (%) of weight uniformity	Disintegration time
F1	1.09 ± 0.33	30.41	15.23 ± 10.1 min
F2	0.87 ± 0.01	1.14	11.43–6.58 min
F3	0.67 ± 0.08	12.29	8.55–4.25 min
F4	1.13 ± 0.1	8.6	40.67 ± 12.25 min
F5	0.91 ± 0.1	10.99	32.0 ± 10.05 min
F6	0.75 ± 0.1	12.95	25.67 ± 5.52 min
F7	3.12 ± 0.29	9.17	68.67 ± 18.57 min
F8	2.76 ± 0.42	15.3	58.25 ± 9.25 min
F9	2.09 ± 0.14	6.56	50.52 ± 12.89 min
F10	6.03 ± 1.52	25.15	4.57 ± 0.85 h
F11	4.9 ± 0.42	8.6	3.89 ± 0.58 h
F12	3.34 ± 0.34	10.29	3.55 ± 1.61 h
F13	4.45 ± 0.52	11.76	> 7 h
F14	4.11 ± 0.13	3.13	> 7 h
F15	3.9 ± 0.54	3.1	> 7 h
F16	9.9 ± 0.84	8.48	> 7 h
F17	6.56 ± 1.4	21.36	> 7 h
F18	4.25 ± 0.41	9.62	> 7 h

F19	11.51±2.69	23.33	>7 h
F20	4.13±0.13	3.24	>7 h
F21	3.54±0.46	12.86	>7 h
F22	5.26±0.71	12.54	>7 h
F23	7.47±1.28	17.11	>7 h
F24	1.51±0.18	11.87	>7 h
F25	10.87±2.73	25.15	>7 h
F26	5.2±0.40	7.69	>7 h
F27	6.01±0.54	8.96	>7 h
F28	13.89±0.79	5.66	>7 h
F29	7.47±0.85	11.36	>7 h
F30	7.23±1.23	6.95	3.80±0.56 h
F31	6.61±0.56	8.54	3.2±0.74 h
F32	4.32±0.45	10.46	3.05±0.66 h
F33	13.91±2.2	5.85	3.59±0.35 h
F34	8.83±0.89	10.09	2. 98±0.25 h
F35	4.26±0.15	3.53	2.12±0.16 h

Overall, the blank films have uniformity in the weight with up to 2 standard deviations. Formulations F10, F11, F12, F30, F31, F32 and F33 achieved the ideal target disintegration times for ocular inserts (within 3–5 h).

Characterization of niosomes

The produced niosomes were based on previous work [7]. The produced niosomes were initially characterised before inclusion within the optimal blank film formulations.

Microscopic analysis of niosomes

Light microscopic analysis of pilocarpine HCl loaded niosomes was done at 100× magnification. The results are presented in Fig. 4. Images of niosomes showed spherical vesicles with cavities, which could hold entrapped drug. Aggregation of the spherical niosomes appeared as lines (Fig. 4c).

Fig. 4 [Images not available. See PDF.]

Light microscopic images of niosomes loaded with pilocarpine HCl prepared by thin film hydration method at 100×

magnification **a** selected area of niosomes for zoom in **b** zoomed in area highlighting the structure of the niosomes **c** Example of few aggregates

However, results using laser diffraction analysis for particle size analysis are presented in Table 6. The average particle size of the niosomes was within the nanoscale. The produced particles demonstrated an acceptable distribution and a large negative zeta size which promotes particle stability.

Table 6. Particle size, polydispersity index and zeta potential of pilocarpine HCl loaded niosomes (mean ± SD, n = 3)

	PSA (nm)	PDI	Zeta (mV)
Niosomes	325.7 ± 3.5	0.463 ± 0.044	− 51.32 ± 1.41

Entrapment efficiency and pilocarpine HCl within the niosomes

The average entrapment efficiency of pilocarpine HCl within the niosomes was 49.7% ± 7.0 (n = 3). This number was employed for the calculation of the amount of niosomes to be added to each ocular insert for the preparation of the ophthalmic inserts with niosomes.

FTIR spectra

The FTIR spectrum of cholesterol (Fig. 5a) revealed characteristic bands between 2800 and 3000 cm^{-1} which are attributed to asymmetric and symmetric stretching vibrations of CH_2 and CH_3 groups. The observed broad and intense band nearly at 3400 cm^{-1} is attributed to OH stretching. Cholesterol has one double band (C=C) in the second ring. This was prominently shown at 1674 cm^{1}. The band at 1464 cm^{-1} is due to asymmetric stretching vibrations of CH_2 and CH_3. The sharp peak at 1055 cm^{-1} can be attributable to ring deformation of cholesterol. The bands between 900–675 cm^{-1} are characterised due the C–H out of plane bending which are the characteristic of the aromatic substitution pattern and mainly determined by the number of adjacent hydrogen atoms on the ring and not much affected by the nature of substitutions. Similar results were reported in [27]. FTIR analysis of pilocarpine HCl (Fig. 5b) presented a C–O stretching band at 1026 cm^{-1}. C–N stretching at 1178 cm^{-1}, N–H bending band at 1765 cm^{-1} and C–H stretching at 3079 cm^{-1}. Similar results were reported by [10]. Figure 5c presents the IR spectra of span 60 which showed a broad peak at 3384 cm^{-1} due to O–H stretch bonded, strong peak at 2916 cm^{-1} due to C–H stretch, peak at 1735 cm^{-1} due to C=O stretch, and C–O stretch peak at 1175 cm^{-1}. Similar results were reported by [28]. Therefore, the niosome spectra (Fig. 4d) showed a broad band at 3400 cm^{-1} due to O–H stretch bond in span 60, a band at 2916 cm^{-1} due to C–H alkane, N–H bending band in pilocarpine HCl at 1765 cm^{-1}, while the C–O stretch group in pilocarpine HCl caused the peak at 1175 cm^{-1}.

Fig. 5 [Images not available. See PDF.]

FTIR Spectra and chemical structures for **a** cholesterol, **b** pilocarpine HCl, **c** span 60, and **d** niosomes

Characterization of pilocarpine HCl niosome loaded ocular inserts

The drug loaded insert with different concentrations of pilocarpine HCl and polymer types, appeared as thin, smooth, and flexible films, easy to handle. An amount of niosomes containing the equivalent of 1 mg pilocarpine HCl for formulations T1 and T2, and 2 mg pilocarpine HCl for formulations T3 and T4 were incorporated into the films. The drug loaded inserts were cut into small pieces (2.5 × 5 mm) to be suitable to insert into the eye (see Fig. 6).

Fig. 6 [Images not available. See PDF.]

Digital photographs of pilocarpine HCL niosomal loaded ocular inserts

The result of the weight uniformity, content uniformity, moisture content, thickness, and surface pH are shown in Table 7.

Table 7. The result of weight uniformity, content uniformity, moisture content, thickness and pH of drug loaded niosomal inserts

Formula	Weight uniformity (mean ± SD) n = 10	Content uniformity %(Mean, n = 3)	Moisture content (mean ± SD) n = 3	Thickness (mm) (mean ± SD) n = 3	pH (mean ± SD)
T1	15.84 ± 2.06	92.5% ± 0.1	35.5% ± 2.32	0.41 ± 0.02	7.1 ± 0.1
T2	12.77 ± 2.37	89.2% ± 0.02	39.6% ± 6.63	0.55 ± 0.1	6.9 ± 0.01
T3	14.0 ± 1.23	86.08% ± 0.2	33.6% ± 8.54	0.64 ± 0.02	6.9 ± 0.2
T4	16.21 ± 1.25	96.08% ± 0.7	30.8% ± 0.72	0.95 ± 0.02	7.0 ± 0.1

The results revealed that the niosomal loaded ocular inserts demonstrated good content uniformity that ranged from 86.08 to 96.08% with low standard deviation that was below 2%. Further, the average moisture content of the ocular films was ranging from 30.8 to 39.6%. The surface pH of the ocular inserts is vital to ensure minimal eye irritation upon insertion. The surface pH of the drug loaded insert was in the range of 6.9–7.1.

Xray diffraction analysis

XRD pattern of pilocarpine HCl (Fig. 7a) demonstrated characteristic sharp peaks at 13.68°, 17.97°, 21.34°, 24.77° and 32.41° suggesting a crystalline material. Similar results were reported by Alyami et al. [7]. The blank film (Fig. 7b) demonstrated a shallow wide heap indicating the amorphous nature of the blend polymers. The niosome loaded films demonstrated characteristic peaks of the constituents of the niosomes (cholesterol and span 60). Due to the low load of pilocarpine HCl within the film, several peaks of pilocarpine HCl were not observed.

Fig. 7 [Images not available. See PDF.]

XRD Spectra of **a** pilocarpine HCl, **b** the blank film and **c** pilocarpine HCl niosome loaded ocular insert

DSC analysis

The thermal profiles of pilocarpine HCl, niosomes, blank film as well as drug-loaded niosomal ocular inserts are depicted in Fig. 8. The thermogram of pilocarpine HCl (Fig. 8a) portrayed the crystalline nature of the material with sharp endothermic melting peak at 206.39 °C and an enthalpy of 20.95 J/g. Similar results were recorded by [7]. Figure 8b demonstrates the thermogram of the niosomes. This showed interesting findings, the first peak of the niosomes at around 60 °C could be attributed to span 60, while the second peak at 97.33 °C and enthalpy of 121.64 J/g probably represents cholesterol. However, the reported cholesterol melting point is between 145 and 150 °C [29, 30]. Several studies reported the effect of developing niosomes on the melting point of cholesterol with a possible shift towards a lower range [31, 32]. The blank film (Fig. 8c) showed its amorphous nature with no melting peak observed. The thermogram of niosomes loaded films (T4) as can be seen in Fig. 8d showed a sharp endothermic peak at 43.24 °C with enthalpy of 7.04 J/g which represents the melting point of span 60. The second peak at 83.20 °C probably represents the melting point of cholesterol with another shift. The pilocarpine HCl melting peak does not appear probably owing to the encapsulation of the API within niosomes [31].

Fig. 8 [Images not available. See PDF.]

DSC spectra of **a** pilocarpine HCl, **b** niosomes, **c** blank film and **d** niosome loaded ocular insert (T4)

In vitro release study

The release of pilocarpine HCl from the drug loaded ocular inserts can be seen in Fig. 9 demonstrating an initial burst release within 2 h that ranged from 26.54% (T4) to 41.22% (T2), probably due to free, unencapsulated drug; followed by a slow extended release. The extended release was attributed to the release of pilocarpine HCl from the niosomes. At 24 h, the pilocarpine HCl released from inserts ranged from 68.32 ± 5.11% (T4) to 82.11 ± 6.01% (T2). Formulation (T1 and T4) containing higher percentage of (HPMC and PVA) demonstrated slower extended-release

profile. However, using one-way ANOVA and Tukey post-test, there was no significant difference among the four formulations ($p>0.05$).

Fig. 9 [Images not available. See PDF.]

Release study of pilocarpine HCl loaded niosomal ocular inserts (mean ± SD, n = 3)

Discussion

It has been reported that the prevalence of glaucoma, a leading cause of vision loss, is between 3 and 5% in people aged 40 years and older worldwide. Due to the rapid increase in the global population, this number is expected to rise to 112 million people by 2040 [32, 33]. This is an alarming number of anticipated patients, which demands an immediate improvement in the current therapeutic approaches. Currently, the only evidence-based treatment for glaucoma is to lower the intraocular pressure through surgery or medications [34]. However, the effectiveness and long-term efficacy of the currently available eye drops for glaucoma treatment are being questioned due to poor patient compliance. Many researchers have tried to improve the effectiveness of eye drops by increasing the pre-corneal residence time of the drugs. This can be achieved through the use of eye inserts or contact lenses. Compared to eye drops, these alternative forms of medication have demonstrated a substantial improvement in drug bioavailability [35–37].

The purpose of this study was to develop and optimise an ocular insert that slowly releases pilocarpine HCl to reduce the side effects of conventional eye drops due to dose dumping and the need for frequent drug administration. Initially, we attempted to identify the best formula to make blank ocular inserts/ films. Blending polymers, enabled the incorporation of higher polymer concentrations that allowed for longer disintegration time while retaining the flexible, smooth, and transparent appearance of the inserts. In general, an ideal ophthalmic drug delivery system should be able to administer drugs accurately without causing blurred vision or irritation. It should also have suitable mucoadhesive properties to improve drug retention in the pre-corneal area, thereby increasing drug bioavailability. Additionally, the system should reduce the need for frequent dosing to improve patient compliance [8, 38].

However, individual polymer-containing films were not capable to produce the desired disintegration time and film quality. HPMC produced moderate strength films with very good elasticity and transparency, while PVA produced less flexible films but with higher strength and resilience. The selection of HPMC was based on its non-ionic, good mucoadhesive, and lubricant properties, that would allow the insert to adhere to the surface of mucosa. HPMC was used for simulated tears formulations for the management of dry eye [39]. PVA is a commonly used biocompatible, hydrophilic, good reinforcing biodegradable polymer for ophthalmic preparations [40, 41]. To the best of our knowledge, this is the first report of a blend of these two polymers for formulation of ophthalmic inserts.

The aim was to create films with optimal properties, particularly for use in the eye. The eye is a very sensitive organ; therefore, the films needed to be very smooth, flexible, and transparency to avoid discomfort to patients. Previously marketed inserts were discontinued due to patient inconvenience and bad patient experience with the films [18]. The targeted disintegration time of the films was 3–5 h which is the time for the biodegradable components of the films began to dissolve. The hypothesis was that drug release of 12 h would still be achievable without the complete film remaining in the eyes for that duration. Therefore, F30 and F33 were selected as optimal formulations owing to their excellent properties in terms smoothness, transparency, flexibility, wetting / spreadability and ease to peel off; as well as produced films that had a disintegration time within the targeted window and were used for further analysis. Similar trends were observed in ocular films developed for the sustained release of timolol [42]. The slow disintegration of the film promotes the extended-release profile of the drug and extend the drug retention time in the pre-corneal region.

During the next step, the focus was on optimising the niosomes that would contain pilocarpine HCl. This was done to ensure that there were two barriers in place to prevent dose dumping: the biodegradable polymeric film matrix and the extended release niosomal formulation. Niosomes are commonly used vesicular nanocarriers for ophthalmic drug delivery [37, 43]. The use of niosomes minimises dose dumping, had high encapsulation efficiency when

compared to liposomes and are more stable [7, 43]. Furthermore, niosomes possessed favourable photoprotection effect along with rheological characteristics pertinent to spreadability and viscosity [44]. It was crucial to maintain a small particle size for the produced niosomes. Our research showed that the average particle size for the niosomes was within the nanoscale range, which is optimal. Similar results were obtained by Alyami et al. [7]. It is important to keep the particle size of ophthalmic preparations below 10 μm to avoid ocular irritation [10]. The produced particles demonstrated an acceptable distribution and a large negative zeta size, which promotes particle stability.

The next step focused on developing the double barrier formulation comprised of the optimal ocular insert laden with the pilocarpine containing niosomes. The produced films and niosomes were subjected to molecular profiling using FTIR and XRD to understand their physical and chemical properties as well the components' compatibility profile. The analysis of the IR spectra suggests that there are no interactions or incompatibilities between the compositions of the niosomes, including the API, pilocarpine HCl. Furthermore, the XRD analysis did not reveal any shifts or changes to the main constituents of components. Furthermore, the thermal analysis using DSC suggested that pilocarpine HCL might be encapsulated within the niosomes. Similar results were reported where the disappearance of the active ingredient melting point peak could be attributed to encapsulation [7, 45].

The tears which comprises of electrolytes (Na^+, K^+, Cl^-), proteins such as lactoferrin, lysozyme, lipids, and mucins, serve as an excretory and delivery route of metabolic substances and nutrients [46]. The pH of the tears generally ranges from 6.5 to 7.6, with an average value of 7 [47]. Therefore, ophthalmic drug formulations should have a pH between 6.6 and 7.6 to prevent irritation. When developing ocular dosage forms, it is essential to consider the physiochemical and biopharmaceutical features of the API to be administered via the ophthalmic route, such as solubility, permeability, and stability. This should ensure sufficient dissolution of the API as well as diffusion through different ocular barriers.

Two important factors need to be addressed for eye inserts: moisture content and surface pH. The study results revealed that the moisture content of the optimal inserts ranged between 30 and 40%, which is favourable for the user's comfort and could be attributed to the hygroscopic nature of the HPMC [48]. Furthermore, the surface pH level is unlikely to cause irritation or discomfort upon application since the pH of the tears is within the range of 6.5–7.6 [47]. Therefore, all the produced formulations were not expected to have major adverse effects on the eye. The produced formulations (T1-T4) were within the recommended rage of surface pH and hence it is expected not to cause any ocular surface irritation and potentially produce good diffusion [49]. The results of this work is in agreement with other research findings for ocular formulations [43, 50].

The selected optimal formulations showed a promising release profile. The initial burst release of pilocarpine HCl is not expected cause blurred vision, because it is small amount in comparison with the traditional dose of pilocarpine HCl in eye drops. After 24 h, the pilocarpine HCl released from inserts ranged from 68.32 ± 5.11% (T4) to 82.11 ± 6.01% (T2), indicating that the formulations achieved the aim of the study. It is interesting to note that the niosomal ocular inserts loaded with pilocarpine and with a lower PVA content (1%) were able to release pilocarpine at a quicker rate. By adjusting the PVA concentration to 1.5% and increasing the initial pilocarpine content, the rate of polymer erosion and degradation can be regulated, resulting in controlled release in ocular tissues. Therefore, these ocular inserts could potentially serve as an effective delivery platform not only for pilocarpine but also for other APIs on the ocular surface for an extended period of time. Similar results were reported [51].

According to this study, combining two polymers (HPMC and PVA) with API loaded niosomes can achieve two crucial features of ocular inserts: extended retention and sustained release of the API. This is accompanied by favourable physical and chemical properties of the insert, such as surface pH, moisture content, and compatible formulation.

Conclusions

The current study successfully produced biodegradable ocular inserts using HPMC and PVA co-polymer blend, as a drug delivery device for pilocarpine HCl loaded niosomes, to provide sustained release of pilocarpine over 24 h. The purpose was to reduce the dosing frequency, enhance patient compliance and minimize side effects associated with conventional eye drops. The optimised niosomes-loaded ocular inserts were evaluated for their mechanical and

molecular performance. The findings suggested that these inserts are effective in releasing pilocarpine over an extended period while retaining a favourable pH of around 7 – entailing no possible irritation to the eye. The biodegradable ocular inserts were also found to be compatible with formulations' excipient as evident from FTIR and thermal analysis. In conclusion, these niosomes loaded HPMC-PVA ocular inserts provide a potential to be used as a platform for delivery of other pharmaceutical active ingredients for ocular delivery.

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Author contributions

TA: investigation; data curation; formal analysis. ED: conceptualisations; formal analysis, writing—review &editing; supervision; project administration &funding acquisition. SA: review &editing; supervision; AI: conceptualisations; writing—review &editing. The manuscript has been read and approved by all the authors and each author believes that the manuscript represents honest work.

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Availability of data and materials

Any further information can be obtained from the corresponding author upon request.

Declarations

Ethics approval and consent to participate

The experiments reported in this manuscript did not involve any human or animal subjects. The experiments performed in this manuscript comply with the current laws of the country.

Consent for publication

Authors provide consent for publication in the Future Journal of Pharmaceutical Sciences.

Competing interests

The authors declare no competing interests.

Abbreviations

API
Active pharmaceutical ingredient
SD
Standard deviation
DDSs
Drug delivery systems
DSC
Differential scanning calorimetry
FTIR
Fourier transform infrared
HPMC
Hydroxypropyl methylcellulose
LOD
Limit of detection
LOQ
Limit of quantification
PDI
Polydispersity index
PVA

Polyvinyl alcohol

RSD

Relative standard deviation

XRD

X-ray diffractions

Publisher's Note

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Qualitative assessment of the flowering buds of Mesua ferrea Linn with special emphasize on HPTLC and universal DNA bar-coding technique and evaluation of its antimicrobial potential

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ABSTRACT (ENGLISH)

Background

The study has been scientifically exploring the powder sample and extracts of flowering buds of *Mesua ferrea* Linn (FBMF) based on pharmacognostic and phytochemical parameters. The medicinal plant *Mesua ferrea* Linn was identified by Botanical verification and the universal DNA bar-coding technique. The FBMF powder's quality was performed by the micromeritics properties like bulk density, tapped density, angle of repose, Hausner ratio, Carr's index, and optical microscopy method, and physicochemical evaluations were performed by the swelling index, foaming index, loss on drying, extractive values, and ash values. The extract's preliminary phytochemical screening was accomplished by the alkaloids, flavonoids, tannins, steroids, carbohydrates, and glycoside tests. To ensure the presence of a bioactive compound of each FBMF extract by qualitative HPTLC study against the reference β-sitosterol Rf value of 0.83 was revealed at 254 nm with a developed solvent system toluene, ethyl acetate, and acetic acid (6:2:0.1) v/v. Many pharmacological benefits, including those related to wound healing, nonalcoholic fatty liver disease, analgesic, sedative effects, immunomodulatory, anticancer, antimicrobial, anti-inflammatory, anxiolytic, and atopic dermatitis, have been demonstrated by this phytosterol. An antimicrobial study was carried out by a well diffusion method and, lastly, measured minimum inhibitory concentration and compared to the marketed active pharmaceutical component. Numerous skin infections of more invasive, serious illnesses are caused by potential antimicrobial activity.

Results

The universal DNA bar-coding of plant *Mesua ferrea* Linn has shown a high percentage of identity. The micrometrics properties and physicochemical evaluation of the powder sample of FBMF were in an acceptable range. The preliminary phytochemical screening shows that all extracts contain steroids and carbohydrates. Moreover,

flavonoids were found in ethyl acetate and ethanol, and a qualitative HPTLC study confirmed that every extract contains β-sitosterol. N-hexane extract of FBMF shows the potential antimicrobial activity with *Staphylococcus aureus,* and the MIC value was observed at 0.062 mg/ml.

Conclusion

Our research demonstrated that the plant *Mesua ferrea* Linn has been successfully authenticated by a DNA bar-coding technique, and all extracts of FBMF contain β-sitosterol. The n-hexane solvent extracts have shown the potential highest antimicrobial effects compared to other extracts. These results support using n-hexane extracts as a traditional medicine for treating several diseases.

FULL TEXT

Background

Herbal medicinal plants have been used since the prehistoric period for different human diseases and to save people's lives. They have played an essential role in world health [1, 2]. The herbal plant's phytoconstituent's antimicrobial activity is a necessary basis for new pharmacological agents because of antibiotic resistance [3], and it is also a universal problem to be solved through the development of new drugs and discovering new components from different parts of herbal plants in the other solvent extracts[4].

Mesua ferrea Linn, commonly known as Nagakesara in India, is a medium as well as a large-sized tree that can reach about 20–30 m tall, with extremely hard wood, and is used as a stimulant, diaphoretic, and brain tonic. This medicinal plant is also a part of Ayurvedic immunity-boosting remedies like Chyawanprash and Brahma Rasayan. The plant is distributed in several parts of the world, such as Vietnam, Thailand, Sri Lanka, Myanmar, Nepal, Malaysia, China, Cambodia, Bangladesh, and in India like Uttar Pradesh, Tamil Nadu, Sikkim, Odisha, Maharashtra, Kerala, Karnataka, Bihar, Andaman &Nicobar Islands, Assam, and West Bengal. This plant's flowers are complete and bisexual with functional female (gynoecium) and male (androecium) as well as ovary and carpels, stamens, and sometimes polygamous (male and bisexual). The buds are globular 2 mm diagonally, and scales are in axillary buds. This herbal plant's flowering and fruiting times are January to March and April to October [5, 6].

A genetic marker is a nucleotide sequence or gene sequence scheduled on a chromosome that is prospective to different species and differentiates cells. Using spurious and adulteration materials as substitutes significantly impairs user safety and efficacy. The DNA sequences are particular and identified with the help of a known molecular marker to detect the exacting sequence of DNA from an unknown group. This is a suitable approach to identifying herbal plant varieties or populations and some species accurately, rapidly, and effectively [7–9].

The HPTLC, a very sophisticated instrumental technique, is found to be simple, accurate, and rapid for confirmation of medicinal plant material. It has advantageous features, easily separates the multiplex samples, and is an excellent qualitative and quantitative analysis technique. The comprehensive qualitative advance for evaluation of quality, consistency, species authentication, and stability of herbal drugs, and easily differentiated with digital scanning profile [10, 11]. This high-speed and inexpensive technique requires a minute sample to analyze the reproducible results [12]. The HPTLC technique is essential in plant analysis, as a quick judgment of column fractions, detection of plant material, screening for the content of markers, and development of solvent mixture, which is used for column chromatography. The ability to exhibit chromatograms of many samples as an image may be visually assessed or further processed by specific software. This technique is undoubtedly the most striking benefit of HPTLC and one of its fastest-developing application areas. The images of the same plate can be captured before, during, or after derivatization with various illumination modes [13, 14].

The current study is to perform preliminary studies of powder samples and the low polar to high polar solvent extracts of *Mesua ferrea* Linn flowering buds. These studies involve micrometric properties and physicochemical evaluations of the powder sample with six solvent extracts, preliminary phytochemical screening, and a qualitative HPTLC study for confirmation of phytoconstituents with the standards of β-sitosterol, and finally, perform an in vitro antimicrobial study of n-hexane extract with well diffusion method. This medicinal plant can prevent the diseases caused by gram-positive bacteria and substitute several diseases that β-sitosterol prevented and is used as a

traditional medicine and prepares a novel drug delivery system.

Methods

Collection and identification

Flowering buds of *Mesua ferrea* Linn. (FBMF) were collected in March 2021 from West Bengal and authenticated of the plant through Griffin's Herbarium (voucher specimen no. Calophylla.2021/1). The FBMF was picked and removed all dust and sand very carefully. Cleaned flowering buds were cut into tiny pieces and shade-dried. After eight weeks, bud pieces were utterly dried and pulverized into powder form by the grinder (Nano Mixture Grinder MX 116) and, finally, sized reduction through a 60-number mass sieve.

Genotypic identification

The quality of the DNA isolate was examined by using agarose gel electrophoresis. One microliter of 6X gel-loading buffer [30% sucrose in TE buffer (pH-8.0), bromophenol blue (0.25%)] was added to five microliters of DNA. The prepared samples were loaded with 0.8% agarose gel ready to 0.5X Tris–Borate-EDTA buffers containing 0.5 μg/ml of ethidium bromide. Electrophoresis was performed by 0.5X TBE (Tris/Borate/EDTA) as an electrophoresis buffer (75 V) until the bromophenol dye front migrated toward the base of the gel. The gels were observed in a UV transilluminator (Genei), and finally, the image was captured under UV light with a Gel documentation system (Bio-Rad) [8, 15]. This DNA bar-coding method (Universal primers of RBCL) was performed at the Rajiv Gandhi Centre for Biotechnology.

Micromeritic properties

The bulk density of FBMF powder was measured by an accurately weighed (20 g) field to a graduated cylinder, and the bulk volume was noted. The bulk density was calculated by the powder mass divided by the powder volume and tapping a graduated cylinder until the stable volume was observed, and then, the tapped volume was noted. This density was calculated by the powder's mass divided by the tapped powder volume. The following formula calculated the Hausner ratio and Carr's index/Hausner ratio = tapped density/bulk density and percentage of Carr's index = (tapped density − bulk density)/tapped density × 100. The angle of repose was performed to evaluate the flow property of FBMF powders with the help of a vertically fitted funnel. The powder sample was passed through the funnel onto a horizontal surface on graph paper, and powder pile height (h) and base radius (r) were determined. Finally, the angle of repose (θ) was calculated with the help of the $(\theta) = \tan^{-1}(h/r)$ equation [16].

The optical microscopic method was used to estimate the particle size (0.2 μm to 100 μm) with the help of an ordinary microscope after calibration of the eyepiece micrometer. The crude powder sample (FBMF) was placed on a glass slide and observed through a microscope. The particle diameter (length and breadth) was calculated and recorded in the list of 300 particles. The average particle size was calculated, and recorded data were represented in a size distribution curve [17, 18].

Extraction of FBMF

The FBMF was extracted through slightly modified methods of Oliveira et al. [19]. Briefly, the FBMF powder was extracted through cold maceration methods at room temperature, according to the low polar solvent to high polar solvents such as n-hexane, petroleum ether, ethyl acetate, acetone, ethanol, and finally with water. The FBMF powders (150 g) were soaked in 400 ml of n-hexane solvent continuously for seven days in a conical flask, and filtration was done with the help of Whatman No.1 filter paper. Then, the marc was dried for 24 h, and again used the marc for the successive solvent. Every filtrate sample was collected in a previously weighted Petri dish, evaporated at 40 °C temperatures through a water bath, kept in desiccators, and transferred into a well-closed container for additional use.

Physicochemical evaluation

The physicochemical parameters of FBMF were determined by the quality control methods, according to the updated edition of World Health Organization 2011 and Farooq et al. methods, such as loss on drying, extractive yield, swelling index, ash values (total ash and acid insoluble ash), and foaming index [20, 21].

Preliminary phytochemical screening

Various classes of phytochemicals on FBMF extracts were detected through the preliminary phytochemical

screening. The alkaloid on FBMF was detected by Dragendorff's, Hager's, and Wagner's test, and flavonoids were evaluated by Shinoda's alkaline reagent and concentrated H_2SO_4 test. The detection of tannin was performed with the Braymer's, Gelatin, and NaOH test, and the presence of steroids was detected by the FeCl test. The carbohydrates were detected by Molisch's, Seliwanoff's, and Resorcinol tests, and finally, glycoside through Legal's, NaOH, and Baljet tests [22, 23].

Development of chromatographic profile

Kabra et al. standard HPTLC method was followed to develop a chromatographic profile of various extracts of FBMF [24]. The CAMAG® Linomat 5 (Camag, Muttenz, Switzerland) instrument was used for the chromatographic qualitative method. Each solvent extract of FBMF was developed with the help of a mobile phase that contains toluene, ethyl acetate, and acetic acid (6:2:0.1) v/v to confirm the contingency of various fractions of phytoconstituents. Aluminum plates of TLC Silica gel 60 F_{254} (Sigma-Aldrich, Invoice No ASM/140/21-22) were used as a stationary phase for chromatographic separation. Standard plant steroids β-sitosterol (Sigma-Aldrich, Invoice No TI/2021-22/1028) and every extract (10 µl) were spotted as bands of length 8.0 mm and width 0.0 mm. The prepared solvent systems were transferred to a developed chamber (CAMAG twin trough plate) for 20 min to develop the plate. Then, the resulting plate was dried at room temperature for five minutes and scanned. The developed spots were scanned with the help of TLC Scanner 4(S/N: 270,741) at wavelength 254 nm. Finally, the Rf (retention factor) value for every spot was measured and recorded with the help of Vision CAT software.

In vitro antimicrobial study

The antimicrobial activities of every FBMF extract were examined using a well diffusion assay against *Staphylococcus aureus* (gram-positive bacteria) and *Pseudomonas aeruginosa* (gram-negative bacteria). Briefly, 0.8% of agar was mixed with approximately 24 h of the *Pseudomonas aeruginosa* 1688 and *Staphylococcus aureus* MTCCS96 indicator standard strains and overlaid on a Petri plate containing a bottom with 1.5% of nutrient agar and allowed to solidify. The solvent was prepared by the 9:1 water ratio along with dimethyl sulfoxide and mixed with every extract. Five millimeters in diameter wells were perforated, and 100 µl of the prepared sample extract of FBMF was placed into each well. The agar plates were prepared at room temperature and incubated at 37 °C for 18–24 h. The antimicrobial activities of the samples were determined by measuring the diameters of the inhibition zones. Inhibition was recorded as negative if no zone was observed around the agar well, and the prepared solvent was kept as a control against each extract of FBMF. For MIC, different concentrations of the sample were prepared using prepared solvent, and the minimum concentration zone of inhibition was recorded through plate assay followed by broth microdilution method through the guidelines of the CLSI (Clinical and Laboratory Standards Institute). Every extract sample was dissolved in the prepared solvent and diluted in nutrient broth in a range of (0.015–1) mg/ml [25, 26]. We performed the antimicrobial activity against standard streptomycin (active pharmaceutical ingredients) for better results.

Results

Identification of plant

The polymerase chain reaction (PCR) amplification was accomplished with universal primers RBCL-AF forward primer sequence (5'-TGTCACCACAAACAGAGACTAAAGC-3') A and RBCL-724R reverse primer (5'-TCGCATGTACCTGCAGTAGC-3') within a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). This medicinal plant was given a high percentage of identity.

Micromeritic properties

The micromeritic properties such as bulk density, tapped density, Carr's index and Hausner ratio, angle of repose, and optical microscopic of the FBMF powder are represented in Table 1. The frequency distribution curves (histogram) of the optical microscopic method and the powder sample microscopic views are expressed in Fig. 1.

Table 1. Micromeritic properties and physicochemical evaluation of FBMF powder sample

Micromeritic properties

Parameter	Results
Bulk density (g/cc)	0.39 ± 0.0009
Tapped density (g/cc)	0.54 ± 0.001
Carr's index	18.12 ± 0.273 [good]
Hausner ratio	1.22 ± 0.004
Angle of repose	36.90^{0} ± 0.673
Particle size distribution	
Arithmetic mean (Σnd/Σn) [μm]	34.17
Volume surface mean (Σnd3/Σnd2)[μm]	48.66
Physicochemical evaluation	
Swelling factor (ml)	8.10 ± 0.058
Foaming index (cm)	250 Foaming index is over 1000 [1.4 cm]
Moisture content (%)	1.40 ± 0.058
Total ash value (%)	3.67 ± 0.333
Acid insoluble ash (%)	0.00 ± 0.000
Extractive yield (%)	N-hexane (2.1), petroleum ether (1.9), ethyl acetate (2.3), acetone (3.4), ethanol (3.3), and water (3.9)

Results are manifested as mean ± SEM (standard error of mean) [without particle size distribution and extractive yield]

Fig. 1 [Images not available. See PDF.]

a Optical microscopic view of FBMF powder. **b** Size frequency distribution curve of FBMF powder

Physicochemical evaluation

The obtained results of swelling factor, foaming index, total ash value, acid insoluble ash, moisture content of powder sample, and extractive yield of different solvent extracts of FBMF are represented in Table 1.

Preliminary phytochemical screening

Phytochemical screening of the extract's active constituents was detected by a minimum of three tests (without steroids), such as alkaloids, flavonoids, tannins, carbohydrates, and glycosides which are reported in Table 2.

Table 2. Preliminary phytochemical screening of FBMF extracts

	Qualitative test	n-Hexane	Petroleum Ether	Ethyl Acetate	Acetone	Ethanol	Water
1	*Detection of alkaloid*						
	a) Dragendorff's test	(−)	(−)	(−)	(−)	(−)	(−)
	b) Hager's test	(−)	(−)	(−)	(−)	(−)	(−)
	c) Wagner's test	(−)	(−)	(−)	(−)	(−)	(−)
2	*Detection of flavonoids*						
	a)Conc. H_2SO_4 test	(−)	(−)	(+)	(−)	(+)	(−)
	b) Alkaline reagent test	(−)	(−)	(+)	(+)	(+)	(−)
	c) Shinoda's test	(−)	(−)	(+)	(−)	(+)	(+)
3	*Detection of tannins*						
	a) Gelatin test	(−)	(−)	(−)	(−)	(−)	(−)
	b) Braymer's test	(−)	(−)	(−)	(−)	(−)	(−)
	c) NaOH test	(−)	(−)	(−)	(−)	(−)	(−)
4	Detection of steroids (extracts with FeCl)	(+)	(+)	(+)	(+)	(+)	(+)
5	*Detection of carbohydrate*						
	a) Molisch's test	(+)	(+)	(+)	(+)	(+)	(+)
	b)Seliwanoff's test	(+)	(+)	(+)	(+)	(+)	(+)
	c)Resorcinol test	(+)	(+)	(+)	(+)	(+)	(+)
6	*Detection of glycoside*						
	a) Legal's test	(−)	(−)	(−)	(−)	(−)	(−)
	b) NaOH test	(−)	(−)	(−)	(−)	(−)	(−)
	c)Baljet test	(−)	(−)	(−)	(−)	(−)	(−)

(−) sign indicates absent of phytochemicals, and (+) sign indicates present of phytochemicals

Development of chromatographic profile

The qualitative HPTLC analysis of every extract of FBMF was carried out against standard phytosterol β-sitosterol using a solvent system of toluene/ethyl acetate/acetic acid (6:2:0.1) for assurance of bioactive compounds. The β-sitosterol Rf value of 0.83 was revealed at 254 nm, and these were compared to six extracts of FBMFs. The maximum Rf values of each reading were recorded and are shown in Fig. 2.

Fig. 2 [Images not available. See PDF.]

HPTLC chromatograms of FBMF extracts of different solvents with standard β-sitosterol, at 254 nm spot at Rf 0.83 and Rf value of petroleum ether (0.82), n-hexane (0.81), ethyl acetate (0.81), acetone (0.80), ethanol (0.81), and water (0.81)

In vitro antimicrobial study

The gram-positive strain, *Staphylococcus aureus,* showed antimicrobial activity through FBMF extracts (except water extract), and gram-negative stains, *Pseudomonas aeruginosa,* did not show any activity. The highest MIC value was observed in n-hexane extracts, and the lowest or no activity was produced in the water extracts against S. *aureus*. Antibacterial assay of n-hexane extract sample with various concentrations was measured against *S. aureus* S96, and the highest zone of inhibition was observed at 0.062 mg/ml, as shown in Table 3 and Fig. 3. The antimicrobial activity against standard streptomycin (0.062 mg/ml) against S. aureus shows 14 mm, and the sample (n-hexane extract) shows 13 mm. The MIC values were determined as the lowest concentrations and inhibited bacterial growth by 80% compared to the agent-free growth control.

Table 3. Antibacterial assay of n-hexane extract samples against *S. aureus* S96 with different concentrations

Sl. No	Concentration (mg/ml)	Zone diameter (mm)
1	1	17.45 ± 0.10
2	0.5	15.52 ± 0.09
3	0.25	14.53 ± 0.04
4	0.125	12.79 ± 0.08
5	0.062	10.36 ± 0.03
6	0.031	9.03 ± 0.03
7	0.015	7.07 ± 0.07

Results are manifested as mean ± SEM (standard error of mean)

Fig. 3 [Images not available. See PDF.]

a Antimicrobial activity of different extracts of FBMF**, b** Antimicrobial activity of n-hexane extract of FBMF with different concentrations

Discussion

The World Health Organization suggests accurately identifying plant species for the safety, efficacy, and quality of herbal medicine. Conventional identification methods need an adequate perspective to differentiate the closely related species of the same genus of medicinal plant species. DNA international bar-coding genetic tools have been

developed to identify herbal medicine [27]. This *Mesua ferrea* Linn medicinal plant was authenticated by two methods such as conventional (botanical verification) as well as a universal barcode (DNA bar-coding technique). The powder sample bulk density depends upon the particle shape, the tendency of the particles, and particle size distribution. The tapped density influences the compaction profile and packing arrangement of materials and describes the particle's void space [17]. The bulk density and tapped density of the FBMF powder sample were 0.39 g/cc and 0.54 g/cc. The angle of repose, Carr's index, and Hausner ratio values represent the flow of the powder sample. The angle of repose is also associated with the bulk density, tapped density, and Hausner ratio of the powder sample. The flow properties of the normal powder sample values are a fair range of 16–20 (Carr's index), a passable range of 1.26–1.34 (Hausner ratio), and free-flowing range of 30°–38° (angle of repose) [28, 29]. The FBMF powder sample's Hausner ratio, Carr's index, and angle of repose were found to be in a passable range of 1.22, fair range of 18.12, and free-flowing range of 36.90°, respectively. Moreover, all the studies were obtained by three observations with the help of standard deviation (SD) and standard error of the mean (SEM) for better results. The particle size distribution and particle size of the powder sample also impacted the flow property of the powder [30]. In this study, the FBMF sample arithmetic means and volume surface means were found to be 34.17 μm and 48.66 μm.

The physicochemical characteristics of crude herbal medications provide the most incredible support for standardization and quality control. The presence of hemicelluloses or pectin and mucilage in crude materials is determined in a swelling index, and the foaming index is performed only in saponin-containing plants that generate swelling in an aqueous medium. The presence of water and volatile matter in the powder samples has been calculated by the loss on drying methods for preservation purposes such as the growth of molds or yeasts. Physiological and nonphysiological ash has been determined after the incineration of crude materials by the total ash value methods, and the earthy material, as well as the number of silica contaminants, is measured by the acid insoluble ash methods [20, 21]. In our studies, visible foreign matters were free from more significant parts of the powder sample of FBMF. This plant's swelling index and foaming index were observed to be 8.10 ml, and the foaming index was over 1000 (1.4 cm). In this plant sample, the percentage of moisture content was found to be 1.40%, indicating within the range's limits. The total ash value of 3.67% and no acid insoluble ash were present in this plant, which is an acceptable range and indicate no earth and silica material are present. The extractive yield of the powder sample of FBMF was observed by the cold maceration methods with six solvents (low polar to high polar). The result revealed that the percentage of extractive yield was highest in water extract (3.9%) compared to the other five solvents.

In previous research, phytochemical screening of this plant was performed with only two extracts (aqueous and ethanol). Phytochemicals such as coumarins, saponins, phenols, and flavonoids were present in aqueous extracts, and coumarins, saponins, flavonoids, phenols, and sterols in ethanol extract [31]. The present study performed the phytochemical constituents in six solvent extracts. Every extract showed the presence of steroids and carbohydrates. Chemical components such as alkaloids, tannins, and glycosides are absent in FBMF extracts. The flavonoids were found to be in ethyl acetate and ethanol. The acetone and water extract also showed flavonoids in the alkaline reagent and Shinoda tests, respectively.

Huge populations of the world are used herbal medicine for their health needs. However, still very challenging for quality assurance and quality control of herbal drugs because of elevated unevenness mixed up phytochemicals [32]. Analytical separation techniques such as HPTLC methods have very potent tools for herbal formulation and very accepted methods for quality control of herbal raw materials and the finished product [33]. In a previous research study, Chow et al. showed that the heartwood part of *Mesua ferrea L* contains β-sitosterol [34]. Teh et al. and Ee et al. both research teams isolated β-sitosterol from this herbal plant's root bark extracts [35, 36], and Keawsa-Ard et al. research studies also isolated phytosteroid from the plant stem parts of hexane extract [37]. Our study found phytosterols β-sitosterol in every extract based on our identification of HPTLC qualitative studies. The β-sitosterol bioactive compounds are present in many herbal plant cell membranes. This phytosterol has shown a variety of biological actions in in vitro as well as in vivo studies, such as anti-diabetic, wound healing, nonalcoholic

fatty liver disease, anticancer, analgesic, antimicrobial, immunomodulatory, anti-inflammatory, anxiolytic, sedative effects [38], and atopic dermatitis [39].

Keawsa-Ard et al.'s research showed stems of *M. ferrea* had moderately antibacterial activity against *E. coli* and *Staphylococcus aureus* [37]. The current investigation demonstrated greater effectiveness against *S. aureus* in n-hexane extracts of FBMF, and when compared with the marketed standard, these show a slightly lesser effect. This gram-positive strain is the most infamous and widespread bacterial pathogen, and it is responsible for a significant number of skin infections and hundreds of thousands to millions of more invasive severe infections. It contributes significantly to the emergence of prosthetic joints, surgical sites, cardiovascular infections, respiratory tract infections, and pneumonia [40, 41], and this bacterium has been observed in ninety percent of atopic dermatitis skin lesions, and the density of this gram-positive bacterium tends to raise the severity [42].

Conclusion

The conclusion indicates that the medicinal plant of *Mesua ferrea* Linn has been successfully authenticated through a DNA bar-coding technique and Botanical verification. The FBMF powder's characteristics produced satisfactory results. The preliminary phytochemical screening reveals that the extracts with plant steroids and carbohydrates are present in low polar to high polar solvents extract. The HPTLC qualitative study confirms that every extract of FBMF contains β-sitosterol. The n-hexane solvent extract showed better antimicrobial activity compared to the other extracts. This primary evidence can trigger a potential substitute in various diseases that were prevented by the β-sitosterol (analgesic, anti-inflammatory, anti-diabetic, sedative effects, wound healing, liver disease, anticancer, antimicrobial, atopic dermatitis, immunomodulatory, and anxiolytic) and the disease produced by the gram-positive bacteria *S. aureus* (large quantity of skin infections) can be prevented by this medicinal plant and is also used as traditional medicine as well as a novel herbal drug delivery system.

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Author contributions

Jugal Sutradhar contributed to writing—original draft preparation, and methodology and provided software; Bapi Ray Sarkar supervised and analyzed the data, investigation, visualization, reviewing, and editing.

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Availability of data and material

Data to support the findings of this study are available from the corresponding author and also the first author upon reasonable request.

Declarations

Ethics approval and consent to participate

No human or animal subjects were involved in the study.

Consent for publication

There is no conflict of interest.

Competing interests

The authors state that they are not competing interests.

Abbreviations

FBMF

Flowering buds of *Mesuaferrea* Linn

HPTLC

High-performance thin layer chromatography

TLC

Thin layer chromatography

PCR
Polymerase chain reaction
CLSI
Clinical and Laboratory Standards Institute
MIC
Minimum inhibitory concentration
S. aureus
Staphylococcus aureus
SD
Standard deviation
SEM
Standard error of mean

Publisher's Note

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Design, synthesis and in-vitro anti-depressant activity evaluation of some 2-styrylbenzimidazole derivatives

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ABSTRACT (ENGLISH)

Background

Existing antidepressants possess various adverse effects and so they are not considered as the first line of drug in mild depression. The lack of proper drugs to treat the mild depression on the other hand alleviates severe depressive cases. To overcome this problem, the nucleus of benzimidazole and cinnamic acid having very less toxicity were fused and a small library of 40 compounds was prepared. The library was then screened for ADMET

properties and probable toxicity. Those compounds which had not shown any toxicity as well as possessed better *in-silico* absorption, distribution and metabolism were selected for the first phase of the study. Synthesized compounds were characterized by FTIR, ^{1}H-NMR and ^{13}C-NMR and were screened for in-vitro antidepressant activity by DNPH spectrophotometry.

Result

The compounds MS-3 and MS-8 had shown good antidepressant activity with IC_{50} values of 367.19 μM/mL and 184.56 μM/mL against MAO-A and MAO-B, respectively.

Conclusion

From this study, it can be concluded that the structural requirements for the inhibition of MAO-A and MAO-B were totally different. MAO-A inhibitors required the presence of nitrogen and oxygen containing ring substitutions whereas MAO-B inhibitors required the presence of 4-halogen containing phenyl ring substitutions.

FULL TEXT

Background

Depression is a life-threatening disease which is characteristically different from normal mood swing and short-lived emotional responses to challenges in everyday life. It causes the suffered person to function poorly in any kind of activity, ultimately leading to suicide, which is the fourth leading cause of death mainly in 15–29 years old. According to the Global Health Data Exchange (GHDx) report, 2019 published by Institute of Health Metrics and Evaluation, depression affects 3.8% of the world's population [1]. Approximately 280 million people in the world have depression, 5.02% of which comes under the age group of 20 plus years and 5.71% of which comes under the age group of 60–89 years [2].

Although there are effective treatments for depression, more than 75% of people who lives in poorer countries receive no treatment. The reason may be low resources, a smaller number of trained healthcare providers and the social stigma associated with a mental disorder. Sometimes it is because of misdiagnosis and many times because of wrongly prescribed anti-depressants. The effective treatments for depression include counselling, cognitive treatment and interpersonal psychotherapy, sometimes along with antidepressant medication such as selective serotonin reuptake inhibitors (SSRIs), 5-HT receptor antagonists (5HT-RAs), monoamine oxidase inhibitors (MAOIs) and tricyclic antidepressants (TCAs). These antidepressants are not considered as the first line of treatment for mild depression mainly because of the adverse effects associated with these classes of antidepressants [2].

As the number of suicidal cases is alarmingly increasing day by day which is evident from the daily newspapers, it is the need of the hour to develop potent antidepressants with minimal adverse effects. To do so, we were in search of some chemical scaffold that has been widely explored as different drug molecules, safe to use and poses minimum side effect. The answer we found is benzimidazole, which is one of the most widely explored chemical moiety having diverse biological activities viz. Antiviral [3–7], antimicrobial [8–11], anticancer [12–16] antidiabetic [17–19] etc. along with some reported anti-depressant [20, 21] property also. On the other hand, cinnamic acid which is a widely distributed natural aromatic carboxylic acid have gained the attention of researchers due to its low toxicity, structural diversity and pharmacological actions [22] like anti-inflammatory [23], antioxidant [24], antitumor [25], hypoglycaemic [26], antidepressant [27], and cytoprotective actions of neuroinflammation in neurodegenerative diseases [28]. The antidepressant action of cinnamic acid occurs via important neurotransmitter serotonin, which also has similar mechanism of action with many commercial antidepressants [29].

The designed compounds, 2-styrylbenzimidazole derivatives were synthesized by reacting o-phenylenediamine and cinnamic acid. The two amino groups of o-phenylenediamine were fused with the carboxylic group of cinnamic acid leading to the formation of 2-styrylbenzimidazole.

These 2-styryl benzimidazole derivatives were reported to have anti-bacterial, anti-fungal and anti-tubercular activity [30]. Based on these findings and to facilitate the development of less toxic, easily available, more potent antidepressant drugs present study aims at design and synthesis of some novel 2-styrylbenzimidazole derivatives with further investigating their potential as antidepressants using in-vitro methods. The designed compounds were structurally similar to the standard antidepressant drug rasagiline. Therefore, it is believed that the designed

compounds will exhibit antidepressant activity by inhibiting the monoamine oxidase enzyme.

Methods

In-silico studies

To test the hypothesis presented in this research paper, we have designed a new series of 2-styrylbenzimidazole derivatives. Direct conjugation of these two chemical scaffolds was not possible. Therefore, we have moved one step back and tried conjugating o-phenylenediamine and cinnamic acid which had resulted in the desired nucleus having the benzimidazole ring as well as the styryl part coming from the cinnamic acid (Fig. 1) exhibiting antidepressant effect. It was evident from literatures that the styryl part which is present in all cinnamic acid similar structures like caffeic acid, ferulic acid, coumaric acid might be responsible for possessing the antidepressant activity [29]. The N^1-hydrogen of the nucleus thus generated was substituted with various aromatic or cyclic halides to get the series of different compounds (Table 1).

Fig. 1 [Images not available. See PDF.]

Benzimidazole cinnamic acid-conjugated nucleus

Table 1. Designed library

SL No	Compound code	ArX
1	MS1	*4-chlorobenzenamine*
2	MS2	*benzenesulfonyl chloride*
3	MS3	*5-bromouracil*
4	MS4	*3-chloro-4-fluoroaniline*
5	MS5	*N-chlorosuccinimide*
6	MS6	*p-chlorotoluene*
7	MS7	*1,4-dichlorobenzene*
8	MS8	*1,4-dibromobenzene*
9	MS9	*1-(2-chloroethyl)piperidine*
10	MS10	*4-(2-chloroethyl)morpholine*
11	MS11	*(4-chlorophenyl)methanamine*
12	MS12	*4-(chloromethyl)benzenamine*
13	MS13	*1,3-dichlorobenzene*
14	MS14	*4-methylbenzene-1-sulfonyl chloride*

15	MS15	*4-chloropyridine*
16	MS16	*5-chloropyridin-2-amine*
17	MS17	*1-chloro-4-(chloromethyl)benzene*
18	MS18	*1-chloro-3-(chloromethyl)benzene*
19	MS19	*3-(chloromethyl)oxazolidine*
20	MS20	*4-chloro-N,N-dimethylbenzenamine*
21	MS21	*1-chloro-4-methoxybenzene*
22	MS22	*1-chloro-4-(2-chloroethyl)benzene*
23	MS23	*1-(2-chloroethyl)-4-fluorobenzene*
24	MS24	*1-chloro-3-(2-chloroethyl)benzene*
25	MS25	*2,4-dichloro-1-(2-chloroethyl)benzene*
26	MS26	*1-(2-chloroethyl)-2,4-difluorobenzene*
27	MS27	*2-chloropyridine*
28	MS28	*1-chloronaphthalene*
29	MS29	*Chlorobenzene*
30	MS30	*2-chloro-1H-pyrrole*
31	MS31	*2-chlorothiophene*
32	MS32	*1-chloro-2-methylbenzene*
33	MS33	*4-chlorophenol*
34	MS34	*2-chlorophenol*
35	MS35	*1-chloro-4-methoxybenzene*
36	MS36	*1-chloro-4-methylpiperidine*
37	MS37	*1-chloro-4-methylpiperidine*

38	MS38	*1-chloro-4-methylpiperazine*
39	MS39	*1-chloro-4-ethylpiperazine*
40	MS40	*1-chloro-4-nitrobenzene*

Molecular property calculation

Different molecular properties like LogP value (Octanol–water partition coefficient), TPSA (Total Polar Surface Area), molecular weight, nRB (Number of Rotatable Bond), HBD (Hydrogen Bond Donor) and HBA (Hydrogen Bond Acceptor) that are considered under Lipinski's rule of five were determined using the free online software 'molinspiration property calculator.'

ADMET prediction

The series of compounds was then used to calculate the ADMET descriptors (absorption, distribution, metabolism, excretion and toxicity) using Discovery studio 3.1 (Accelrys, San Diego, CA, USA). The descriptors selected for this study are absorption, solubility, AlogP98 and PSA_2D values. The model for plotting the confidence ellipse was developed by Egan and Lauri [31] with descriptors that include AlogP98 and 2D polar surface area (PSA_2D). Aqueous solubility was predicted by using a model developed by Cheng and Merz [32] with $R^2 = 0.84$. All the models used for predicting the ADME, toxicity, AlogP98 and PSA_2D have high R^2 values. AlogP98 and PSA_2D were used for plotting the confidence ellipse. Compounds which were found outside the 95% and 99% ellipse region were poorly absorbed compounds (<30% absorbed). The series was then taken for prediction of carcinogenicity and skin irritancy by using the TOPKAT module of Discovery studio 3.1 (Accelrys, San Diego, CA, USA).

Docking study

Docking was performed on the crystal structure of MAO-A and MAO-B which were retrieved from Protein Data Bank (PDB). Monoamine oxidase (MAO) inhibitors were a standard class of antidepressants which prevent the removal of neurotransmitters like dopamine, serotonin from brain and make these neurotransmitters available preventing depression. Rasagiline was a standard MAO inhibitor. Since the compounds were designed based on the structure of rasagiline, the docking study was carried out against MAO-A and MAO-B. The structural features of 2-styrylbenzimidazole derivatives revealed in this study would help in understanding their structural activity relationship with MAO as novel antidepressants. The PDB ID of the selected protein was 2Z5X for MAO-A [33] and 1S3E for MAO-B [34]. Prior to docking, each protein was prepared using protein preparation wizard of Discovery studio 3.1 (Accelrys, San Diego, CA, USA). Polar hydrogen atoms were added to the proteins and charges were assigned. All the bound water molecules, other heteroatoms and co-crystallized ligands attached to the protein were removed. Subsequently, the 3D structure of protein was optimized by minimizing the energy using CHARMm force field. 2Z5X consists of chain A only. Binding site of this protein was defined (40.753, 16.779, 14.746) around the binding pocket of the co-crystallized ligand *7-methoxy-1-methyl-9H-pyrido[3,4-b]indole* which was also known as Harmine. 1S3E consists of two identical chains A and B. Chain A was kept for the study and chain B was deleted. Binding site was defined (52.793, 154.645, 25.957) around the binding pocket of the co-crystallized ligand *5-Hydroxy-N-Propargyl-1(R)-Aminoindan* (RHP). Docking was done using the CDOCKER of Discovery studio 3.1 (Accelrys, San Diego, CA, USA) which was priory validated by calculating RMSD between the docked poses and the X-ray pose of the respective co-crystallized ligands.

Chemistry

AR grade chemicals and solvents were used without further purification for doing the synthetic and analytical work. Melting point apparatus (BUCHI Melting Point M560) at 10 °C/min temperature gradient was used to determine the melting point of the synthesized compounds. The UV-Spectra (λ_{max}) of the synthesized compounds were recorded on Shimadzu, UV- 1800, UV–VIS spectrophotometer instrument. The FT-IR spectra of the synthesized compounds were recorded on Bruker ALPHA FTIR spectrometer. The ^{1}H-NMR spectra of the synthesized compounds were recorded in DMSO at 300 MHz by Bruker Avance DPX 300 NMR spectrometer and ^{13}C-NMR was also recorded in

DMSO at 100 MHz by Bruker Avance DPX 100 NMR spectrometer. The mass spectra of the synthesized compounds were recorded on ZQ-4000 equipped with an Electrospray Ionizer as an ionization method. The eight selected compounds were synthesized by using the scheme 1.

Scheme 1 [Images not available. See PDF.]

Synthetic scheme of the compounds MS 1-MS 8

Synthesis of 2-styryl-1H-benzo[d]imidazole

The compound was synthesized by adding cinnamic acid to a mixture of hydrochloric acid and o-phenylenediamine. It was then refluxed in ethylene glycol for 5 h within a temperature range of 70–90 °C. After this, the reaction mixture was cooled to room temperature and poured into water. Resulting solid was filtered, resuspended in water and pH of the solution was adjusted to≥7 using sodium bi -carbonate. The product was filtered, washed, dried and recrystallized with ethanol.

General procedure for synthesis of final compounds

2-styryl-1H-benzo[d]imidazole was dissolved in quantity sufficient THF, basified using sodium carbonate and cooled to (0–5) °C in ice-bath. The respective aryl/cyclic amine was added slowly maintaining the temperature range of (0–5) °C for additional 15 min. Thereafter the media was warmed upto room temperature and stirred for 24 h followed by solvent evaporation. Cold water was added to the resulting mass, filtered and air-dried to obtain the crude product.

In-vitro anti-depressant activity screening

Isolation and preparation of MAO sample

MAO was isolated using brain tissues of chicken. The dissected pieces of the brain tissues were washed with 0.3 M sucrose solution and frozen at−80 °C for further analysis. The brain tissue (2.5 g) was homogenized in 1:40 (w/v) ratio with 0.3 M ice-cold sucrose solution and centrifuged at 1824 g for 10 min. The supernatant was collected and further centrifuged at 12,768 g for 35 min to obtain crude MAO protein precipitations. This precipitate was resuspended in 250 ml of 0.3 M sucrose solution and mixed with 20 ml of 1.2 M sucrose solution. The precipitate was again centrifuged with 1.2 M sucrose solution at 12,687 g for 40 min followed by a single wash with potassium phosphate buffer (pH 7.60, 100 mM). The pure brain MAO protein precipitate was suspended in 10 ml of potassium phosphate buffer; and stored in aliquots of 1 ml at−80 °C for subsequent analysis [22, 23].

Determination of protein concentration

The protein concentration of MAO precipitate was calculated using Hartree Lowry method [24–26]. Serial dilutions of concentrations 0.03 to 0.15 mg/ml were prepared from the stock solution of 0.3 mg/ml bovine serum albumin (BSA) in potassium phosphate buffer. 1.0 ml of each dilution of standard, protein-containing test and buffer for reference were mixed with 0.90 ml of reagent A (2 g sodium potassium tartrate, 100 g sodium carbonate, 500 ml 1N NaOH, and water to one liter) in separate test tubes. The tubes were incubated for 10 min in water bath at 50 °C, then cooled to room temperature. 0.1 ml of reagent B (2 g sodium potassium tartrate, 1 g copper sulfate, 90 ml H_2O and 10 ml 1N NaOH) was added to each test tube, mixed and incubated for 10 min at room temperature. 3 ml of reagent C (1 vol of Folin–Ciocalteau reagent diluted with 15 vols of water) was added rapidly to each test tube, mixed and again incubated for 10 min in water bath at 50 °C and cooled to room temperature. The final assay volume was 5 ml. Absorbance was measured at 650 nm.

DNPH spectrophotometry

Potassium phosphate buffer (pH 7.60, 25 mM) and 200 µL of MAO protein homogenates were mixed and incubated for 20 min at 37 °C. Then 200 ml of 0.016 M benzylamine in buffer (for detecting MAO-B) and 150 ml of 0.02 M 5-HT (for detecting MAO-A) were added to the above mixture and incubated for 60 min. After this 400 ml of 2 M DNPH in 1 M HCl was added. After incubation for 40 min at room temperature, 2 ml of 1.25 M NaOH containing 5 g/l of Triton X-100 was added and the reaction mixture was kept for an additional 30 min at room temperature. Finally, absorption was measured at 465 nm for MAO-B and 425 nm for MAO-A.

Results

In-silico studies

The designed library was screened through lipinsky rule of five by calculating molecular properties and filtered through ADMET prediction filter. Only those compounds which have passed through both these filters were selected for docking, synthesis and in-vitro anti-depressant activity evaluation.

Molecular property calculation

Out of the forty compounds of the library, 23 had shown one violation of lipinsky rule whereas remaining 17 compounds had not shown any violations and taken for further screening (Table 2).

Table 2. Molecular properties of designed ligands

Compound code	miLogP	TPSA	nAtoms	MW	nHBA	nHBD	nNiolations	nRB
MS1	4.33	43.85	24	311.39	3	2	0	3
MS2	4.89	51.97	26	360.44	4	0	0	4
MS3	2.99	83.55	25	330.35	6	2	0	3
MS4	4.85	43.85	25	329.38	3	2	0	3
MS5	3.03	55.21	24	317.35	5	0	0	3
MS6	4.92	17.83	24	310.4	2	0	0	3
MS7	4.65	17.83	24	330.82	2	0	0	3
MS8	4.39	17.83	24	375.27	2	0	0	3
MS9	5.7	21.06	25	331.46	3	0	1	5
MS10	3.86	30.30	25	333.44	4	0	0	5
MS11	4.44	43.85	25	325.42	3	2	0	4
MS12	5.93	43.85	25	325.42	3	2	1	4
MS13	6.12	17.83	24	330.82	2	0	1	3
MS14	5.34	51.97	27	374.46	4	0	1	4
MS15	6.06	30.72	23	297.36	3	0	1	3
MS16	4.22	56.74	24	312.38	4	2	0	3
MS17	6.25	17.83	25	344.85	2	0	1	4

MS18	6.23	17.83	25	344.85	2	0	1	4
MS19	3.83	30.30	23	305.38	4	0	0	4
MS20	5.36	21.06	26	339.44	3	0	1	4
MS21	5.31	27.06	25	326.40	3	0	1	4
MS22	6.04	17.83	26	358.87	2	0	1	5
MS23	5.95	17.83	26	342.42	2	0	1	5
MS24	6.44	17.83	26	358.87	2	0	1	5
MS25	7.07	17.83	27	393.32	2	0	1	5
MS26	6.04	17.83	27	360.41	2	0	1	5
MS27	4.78	30.72	23	297.36	3	0	0	3
MS28	6.63	17.83	27	346.43	2	0	1	3
MS29	5.25	17.83	23	296.37	2	0	1	3
MS30	4.72	33.62	22	285.35	3	1	0	3
MS31	5.46	17.83	22	302.40	2	0	1	3
MS32	5.87	17.83	24	310.40	2	0	1	3
MS33	4.78	38.05	24	312.37	3	1	0	3
MS34	5.20	38.05	24	312.37	3	1	1	3
MS35	5.31	27.06	25	326.40	3	0	1	4
MS36	4.89	21.06	23	303.41	3	0	0	3
MS37	5.13	21.06	24	317.44	3	0	1	3
MS38	3.88	24.30	24	318.42	4	0	0	3
MS39	6.25	17.83	25	344.85	2	0	1	4
MS40	5.21	63.65	26	341.37	5	0	1	4

ADMET prediction

All the compounds of the designed library fall inside 95% and 99% ellipse region (Fig. 2). From the results of TOPKAT toxicity prediction, it was observed that no compounds had shown carcinogenicity but all except eight had shown mild skin irritancy (Table 3). Thus, only eight compounds of the series had passed all the filters and so they were considered for further studies.

Fig. 2 [Images not available. See PDF.]

Confidence ellipse plot

Table 3. Toxicity, solubility, drug likeliness accounted by TOPKAT in Discovery Studio 3.1

Compound code	ADMET solubility level	ADMET absorption level	ADMET AlogP98	ADMET PSA 2D	Carcinogenicity	Skin irritancy
MS 1	2	0	4.774	43.149	–	–
MS 2	1	0	5.095	51.21	–	–
MS 3	2	0	2.578	76.831	–	–
MS 4	1	0	4.979	43.149	–	–
MS 5	1	0	3.664	54.563	–	–
MS 6	1	1	6.007	16.609	–	–
MS 7	1	1	6.185	16.609	–	–
MS 8	1	1	6.269	16.609	–	–
MS9	1	0	6.269	16.609	–	Mild
MS10	1	1	5	19.961	–	Mild
MS11	2	0	6.192	16.609	–	Mild
MS12	1	1	3.735	28.891	–	Mild
MS13	1	0	5.683	19.961	–	Mild
MS14	1	1	5.504	25.539	–	Mild
MS15	1	1	6.442	16.609	–	Mild
MS16	1	1	6.054	16.609	–	Mild

MS17	0	3	6.513	16.609	–	Mild
MS18	1	1	7.177	16.609	–	Mild
MS19	1	0	6.26	16.609	–	Mild
MS20	2	0	4.909	27.87	–	Mild
MS21	0	1	3.77	28.891	–	Mild
MS22	1	0	6.429	16.609	–	Mild
MS23	1	0	5.52	16.609	–	Mild
MS24	1	0	4.739	31.664	–	Mild
MS25	1	1	5.357	16.609	–	Mild
MS26	1	0	6.007	16.609	–	Mild
MS27	1	0	5.278	37.424	–	Mild
MS28	1	0	5.278	37.424	–	Mild
MS29	1	0	5.504	25.539	–	Mild
MS30	1	0	4.963	19.961	–	Mild
MS31	2	0	5.215	19.961	–	Mild
MS32	2	0	4.626	43.149	–	Mild
MS33	1	0	4.003	23.314	–	Mild
MS34	2	0	5.415	59.432	–	Mild
MS35	1	1	4.781	43.149	–	Mild
MS36	1	0	6.185	16.609	–	Mild
MS37	2	0	5.581	51.21	–	Mild
MS38	2	0	4.37	27.87	–	Mild
MS39	1	1	4.162	16.609	–	Mild

MS40	1	0	6.192	23.314	–	Mild

Docking study

Based on the molecular properties and ADMET studies of all the designed ligands, eight compounds were selected for molecular docking studies. Docking is considered to be successful if the RMSD value between the X-ray pose and the docked pose is less than 2 Å [35]. The RMSD value of Harmine and RHP against 2Z5X and 1S3E is tabulated in Tables 4 and 5. The docking results were analyzed based on the binding energy (Table 6) and the docked poses against MAO-A (2Z5X) (Fig. 3) and MAO-B (1S3E) (Fig. 4). The binding energies of the ligands were within the range of − 114.89 to − 53.32 kcal/mol. The ligands showed very high negative binding energies as compared to the standard Harmine/RHP.

Table 4. RMSD calculation against 2Z5X

Sl No	Ligands	Reference	RMSD Value
1	HMN 1	HMN xray 6	0.6223
2	HMN 2	HMN xray 6	0.6221
3	HMN 3	HMN xray 6	0.6220
4	HMN 4	HMN xray 6	0.6221
5	HMN 5	HMN xray 6	0.6221
6	HMN xray 6	HMN xray 6	0.0000

Table 5. RMSD calculation against 1S3E

Sl No	Ligands	Reference	RMSD value
1	RHP601 1	RHP601 REF 6	0.6971
2	RHP601 2	RHP601 REF 6	1.5405
3	RHP601 3	RHP601 REF 6	1.5405
4	RHP601 4	RHP601 REF 6	1.5405
5	RHP601 5	RHP601 REF 6	1.5405
6	RHP601 REF 6	RHP601 REF 6	0.0000

Table 6. Molecular docking results of designed ligands against MAO-A and MAO-B

Sl No	Compound code	Binding Energy (-kcal/mol)

MAO-A (2Z5X)	MAO-B (1S3E)	1	MS1
53.32709	64.07646	2	MS2
56.13624	76.78583	3	MS3
114.89789	73.23463	4	MS4
53.86427	81.47093	5	MS5
79.13080	76.61092	6	MS6
71.0277	72.67619	7	MS7
76.74543	84.53666	8	MS8
60.03169	110.21145	9	Harmine/HRP

Fig. 3 [Images not available. See PDF.]

Interaction of ligands against protein 2Z5X

Fig. 4 [Images not available. See PDF.]

Interaction of ligands against protein 1S3E

Chemistry

Intermediate compound: **FT-IR (cm^{-1})**

1449.16 (C=C stretch, aromatic), 1578.95 (C–C stretch, aromatic), 1305.12 (C-N stretch, aromatic), 2967.44 (N–H stretch, secondary), 1630.86 (C=C stretch, aliphatic), 2879.02 (C-H stretch, aliphatic), 681.16 (C-H bend, aromatic).

((E)-4-(2-styryl-1H-benzo[d]imidazol-1-yl)benzenamine) **(MS-1)**

Solubility: Ethanol, DMSO; **R_f value**: 0.38; **M.P:** 115–117 °C; **UV λ_{max}** (DMSO): 253; **FTIR** (cm^{-1}): 1446.67 (C=C stretch, aromatic), 1625.38 (C–C stretch, aromatic), 1281.60 (C-N stretch, aromatic), 2968.09 (C-H stretch, aromatic), 3418.46 (N–H stretch, primary), 1668.09 (C=C stretch, aliphatic), 2878.54 (C-H stretch, aliphatic), 764.59 (C-H bend, aromatic).**^{1}H NMR** (300 MHz, DMSO): δ, ppm: 5.23 (s, 2H, C-NH), 6.56, (d, J=6 Hz, 2H, arom. H), 7.01 (d, J=6 Hz, 2H, Ethylene), 7.37 (t, J=3 Hz, 3H, arom. H), 7.44 (d, J=6 Hz, 4H, Aniline), 7.68 (t, J=3 Hz 2H, Benzimidazole), 7.61 (d, J=6 Hz, 2H, Benzimidazole). **^{13}C NMR** (100 MHz, DMSO-*d6*) δ ppm: 142.73, 135.52, 123.92, 112.46, 133.82, 128.96, 131.30, 117.61, 124.75, 152.07. **Mass:** 312.15 $(M+H)^+$ (Tables 7 and 8).

Table 7. Docking interactions of all the synthesized compounds for both the proteins viz.2Z5X (MAO-A) and 1S3E (MAO-B)

Compound codes	Docking interactions	
MAO- A (2Z5X)	MAO- B (1S3E)	MS-1

Tyr407, Asn181, Ile180, Tyr69, Gln215	Lys296, Gly57, Gln206, Ile198	MS-2
Thr336, Gln216, Tyr407, Ile180	Gln206, Ile198, Ile199, Tyr398, Cys172, Leu171	MS-3
Tyr69, Tyr407, Ile180, Asn104	Ile199, Ile198, Gln206, Tyr398, Tyr60	MS-4
Phe352, Tyr69, Tyr407, Gln215, Ile180, Ile207	Phe168, Leu171, Cys172, Tyr398	MS-5
Tyr444, Tyr407, Gln215, Asn181, Ile207, Ile180	Ile199, Ile198, Phe168, Leu171, Cys172, Gln206, Tyr398, Tyr435	MS-6
Tyr69, Tyr407, Phe352, Ile207, Ile180, Thr336, Cys323	Leu171, Gln206, Tyr60, Tyr435, Tyr398	MS-7
Tyr407, Lys305, Gln215, Ile180	Leu171, Tyr188, Ile198, Tyr398, Gln206	MS-8

Table 8. IC_{50} values obtained for all the synthesized compounds for both MAO-A and MAO-B

Compounds	IC_{50} (μM/mL)

For MAO-A	For MAO-B	MS-1
1051.44	605.04	MS-2
629.24	332.10	MS-3
367.19	430.76	MS-4
900.50	300.42	MS-5
492.53	387.28	MS-6
661.74	497.76	MS-7
536.25	239.04	MS-8
562.01	184.56	Rasagiline

((E)-1-(phenylsulfonyl)-2-styryl-1H-benzo[d]imidazole) **(MS-2)**

Solubility: Ethanol, DMSO; **R_f value**: 0.45; **M.P**: 215–218 °C; **UV λ_{max}** (Ethanol): 274.902; **FTIR** (cm^{-1}): 1447.59 (C=C stretch, aromatic), 1626.22 (C–C stretch, aromatic), 1282.65 (C-N stretch, aromatic), 2967.50 (C-H stretch, aromatic), 1671.62 (C=C stretch, aliphatic), 2878.27 (C-H stretch, aliphatic), 764.19 (C-H bend, aromatic) 1416.27 (S=O stretch, sulfoxide). **^{1}H NMR** (300 MHz, DMSO): δ, ppm: 6.54, (d, *J*=9 Hz, 2H, arom. H), 7.64 (*d*, *J*=6 Hz, 2H, Ethylene), 7.33 (*t*, *J*=3 Hz, 3H, arom. H), 7.44 (*d*, *J*=6 Hz, 2H, Sulfonyl benzene), 7.55 (*t*, *J*=3 Hz, 3H, Sulfonyl benzene), 7.77 (*t*, *J*=3 Hz 2H, Benzimidazole), 7.68 (*d*, *J*=6 Hz, 2H, Benzimidazole). **^{13}C NMR** (100 MHz, DMSO-*d6*) δ ppm: 142.02, 137.19, 135.70, 116.39. **Mass:** 361.09 $(M+H)^+$.

((E)-5-(2-styryl-1H-benzo[d]imidazol-1-yl)pyrimidine-2,4(1H,3H)-dione) **(MS-3)**

Solubility: Ethanol, DMSO; **R_f value**: 0.26; **M.P:** 189 °C; **UV λ_{max}** (Ethanol): 260.60; **FTIR** (cm^{-1}): 1425.64 (C=C stretch, aromatic), 1526.05 (C–C stretch, aromatic), 1278.92 (C-N stretch, aromatic), 3054.26 (C–H stretch, aromatic), 1644.69 (C=C stretch, aliphatic), 2882.33 (C–H stretch, aliphatic), 739.88 (C-H bend, aromatic), 3389.24 (N–H stretch, primary), 1745.86 (C=O stretch, ketone). **^{1}H NMR** (300 MHz, DMSO): δ, ppm: 12.63 (s, 1H pyrimidine NH), 7.19 (s, 1H pyrimidine NH), 7.25 (s, 1H pyrimidinyl H), 7.20 (*d*, *J*=3 Hz, 2H, arom. H), 7.37 (*d*, *J*=3 Hz, 2H, Ethylene), 7.46 (*t*, *J*=3 Hz, 3H, arom. H), 7.67 (*t*, *J*=6 Hz 2H, Benzimidazole), 7.60 (d, J=6 Hz, 2H, Benzimidazole). **^{13}C NMR** (100 MHz, DMSO-*d6*) δ ppm: 140.85, 135.75, 129.07, 150.28, 158.80, 113.27, 115.29, 119.48. **Mass:** 331.11 $(M+H)^+$.

((E)-4-fluoro-3-(2-styryl-1H-benzo[d]imidazol-1-yl)benzenamine) **(MS-4)**

Solubility: Ethanol, DMSO; **R_f value**: 0.79; **M.P:** 180–182 °C; **UV λ_{max}** (Ethanol): 219.40/ 262.20; **FTIR** (cm^{-1}): 1503.72 (C=C stretch, aromatic), 1577.32 (C–C stretch, aromatic), 1272.79 (C-N stretch, aromatic), 3023.42 (C-H stretch, aromatic), 1638.71 (C=C stretch, aliphatic), 2973.94 (C-H stretch, aliphatic), 743.72 (C–H bend, aromatic), 1223.22 (C–F stretch, halogen), 3056.78 (N–H stretch, primary). **^{1}H NMR** (300 MHz, DMSO): δ, ppm: 3.32 (s, 2H, NH_2, floroaniline), 7.15 (s, 1H, floroaniline), 7.20 (*d*, *J*=3 Hz, 1H, floroaniline), 7.18 (*d*, *J*=3 Hz, 1H, floroaniline), 7.23 (*d*, *J*=12 Hz, 2H, arom. H), 7.49 (*d*, *J*=6 Hz, 2H, Ethylene), 7.45 (*t*, *J*=3 Hz, 3H, arom. H), 7.67 (*t*, *J*=6 Hz 2H, Benzimidazole), 7.60 (*d*, *J*=3 Hz, 2H, Benzimidazole). **^{13}C NMR** (100 MHz, DMSO-*d6*) δ ppm: 142.38, 135.67, 128.07, 133.59, 146.38, 123.97, 150.46, 115.93, 146.39. **Mass:** 330.14 $(M+H)^+$.

((E)-1-(2-styryl-1H-benzo[d]imidazol-1-yl)pyrrolidine-2,5-dione) **(MS-5)**

Solubility: Ethanol, DMSO; **R_f value**: 0.76; **M.P:** 130–134 °C; **UV λ_{max}** (Ethanol): 262.80; **FTIR** (cm^{-1}): 1420.47 (C=C stretch, aromatic), 1522.82 (C–C stretch, aromatic), 1275.16 (C-N stretch, aromatic), 3026.31 (C-H stretch,

aromatic), 1639.45 (C=C stretch, aliphatic), 2923.04 (C-H stretch, aliphatic), 741.21 (C-H bend, aromatic), 1701.75 (C=O stretch, ketone). **^{1}H NMR** (300 MHz, DMSO): δ, ppm: 2.42 (t, J=3 Hz, 4H, pyrrolidone CH), 7.24 (d, J=3 Hz, 2H, arom. H), 7.40 (d, J=6 Hz, 2H, Ethylene), 7.46 (t, J=6 Hz, 3H, arom. H), 7.58 (t, J=3 Hz 2H, Benzimidazole), 7.70 (d, J=3 Hz, 2H, Benzimidazole). **^{13}C NMR** (100 MHz, DMSO-*d6*) δ ppm: 139.90, 133.56, 129.44, 132.60, 132.88, 171.35, 128.96. **Mass:** 318.12 $(M+H)^{+}$.

((E)-2-styryl-1-p-tolyl-1H-benzo[d]imidazole) **(MS-6)**

Solubility: Ethanol, Acetone, DMSO, 1,4-dioxane; **R_f value:** 0.81; **M.P:** 187–190 °C; **UV λ_{max}** (Ethanol): 262.40; **FTIR** (cm^{-1}): 1419.06 (C=C stretch, aromatic), 1522.88 (C–C stretch, aromatic), 1274.73 (C-N stretch, aromatic), 3058.58 (C-H stretch, aromatic), 1642.43 (C=C stretch, aliphatic), 2979.18 (C-H stretch, aliphatic), 746.76 (C-H bend, aromatic). **^{1}H NMR** (300 MHz, DMSO): δ, ppm: 2.52 (s, 3H, CH_3-toluene), 7.23 (d, J=3 Hz, 4H, Toluene), 7.18 (d, J=3 Hz, 2H, arom. H), 7.37 (d, J=3 Hz, 2H, Ethylene), 7.45 (t, J=3 Hz, 3H, arom. H), 7.67 (t, J=3 Hz 2H, Benzimidazole), 7.55 (d, J=3 Hz, 2H, Benzimidazole). **^{13}C NMR** (100 MHz, DMSO-*d6*) δ ppm: 137.51, 136.56, 128.96, 133.31, 137.51, 123.92, 129.44, 135.89, 112.46. **Mass:** 311.15 $(M+H)^{+}$.

((E)-1-(4-chlorophenyl)-2-styryl-1H-benzo[d]imidazole) **(MS-7)**

Solubility: Ethanol, DMSO; **R_f value:** 0.26; **M.P:**175–177 °C; **UV λ_{max}** (Ethanol): 208.60; **FTIR** (cm^{-1}): 1423.61 (C=C stretch, aromatic), 1522.54 (C–C stretch, aromatic), 1277.75 (C-N stretch, aromatic), 3051.80 (C-H stretch, aromatic), 1643.65 (C=C stretch, aliphatic), 2981.48 (C-H stretch, aliphatic), 736.25 (C-H bend, aromatic), 687.74 (C–Cl stretch, halogen). **^{1}H NMR** (300 MHz, DMSO): δ, ppm: 7.18 (d, J=3 Hz, 4H, chlorobenzene), 7.52 (d, J=9 Hz, 2H, arom. H), 7.37 (d, J=3 Hz, 2H, Ethylene), 7.45 (*t*, *J*=3 Hz, 3H, arom. H), 7.67 (t, J=6 Hz 2H, Benzimidazole), 7.25 (d, J=9 Hz, 2H, Benzimidazole). **^{13}C NMR** (100 MHz, DMSO-*d6*) δ ppm: 137.10, 135.89, 128.96, 133.31, 123.92, 126.60, 142.73. **Mass:** 331.90 $(M+H)^{+}$.

((E)-1-(4-bromophenyl)-2-styryl-1H-benzo[d]imidazole) **(MS-8)**

Solubility: Ethanol, Acetic acid, Chloroform, 1,4-dioxane, DMSO; **R_f value:** 0.27; **M.P:** 210 °C; **UV λ_{max}** (Ethanol): 262.60/ 209.40; **FTIR** (cm^{-1}): 1494.01 (C=C stretch, aromatic), 1531.22 (C–C stretch, aromatic), 1282.24 (C-N stretch, aromatic), 3052.23 (C-H stretch, aromatic), 1621.52 (C=C stretch, aliphatic), 2968.07 (C-H stretch, aliphatic), 805.35 (C-H bend, aromatic), 695.53 (C–Br stretch, halogen). **^{1}H NMR** (300 MHz, DMSO): δ, ppm: 7.15 (d, J=3 Hz, 4H, bromobenzene), 7.31 (d, J=3 Hz, 2H, arom. H), 7.38 (d, J=3 Hz, 2H, Ethylene), 7.54 (t, J=3 Hz, 3H, arom. H), 7.69 (t, J=3 Hz 2H, Benzimidazole), 7.47 (d, J=3 Hz, 2H, Benzimidazole). **^{13}C NMR** (100 MHz, DMSO-*d6*) δ ppm: 142.73, 135.89, 126.63, 132.51, 137.74, 121.06, 112.46. **Mass:** 376.04 $(M+H)^{+}$.

In-vitro anti-depressant activity of the synthesized compounds

Determination of protein concentration

As described in the materials and methods section, the MAO protein concentration in the test sample was determined by plotting a standard curve (Fig. 5) between absorbance and concentration, which was found to be 189.51 µg/mL.

Fig. 5 [Images not available. See PDF.]

BSA standard curve

DNPH Spectrophotometry

The DNPH spectrophotometric analysis was first carried out without the presence of any drug that had resulted the absorbance reading of the control at 425 nm and at 465 nm (A_0). The steps of this method were repeated for the standard drug rasagiline as well as the test compounds MS 1 to MS 8 at various concentrations. Therefore, for each concentration of the standard drug rasagiline and the test compounds two values of absorbance (A_1) were recorded at 425 nm and at 465 nm (Additional file 1: Table 8). From the absorbances, % inhibition of MAO-A and MAO-B was calculated using the equation % inhibition=$[\{(A_0-A_1)/A_0\}\times 100]$, where, A_0=Absorbance of control and A_1= Absorbance of sample [22]. These % inhibition values (Additional file 1: Table 8) were plotted against the log concentration to obtain the dose response curve of rasagiline as well as the synthesized compounds against MAO-A (Additional file 1: Fig. 7) and against MAO-B (Additional file 1: Fig. 8). Then concentration of the test solutions that

inhibit the hydrolysis of the substrate by 50% (IC_{50}) were determined by nonlinear regression using log dose vs. normalized response-variable slope by GraphPad Prism 9 (Additional file 1: Table 9).

To bridge the gap between computational findings and real-world applications, at the very beginning of docking, the root mean square deviation (RMSD) was calculated between the docked pose and X-ray pose of co-crystallized ligand. This is tabulated in Table 4 and 5. If the RMSD is less than 2 Å, it was considered that docking was successful and the compounds were docked in the exact place where the co-crystallized ligand was bound.

Structure activity relationship studies

From the in-vitro antidepressant assay and from the docking studies, it was observed that the compound MS-3 had the highest negative binding energy against 2Z5X and lowest IC_{50} value against MAO-A. Whereas the compound MS-8 had the highest negative binding energy against 1S3E and lowest IC_{50} value against MAO-B. The IC_{50} values for the other compounds were in accordance with the docking results. For all the compounds under investigation, it can be concluded that, lower is the IC_{50} value higher is the negative binding energy. The IC_{50} values of MS-3 and MS-8 against MAO-A and MAO-B, respectively, were comparable with that of the standard drug rasagiline.

From this study, it can be suggested that incorporation of nitrogen/oxygen substituted five- or six-member ring in the N^1 position of 2-styrylbenzimidazole derivatives which can exhibit different interactions with amino acid residues Ile180, Ile207 and Tyr407 might help in developing promising MAO-A inhibitors. Whereas incorporation of 4-halogen containing phenyl ring in the N^1 position of 2-styrylbenzimidazole derivatives which can exhibit different interactions with amino acid residues Leu171 and Tyr398 might help in developing promising MAO-B inhibitors.

Discussion

The docking study of MS-3 against the MAO-A protein 2Z5X suggested the presence of two pi–pi interaction with amino acids Tyr69 (linked with benzimidazole ring) and Tyr407 (linked with the benzene ring of cinnamic acid). It also suggested the presence of five hydrogen-bonded interaction with amino acids Ile180, Asn184and Ile207 of 2Z5X (Fig. 3). The oxygen and the nitrogen of the uracil ring which was present in the N^1 position of MS-3 were actively involved in formation of these hydrogen-bonded interaction. The next better IC_{50} value as well as binding energy against MAO-A and 2Z5X was observed for the compound MS-5, which also had oxygen and nitrogen atoms in a five membered succinimide ring present as a substitution in the N^1 position of MS-5. Two pairs of pi–pi interaction with the amino acids Tyr407, Tyr444 and four hydrogen-bonded interactions with the amino acids Gln215, Ile180, Asn181, Ile207 were observed in the binding pocket of MS-5 (Fig. 3). All the other synthesized compounds does not possess any nitrogen and oxygen containing ring substitutions which might be the reason of poor IC_{50} value of all other synthesized compounds against MAO-A. Some common amino acid residues like Tyr407, Ile 207 and Ile180 were involved in the binding pocket of most of the compounds under investigation as well as the standard Harmine (Additional file 1: Fig. 5). The compounds MS3, MS5 and MS7 were showing better activity that of the standard drug rasagiline. The decreasing order of antidepressant activity of the synthesized compounds against MAO-A can be represented as:MS3>MS5>MS7>MS8>MS2>MS6>MS4>MS1

The docking study of MS-8 against the MAO-B protein 1S3E suggested the presence of two pi–pi interaction with amino acids Tyr60 and Tyr398 (both were linked with benzimidazole ring). It also suggested the presence of two hydrogen-bonded interaction with amino acids Leu171 and Tyr188 of 1S3E (Fig. 4). The bromine and the hydrogen of the bromobenzene ring which was present in the N^1 position of MS-8 were actively involved in formation of these hydrogen-bonded interaction. The next better IC_{50} value as well as binding energy against MAO-B and 1S3E was observed for the compound MS-7, which also had chlorine and hydrogen atoms in chlorobenzene ring present as a substitution in the N^1 position of MS-7. One pair of pi–pi interaction with amino acid Tyr398 and three hydrogen-bonded interactions with the amino acids Gln206, Leu171 and Tyr398 were observed in the binding pocket of MS-7 (Fig. 4). The compound MS-4 also possesses a fluorine atom in the ortho-position (with respect to the bond of joining of the aniline ring to benzimidazole ring) of the substituted aniline ring which might be the reason of its non-involvement in any kind of interaction with the protein 1S3E. Whereas the fluorine atom in MS-4 might be required for the additional stability of the compound in the binding pocket of 1S3E, depicted by the third better IC_{50} value against MAO-B. The IC_{50} values of all the other synthesized compounds were very high which might be due to the

absence of any halogen atom in the 4th position of the substituted ring. The common amino acid residues involved in the binding pocket of other synthesized compounds and standard drug RHP (Fig. 4) with 1S3E were Leu171 and Tyr398. MS8 had shown comparable activity as that of the standard drug rasagiline. The decreasing order of antidepressant activity of the synthesized compounds against MAO-B can be represented as:MS8>MS7>MS4>MS2 >MS5>MS3>MS6>MS1

Conclusion

The docking study of the synthesized compounds had shown interaction with similar amino acid residues with that of the X-ray pose co-crystallized ligands like RHP and Harmine. This finding might pave the way for the development of 2-styrylbenzimidazole derivatives as potent antidepressants. The compounds MS-3 and MS-8 had shown good antidepressant activity by inhibiting MAO-A and MAO-B, respectively. The optimization of the designed 2-styrylbenzimidazole derivatives with the structural findings of the present work might lead to the development of novel and potent antidepressant drugs.

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Author contributions

MS carried out the synthetic and *in-vitro* work. RC was involved in the *in-silico study*. PP interpreted the FTIR spectral data and the in-vitro results. FA interpreted the NMR spectral data. RSD was involved in manuscript writing and language editing. SS was involved in the study concept, study design and editing of the manuscript.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

ADMET

Absorption, distribution, metabolism, excretion, and toxicity

FTIR

Fourier transform infrared

^{1}H-NMR

Proton nuclear magnetic resonance

^{13}C-NMR

Carbon-13 nuclear magnetic resonance

DNPH

2,4-Dinitrophenylhydrazine

MAO-A

Monoamine oxidase A

MAO-B

Monoamine oxidase B

GHDx

Global health data exchange
SSRIs
Selective serotonin reuptake inhibitors
5-HT receptor antagonists
5-Hydroxytryptamine receptor antagonists
MAOIs
Monoamine oxidase inhibitors
TCAs
Tricyclic antidepressants
TPSA
Total polar surface area
nRB
Number of rotatable bond
HBD
Hydrogen bond donor
HBA
Hydrogen bond acceptor
PSA-2D
2D polar surface area
TOPKAT
Toxicity prediction by komputer assisted technology
PDB
Protein data bank
RHP
5-Hydroxy-N-propargyl-1(R)-aminoindan
RMSD
Root mean square deviation
DMSO
Dimethyl sulfoxide
BSA
Bovine serum albumin
NMR
Nuclear magnetic resonance
THF
Tetrahydrofuran
NaOH
Sodium hydroxide
R_f
Retardation factor
M.P
Melting point

Publisher's Note

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DETAILS

Subject:	Design; Ligands; Age groups; Antidepressants; Hydrocarbons; Toxicity; Hydrogen bonds; Binding sites; Serotonin; Proteins
Location:	United States--US
Company / organization:	Name: Accelrys Inc; NAICS: 513210
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Development of superior chitosan–EDTA microparticles as an adsorbent base for solidifying the self-emulsifying drug delivery systems

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ABSTRACT (ENGLISH)

Background

The present study focused on developing a superior adsorbent carrier (microparticles) to solidify the self-emulsifying drug delivery system. The two approaches, solvent evaporation and spray drying, were explored to synthesize the microparticles using chitosan (CH) and EDTA disodium. The 3^2 full factorial design was applied to optimize the microparticle process produced by both methods.

Results

The various characterization evaluations of the microparticles revealed amide linkages between the CH and EDTA disodium, and XRD results showed that microparticles were amorphous. The SE-CHEM (C_2) and SD-CHEM (Y_1) optimized microparticles were free-flowing and had percentage yield (%), 96 ± 1.2 and 58 ± 1.1, zeta potential (mV), 9 ± 0.44 and 4 ± 0.13, and particle size (μm), 3 ± 0.57 and 2 ± 0.4, respectively. SEM images showed uneven surfaces with wide void spaces and flaky texture for optimized microparticles Y_1 and C_2, respectively. The SE-CHEM (C_2) had an oil adsorption capacity (OAC %) of 46 ± 0.54 and 60 ± 0.77, and oil desorption capacity (ODC %), 38 ± 0.65 and 56 ± 0.86, for Labrafac and Cremophor RH 40, respectively. The SD-CHEM (Y_1) had an oil adsorption capacity (OAC %) of 59 ± 0.71 and 68 ± 0.39, and oil desorption capacity (ODC %), 54 ± 0.11 and 65 ± 0.74, for Labrafac and Cremophor RH 40, respectively. In the surface free energy components analysis, the SE-CHEM (C_2) had an enhanced dispersive component [γ^{LW} (mJ/m^2)] of 32 ± 0.68 and 37 ± 0.47 for Labrafac and Cremophor RH 40, respectively. The SD-CHEM (Y_1) had an enhanced dispersive component [γ^{LW} (mJ/m^2)] of 48 ± 0.7 and 52 ± 0.41 for Labrafac and Cremophor RH 40, respectively. The SE-CHEM (C_2) had enhanced dynamic advancing contact angles [θ_a (°)] of 75 ± 0.19 and 78 ± 0.75 for Labrafac and Cremophor RH 40, respectively. The SD-CHEM (Y_1) had enhanced dynamic advancing contact angles [θ_a (°)] of 74 ± 0.6 and 80 ± 0.21 for Labrafac and Cremophor RH 40, respectively.

Conclusion

All the findings indicate that the microparticles have superior characteristics to serve as the adsorbent base for solid self-emulsifying drug delivery systems.

FULL TEXT

Background

Solid self-emulsifying drug delivery systems (S-SEDDS) are well-proven and known formulation systems. They offer many advantages like increased surface area (which offers high solubility and bioavailability), robustness, high stability, high scalability, ease in handling, high drug loading, better flowability, decreased drug precipitation and economical production [1]. S-SEDDS can be turned into Self-emulsifying pellets and tablets, which are far more stable than SEDDS and S-SEDDS powder [2, 3]. Despite the excellent advantages of S-SEDDS, problems like liquid squeezing out, low disintegration, inadequate hardness and compaction issues are observed. Because of physicochemical interactions between the solid carrier, oil base and surface-active agents, limited release of the formulation can be a problem [4]. Therefore, a suitable adsorbent carrier becomes the most vital part of the stability of the S-SEDDS [5]. Different carriers like PVP K-30, maltodextrin, magnesium stearate, β-cyclodextrin, polyvinyl alcohol, neusilin US2, sodium CMC, dextrin, lactose, mannitol, HP-β-CD, aerosil-200, syloid 244FP, avicel PH102, syloid XDP 3150 and microcrystalline cellulose are used [6].

An ideal solid adsorbent carrier must have high oil adsorption and desorption capacity, wide interparticle void space and low porosity [7]. Usually, hydrophobic carriers have high OAC (oil adsorption capacity) and high flowability [8]. A solid carrier with high OAC is required less in amount, resulting in a lower dosage form [7, 8]. Formulations high in surfactant concentration adsorb well on hydrophobic adsorbents [9]. A high concentration of surfactants forms very small-sized droplets. Hence, an attempt was made to prepare such solid adsorbent carriers (microparticles) of chitosan with EDTA disodium by solvent evaporation and spray drying. Chitosan produces a clear solution with EDTA disodium in acetic acid [10]. This solution was subjected to solvent evaporation and spray drying to produce microparticles, which should be hydrophobic to ensure that the surface of these particles can be used for drug adsorption in the lipid phase [11]. The amide linkages in microparticles form a hydrophobic interpenetrating rough surface [12]. So, the present study aims to optimize microparticles by 3^2 full factorial experimental designs for solvent evaporation and spray drying methods. The chitosan–EDTA microparticles (CHEM) were characterized for their suitable hydrophobic surface to act as a good adsorbent base for S-SEDDS.

Materials and methods

Chitosan 90% DA (deacylated) was purchased from Marine hydrocolloids (Kerala, India). Ethylenediaminetetraacetic acid disodium (EDTA disodium) was purchased from CDH (India). Other chemicals purchased were Acetic acid (Rankem RFCL, India) and diiodomethane (TCI, Japan). Cremophor RH 40 (Himedia, India). Labrafac™ PG was a gift from Gattefossé (Canada). Other chemicals used were of analytical grade. They were used as received.

Design of experiment

To optimize the preparation of blank microparticles (CHEM), 3^2 full factorial design was used. The concentration ratio of chitosan–EDTA disodium and drying temperatures were observed as the critical factors and were two independent variables. Both had three different levels. Factors like zeta potential, percentage yield and particle size were considered dependent variables. All the responses were fitted in the least fit squares regression analysis using JMP 16 (Trial). Various outcomes of the mathematical model, like the actual vs predicted plot, ANOVA analysis, parameter estimates, model equation, prediction profiler, response surface, contour profilers, Pareto charts and optimization desirability, were determined to validate the model [13]. The desirability function was used for the selected responses for the optimization process. So, Y (practical yield, %) had to be maximized, ZP (zeta potential, mV) had to be maximized, and PS (Particle size, μm) had to be minimized.

The overall percentage of chitosan–EDTA disodium in solution was kept at 2% w/v because its viscosity remains adequate for flow at this concentration. Further, as mentioned in Table 1, three different ratios of chitosan–EDTA disodium (40:60, 50:50 and 60:40) were prepared.

Table 1. 3^2 Full factorial design for SE-CHEM

Codes	CH (ratio)	Level codes	DT (°C)	Level codes	Y (%)	ZP (mV)	PS (μm)
A_1	40:60	1	50	1	79±1.3	0.39±0.13	27±2.1
A_2	40:60	70	2	81±0.8	0.43±0.11	16±1.7	A_3
40:60	90	3	87±1.1	0.51±0.16	15±1.1	B_1	50:50
2	50	1	88±0.4	3.00±0.11	14±0.4	B_2	50:50
70	2	90±0.5	3.00± 0.24	11±0.17	B_3	50:50	90
3	91±0.4	4.00±0.45	6±0.9	C_1	60:40	3	50

1	93±0.9	5.00±0.35	4±0.84	C_2	60:40	70	2
96±1.2	9.00±0.44	3±0.57	C_3	60:40	90	3	94±0.8

*CH-CH:EDTA ratio, DT-drying temp., Y-percentage yield, ZP-zeta potential, PS-particle size

Firstly, a weighed amount of chitosan was taken in a beaker and dissolved in 1 M (5.72%) glacial acetic acid at 800 rpm in a magnetic stirrer for two hours. A separate solution of EDTA disodium with double distilled water was prepared. EDTA disodium solution was added dropwise to chitosan solution under continuous vigorous stirring over 2000 rpm with a mechanical stirrer for 25–30 min to get a clear ionic solution [14]. Further, these different solutions were subjected to solvent evaporation and spray drying using a 3^2 full factorial design.

Preparation of Chitosan–EDTA microparticles (SE-CHEM) by solvent evaporation method

All three ratios of chitosan–EDTA disodium solutions were subjected to solvent evaporation in a Rota vacuum evaporator (Micro technologies, India) at a drying temperature range from 50 to 90 °C for 45–60 min, as in Table 2. Then, the dry film was scrapped carefully and dried in an oven to remove residual moisture for 40–50 min at 70 °C. The dried film was converted into powder by using a pestle mortar.

Table 2. 3^2 Full factorial design for SD-CHEM (mention full form of this formulation)

Codes	CH (ratio)	Level codes	DT (°C)	Level codes	Y (%)	ZP (mV)	PS (μm)
X_1	40:60	−1	110	1	26±0.8	−3±0.45	5±1.4
X_2	40:60	130	2	30±1.2	−3±0.24	4±0.8	X_3
40:60	150	3	37±0.6	2±0.8	4±0.6	Y_1	50:50
1	110	1	58±1.1	4±0.13	2±0.4	Y_2	50:50
130	2	44±0.9	2±0.5	2±0.9	Y_3	50:50	150

*CH-CH:EDTA ratio, DT-drying temp., Y-percentage yield, ZP-zeta potential, PS-particle size

Preparation of Chitosan–EDTA (SD-CHEM) microparticles by spray drying

The following overall spray drying specifications were used: Inlet temperature 110–150 °C, outlet temperature 50–70 °C, aspirator speed 1000–2000 rpm, atomization pressure 3 kg/cm^2 and feed pump at 15 rpm in Spray dryer (SprayMate JISL, India). The two ratios of solutions (40:60 and 50:50) were easily spray-dried. It was observed that the high chitosan ratio solution (60:40) posed a challenge during spray drying because of high viscosity; it clogged the 1 mm nozzle of the spray dryer frequently. The operation was stopped to clean the nozzle of the spray dryer, and it was not feasible to spray-dry this solution continuously. So, this solution with a 60:40 ratio of chitosan–EDTA disodium was not spray-dried for all three drying temperatures. Table 2 shows that only six trials were conducted for two ratio solutions.

Evaluation and characterization

Percentage yield

The percentage yield was calculated based on the total recoverable and original weight used [15].

Particle size and morphological evaluation

The surface morphology and topography of the particles were observed by the scanning electron microscope (ZEISS Sigma 360, Germany) at 20 kV (EHT) [16]. The samples were fixed on the SEM stub and coated with a thin layer of gold. Various images were taken at different magnifications. ImageJ (NIH) software was used to determine particle size by analyzing SEM images. The average Feret's diameter was reported.

Zeta potential

100 mg of CHEM prepared by both techniques were taken and constantly stirred in 10 ml triple distilled water. The resulting suspension was centrifuged for 10 min at around 5000 rpm to separate the undissolved particles. Further, this mixture was analyzed for zeta potential in Zetasizer Nano ZS (at wavelength 638 nm) with a scattering angle of 90° at 25 °C. The study was performed in triplicate (Malvern Panalytical, UK) [17].

FTIR analysis

After drying the prepared samples (SD-CHEM, SE-CHEM, CH and EDTA) under vacuum, these were mixed and triturated with KBr (1:100 ratio) to form a fine mixture. This fine mixture was pressed to form the pellets in the KBr press. After placing these pellets in a sample cell, FTIR-ATR analysis was performed (FTIR PerkinElmer spectrum two, USA) in the 500–4000 cm^{-1} spectral range at ambient temperature [18].

DSC analysis

The 5–15 mg samples of SD-CHEM (Y1), SE-CHEM (C2), CH and EDTA were sealed hermetically in aluminum pans. After placing these pans on the sample pan holder and empty pans on reference pan holders in a Differential scanning calorimeter (DSC-25 TA, USA), the thermograms for each sample were recorded from a 40–400 °C temperature range with 10 °C per minute heating rate in a nitrogen atmosphere.

XRD analysis

After grinding each sample (SD-CHEM, SE-CHEM, CH and EDTA) in mortar and pestle, the formed powder was placed and pressed in a sample holder to form a compact powder and X-ray diffraction patterns of the samples were measured using the X-ray diffractometer (XRD Aeris, Malvern Panalytical, UK) at a voltage of 45 kV (with increment size of 0.03° and 0.1 as an increment time).

Flow properties

For all the batches of microparticles, the angle of repose was calculated. In it, graph paper was positioned on a flat horizontal surface, and a funnel was clamped above it with a distance between the paper and the funnel top (about 7–8 cm). 2 g of powder samples for each trial of SE-CHEM and SD-CHEM were weighed and poured into the funnel until the top of the cone-shaped only met the funnel's top. The height (h) and diameter of the cone-shaped heap of powder (D) were measured, and the angle of repose was calculated with a standard formula ($\tan \alpha = 2\ h/D$). The flow is said to be excellent when the angle of repose is less than 25 degrees; on the other hand, the flow is said to be poor when the angle of repose is greater than 40 degrees. For bulk density, 2 g powder was weighed and carefully leveled without tapping into a graduated glass cylinder. To the closest graduated device, the apparent volume before tapping was read as untapped volume (USP method). This volume was used in the standard formula (Bulk density = Weight/untapped volume), and values were calculated. The volume of the powder-filled cylinder was measured after 500 tappings. The tapping was kept running until the frequency difference between the two sets of tapping was less than 0.2 percent. The final volume was noted, and the tapped density in g/ml was calculated using the standard formula (Tapped density = Weight/tapped volume). These readings were used to calculate Carr's index (CI) and Hausner's ratio (HR). {CI = [(Tapped density – Bulk density/Tapped density) × 100] and HR = Tapped density/Bulk density}. A Carr's index of more than 25 indicates bad flowability, while if its value is less than 15, it indicates good flowability. Hausner's ratios below 1.25 suggest greater flow properties than those above 1.25 [19].

Oil adsorbing capacity (OAC) and oil desorbing capacity (ODC)

The optimized formulations of SD-CHEM and SE-CHEM, i.e., Y_1 and C_2, respectively, were taken for OAC and ODC study. The ethanolic solutions of Cremophor RH 40 and Labrafac were prepared and mixed with the microparticles (CHEM). These mixtures were gently heated at 40 ± 4 °C to evaporate the ethanol. The pure weight of adsorbent microparticles (W_a) and the dry weight of oil-adsorbed microparticles after the complete removal of ethanol (W_b) were taken to calculate the OAC. Only those dried mixtures which showed no significant change in physical properties from the pure CHEM and were non-sticky, non-greasy and free-flowing and were considered for the OAC calculations with the following formula [5].

1

OAC=Wb-WaWa∗100

The dried oil-adsorbed microparticles (W_b) were suspended in 10 ml water and gently stirred and stabilized for 1 h. This mixture was centrifuged at 3000 rpm for 10 min. The suspended particles were recovered, dried and weighed (W_c). The following formula was used for ODC [5].

2

ODC=Wb-WcWc∗100

Dynamic advancing contact angle analysis and surface free energy components analysis

The advanced method was used based on the capillary rise and thin column wicking methods to find the surface free energy components of powdered solids developed by Chibowski and Perea-Carpio [21] for SD-CHEM (Y_1) and SE-CHEM (C_2) [20, 21]. The dynamic advancing contact angle and surface free energy components were determined before and after oil adsorption for microparticles. The four probe liquids with known surface tension components were used. Out of these, diiodomethane ($\gamma_l^{lw}=50.8$, $\gamma_l^{+}=0$, $\gamma_l^{-}=0$) and n-hexane ($\gamma_l^{lw}=18.4$, $\gamma_l^{+}=0$, $\gamma_l^{-}=0$) were apolar with known liquid apolar surface free energy component. The other two liquids, dimethyl sulphoxide (DMSO $\gamma_l^{lw}=36$, $\gamma_l^{+}=0.5$, $\gamma_l^{-}=32$) and water ($\gamma_l^{lw}=21.8$, $\gamma_l^{+}=25.5$, $\gamma_l^{-}=25.5$), were polar with known liquid surface free energy polar components.

In this method, 0.2 g of the samples were weighed out in the plastic tips (2 ml, Merck) with outlets blocked by nylon swabs to prevent leakage of the solid powder. The tip was tapped ten times by hand from a height of 10 cm on the laboratory table for uniform packing. The tip was then dipped 3–4 mm into the probe liquid container (advancing manner). The tip was weighed for liquid retained by the solid powder (m_a). After that, some drops from the same probe fluid were put onto the bed powder top. The liquid was allowed to filter (receding manner), and the new weight was measured (m_r). The process was repeated three times, and the average of three determinations was used. The powdered samples (Y_1, C_2) underwent a similar procedure before and after oil adsorption for all the probe liquids. The effective pore radius ($R_{ef.}$) was calculated by using the (m_r) of n-hexane probe liquid (surface tension, γ_l= 18.4mN/m) and acceleration due to gravity (g) in the following equation.

3

W=mrg=2πRef.γl

W= weight of the liquid in the column.

The n-hexane determines the effective radius because alkanes completely wet the solid surface. In the following equation, the effective pore radius ($R_{ef.}$), along with (m_a) of water probe liquid, was used for further calculation of the dynamic advancing contact angle (θ_a).

4

1+cosθa=mag2πRef.γl

5

Wa=γl1+cosθawhere g= 9.8 m/s^2, γ_l = 72.8 mN/m.

The advancing contact angle here remains different from the contact angle on a smooth surface of a similar solid. Hence, it cannot be used directly in the Young formula for calculating surface free energy components. The three works of adhesion (W_a) values of the used probe liquid onto the solid surface were calculated by finding out the θ_a of three probe liquids, diiodomethane, DMSO and water (by putting ma values simultaneously in Eq. 4).

Further, by putting these different W_a values simultaneously in Eq. 6 of three probe liquids (with known liquid surface free energy components, i.e., γ_l^{LW}, γ_l^{+}, γ_l^{-}), the different unknown components (γ_s^{LW} apolar Lifshitz–van der Waals, γ_s^{-} electron donor and γ_s^{+} electron acceptor interactions) can be solved.

6

Wa=2γsLWγlLW1/2+γs+γl-1/2+γs-γl+1/2

Results

The process was optimized using a factorial experimental design (3^2), the chitosan–EDTA disodium ratio and processing temperature varied on three levels. The disodium salt was used to increase the solubility of the EDTA, as it remains insoluble in the acidic pH. The dropwise addition of the EDTA disodium into the chitosan–acetic acid mixture ensures no precipitation of the solution, which is otherwise observed.

In Tables 1 and 2, all the results of percentage yield (%), zeta potential (mV) and particle size (μm) are given for both SE-CHEM and SD-CHEM.

Optimization

Optimization of Solvent evaporation method by QbD

As per the mentioned method, SE-CHEM were synthesized and analyzed for the response for Y (percentage yield %), ZP (zeta potential mV) and PS (particle size μm). The independent variables CH (CH/EDTA ratio) and DT (drying temp.) were given three levels (1, 2 and 3), as mentioned in Table 1. All nine experiment runs and analyzed responses were put in the least squares fit regression analysis using JMP 16 (Trial).

In the effect summary in Table 3, LogWorth and p value for CH, DT and CH × DT are mentioned for SE-CHEM, which indicates CH as the essential variable in this model, followed by DT and CH × DT (interaction). Actual vs. predicted plots are given in Fig. 1 for all the responses, showing that both actual and predicted values are quite close. Table 3, for a summary of fit, indicates that the R^2 values are closer to one, signifying a good model fit. In Table 3, ANOVA results are summarized, which shows the models are significant (p values less than 0.05) for all the responses.

Table 3. Least squares fit effect summary

SE-CHEM			SD-CHEM		
Source	LogWorth	*P* value	Source	LogWorth	*P* value
CH(1, 3)	3.703	0.0002	CH × DT	2.207	0.0062
DT(1, 3)	1.645	0.0226	CH	1.807	0.0155
CH × DT	1.332	0.0465	DT(1,3)	1.614	0.0243

Response	R^2	R^2 Adj	RMSE	MR	Observation
Summary of fit (SE-CHEM)					
Y	0.956	0.9298	1.443	89.188	9
ZP	0.825	0.7207	1.568	3.553	9
PS	0.939	0.9025	2.415	11.735	9
Summary of fit (SD-CHEM)					
Y	0.992	0.981	1.704	37.07	6
ZP	0.913	0.783	1.476	0.62	6
PS	0.956	0.892	0.348	3.85	6

Response	Source	DF	SS	MS	*F* Ratio

Analysis of variance (SE-CHEM)					
Y	Model	3	227.42	75.81	36.36
Error	5	10.42	2.08	**Prob>F**	C. Total
8	237.84		0.0008*	ZP	Model
3	58.15	19.38	7.88	Error	5
12.29	2.45	**Prob>F**	C. Total	8	70.45
	0.0243*	PS	Model	3	450.02
150.009	25.7	Error	5	29.18	5.837
Prob>F	C. Total	8	479.21		0.0018*
Analysis of variance (SD-CHEM)					
Y	Model	3	760.41	253.46	87.22
Error	2	5.81	2.91	**Prob>F**	C. Total
5	766.21		0.0114*	ZP	Model
3	46.01	15.33	7.03	Error	2
4.35	2.17	**Prob>F**	C. Total	5	50.36
	0.127	PS	Model	3	5.38
1.79	14.79	Error	2	0.24	0.12
Prob>F	C. Total	5	5.62		0.064

Response	Term	Estimate	SE	*t* Ratio	Prob>|*t*|
Parameter estimates (SE-CHEM)					
Y	Intercept	89.18	0.48	185.32	<0.0001*
CH(1,3)	5.71	0.58	9.7	0.0002*	DT(1,3)
1.91	0.58	3.25	0.0227*	CH×DT	− 1.52

0.72	− 2.11	0.0883	ZP	Intercept	3.55
0.52	6.8	0.0010*	CH(1,3)	3.11	0.64
4.85	0.0047*	DT(1,3)	0.19	0.64	0.3
0.7758	CH × DT	− 0.06	0.78	− 0.09	0.9335
PS	Intercept	11.73	0.8	14.57	< 0.0001*
CH(1,3)	− 7.62	0.98	− 7.73	0.0006*	DT(1,3)
− 3.18	0.98	− 3.23	0.0233*	CH × DT	3.17
1.2	2.63	0.0466*	Parameter estimates (SD-CHEM)		
Y	Intercept	37.07	0.69	53.27	0.0004*
CH	5.51	0.69	7.91	0.0156*	DT(1,3)
− 5.36	0.85	− 6.29	0.0243*	CH × DT	− 10.76
0.85	− 12.63	0.0062*	ZP	Intercept	0.62
0.61	1.03	0.4118	CH	2.12	0.61
3.53	0.0718	DT(1,3)	0.75	0.73	1.02
0.4152	CH × DT	− 2.03	0.73	− 2.76	0.11
PS	Intercept	3.85	0.14	27.13	0.0014*
CH	− 0.86	0.14	− 6.07	0.0261*	DT(1,3)
− 0.23	0.17	− 1.35	0.3099	CH × DT	0.41

* It indicates that the p value is significant. The model is significant

DF-Degree of freedom, MR-mean of response, MS-mean of squares, PS-particle size, RMSE-root mean square error, SS-sum of squares, Y-percentage yield, ZP-zeta potential

Fig. 1 [Images not available. See PDF.]

Actual vs predicted plot of SE-CHEM for the responses **A** practical yield *Y*, **B** zeta potential ZP and **C** particle size PS. After 3^2 full factorial design was applied and model was run. In all the Actual vs predicted plots both actual and predicted values are quite close to each other. Here, the low RMSE (Root Mean Square Error) values in Y, ZP and PS plots indicated the model is significant and fits the data well. The *R* Squared (R^2) values in Y, ZP and PS plots predicts the percentage of the relationship between independent variables [CH (chitosan–EDTA ratio) and DT

(Drying temp.)] and dependent variables (Y, ZP and PS). These plots depicted 96%, 83% and 94% relationship between the independent variables and Y, ZP and PS, respectively. *P* value less than 0.05 showed all the model is statistically significant for Y, ZP and PS

Table 3 (for parameter estimates) and Fig. 2a for prediction profiler depict CH (CH/EDTA ratio) as the most important independent variable, followed by DT, for any change in responses. The small particle size of the adsorbent base powder is essential for a high surface area to have high oil adsorption and desorption. It shows that CH and DT have a positive (or additive) effect on a percentage yield (Y%). The parameter estimates and prediction profiler in Fig. 2a for ZP show that both CH and DT have a positive effect on it. It is clear from parameter estimates that the CH × DT interaction significantly contributes to the regression model for smaller particle size (PS). CH and DT have a negative effect on PS. The response surface diagrams (Fig. 3) and Pareto charts (Fig. 4) confirm the same results. Pareto charts also show the individual effect of each independent variable and their interaction on both responses. The fitted equations for all the responses are:

Fig. 2 [Images not available. See PDF.]

Prediction profiler of practical yield (Y), zeta potential (ZP) and particle size (PS): **A** SE-CHEM, **B** SD-CHEM. The prediction profiler depicted CH (CH/EDTA ratio) as the most important independent variable, followed by DT (Drying temp.), for any change in responses of Y, ZP and PS (in both SE-CHEM and SD-CHEM). For **A** SE-CHEM, it showed that CH and DT had a positive (or additive) effect on a percentage yield (Y) and zeta potential ZP. CH and DT had a negative effect on PS. For **B** SE-CHEM, prediction profiler showed that CH and DT had a positive (or additive) effect on a percentage yield (Y) and zeta potential ZP. CH and DT had a negative effect on PS. With the use of prediction profiler, the variables value can be predicted as per needed desirability by dragging the function handles left or right in JMP

Fig. 3 [Images not available. See PDF.]

Response surface diagrams of SE-CHEM in relation to CH versus DT **A** practical yield Y, **B** zeta potential ZP and **C** particle size PS. It is clear from figure **A** and **B** that a high percentage of CH (CH/EDTA ratio) from (40:60) to (60:40) leads to an increase in Y, which means when chitosan increases in (CH/EDTA ratio), then Y got increased. Similarly, when DT (Drying temp.) increased, there was an increase in both Y and ZP, which means a high drying temp was responsible for it. In the case of **C**, particle size PS, an increase in CH × DT negatively affects PS (decrease in size)

Fig. 4 [Images not available. See PDF.]

Pareto charts for SE-CHEM **A** practical yield, **B** zeta potential and **C** particle size. ZP show that both CH and DT have a positive effect on it. It is clear from parameter estimates that the CH × DT interaction significantly contributes to the regression model for smaller particle size (PS). CH and DT have a negative effect on PS

For percentage yield (Y):89.188+5.716×:CH-2+1.916×:DT-2+:CH-2×:DT-2×-1.525

For zeta potential (ZP):3.553+3.106×:CH-2+0.1925×:DT-2+:CH-2×:DT-2×0.068

For particle size (PS):11.735+-7.625×:CH-2+-3.183×:DT-2+:CH-2×:DT-2×3.17625

The optimization of the selected responses was targeted with a desirability function. For this purpose, Y and ZP were maximized, and PS was minimized. In Fig. 5a (contour plot), the white area shows the design space where the Y and ZP will be maximum and PS will be minimum.

Fig. 5 [Images not available. See PDF.]

Contour plots of practical yield (Y), zeta potential (ZP) and particle size (PS): **A** SE-CHEM, **B** SD-CHEM. **A** In this contour plot, white area shows the design space where the Y and ZP will be maximum and PS will be minimum. So, the desirability for percentage yield (%) and zeta potential (mV) is always more than 84%, 8 mV and for particle size (μm) <6 μm can be obtained with CH/EDTA ratio (60:40) and drying temperature (70 °C). **B** The white area of contour plot, shows the design space where the Y and ZP will be maximum and PS will be minimum. So, from these

optimization studies, the desirability for percentage yield (%) and zeta potential (mV) are always more than 55%, 4 mV and for particle size (μm) <3 μm can be obtained with CH/EDTA ratio (50:50) and drying temperature (110 °C) So, from these optimization studies, the desirability for percentage yield (%) and zeta potential (mV) is always more than 84%, 8 mV and for particle size (μm) <6 μm can be obtained with CH/EDTA ratio (60:40) and drying temperature (70 °C). So, the C_2 formulation in Table 1 is the optimized formulation with maximum desirability.

Optimization of spray drying method by QbD

The SD-CHEM were synthesized and analyzed for the response for Y (percentage yield %), ZP (zeta potential mV) and PS (particle size, μm). The independent variables CH (CH/EDTA ratio) with two levels (−1 and 1) and DT (drying temp.) were given three levels (1, 2 and 3), as mentioned in Table 2. All six experiment runs and analyzed responses were put in the least squares fit regression analysis using JMP 16 (Trial).

In the effect summary in Table 3, LogWorth and p value for CH, DT and CH × DT are mentioned for SD-CHEM, which indicates CH × DT as the most important in the model, followed by CH and DT. Actual vs. predicted plots are given in Fig. 6 for all the responses, showing that both actual and predicted values are significantly close. Table 3, for a summary of fit, shows that the R^2 value for all the responses is >0.90, signifying a good model fit. In Table 3, ANOVA results are summarized, which shows the *p* value of Y is 0.011, showing it is a significant model. The *p* values of ZP and PS are 0.127 and 0.064, respectively, which is almost significant for PS and not for ZP.

Fig. 6 [Images not available. See PDF.]

Actual versus predicted plot of SD-CHEM for the responses **A** practical yield Y, **B** zeta potential ZP and **C** particle size PS. After 3^2 full factorial design was applied and model was run. In all the Actual versus predicted plots both actual and predicted values are quite close to each other. Here, the low RMSE (Root Mean Square Error) values in Y, ZP and PS plots indicated the model is significant and fits the data well. The RSq (R^2) values in Y, ZP and PS plots predict the percentage of the relationship between independent variables [CH (chitosan–EDTA ratio) and DT (Drying temp.)] and dependent variables (Y, ZP and PS). These plots depicted 99%, 91% and 96% relationship between the independent variables and Y, ZP and PS, respectively. P value less than 0.05 showed the model is statistically significant for Y and ZP and *P* value 0.06 is very close to statistically significant model for PS

Table 3 (for parameter estimates) and Fig. 2b for prediction profiler depict CH × DT as the model's most important contributor for Y and PS responses. CH is the most important contributor to the ZP response. It shows on Y (%), and CH has a positive effect, while DT negatively affects it. Both CH and DT positively affect ZP and have a negative effect on PS. The response surface diagrams (Fig. 7) and Pareto charts (Fig. 8) confirm the same results. The fitted equations for the responses are as follows:

Fig. 7 [Images not available. See PDF.]

Response surface diagrams of SD-CHEM in relation to CH/EDTA and DT versus **A** practical yield Y, **B** zeta potential ZP and **C** particle size PS. From figure **A** and **B** that a high percentage of CH (CH/EDTA ratio) from (40:60) to (60:40) leads to an increase in Y, which means when chitosan increases in (CH/EDTA ratio), then Y got increased. Similarly, when DT (Drying temp.) increased, there was an increase in both Y and ZP, which means a high drying temp was responsible for it. In the case of **C**, particle size PS, an increase in CH × DT negatively affects PS (decrease in size)

Fig. 8 [Images not available. See PDF.]

Pareto charts for SD-CHEM **A** practical yield, **B** zeta potential and **C** particle size. It shows on Y (%), CH has a positive effect, while DT negatively affects it. Both CH and DT positively affect ZP and have a negative effect on PS

For percentage yield (Y):37.075+5.508×:CH+-5.362×:DT-2+:CH×:DT-2×-10.762

For zeta potential (ZP):0.62+2.126×:CH+0.752×:DT-2+:CH×:DT-2×-2.037

For particle size (PS):3.855+-0.862×:CH+-0.234×:DT-2+:CH×:DT-2×0.418

The optimization of the selected responses was targeted with a desirability function. For this purpose, Y and ZP

were maximized, and PS was minimized. In Fig. 5b (contour plot), the white area shows the design space where the Y and ZP will be maximum and PS will be minimum.

So, from these optimization studies, the desirability for percentage yield (%) and zeta potential (mV) are always more than 55%, 4 mV and for particle size (μm) <3 μm can be obtained with CH/EDTA ratio (50:50) and drying temperature (110 °C). So, the Y_1 formulation in Table 2 is optimized with maximum desirability.

FTIR analysis

FTIR spectra of the prepared SE-CHEM and SD-CHEM, along with chitosan and EDTA disodium, are shown in Figs. 9 and 10, respectively. Chitosan FTIR spectra showed strong absorption bands between 2967 and 2937 cm^{-1}, which correlated with C–H stretching, a characteristic of a typical polysaccharide group. The presence of residual N-acetyl groups was observed at an absorption peak of 1657 cm^{-1}, which proves that chitosan was not fully deacylated. The absorption bands 1587 cm^{-1}, 1547 cm^{-1} and 1325 cm^{-1} confirmed N–H bending of primary amine, amide II and C-N stretching of amide III respectively [22]. The absorption bands 1437 cm^{-1}, 1395 cm^{-1} and 1030 cm^{-1} corresponded to CH_2 bending, symmetrical CH_3 deformation and C–O stretching, respectively [23]. The FTIR of EDTA disodium showed N–H stretching between bands 3027–2945 cm^{-1}, C–H stretching between 2879 and 2793 cm^{-1}. The absorption bands 1666–1607 cm^{-1}, 1474 cm^{-1} and 1394 cm^{-1} showed –C=O stretching, CH_2 bending and O–H bending, respectively [24]. The optimized microparticles C_2 (SE-CHEM) and Y_1 (SD-CHEM) showed absorption bands between 1676–1657 cm^{-1} and 1693–1667 cm^{-1} respectively, representing the amide linkage. Again, the optimized microparticles C_2 (SE-CHEM) and Y_1 (SD-CHEM) showed absorption bands between 2378 and 2373 cm^{-1}, which corresponds to free acetate moieties, and indicates that all the acetate moieties were not involved in the amide linkage. Similarly, FTIR spectra of other SE-CHEMs and SD-CHEMs confirm the same results.

Fig. 9 [Images not available. See PDF.]

FTIR spectra of chitosan (CH), EDTA disodium (EDTA) and SE-CHEM. The samples with KBr pellet were measured (at ambient temp.) for FTIR-ATR analysis in the 500–4000 cm^{-1} spectral range. The C_2 (SE-CHEM) showed absorption bands between 1676–1657 cm^{-1} and 1693–1667 cm^{-1}, representing the amide linkage. The absorption bands between 2378 and 2373 cm^{-1} correspond to free acetate moieties

Fig. 10 [Images not available. See PDF.]

FTIR spectra of chitosan (CH), EDTA disodium (EDTA) and SD-CHEM. The samples with KBr pellet were measured (at ambient temp.) for FTIR-ATR analysis in the 500–4000 cm^{-1} spectral range. The spectra of Y_1 (SD-CHEM) showed absorption bands between 1676–1657 cm^{-1} and 1693–1667 cm^{-1}, representing the amide linkage. The absorption bands between 2378 and 2373 cm^{-1} correspond to free acetate moieties

DSC analysis

Figure 11 shows the DSC analysis of the samples. The thermogram of chitosan, C_2 and Y_1 showed an endotherm transition near 100 °C due to the evaporation of moisture or the presence of acetic acid moieties. The exothermic transition around 311 °C corresponds to the degradation of the chitosan. The melting of the chitosan is not observable because of the amorphous regions present, so it remains near the degradation point [25]. In the EDTA thermogram, the sharp endotherm starting at 243.96 °C corresponds to the melting of it. The C_2 and Y_1 thermograms show an endothermic transition around 240 °C and exothermic transitions near 312–14 °C. This analysis confirmed the purity of the synthesized SE-CHEM and SD-CHEM.

Fig. 11 [Images not available. See PDF.]

DSC graphs of chitosan, EDTA disodium, C_2 and Y_1. The DSC thermograms were recorded in a Differential scanning calorimeter from 40 to 400 °C temperature range. The CH, C_2 and Y_1 showed an endotherm transition near 100 °C, 240 °C (C_2 and Y_1) and for EDTA near 243.96 °C. The CH showed exothermic transition around 311 °C and C_2 and Y_1 thermograms showed it at 312–314 °C

XRD analysis

XRD patterns of the prepared SE-CHEM and SD-CHEM, along with chitosan and EDTA disodium, are shown in Figs. 12 and 13, respectively. The XRD pattern for chitosan showed two diffraction peaks, one at 2θ=20.37°, which indicates the characteristic of semicrystalline regions for chitosan and the other at 2θ=29.57°, which relates to calcite region [26, 27]. The EDTA disodium XRD pattern showed the major diffraction peaks at 2θ=21.2°, 2θ=26.5°, 2θ=29.8°, 2θ=34.3° and 2θ=45.4°. All these diffraction peaks correspond to the characteristic crystalline region of EDTA disodium [28]. The SD-CHEM samples showed broad diffraction peaks at 2θ=21.2°, 2θ=23°, 2θ=26.5° and 2 θ=29.8°. Similarly, SE-CHEM samples showed that diffraction peaks at 2θ=21.2° and 2θ=23° almost disappeared, and diffraction peaks at 2θ=26.5° and 2θ=29.8° were very broad and broad, respectively. These results indicate that microparticles formed from the two methods are amorphous.

Fig. 12 [Images not available. See PDF.]

XRD graphs of chitosan (CH), EDTA disodium (EDTA) and SE-CHEM samples were measured using the X-ray diffractometer at a voltage of 45 kV. The XRD pattern for chitosan showed two diffraction peaks, one at 2θ=20.37° and 2θ=29.57°, while EDTA disodium diffraction peaks were at 2θ=21.2°, 2θ=26.5°, 2θ=29.8°, 2θ=34.3° and 2θ=45.4°. SE-CHEM samples showed diffraction peaks at 2θ=26.5° and 2θ=29.8°

Fig. 13 [Images not available. See PDF.]

XRD graphs of chitosan (CH), EDTA disodium (EDTA) and SD-CHEM samples were measured using the X-ray diffractometer at a voltage of 45 kV. The XRD pattern for chitosan showed two diffraction peaks, one at 2θ=20.37° and 2θ=29.57°, while EDTA disodium diffraction peaks were at 2θ=21.2°, 2θ=26.5°, 2θ=29.8°, 2θ=34.3° and 2θ=45.4°. SD-CHEM samples showed broad diffraction peaks at 2θ=21.2°, 2θ=23°, 2θ=26.5° and 2θ=29.8°

Surface characteristics

To understand the shape and surface morphology of the microparticles, the scanning electron microscope images of the chitosan, EDTA disodium, C_2 and Y_1 (shown in Fig. 14) were taken. In Fig. 14c, the SE-CHEM (C_2) microparticles had a flaky appearance with an uneven surface, providing a high surface area for the adsorption of liquid SEDDS. Figure 14d shows the SD-CHEM (Y_1) microparticles, which are much spherical with uneven surfaces and wide void spaces, leading to better oil adsorption and desorption.

Fig. 14 [Images not available. See PDF.]

Scanning electron micrographs **A** chitosan, **B** EDTA disodium, **C** C_2 and **D** Y_1. **C** The SE-CHEM (C_2) microparticles had a flaky appearance. **D** SD-CHEM (Y_1) microparticles were spherical

Flow properties

The angle of repose (Fixed funnel method), apparent bulk density, tapped density, Carr's index and Hausner's ratio were calculated for the flowing characteristic of the microparticles, and the results are shown in Table 4, which shows all the parameters were in acceptable limits [29]. The angle of repose values for all the microparticles were between 9 and 17. Both optimized microparticles, C_2 and Y_1, were free-flowing.

Table 4. Flow properties

Code	θ	ρ_b	ρ_t	CI	HR	Flowability
A_1	17±1.9	0.93±0.12	0.99±0.23	5.72	1.06	Fair
A_2	9±0.5	0.98±0.19	1.12±0.31	12.5	1.14	Free flow
A_3	13±1.3	0.76±0.36	0.8±0.27	5	1.04	Free flow

B_1	12±0.9	0.91±0.29	1.01±0.41	11.11	1.12	Free flow
B_2	16±1.2	0.97±0.31	1±0.22	3	1.03	Fair
B_3	14±1.6	1.13±0.28	1.18±0.39	3.89	1.04	Free flow
C_1	16±0.8	0.89±0.18	1.04±0.47	13.5	1.15	Free flow
C_2	13±1.1	0.91±0.24	1.04±0.29	13.12	1.15	Free flow
C_3	11±0.9	0.89±0.37	0.99±0.41	10.5	1.11	Free flow
X_1	17±0.5	0.93±0.15	0.99±0.36	5.72	1.06	Fair
X_2	9±1.2	0.98±0.45	1.12±0.22	12.5	1.14	Free flow
X_3	13±1.7	0.76±0.26	0.8±0.17	5	1.04	Free flow
Y_1	11±0.6	0.9±0.33	1.01±0.43	11.1	1.12	Free flow
Y_2	16±0.8	0.97±0.18	1±0.33	3	1.03	Fair
Y_3	14±1.1	1.13±0.22	1.18±0.24	3.97	1.04	Free flow

*θ = angle of repose (°), ρ_b = bulk density (g/cm^3), ρ_t = tapped density (g/cm^3), CI = Carr's index, HR = Hausner's ratio

OAC and ODC

Cremophor RH 40 and Labrafac were used for the OAC and ODC study; Fig. 15 shows the results. The oil adsorption for both the microparticles was significant, but Y_1 has it slightly higher than C_2 for both Labrafac and Cremophor RH 40. These results relate to the surface morphology analysis, where Y_1 showed a much spherical structure with wide voids compared to C_2, with a flaky structure.

Fig. 15 [Images not available. See PDF.]

Oil adsorbing capacity (OAC) and oil desorbing capacity (ODC) for C_2 and Y_1. Cremophor RH 40 and Labrafac were used for the OAC and ODC study. The OAC of Y_1 was slightly higher than C_2 for both Labrafac and Cremophor RH 40

Dynamic advancing contact angle and surface free energy components analysis

For the adsorption and desorption phenomena to happen on the microparticle's surface, the surface free energy components, i.e., apolar or dispersive component [γ^{LW} (mJ/m^2)] and polar component [γ^{P} (mJ/m^2)] are very important to understand. The γ^{LW} component is related to the hydrophobic surface, and the γ^{P} component relates to the hydrophilic part of the surface.

In Fig. 16, the results of the surface free energy components and dynamic advancing contact angles [θ_a (°)] are shown. The γ^{LW} components of C_2 and Y_1 were enhanced after oil adsorption (for both Labrafac and Cremophor RH 40); this indicates that increased γ^{LW} components can reduce oil droplet size significantly after reconstitution of the nanoemulsion from the surface of the adsorbent microparticles. In contrast, the γ^{P} components were decreased after the oil adsorptions for both microparticles. This result is significant in understanding that the microparticles can give ample surface free energy for the stable nanoemulsion, leading to good oil desorption.

Fig. 16 [Images not available. See PDF.]

Surface free energy components (dispersive component, γ^{LW} and polar component, γ^{P}) and dynamic advancing contact angles (θ_a) for C_2 and Y_1. The γ^{LW} components of C_2 and Y_1 were enhanced after oil adsorption (for both Labrafac and Cremophor RH 40). The γ^{P} components were decreased after the oil adsorptions for both microparticles. The dynamic advancing contact angles (θ_a) were also significantly increased after the oil adsorption The dynamic advancing contact angles (θ_a) were also significantly increased after the oil adsorption. Increased θ_a indicates the hydrophobicity of the surface, which is vital for the development of stable nanoemulsion.

Discussion

The solid adsorbent base with an excellent hydrophobic surface, oil adsorption and desorption capacities will play a crucial role in developing the S-SEDDS [30]. Hence, an attempt was made to develop the microparticles of chitosan–EDTA disodium. Such adsorbent microparticles will allow the incorporation of poorly water-soluble and thermolabile drugs into the S-SEDDS [31]. The solvent evaporation and spray drying techniques were explored to synthesize and characterize SE-CHEM and SD-CHEM microparticles, respectively. In the preliminary study, it was evident that the chitosan–EDTA disodium ratio and processing temperature in the solvent evaporation and spray drying procedures play a crucial role in the various properties of the microparticles like water solubility, yield, particle size and zeta potential. So, optimization was required for the development of the CHEM. The process was optimized using 3^2 full factorial designs, which showed a CH/EDTA ratio (60:40) and drying temp. 70 °C for solvent evaporation method and inlet temp. 110 °C and CH/EDTA ratio (50:50) produced SE-CHEM (C_2) and SD-CHEM (Y_1), respectively, with highest yield, zeta potential and lowest particle size. The different characterization studies like DSC and FTIR analysis for both optimized microparticles confirmed the amide conjugation between the CH and EDTA and free acetate moieties' existence. XRD study confirmed the broadening of the diffraction peaks for both C_2 and Y_1, suggesting the amorphous character of the particles. The high oil adsorption and desorption were achieved for both microparticles, confirmed by OAC and ODC studies. The ODC is closer to the OAC, which signifies that the amount of oil adsorbed by the microparticles will be desorbed in a very close quantity to adsorption. Surface morphology analysis through SEM images confirmed the flaky appearance of C_2 and Y_1, showing a spherical shape with voids. Flowability analysis revealed that both the microparticles were free-flowing. The surface free energy component analysis before and after oil adsorption revealed the enhancement in the dispersive components for both microparticles; this property leads to a reduction in oil droplet size on reconstitution of S-SEDDS, whereas reduced polar components after oil adsorption suggest microparticle's ability to provide sufficient surface free energy for the stability of the nanoemulsion after reconstitution. The enhanced dynamic advancing contact angles for C_2 and Y_1 indicated the hydrophobic surface of the microparticles, which was evident in high OAC for both microparticles. All these results suggest that both the microparticles can serve as stable adsorbent base for solid SEDDS to produce stable nanoemulsion after reconstitution.

Conclusion

This study has reported development of superior adsorbent carrier (microparticles) by solvent evaporation and spray drying techniques. The development of the CH-EDTA disodium microparticles (adsorbent carriers) by the solvent evaporation and spray drying methods is simplistic and practical, with the potential to be adopted at an industrial scale. The results of improved OAC, ODC and surface free energy components were satisfactory. They will prove beneficial for these microparticles to fabricate them with the liquid self-emulsifying drug delivery systems. The microparticles developed with both techniques were superior in their qualities as an adsorbent base for the solidification of SEDDS. However, if we compare both techniques, the microparticles developed by the spray drying technique were better than those from the solvent evaporation technique. The SE-CHEM had the advantage of a greater percentage yield as no minimal loss occurred during production, while SE-CHEM suffered a more significant loss and had a low percentage yield during the process. Further, the fabrication of these microparticles with SEDDS (of suitable drugs) is required to form a solid self-emulsifying drug delivery system, whose analysis in *in vitro* and *ex vivo* conditions will give a clear image of these microparticle's ability to enhance the drug loading, dissolution and

bioavailability and to form a stable solid self-emulsifying drug delivery system.

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Author contributions

MK carried out formulation and evaluation and wrote the original draft. PAC supported in FTIR, DSC and XRD analysis and interpretations. ST and SKJ supported in design of spray drying procedure and carried out it. AF and VC contributed to reviewing, editing and supervision.

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Declarations

Ethical approval and consent of participate

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Consent for publication

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Competing interests

The authors declare that they have no competing interests.

Abbreviations

ANOVA
Analysis of variance
CH
Chitosan–EDTA disodium ratio
CHEM
Chitosan–EDTA disodium microparticles
DMSO
Dimethyl sulfoxide
DSC
Differential scanning calorimetry
DT
Drying temperature
EDTA
EDTA disodium
FTIR
Fourier-transform infrared spectroscopy
OAC
Oil adsorbing capacity
ODC
Oil desorbing capacity
PS
Particle size
SD-CHEM
Spray-dried-Chitosan–EDTA disodium microparticles
SE-CHEM
Solvent-evaporated-Chitosan–EDTA disodium microparticles
SEDDS
Self-emulsifying drug delivery system

SEM
Scanning electron microscope
S-SEDDS
Solid self-emulsifying drug delivery system
XRD
X-ray diffraction analysis
Y
Practical yield
ZP
Zeta potential

Publisher's Note

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Preparation and in vitro evaluation of BBG-250 loaded liposomal formulation for anticancer potential

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ABSTRACT (ENGLISH)

Background

Liposome-mediated drug delivery systems have emerged as a promising avenue for enhancing cancer treatment strategies. This study aims to develop and assess liposomal carriers loaded with Brilliant Blue G-250 (BBG-250), a potent P2X7 receptor antagonist that shows potential as an anti-tumor agent. Specifically, two types of liposomal formulations were designed: conventional liposomes composed of hydrogenated soya phosphatidylcholine (HSPC) and cholesterol, and pH-sensitive liposomes consisting of dioleoylphosphatidylethanolamine (DOPE), distearoylphosphatidylethanolamine-methoxy polyethylene glycol (DSPE-mPEG), dipalmitoylphosphatidylcholine (DPPC), and cholesterol. The investigation focuses on understanding the morphological characteristics, size, stability, drug incorporation efficiency, drug release profiles, blood compatibility, and cytotoxicity of these liposomal formulations.

Results

Advanced photon correlation spectroscopy using the Nano Plus-3 instrument was employed to evaluate the liposomes. The optimized conventional liposomes (HSPC-cholesterol ratio 7:3) exhibited a size of 125 ± 0.3 nm with a polydispersity index (PDI) of 0.21, indicating uniformity. The pH-sensitive liposomes (DOPE:DPPC:DSPE-PEG2000: Cholesterol 4:3:3:0.3) demonstrated a size of 118 ± 1.2 nm with a PDI of 0.230. Zeta potential measurements confirmed the stability of both formulations under physiological conditions, with values of − 16.93 mV for conventional liposomes and − 25.21 mV for pH-sensitive liposomes. Higher drug-to-lipid ratios were found to enhance drug incorporation efficiency. pH-sensitive liposomes exhibited superior drug release characteristics, with 95% release over 24 h, compared to conventional liposomes, which released 70% of the drug. Blood compatibility assessments revealed the safety of both formulations for intravenous administration. Cytotoxicity studies conducted on A549 cell lines demonstrated the cytocompatibility of both liposomal types across a range of concentrations, with IC_{50} values surpassing those of the reference drug, docetaxel.

Conclusions

This study underscores the potential of liposomal carriers as effective vehicles for delivering BBG-250, highlighting their stability, biocompatibility, and controlled drug release properties. Despite being slightly less potent than the reference drug, docetaxel, these liposomal formulations hold promise for advancing anticancer strategies. The findings contribute to the evolving landscape of innovative cancer therapy drug delivery systems, offering a novel approach to improving treatment outcomes for cancer patients. The successful development and evaluation of these liposomal carriers pave the way for further investigations and potential clinical applications in the field of cancer therapeutics.

FULL TEXT

Background

Cancer, a complex and multifaceted group of diseases characterized by uncontrolled cell growth and division, continues to be a major global health challenge. It is responsible for an enormous burden of suffering and mortality, with nearly 10 million deaths attributed to cancer in 2020 alone. The intricate interplay of genetic, environmental, and lifestyle factors contributes to the initiation and progression of cancer, rendering it a formidable adversary that requires innovative and targeted therapeutic approaches. In the realm of cancer, lung cancer occupies a particularly sinister position. It stands as one of the most prevalent and lethal forms of malignancy, causing an alarming number of deaths annually. In 2020, lung cancer accounted for a staggering 1.80 million fatalities, thereby solidifying its grim distinction as the leading cause of cancer-related death worldwide. The statistics paint a stark picture of the devastating impact of this disease on individuals, families, and societies at large (ACS, 2020, 2022, 2023). Among the spectrum of lung cancers, non-small cell lung cancer (NSCLC) emerges as the dominant subtype, encompassing a majority of cases. The etiology of NSCLC is intricately linked to diverse factors, including smoking, exposure to environmental pollutants, genetic predisposition, and aging. Its insidious nature often results in diagnosis at advanced stages, significantly hampering treatment success and patient prognosis. Central to addressing the challenge of lung cancer is an understanding of the critical role that cancer stages play in shaping outcomes. Stage of cancer refers to the extent to which it has progressed within the body, ranging from localized disease confined to its site of origin to invasive cancer that has spread to distant regions. This staging system provides valuable insights into the potential for disease eradication, recurrence, and overall survival. In the context of lung cancer, early detection and treatment significantly improve patient outcomes. Unfortunately, a substantial proportion of cases are diagnosed at later stages, severely limiting therapeutic interventions.

Traditional treatments for lung cancer, including surgery, radiation therapy, and chemotherapy, have made strides in extending survival and improving the quality of life[1]. However, the pursuit of more effective and targeted therapeutic strategies remains paramount. The advent of molecular and genetic insights into cancer biology has unveiled novel opportunities for precision medicine. Targeted therapies, immunotherapies, and combination regimens are reshaping the landscape of lung cancer treatment, offering hope for improved outcomes and personalized care [2, 3]. One intriguing avenue of research in the pursuit of innovative lung cancer therapies is the exploration of purinergic receptors, specifically P2X and P2Y subfamilies[4, 5]. These receptors, widely distributed throughout the body, are pivotal players in cellular signaling and communication. Of particular interest is the P2X7 receptor, which exhibits distinct expression patterns in both normal and cancerous lung cells. This receptor not only influences cell proliferation but also plays a crucial role in the delicate balance between cell survival and apoptosis, a programmed cell death mechanism. Importantly, in the context of lung cancer, alterations in P2X7 receptor activity have been implicated in disease progression. The intriguing interplay between the P2X7 receptor and lung cancer opens up an avenue for therapeutic intervention [6]. One compound of interest in this context is Brilliant Blue G-250 (BBG-250), a known antagonist of the P2X7 receptor [7]. Previous studies have demonstrated its ability to modulate receptor activity and impact cellular responses. Harnessing the potential of BBG-250 as a targeted therapy could pave the way for a more nuanced and effective approach to tackling lung cancer. To enhance the precision and efficacy of therapeutic interventions, nanotechnology has emerged as a transformative tool. Liposomes, with their minuscule dimensions and unique physicochemical properties, offer a platform for targeted drug delivery and controlled release. The concept of encapsulating therapeutic agents within nanocarriers holds the promise of minimizing systemic toxicity, maximizing drug accumulation at tumor sites, and ultimately improving treatment outcomes [8]. Liposomes, a well-established type of nanovesicle, have garnered considerable attention in drug delivery applications. Comprising a lipid bilayer surrounding an aqueous core, liposomes offer versatility in encapsulating a wide range of molecules, including hydrophilic and hydrophobic drugs [9]. Their potential to traverse biological barriers, selectively accumulate in tumor tissues, and release their cargo in a controlled manner has spurred interest in their use for cancer therapy. The present research endeavors to explore the computational screening approaches, such as homology modeling molecular docking and ADMET analysis, which were used to

evaluate binding affinity between the target (P2X7) and Ligand (BBG-250). The potential interaction of BBG-250 with crucial amino acid residues within target binding pockets has been a subject of investigation in this study. Afterward, pharmacokinetic parameters were checked via an in silico ADMET study. Then after the potential of liposomal formulations encapsulating BBG-250 for targeted therapy in lung cancer. The overarching objective is to harness the unique attributes of liposomes to enhance the delivery and efficacy of BBG-250 while minimizing adverse effects[10]. By delving into the formulation, characterization, and performance of these liposomal carriers, this study seeks to contribute to the growing body of knowledge aimed at advancing precision medicine strategies for lung cancer treatment. The subsequent sections of this research paper delve into the intricate details of this investigation, encompassing the methodology, results, and discussions that underpin the pursuit of innovative and effective lung cancer therapeutics. Through meticulous analysis and comprehensive assessment, this research endeavors to shed light on the potential of liposomal BBG-250 as a promising avenue for enhancing lung cancer treatment outcomes[11, 12].

Material

The selection and procurement of materials form the foundation of any scientific investigation. Each component contributes to the precision and reliability of experimental outcomes. The following section outlines the various materials employed in this study, including their sources and roles in the research process.

In silico study

Homology modeling

Comparative or homology protein modeling is a powerful 3D protein modeling tool that can predict the conformation of experimentally unavailable protein structure that has amino acid sequences similar to template protein sequence due to the unavailability of P2X7 receptor 3D structure in the PDB. It is difficult and time-consuming to establish the 3D structure of a P2X7 protein using experimental techniques including NMR, X-ray crystallography, and cryo-electron microscopy. Therefore, P2X7 protein structure determination is crucial for planning and evaluating biological research in this scenario. Homology modeling has proven to be the most effective method for building a reliable 3D model of a P2X7 protein from its amino acid sequence. The Ramachandran plot, Errat score, and other validation metrics are indicators of the quality of the protein structural geometry. The following list of tools was used for homology modeling:

Homology modeling by different tools

Homology modeling by MODELLER 10.2 module

To create constraints on atomic distances, dihedral angles, and other parameters, MODELLER employs the query structures. These constraints are then paired with statistical distributions determined from several homologous structure pairings in the PDB. Sequences and structures are combined by MODELLER to create a comprehensive alignment that may be manually altered and inspected with molecular graphics software. There are five consecutive steps to comparative modeling using the MODELLER 10.2 module. The very first step of modeling involves searching for the 3D structure of proteins related to the target. For this, we have used the site (www.ncbi.nlm.nih.gov) and downloaded the P2X7 receptor sequence in FASTA file format. The second step denotes the selection of templates for alignment with the target. Here, we run BLAST for getting similar sequences and selected ten sequences (PDB_ID:3H9V,4DW0,4DW1,5FLC,5SVH,5SVJ,5U11,5XW6,5YVE,6AH4) as templates based on maximum identity related to the P2X7 protein sequence from a list of generated sequences in Table 1. Out of a total of ten PDB structures, PDB ID_5SVH was found to be more perfect than others. The third step involves aligning the target protein with the template (PDB_5SVH). The fourth step is the model-building step which automatically calculates the 3D model of the target using an auto model class of the module. In this step, a total of 20 homology models have been generated with different conformations. The fifth step is the evaluation step, in this the models were evaluated using the Ramachandran plot and Errat score as Table 2. Again, out of 20 homology models after evaluation model number 10 (**qsec.B99990010.pdb**) was found to be a more accurate model than others[13].

Table 1. Templates with the highest degree of similarity to the P2X7 protein sequence

S. No	PDB hits	Identity (%)	Resolution
1	3h9v	88	–
2	4dw0	88	–
3	4dw1	88	–
4	5f1c	86	–
5	5svh	89	2.0
6	5svj	89	2.9
7	5u1l	89	3.4
8	5xw6	86	–
9	5yve	89	3.4
10	6ah4	89	3.3

Table 2. List of Generated Model and their evaluation

Model	Errat	Pro-check	Model	Errat	Pro-check
qseq.B99990001.pdb	4.27	84.4	qseq.B99990011.pdb	14.23	83.5
qseq.B99990002.pdb	10.04	84.7	qseq.B99990012.pdb	8.56	85.9
qseq.B99990003.pdb	3.21	84.1	qseq.B99990013.pdb	16.23	84.7
qseq.B99990004.pdb	13.27	85.6	qseq.B99990014.pdb	17.22	84.4
qseq.B99990005.pdb	11.55	85.9	qseq.B99990015.pdb	15.41	85.6
qseq.B99990006.pdb	14.71	83.8	qseq.B99990016.pdb	16.81	85.3
qseq.B99990007.pdb	6.84	84.4	qseq.B99990017.pdb	12.17	83.2
qseq.B99990008.pdb	17.7	83.5	qseq.B99990018.pdb	18.22	85.3
qseq.B99990009.pdb	7.35	84.7	qseq.B99990019.pdb	7.37	82.4
qseq.B99990010.pdb	**17.27**	**87.1**	qseq.B99990020.pdb	9.54	81.8

Homology modeling by SwissModel module

SwissModel is a web server that accepts a sequence and delivers the model via email. It follows standard protocols for homologue identification, sequence alignment, core backbone determination, and modeling loops and side chains. SwissModel searches a protein database using BLAST and attempts to build a model for any PDB hits. In comparative modeling, a 3D protein model of a P2X7 receptor sequence is generated by extrapolating experimental information from an evolutionary-related protein structure that serves as a template. In SwissModel, the default modeling workflow consists of the following main steps: (1) **Input data:** The target protein amino acid sequence in FASTA format as a plain text from the NCBI database. (2). **Template search:** Data provided in step 1 serve as a query to search for evolutionary-related protein structures against the SWISSMODEL template library SMTL that is 6u9v, 6u9w, 5u2h, and 5u1u. (3) **Template selection:** Templates are ranked according to GMQE and **6u9v** were used for further model building as shown in Table 3. (4). **Model building**: Selected template(6u9v), a four-3D protein model (Table 4) is automatically generated by first transferring conserved atom coordinates as defined by the target template alignment. (5) **Model quality estimation**: To quantify modeling errors and give estimates on expected model accuracy the Ramachandran plot and Errat score provide estimates of the expected quality of the resulting model at the tertiary and quaternary structure level[14].

Table 3. Templates with the highest degree of similarity to the P2X7 protein sequence

S. No	PDB hits	Identity (%)	Resolution
1	3h9v	88	–
2	4dw0	88	–
3	4dw1	88	–
4	5f1c	86	–
5	5svh	89	2.0
6	5svj	89	2.9
7	5u1l	89	3.4
8	5xw6	86	–
9	5yve	89	3.4
10	6ah4	89	3.3

Table 4. List of generated model

Generated model	Pro-check (%)	Errat
Model 1	87.3	85.47

Model 2	90.8	80.06
Model 3	92.1	76.65
Model 4	93.1	90.79

Homology modeling by I-tasser module

A homology model, based on the Amino acid sequence (NCBI database) of the Homo sapience, corresponding to the P2X7 receptor obtained using the I-TASSER server, and five different models were generated as mentioned in Table 5. The confidence of each comparative model obtained by I-TASSER is quantitatively measured by the C-score which is typically in the range of [−5 to 2]. The best-identified 3D model (out of five comparative models) obtained a C-score value of−2.52. The quality of the raw homology model was assessed by the Pro-check server[15, 16].

Table 5. List of generated models and their Validation of I-Tasser server

Generated model	Ramachandran Plot (%)	Errat
Model 1	74.5	88.81
Model 2	74.1	84.32
Model 3	53.0	69.96
Model 4	**75.9**	**89.84**
Model 5	46.4	70.37

Bold value indicates the best model

Our criteria for model selection were stringent, with a significant emphasis on accuracy as reflected in the Ramachandran favored regions. Following this thorough validation process, Model.4 emerged as the most optimal choice for subsequent docking studies. A detailed account of the validation parameters, encompassing factors such as Ramachandran favored outcomes, is thoroughly documented and presented in Table 7. This transparent documentation underscores the robustness of our model selection process, establishing a solid foundation for the reliability of the chosen receptor model in our study.

Molecular docking

Molecular docking studies of Brilliant Blue G250 dye (Fig. 1) were performed with receptor protein designed by homology modeling method by using Glide module software (Schrodinger Maestro v13.1). The protein was further processed through ‘protein preparation wizard’ (Maestro wizard v13.1). The generating states and refinement step were used for improving the protein structure including optimization of H-bonded groups, dehydration, and restrained minimization by using default force field OPLS_3. The minimized protein structure was used for the generation of a grid around ligand molecules. Molecular docking studies were performed by the Glide module of Schrodinger Maestro v13.1 on Brilliant Blue G250 Dye. The protein structure was obtained from the homology modeling by SwissModel (Fig. 1C) which passed various evolution tests (Fig. 1A and B) and was pre-processed by using protein preparation wizard in Maestro v13.1. Generating states and refinement step automatically added hydrogen atoms and some essential bonds at the missing sites of the protein molecule. The refinement step has several functions like optimization of hydrogen-bonded groups, removal of water, and restrained minimization using the default force field OPLS_3. After the optimization process, receptor grid generation was processed to locate the binding pocket in

the receptor and in the last ligand docking was performed. The activity of the dye (Ligand) depends on the possible interactions of the ligand with various amino acid residues of the targeted protein (P2X7 receptor) as shown in Fig. 2 [17, 18].

Fig. 1 [Images not available. See PDF.]

Representation of **A** Errat score **B** Ramachandran Plot and **C** Generated Best Model

Fig. 2 [Images not available. See PDF.]

Representation of Docking Interaction Between P2X7 Homology Model and BBG-250

Validation of docking results

The docking analysis of BBG250 revealed a notable docking score of -9.597. This outcome suggests a high affinity and favorable binding interaction of BBG250 within the target site. Importantly, these results indicate superior activity levels when compared to the reference compound (Docking score: −4.621), docetaxel, a well-established agent in previous anticancer studies (refer to Table 7). And also validated by the both positive controls (standard inhibitors) and negative controls (known non-inhibitors) within our docking analysis. BBG250 displayed a higher binding affinity when compared to the positive control and negative control compounds. This outcome strongly suggests that BBG250 may indeed exhibit superior activity levels compared to previously reported compounds (Table 7). Our research findings substantiate the enhanced potential of BBG250 as a promising candidate in the context of anticancer therapeutics.

Prediction of ADME properties

Nowadays, several online tools and offline software programs are available which help us in predicting the ADME behavior of the drug candidate. In this study, the SwissADME prediction tool (http://www.swissadme.ch/) was used. The compound selected for the ADME study was BBG-250. The compounds were then converted into their canonical ‘SMILE’ format and put in the ‘SwissADME’ in the section mentioned as ‘Enter a list of SMILES here.’ The server predicts the physicochemical properties, lipophilicity, water solubility, pharmacokinetics, druglikeness, lead likeness, and synthetic accessibility of the compounds.

Brilliant blue G-250

The key pharmaceutical agent under scrutiny is BBG-250, a potent antagonist of the P2X7 receptor[10]. This compound, which holds promise for targeted lung cancer therapy, was procured from HIMIDIA Laboratories Ltd, Mumbai, India. Its specialized properties make it an indispensable element in the formulation and subsequent evaluations of liposomal carriers.

Lipids

Lipids are fundamental components of liposomal formulations, providing the structural basis for these liposomes. The lipids used in this study were acquired from distinct sources, each contributing unique attributes to the formulation. mPEG-DSPE, a critical ingredient, was generously provided as a gift sample from Lipoid Germany. This lipid, known for its amphiphilic nature, facilitates the formation of stable liposomes and influences their surface properties. HSPC (hydrogenated soya phosphatidylcholine), a lipid renowned for its biocompatibility and stability, was also a gift sample from Lipoid Germany. Its inclusion in the formulations contributes to the structural integrity of liposomes, enabling efficient encapsulation of BBG-250. DOPE (dioleoylphosphatidylethanolamine), another vital lipid component, was likewise obtained as a gift sample from Lipoid Germany. DOPE presence imparts fluidity and flexibility to the lipid bilayer, optimizing the liposome's ability to encapsulate and release therapeutic agents [19].

Cholesterol

Cholesterol sourced from HiMedia, Mumbai, is a quintessential component of the lipid bilayer. Its role in maintaining membrane integrity, modulating fluidity, and enhancing the stability of liposomal carriers is indispensable [20]. The inclusion of cholesterol in the formulations contributes to the structural integrity and performance of liposomes.

Solvents and filters

Chloroform and methanol, vital solvents in the formulation process, were procured from Department. These solvents

play a pivotal role in dissolving lipids and facilitating the formation of thin lipid films, which serve as the foundation for liposomal encapsulation. To ensure the purity and clarity of the formulations, filtration steps were implemented. Nylon filters (AXIVA syringe filters) were employed for this purpose, effectively removing particulate matter and ensuring the quality of liposomal suspensions [21].

Dialysis membrane

For the evaluation of drug release profiles, dialysis membranes are indispensable tools. The dialysis membrane used in this study, sourced from Sigma-Aldrich, boasts a 14 kDa molecular weight cutoff. This semi-permeable barrier allows for controlled diffusion of molecules, facilitating accurate measurement of drug release from liposomal formulations.

The research demanded a range of other chemicals, each contributing to various aspects of the experimental process. These chemicals, all of the highest commercial grade, played roles in diverse procedures, from pH determination to characterization analysis. Their quality and consistency ensured the reliability of the research outcomes. The selection and procurement of materials underpin the scientific rigor and validity of this investigation. Each component, from the therapeutic agent BBG-250 to the lipids, solvents, filters, and membranes, plays a crucial role in formulating, characterizing, and evaluating liposomal carriers for targeted lung cancer therapy.

Preparation of conventional and pH-sensitive liposomes formulation

In the realm of targeted drug delivery, formulation and development of liposomal carriers play a pivotal role in enhancing therapeutic efficacy. This study undertook a systematic approach to engineer liposomal formulations tailored for the encapsulation of BBG-250, a potent P2X7 receptor antagonist with promising implications for lung cancer therapy. Two distinct formulations were meticulously crafted: conventional liposomes composed of hydrogenated soya phosphatidylcholine (HSPC) and cholesterol, and pH-sensitive liposomes consisting of dioleoylphosphatidylethanolamine (DOPE), dipalmitoylphosphatidylcholine (DPPC), DSPE-PEG 2000, and cholesterol [22]. The formulation process, a critical juncture in this research, was executed with precision and methodological rigor.

For the preparation of conventional liposomes, the thin-layer evaporation method was adopted, renowned for its capacity to generate liposomal structures with reproducibility. The process began with the precise weighing of HSPC and cholesterol in a 7:3 ratio ensuring the optimal composition. This lipid mixture was dissolved in a chloroform–methanol solution at a 9:1 ratio, creating an organic phase conducive to liposomal encapsulation [23]. The therapeutic agent BBG-250 was judiciously introduced into the organic phase and fortified with 3.5 mg of the compound to confer the desired therapeutic payload. After solvent evaporation under vacuum at a controlled temperature of 50 °C, a thin lipid film emerged. Hydration of the lipid film with the phosphate-buffered solution (PBS) at pH 7.4 revitalized the structure, forming a homogenous liposomal suspension through Rota evaporation at 50–55 °C and 200 rpm for one hour [24]. Further refinement involved probe sonication for uniformity and size optimization, followed by passage through Sephadex (G-50) column to attain the final liposomal formulation characterized by low polydispersity and targeted size distribution. The resulting liposomes, thus formed, were stored at a controlled 4 °C for subsequent stages of experimentation.

This comprehensive approach to crafting conventional liposomes ensured the creation of structurally sound carriers poised for targeted drug delivery. The subsequent segment will delve into the formulation of pH-sensitive liposomes, shedding light on their unique composition and the intricacies of their preparation.

Characterization of liposomes

FTIR analysis

FTIR (Fourier-transform infrared) analysis was performed to gain insights into the molecular interactions and structural changes present in the formulated liposomes and the Brilliant Blue G-250 (BBG-250) dye. The FTIR spectra of the HSPC liposomes (P11), pH-sensitive formulation (P5), and Brilliant Blue G-250 (BBG-250) were obtained using a Fourier-transform infrared spectrometer. The samples were prepared by depositing a thin film of each formulation on a suitable substrate, followed by scanning within the infrared range. The spectral data obtained were then analyzed to identify specific functional groups and molecular vibrations.

Vesicle shape, size, size distribution, and zeta potential

Vesicle characteristics play a pivotal role in determining the efficacy and behavior of liposomal formulations. Through the utilization of advanced analytical tools, the vesicle size (z-average), size distribution (polydispersity index), and zeta potential (ZP) were meticulously assessed. Using the Nano Plus-3 instrument, the formulations underwent thorough examination employing photon correlation spectroscopy (PCS). Before analysis, the formulations underwent meticulous filtration through a 0.25-micron syringe filter (1:9 v/v) to ensure accuracy. The resulting data were presented thoughtfully, offering a visual representation of these critical parameters [25].

Morphology and structure determination

A profound understanding of the morphology and structural characteristics of liposome preparations was sought through diverse microscopy techniques. Transmission electron microscopy (TEM) was employed to capture distinct features. Glass-mounted HSPC and pH-sensitive liposomes were meticulously examined under phase contrast microscopy, allowing detailed observations at optimal magnification. These observations were thoughtfully documented through photomicrographs, unveiling the distinctive visual attributes of each formulation. Furthermore, a comprehensive TEM study was conducted, involving the creation of thin films on carbon-coated copper grids. These grids, housing the liposome films, were meticulously air-dried and subjected to TEM analysis, providing valuable insights into their internal architecture at magnifications enabled by an accelerating voltage of 200 kV. The resulting images offered a deeper understanding of the structural organization of liposomes [26].

pH determination

The inherent pH values of both types of liposomal formulations were critically assessed, serving as a vital indicator of their stability and compatibility. Measurements were carried out using a precise Elico pH meter, maintaining a controlled temperature of 25 °C ± 0.5 °C. Ensuring the robustness of the findings, the pH determination was conducted in triplicate, reinforcing the accuracy and reliability of the obtained results [27].

Percentage entrapment efficiency

The encapsulation efficiency of the liposomal formulations was quantitatively determined to offer insights into their drug incorporation capabilities. Employing a meticulously designed protocol, liposome pellets underwent dissolution in PBS with 3% Triton X-100, followed by sonication and ultracentrifugation. The resultant process enabled the quantification of percentage entrapment efficiency (%EE). The centrifugation steps, conducted under precise conditions, contributed to accurating measurements. The derived values of %EE were calculated employing established formulas (Eq. 1), providing a quantitative assessment of the formulations' drug incorporation efficacy[28].

1

$$\%EE = \frac{Weightofinitialdrug - WeightoffreedrugWeightofinitialdrug}{} \times 100$$

In vitro drug release study

The in vitro drug release profiles of the formulations were meticulously examined, employing a well-established dialysis bag method. In this procedure, the formulations were introduced into dialysis bags, which were immersed in a controlled environment consisting of PBS with a pH of 6.4. Maintained under conditions of controlled temperature (37 °C ± 0.5 °C) and gentle stirring, the formulations' drug release was monitored over 24 h. At regular intervals, samples were withdrawn, and their drug release was quantified through UV spectroscopy. The cumulative drug release data provided valuable insights into controlled release kinetics of the liposomal carriers [29].

Blood compatibility analysis

Evaluating the formulations' compatibility with blood coagulation mechanisms, the PT (prothrombin time) and APTT (activated partial thromboplastin time) assays were deployed. Fresh blood samples, collected in ACD-containing tubes, underwent centrifugation to obtain platelet-poor plasma (PPP). This plasma was subsequently mixed with samples from the formulations, and after an incubation period, PT and APTT were assessed through coagulation analyzer reagent kits. The meticulous evaluation of coagulation parameters allowed for a comprehensive understanding of the formulations' impact on blood coagulation mechanisms, thereby ensuring their biocompatibility [30].

MTT assay: cell viability assessment

The cytotoxic potential of the optimized HSPC and pH-sensitive liposomes was evaluated through MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assay, a widely accepted method for gaging cell viability. Employing A549 cell lines as a model, cells were cultured in 96-well tissue culture plate with cell density of 104 cells/well and were allowed to adhere for 24 h. Following the cell adhesion, cell media was replaced with fresh media containing the treatment sample (2.5, 5, 10, 20, 40, 80 µg/mL) and was incubated for 24 h. Following the incubation, 20 µL of MTT solution (5 mg/mL) was added in each well and was incubated for 3 h. The media was aspirated and 100 µL of dimethylsulfoxide was accurately pipetted into each well to dissolve the formed formazan crystals. The optical density in each well was observed using ELISA microplate reader (M/s BioTek, Vermont, USA) at 550 nm. Thus, based on the optical density observed, % cell viability was calculated (Eq. 2)[31].

2

$$\%\text{Cellviability}=\frac{O.D_{\text{treatment}}-O.D_{\text{blank}}}{O.D_{\text{control}}-O.D_{\text{blank}}}\times 100$$

$O.D_{\text{treatment}}$, $O.D_{\text{blank,}}$, and $O.D_{\text{control}}$ are the optical densities observed for the treatment, blank (without cells), and the control (without treatment) group, respectively.

Incorporating a diverse array of methodologies, these characterization efforts contributed to a profound understanding of the structural, functional, and biocompatible attributes of liposomal formulations. Each subheading delved into specific facets of the formulations, offering valuable insights that collectively contribute to a holistic comprehension of their performance and potential applications.

Results

In silico analysis

A total of 29 protein structures were predicted using computational modeling tools, including MODELLER 10.2, I-Tasser, and SwissModel. Among these models, the SwissModel-generated model.4 exhibited superior accuracy, as evidenced in Fig. 1, thereby warranting its selection for subsequent docking analyses. Docking studies were conducted across five grid configurations to investigate the binding cavity of the P2X7 receptor, which was homology-modeled using SwissModel. The results are depicted in Fig. 1C, highlighting specific interactions, such as two hydrogen bonds and two Pi-Pi stacking interactions, depicted in distinct colors in Fig. 2. The ligand, Brilliant Blue G dye (BBG250), demonstrated binding interactions with amino acid residues Glu66, Phe97, Tyr111, and Pro112. The XP docking score for this interaction was determined to be −9.597, An estimated high binding affinity between the ligand (in this case, Brilliant Blue G dye or BBG250) and the P2X7 receptor is indicated by a docking score value of −9.597. The arbitrary negative sign simply denotes that a lower energy state is connected to a more stable complex. A result of −9.597 means that there is a strong and favorable binding interaction between BBG250 and the P2X7 receptor, indicating that there is a high probability of the ligand binding to the receptor and creating a stable complex. In comparison, the reference drug, docetaxel, exhibited a docking score of -4.921, indicating lower affinity than BBG250. The observed interactions between BBG250 and the P2X7 receptor, as outlined in Table 6, play a pivotal role in its inhibitory activity. These findings underscore the potential of BBG250 as a candidate for modulating the P2X7 receptor.

Table 6. Amino Acid residues interaction with BBG-250

Ligand	Protein	DOCK Score	H-bond	Pi-Pi stacking	Salt bridge
BBG 250	P2X7	−9.597	2	2	0

The results of the *In silico* ADME study are shown in Tables 7, 8, 9, 10, 11 The physicochemical properties of BBG-250 are reported in Table 7, indicating that BBG-250 possesses molecular weight>500, several hydrogen bond acceptors between 7, several hydrogen bond donors between 1, several rotatable bonds between 15, and a molar refractivity is 233.61. The lipophilicity profile of selected compounds is reported in Table 8, which suggested that BBG-250 possesses reasonable lipophilic character BBG-250 did not cross the blood–brain barrier. To check the

druglikeness parameter, Lipinski &Ghose filter is applied. BBG-250 violet the two Lipinski rule and four Ghose rules (Tables 11, 12, 13). It was observed from the above study that BBG-250 has high GI absorption (Table 10), does not cross the blood–brain barrier, has water solubility (Table 9), considerable lipophilic profile and moderate synthetic accessibility. BBG-250 did not follow the Lipinski rule because of its higher molecular weight (>500). The accessibility of the compound (BBG-250) was similar to that of the reference drug, docetaxel.

Table 7. Validation of docking result

Ligand	Protein	DOCK Score
Reference compounds		
Docetaxel	P2X7	−4.921
Positive control (Non-inhibitors)		
ATP (Adenosine triphosphate)	P2X7	−6.476
BzATP (2′,3′-O-(4-benzoylbenzoyl) adenosine 5′-triphosphate)	P2X7	−5.494
Negative control (Inhibitors)		
A-438079	P2X7	−4.499
AZ11645373	P2X7	−5.595
KN-62	P2X7	−4.957

Table 8. Generated refine model

Model	GDT-HA	RMSD	MolProbity	Varify3D	Errat score	Clash score	Poor rotamers	Rama favored
Initial	1.0000	0.000	1.187	54.56%	90.7955	2.0	1.1	96.8
MODEL 1	0.9705	0.363	1.560	59.55%	93.5943	11.1	0.0	98.1
MODEL 2	0.9753	0.357	1.532	53.82%	88.5714	10.3	0.4	98.4
MODEL 3	0.9769	0.344	1.567	52.87%	88.172	11.2	0.4	98.1
MODEL 4	**0.9697**	**0.363**	**1.574**	**61.78%**	**88.1533**	**11.4**	**0.4**	**98.7**

MODEL 5	0.9721	0.378	1.532	58.60%	87.8571	10.3	0.4	98.4

Bold value indicates the best model

Table 9. Results of ADME study (Physicochemical properties)

S.No	Name	Formula	Mol. Wt. (g/mol)	No. rot bonds	No. H-bond acceptor	No. H-bond donors	Molar refrac-tivity
1	BBG-250	C47H48N3NaO7S2	854.02	15	7	1	233.61
2	Docetaxel	$C_{43}H_{53}NO_{14}$	807.9	14	14	5	205.25

Table 10. Lipophilicity profile of BBG-250

Sl No	Compound name	Log Po/w (XLOGP3)	Log Po/w (WLOGP)	Log Po/w (MLOGP)
1	BBG-250	8.21	10.82	5.67
2	Docetaxel	2.81	2.94	1.06

Table 11. Water solubility profile of BBG-250

S No	Compound name	Log S (ESOL)	Solubility (mg/ml)	Class
1	BBG-250	−9.69	1.75e−07	Poorly soluble
2	Docetaxel	−5.85	1.15e−03	Moderately soluble

Table 12. Pharmacokinetics results

Sl No	Compound name	GI absorpti-on	BBB perme-ant	CYP1 A2 inhibit-or	CYP2 C19 inhibit- or	CYP2 C9 inhibit-or	CYP2 D6 inhibi t-or	CYP3 A4 inhibi t-or
1	BBG-250	Low	No	No	No	No	No	No
2	Docetaxel	Low	No	No	No	No	No	Yes

Table 13. Results of druglikeness, leadlikeness

Sl No	Compound name	Druglikeness			Leadlikeness ; violation
Lipinski rule; Violation	Ghose rule; violation	Bioavaila- bility score	1	BBG-250	2
4	0.17	3.5	2	Docetaxel	2

Solubility class: Log S scale. Insoluble <−10 <Poorly <−6 <Moderately <−4 <Soluble <−2 Very <0 <Highly. Lipinski (Pfizer) filter: MW≤500, MLOGP≤4.15, N or O≤10, NH or OH≤5; Ghose filter: 160≤MW≤480, -0.4≤WLOGP≤5.6, 40≤MR≤130, 20≤atoms≤70; Leadlikeness: 250≤MW≤350, XLOGP≤3.5, Number of rotatable bonds≤7; Synthetic accessibility: from 1 (very easy) to 10 (very difficult).

FTIR spectrum

The FTIR spectrum of HSPC liposomes (P11) revealed characteristic peaks corresponding to various functional groups present in the formulation. Notably, the presence of strong absorption bands around 2920 cm^{-1} and 2850 cm^{-1} indicated the presence of CH stretching vibrations, typically found in lipid chains. The absorption bands around 1736 cm^{-1} indicated the presence of carbonyl (C=O) stretching vibrations, further confirming the presence of lipid components. Additionally, absorption peaks around 1230 cm^{-1} and 1100 cm^{-1} indicated the presence of phosphodiester groups in the formulation, which is consistent with the presence of phospholipids in liposomal formulations (Fig. 3a).

Fig. 3 [Images not available. See PDF.]

a FTIR spectrum of HSPC liposomes (P11); **b** FTIR spectrum of pH-sensitive formulation (P5); **c** FTIR spectrum of Brilliant Blue G-250 (BBG-250); **d** Particle size of formulation (P5) 118 nm; **e** Zeta potential of formulation (P5) − 25 mV; **f** Microscopic image of formulation (P5); **g** TEM image of formulation (P5); **h** In vitro drug release of formulation pH-sensitive (P5) and HSPC liposomes (P11) in phosphate buffer (pH 6.4); **i** Graph between % viability and concentration of formulation pH-sensitive (P5) and HSPC liposomes (P11), free dye, Docetaxel (DTX)

The FTIR spectrum of the pH-sensitive formulation (P5) exhibited distinct peaks corresponding to the specific functional groups present in the formulation. Notably, the absorption bands around 1736 cm^{-1} indicated the presence of carbonyl (C=O) stretching vibrations, characteristic of lipid components. The absorption bands around 1650 cm^{-1} indicated the presence of amide I bond, suggesting the incorporation of protein-like components, such as DSPE-PEG_{2000}, within the formulation. Furthermore, absorption peaks around 1100 cm^{-1} and 1050 cm^{-1} indicated the presence of P=O stretching vibrations, confirming the presence of phospholipids (Fig. 3b).

The FTIR spectrum of Brilliant Blue G-250 (BBG-250) showed distinct absorption bands characteristic of the dye molecule. Notably, the absorption peak around 1600 cm^{-1} indicated the presence of aromatic C=C stretching vibrations, confirming the aromatic nature of the dye. The absorption bands around 1300 cm^{-1} and 1200 cm^{-1} indicated the presence of aromatic C-N stretching vibrations, further confirming the structure of the dye (Fig. 3c).

FTIR analysis provided valuable insights into the molecular composition and interactions within the HSPC liposomes, pH-sensitive formulation, and Brilliant Blue G-250 dye. The characteristic peaks observed in the spectra confirmed the presence of specific functional groups and molecular vibrations, further supporting the formulation and

characterization of the liposomal delivery system and the dye.

Vesicle shape, size, size distribution, and zeta potential

The quantitative incorporation of BBG-250 into two distinct liposomal formulations was the focal point of the current study. One formulation comprised hydrogenated soya phosphatidylcholine (HSPC) and cholesterol, while the other featured dioleoylphosphatidylethanlamine (DOPE), DSPE-mPEG, DPPC, and cholesterol. A thin film method was employed to create these liposomes, with specific lipid ratios determining their properties. The HSPC and cholesterol ratio of 7:3 yielded liposomes with a vesicular size of 118 ± 1.2 and a polydispersity index (PDI) of 0.316. Similarly, the DOPE, DPPC, DSPE-PEG_{2000}, and cholesterol ratio of 4:3:3:0.3 resulted in liposomes with a vesicular size of 125 ± 1.6 and PDI of 0.165 (Fig. 3 d, e).

Morphology and structure determination

Transmission electron microscopy (TEM) examination revealed the morphology and structural attributes of the formulated HSPC and pH-sensitive liposomes. The TEM images showcased spherical vesicles with uniform size distribution for both formulations. HSPC liposomes exhibited sizes ranging from approximately 100–150 nm, while pH-sensitive liposomes displayed slightly larger sizes spanning 120–180 nm, potentially due to additional components. The lipid bilayer membrane of both liposomal types appeared intact and continuous, affirming their stability and suitability for drug encapsulation and delivery. The TEM results affirmed the successful formation of well-defined liposomal structures, underlining their potential for effective targeted drug delivery applications (Fig. 3f, g).

Analysis of pH levels

The pH values of formulated HSPC (P11) and pH-sensitive liposomes (P5) were measured and found to be 7.3 and 7.8, respectively. These pH values signify the intravenous safety of the liposomal formulations, highlighting their compatibility within physiological pH ranges.

Percentage drug efficiency

The entrapment efficiency of plain dye and HSPC liposome (P11) was determined to be 97% and 70%, respectively. In contrast, the pH-sensitive liposomes (P5) exhibited an entrapment efficiency of 95%. These findings underscore the formulations' capacity to encapsulate the drug effectively.

In vitro drug release studies

The In vitro drug release profiles of both optimized formulations were assessed using a dialysis tube method. The optimized HSPC formulation (P11) displayed a drug release profile of 84.95% over 24 h in PBS 6.4, while the optimized pH-sensitive liposomes (P5) exhibited a drug release of 95% over the same period. These release profiles are visually presented in Fig. 3h, providing a comprehensive depiction of the formulations'-controlled drug release kinetics (Table 14).

Table 14. Optimized formulation of conventional and pH-sensitive formulation

Formulation code	Lipid ratio	Drug (mg)	Sonication time	Particle size (nm)	PDI	Entrapment efficiency	Zeta potential
P1	3:2:3:0.3	4	4 min	325 ± 1.6	0.230	49%	−53
P2	3:3:3:0.3	4	4 min	370 ± 0.2	0.165	52%	−55
P3	3:4:3:0.3	4	4 min	390 ± 1.3	0.599	75%	−37

P4	4:2:3:0.3	4	4 min	156±1.4	0.230	72%	−20
P5	**4:3:3:0.3**	**4**	**4 min**	**118±1.2**	**0.165**	**78%**	**−25**
P6	4:4:3:0.3	4	4 min	290±0.8	0.599	65%	−47
P7	5:2:3:0.3	4	4 min	328±0.6	0.230	69%	−30
P8	5:3:3:0.3	4	4 min	425±1.2	0.165	70%	−22
P9	5:4:3:0.3	4	4 min	609±0.6	0.599	45%	−41
P10	8:2:0.3	4	4 min	425±0.6	0.215	**35**	−59
P11	**7:3:0.3**	**4**	**4 min**	**125±0.3**	**0.316**	**64**	**−28**
P12	6:4:0.3	4	4 min	356±0.9	0.366	**52**	−38

Lipid Ratio P1-P9 (DPPC: DOPE: Cholesterol: DSPE-PEG_{2000}); P10-P12 (HSPC: Cholesterol: DSPE-PEG_{2000})
Bold letters highlight the highest drug release in both formulations, whether HSPC or pH-sensitive

Blood compatibility analysis

The blood compatibility of the liposomal formulations was assessed through PT and APTT analyses, evaluating their impact on the extrinsic and intrinsic pathways of blood coagulation. The PT and APTT times for the optimized pH-sensitive formulation (P5) were 12 s and 43 s, respectively, while the HSPC liposomal formulation (P11) exhibited 14 s and 46 s, respectively. These times, slightly lower than the pH-sensitive formulation, demonstrated a normal safe margin compared to the negative control (saline). The findings as in Table 15 indicated that the liposomal formulations were not disrupted the blood coagulation pathways, thus confirming their biosafety.

Table 15. Coagulation assay by prothrombin time (PT) and activated partial thromboplastin time (APTT) assessment

S. No	Formulation	APTT Assay	PT assay
1	Normal patient value	45 s	15 s
2	P5	43 s	12 s
3	P11	46 s	14 s

MTT assay: cell viability assessment

The cytotoxicity of HSPC and pH-sensitive liposomes was assessed through an MTT assay on A549 cell lines. The results revealed that both liposomal formulations exhibited cytocompatibility across all concentrations tested. Notably, the MTT profiles illustrated that HSPC and pH-sensitive liposomes displayed significant toxicity toward A549 cell lines compared to blank media. The calculated IC_{50} values further emphasized the formulation's cytotoxic effects, with pH-sensitive formulation, conventional formulation, free drug, and docetaxel having IC_{50} values of 31.6181, 30.2012, 38.8108, and 4.72867, respectively (Fig. 3i, Table 16).

Table 16. Concentration, viability and IC_{50} of formulation pH-sensitive (P5) and HSPC liposomes (P11), free dye, Docetaxel (DTX)

Conc. (μg/mL)	P5		P11		Free Dye		DTX	
% viability	SD	% viability	SD	% viability	SD	% viability	SD	2.5
94.88542	4.718652	93.40623	4.654433	98.67076	1.0087	59.71	0.61	5
90.52881	5.725947	89.45039	5.152342	91.2	0.702183	57.6	0.85	10
86.21429	5.132336	86.23587	4.221246	90.0381	1.165957	51.95	0.87	20
79.00585	4.603671	77.53959	5.348681	83.30548	0.741875	38.04	1.27	40
54.26522	4.212066	54.25856	4.32278	58.35145	5.786023	32.16	1.74	80
43.68431	4.356637	44.89444	4.736364	46.88714	0.483777	26.85	0.57	IC_{50} (μg/mL)

The comprehensive experimental results elucidated the vesicular attributes, drug incorporation efficiency, drug release behavior, blood compatibility, and cytotoxicity profiles of the developed liposomal formulations. These findings collectively contribute to a comprehensive understanding of the formulation’s potential biomedical applications and safety considerations.

Discussion

The binding of BBG-250 to the inter-subunit allosteric pocket of the P2X7 receptor in a noncompetitive manner has been established as a safe approach for human administration. In this study, before doing the formulation study we proved interaction between P2X7 and BBG-250 through Docking Study (Fig. 1), and computational ADME study was observed from the above study (Tables 7, 8, 9, 10, 11, 12) that BBG-250 has high GI absorption, not crosses the blood–brain barrier, having water solubility, considerable lipophilic profile. BBG-250 did not follow the Lipinski rule because of higher molecular weight (>500), so due to this inference, we aimed to explore the potential of liposomal formulations for delivering BBG-250, focusing on formulation comparison, drug release, blood compatibility, and in vitro cytotoxicity. Our findings shed light on the effectiveness of liposomal encapsulation and its implications for antitumoral activity. Formulation optimization is crucial for successful drug delivery. We compared two liposomal formulations: a conventional one composed of HSPC and cholesterol in a 7:3 ratio, and a pH-sensitive formulation consisting of DPPC, DOPE, DSPE-PEG_{2000}, and cholesterol in a 4:3:3:0.3 ratio, both incorporating BBG-250. Microscopic analysis confirmed the vesicles' spherical morphology and uniform size distribution. Size plays a pivotal role in liposome behavior within biological systems. To ensure effective delivery and prevent undesirable accumulation, liposome size is typically maintained below 200 nm. In our study, the conventional and pH-sensitive

formulations exhibited sizes of 118 ± 1.2 nm and 125 ± 0.3 nm, respectively, optimizing their potential for tumor targeting while avoiding lung deposition and macrophage clearance. Zeta potential reflects the stability of liposomal suspensions under physiological conditions. The zeta potentials of the conventional and pH-sensitive formulations were − 25 and − 28 mV, respectively, suggesting adequate stability due to the repulsion between particles. The entrapment efficiency and drug loading of both formulations increased with higher drug-to-lipid ratios, indicative of the formulation's capacity to accommodate BBG-250. This finding supports the feasibility of tailoring drug loading based on therapeutic requirements. In vitro, drug release profiles are critical indicators of formulation performance. pH-sensitive and conventional liposomes exhibited superior drug release, reaching 95% and 74.95%, respectively, within 24 h, compared to the plain drug solution. This enhanced drug release kinetics in the pH-sensitive formulation aligns with its potential for controlled and targeted drug delivery.

Blood compatibility is a crucial factor for intravenous administration. Our study demonstrated the safety of both conventional and pH-sensitive liposomal formulations in terms of blood coagulation parameters. This highlights their potential for clinical translation and systemic administration. Cytotoxicity assessment using A549 cell lines revealed the effectiveness of both HSPC and pH-sensitive liposomes at various concentrations. The calculated IC_{50} values of 30.2012 ug/ml and 31.6181 ug/ml for HSPC and pH-sensitive liposomal formulations, respectively, indicate their significant toxicity compared to blank media. Notably, the IC_{50} values were approximately 7–eightfold higher than that of docetaxel, a reference drug. In conclusion, nanoscale liposomal formulations offer promising potential for cancer therapy due to their efficient drug delivery and tumor cell penetration capabilities. Our results indicate that liposomes composed of natural SL can serve as stable, biocompatible carriers for BBG-250 delivery, demonstrating the potential for cancer treatment, albeit with less potency compared to the reference drug, docetaxel. Future research could focus on further enhancing liposomal formulation characteristics to optimize antitumoral activity and clinical translatability.

Conclusion

In conclusion, this study establishes the safe and noncompetitively binding nature of BBG-250 to the inter-subunit allosteric pocket of the P2X7 receptor for human administration. Computational ADME analysis revealed favorable pharmacokinetic characteristics for BBG-250, while subsequent exploration into liposomal formulations demonstrated their potential as effective carriers for the compound. Both conventional and pH-sensitive liposomes exhibited promising characteristics, including uniform size distribution, stability, and controlled drug release. Notably, pH-sensitive liposomes demonstrated superior drug release kinetics. These findings underscore the potential of liposomal delivery systems for BBG-250, offering a viable avenue for enhancing cancer therapy. Further refinement of these formulations could lead to optimized treatment strategies in the future.

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Author contributions

All authors, including Twinkle Gupta and Vivek Yadav (formulation and data collection), Sakshi Soni and Sanyog Jain (data collection and interpretation), and Vandana Soni and Sushil Kumar Kashaw (supervisors and manuscript writing), have read and approved the manuscript.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Ethics approval and consent to participate

No animal experiments were performed in this work,

Consent for publication

NA.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

BBG-250
Brilliant Blue G-250
HSPC
Hydrogenated soya phosphatidylcholine
DOPE
Dioleoylphosphatidylethanolamine
DSPE-mPEG
1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]
DPPC
Dipalmitoylphosphatidylcholine
PBS
Phosphate-buffered solution
PDI
Polydispersity index
TEM
Transmission electron microscopy
PT
Prothrombin time
APTT
Activated partial thromboplastin time
MTT
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
IV
Intravenous
DTX
Docetaxel

Publisher's Note

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The possible anti-inflammatory effect of extra virgin olive oil with colchicine in treatment of resistant cases of familial Mediterranean fever in a cohort of pediatric Egyptian patients

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ABSTRACT (ENGLISH)

Background

People of Mediterranean descent are primarily affected by the autoinflammatory genetic condition known as familial Mediterranean fever (FMF). The disease is resistant to colchicine therapy in 10–20% of patients. Numerous recent animal studies showed promising results of extra virgin olive oil (EVOO) to control inflammation. The objective of this

study was to assess the effectiveness of combining EVOO with colchicine in the treatment of colchicine-resistant familial Mediterranean fever (CRFMF) patients.

Results

Both the frequency of episodes and inflammatory indicators significantly decreased after a three-month course of daily EVOO treatment with colchicine. The average erythrocyte sedimentation rate (ESR) of patients was 78.6 mm/h before the EVOO administration, and it dropped to 27.8 mm/h, after that. Additionally, after taking EVOO, the mean serum amyloid A (SAA) decreased from 123.82 mg/dl to 59.78 mg/L. Also, the average C-reactive protein (CRP) decreased from 34.22 to 7.84 mg/dl following its administration; the mean nucleotide-binding domain, leucine-rich-containing family, and pyrin domain-containing-3 (NLRP3) level decreased from 134.92 to 64.23 pg/ml. The mean caspase-1 level decreased from 7.8 to 4.98 ng/ml; and the mean levels of cytokines, interleukin 6 (IL-6), interleukin 1 beta (IL-1 β), and tumor necrosis factor-alpha (TNF-α) decreased from 9.8, 18.14, and 52.7 pg/ml, respectively, to 5.95, 12.51, and 29.39 pg/ml. Finally following the administration of EVOO, there was a notable overall improvement in the quality of life of (CRFMF) patients.

Conclusion

EVOO demonstrated a significant positive impact when paired with the tolerated dosage of colchicine in the management of CRFMF. Improvements were observed in both clinical and laboratory settings, including a reduction in the attack frequency and serum levels of inflammatory markers, such as NLRP3, caspase-1, ESR, CRP, IL-1β, IL-6, and TNF-α without any negative side effects.

FULL TEXT

Background

The autoinflammatory condition known as familial Mediterranean fever (FMF) is represented by repeated episodes of fever, polyserositis, arthritis, and skin manifestations as well as a strong acute-phase response lasting 12–72 h [1, 2]. It is the most prevalent autoinflammatory illness globally [3, 4]. In 1945, the illness was initially referred to as “benign paroxysmal peritonitis.” People in the Mediterranean region, such as Arabs, Greeks, Italians, and Turks, are primarily affected by the disease, but, throughout the twentieth century, migration and travel have increased its incidence [5, 6]. Prodromal signs of FMF include pain in the area where the flare is expected to occur one or two days before symptoms manifest. FMF fever is usually recurring and of a high degree (>38 °C). It usually rises quickly for one to three days, reaching a plateau then falls off [7].

Severe abdominal pain and stiffness are brought on by peritoneal inflammation (aseptic peritonitis), which starts locally and spreads throughout the body. It is possible to develop pericarditis or pleuritis, in the form of unilateral chest pain [8]. When FMF attacks occur in children, arthritis is frequently the accompanying symptom. It is usually monoarticular and commonly affects the big joints in the lower limbs such as ankles and knees. These arthritis symptoms can arise during FMF attacks and may also occur intermittently between episodes [9]. The dermatological manifestation of FMF is the skin lesions that resemble warm, painful erysipelas on the lower limb. Renal amyloidosis is the main FMF consequence that leads to end-stage renal failure [10, 11].

FMF arises from a mutation in the MEFV gene found on chromosome 16's short arm (p). This specific gene is responsible for producing pyrin protein, comprising 781 amino acids [12].

Over the past few years, evidence linking inflammation to FMF has grown. Pyrin is a member of the cytosolic pattern recognition receptors (PRRs) group expressed in neutrophils, eosinophils, monocytes, dendritic cells, and synovial fibroblasts. It is responsible for regulating innate immune responses upon detecting specific signals from pathogens or host-derived danger molecules, known as pathogen/danger-associated molecular patterns. When activated, pyrin, along with other receptors, forms inflammasomes, which are multiprotein signaling complexes. These inflammasomes recruit and activate caspase-1, an enzyme involved in promoting inflammation. Active caspase-1 facilitates the proteolytic maturation and secretion of cytokines, such as interleukin (IL)-1β that triggers a form of cell death characterized as pyroptosis, which is necrotic in nature [13, 14]. Mutation of MEFV genes disrupts pyrin protein and its function leads to unrestricted pyrin activity, causing excessive production of the NLRP3 inflammasome which in turn triggers the complete inflammatory cascade. This uncontrolled inflammation is

responsible for the characteristic febrile inflammatory episodes seen in FMF [15]. Therefore, addressing these inflammatory pathways can stop attacks from happening, return inflammation to normal in between episodes, and stop amyloidosis from developing [16].

The clinical presentation of FMF varies greatly, depending on the specific sequence variations found in the MEFV gene [17]. According to the European Alliance of Associations for Rheumatology (EULAR) recommendations, the objective of treating FMF is to achieve control over acute episodes, reduce chronic and subclinical inflammation, and prevent consequences, primarily renal amyloidosis that leads to renal failure [18].

Since 1972, colchicine has remained the primary treatment for FMF. This alkaloid compound inhibits a variety of cellular processes, including cell adhesion, microtubule assembly, and inflammasome activation. Administration of colchicine ultimately helps prevent FMF flare-ups in patients receiving this treatment [19]. However, approximately 10–20% of FMF patients exhibit resistance to colchicine. Colchicine resistance is typically described as experiencing one or more attacks per month even though receiving the maximum tolerated dose for six months, while others may only partially respond or be intolerant to high doses of colchicine. This poses a challenge for rheumatologists in managing such cases [20].

On the other hand, macrophages are crucial components of the immune system, playing a central role in the regulation of infections and inflammatory processes through their involvement in both innate and adaptive immune responses [21]. Regrettably, FMF patients with MEVF mutations experience an overabundance of macrophage activation. When macrophages are stimulated, they release pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α [22].

Extra virgin olive oil (EVOO) is commonly used as a dietary supplement or an over-the-counter remedy for various health benefits. EVOO comprises over 200 distinct chemical compounds, encompassing fatty acids, sterols, terpenoids, carotenoids, tocopherols, flavonoids, and olive polyphenols such as tyrosyl, hydroxytyrosol, oleuropein, oleacein, olive ligstroside, and oleocanthal (OC) [23]. Among these polyphenols, OC has garnered significant scientific attention due to its intriguing biological activities, despite representing only 10% of the olive's polyphenol content (100–300 mg/kg olive oil). This variability is influenced by various factors, including the type of olive cultivars, the environmental conditions in which the olives are grown, agricultural practices employed, the maturity of the olives, the methods used for processing the olives into oil, as well as the storage and heating conditions [24].

Research has revealed that OC exhibits potent anti-inflammatory properties [25]. Given that the development of numerous chronic diseases involves both inflammatory and oxidative components. OC was shown to be able to block catabolic genes like Matrix metalloproteinase-13 and ADAM Metallopeptidase with Thrombospondin Type 1 Motif (ADAMTS-5) as well as inflammatory genes like IL-6, IL-8, cyclo-oxygenase-2, nitric oxide synthase-2, macrophage inflammatory protein-1 alpha, TNF-α, and lipocalin-2 in osteoarthritis chondrocytes and macrophages studies. These findings demonstrated the compound's significance in the management of rheumatic and inflammatory disorders by controlling the NF-kB and mitogen-activated protein kinases pathways [26]. Additional recent data demonstrated that when EVOO polyphenolic extract OC and oleoresin were added to human synovial SW982 cell line, a substantial decrease in IL-1β-induced TNF-α and IL-6 production was observed [27].

Oleocanthal shows promise as a potential agent for preventing such conditions. It is worth noting that oleocanthal may work with other bioactive compounds present in EVOO to maximize its therapeutic potential. Additionally, several studies have suggested that combining colchicine with biological therapy targeting FMF inflammatory markers can be valuable for patients who do not respond adequately [28, 29]. However, the high cost associated with lifelong treatment using biological therapy and concerns about safety, [30] including an increased risk of cancer and infection, have prompted the need for alternative approaches [31]. Therefore, our study aims to investigate the effect of using extra virgin olive oil, which is rich in polyphenols, in combination with colchicine to achieve better control of attacks in FMF patients who have not responded to colchicine treatment alone clinically and laboratory, furthermore to determine the molecular mechanisms by which EVOO exerts its effect if found.

Patients and methods

This study was a single-center prospective single-arm study design. A total of 50 patients were assessed for

eligibility in this single-arm study, 20 patients were excluded (17 patients did not meet the inclusion criteria, and three patients refused to consume olive oil due to taste preferences). Then, 30 FMF patients continued the study. Among the participants, there were 14 males and 16 females who were being monitored at the Pediatric Rheumatology Outpatient Clinic of Children's Hospital (Pediatrics Specialized Hospital) at Cairo University. The study has lasted three months, starting in October and concluding at the end of December. The patients included in the study were children ≤ 16 years old, diagnosed with FMF based on the Yalcinkaya new FMF criteria [32] and showing +ve MEFV gene mutation who had at least one attack per month despite adhering to the maximum tolerated dose of colchicine treatment (up to 2 mg/day). The researchers assessed the participants' compliance by asking them and their relatives, and by counting the number of the remaining medication in the strip inside the package with the patient at each visit. It was observed that despite the patients taking their medications correctly, their response to colchicine was still limited, and hence, they were classified as colchicine resistance FMF patients [33].

Patients over 16 years old or those with conditions that could impact compliance with the treatment protocol were excluded from the study. Patients receiving concurrent medications with biological or disease-modifying antirheumatic drug (DMARD), systemic steroids, or those with various autoimmune and other autoinflammatory diseases that could affect FMF activity, such as systemic lupus or juvenile idiopathic arthritis, Crohn's disease, ulcerative colitis, diabetes mellitus, gastrointestinal diseases, active ischemic cardiovascular disease, congestive heart failure, renal amyloidosis, or chronic renal failure, were also excluded.

Following the protocol's (N.320.2023) approval by Clinical Research Ethics Committee and the acquisition of informed consent from each patient's parents. A thorough history was obtained of each patient, including the age at which symptoms first appeared, the age of diagnosis, the length of the illness, the characteristics of attacks (frequency, duration, and clinical signs), and the dosage of colchicine the patient had taken. Quality of life (QOL), baseline data, and a physician checkup were completed during the first visit and evaluated again at the end of the study. The patient data confirmed the MEFV gene alterations.

Intervention and assessment

Over three months, the patients in the study received the maximum tolerated dose of colchicine (COLCHICINE OPO CALCIUM, France) along with a daily oral dose of (15–30 ml) of EVOO [34]. The specific EVOO used was chosen by an expert based on its high content of polyphenolic compounds, which are recognized by their characteristic smell and strong taste on the back of the tongue. The selected EVOO was obtained from ELSALHYA company at the Ministry of Agriculture outlet in Dokki, Cairo. To preserve the phenolic compounds, EVOO was stored in dark glass containers to protect it from light.

The patients were given instructions to maintain diaries, recording any concomitant medications used, and FMF attacks experienced, including their duration and affected sites, the need for analgesics, hospital visits during attacks, and the number of school days missed due to pain or FMF-related attacks. Additionally, one of the investigators was responsible for contacting the patients to ensure treatment compliance and to assist in interpreting symptoms as true attacks or not.

To evaluate QOL, the patients' pre- and post-treatment assessments were conducted using the Arabic-translated version of the PedsQL 4.0 Generic Core Scales (Mapi Research Trust, Lyon, France) [35]. This self-report scale covered physical functioning (eight items), emotional functioning (five items), social functioning (five items), and school functioning (five items). The scale items were scored on a 5-point Likert response scale ranging from “never a problem” to “almost always a problem.” The scores were reverse scored and linearly transformed to a 0–100 scale, where higher scores indicated a better quality of life.

Blood samples were collected from the patients at the beginning and the end of three months. ESR was measured, and then, samples were allowed to clot before being centrifuged at 10,000 rpm for 20 min. The separated serum was frozen and stored at -80°C until further analysis. The laboratory tests conducted on the serum samples included CRP screening using the latex agglutination test [36] and confirmation via nephelometry analysis, serum amyloid A (SAA) analysis using ELISA kits catalog no. CSB-E08589h supplied by (Cusabio, USA) according to the

manufacturer's instruction [37]. IL-1β levels catalog no. MBS012415, IL-6 levels catalog no. MBS355306, TNF-α levels catalog no. MBS2502004, NLRP3 levels by NACHT, LRR, and PYD domains-containing protein 3 (NLRP3/C1orf7/CIAS1/NALP3/PYPAF1) catalog no. MBS917009, Caspase-1 catalog no. MBS264676. All kits of IL-1β, IL-6, TNF-α, NLRP3, and Caspase-1 were ELISA kits supplied by (MyBioSource, S, USA) according to manufacturer instructions.

Statistical analysis

Sample size calculation

To obtain a two-sided 95% confidence interval, a total of 30 patients were required based on a previous study [38]. Initially, thirty-three FMF patients were included but three patients discontinued their participation, resulting in a dropout rate of 1%. The sample size estimation was conducted using the Epi info7 statistical package, with a power of 95% and a type I error of 0.05. The data collected at the end of the study were coded and entered into the Statistical Package for the Social Sciences (SPSS) version 26 (IBM Corp., Armonk, NY, USA). Quantitative data were summarized using measures such as mean, standard deviation, median, minimum, and maximum, while categorical data were summarized using frequency (count) and relative frequency (percentage). For comparing serial measurements within each patient (before and after), a paired t test was used for normally distributed quantitative variables, whereas the nonparametric Wilcoxon signed-rank test was employed for non-normally distributed quantitative variables [39]. *P* values less than 0.05 were considered statistically significant.

Results

Participant characteristics

All participants in this study showed genetic mutation as the following: The allelic frequency of MEFV mutations was M694V (36.6%), M694I (30%), E148Q (20%), and M680I (13.3%).

As illustrated in Fig. 1 ($n=30$), patients were included in this study, 16 females (53.3%) and 14 males (46.7%) with a mean age of 6.25 ± 3.07 years at diagnosis, and a mean disease duration of 5.54 ± 3.19 years.

Fig. 1 [Images not available. See PDF.]

The percentage of male and female participants

Clinical presentation data

The main symptoms of the studied group during periods of active disease were abdominal pain (100%), arthralgia (80%), fever (53.3%), and pleurisy (46.7%) Fig. 2.

Fig. 2 [Images not available. See PDF.]

The percentage of the main symptoms observed within the studied group

Numbers of attacks before and after receiving EVOO

All participants were taking the maximum tolerated dose of colchicine that was not enough to control the attacks and they were in need for frequent intake of analgesics. However, they exhibited resistance to these treatments, experiencing frequent attacks at varying intervals. These ranged from two attacks every two weeks (four attacks per month) for 6.7% of participants, to two attacks every four weeks (two attacks per month) for 23.3% of participants. Most participants experienced one attack every two weeks (33.3%) or one attack every three weeks (33.3%) before starting a daily regimen of extra virgin olive oil (EVOO), as shown in Fig. 3A. After three months of daily EVOO consumption, a reduction in the number of attacks was observed. Specifically, 40% of participants had one attack every four weeks, while 36.7% experienced one attack every six weeks, as depicted in Fig. 3B.

Fig. 3 [Images not available. See PDF.]

A and **B** Comparison between numbers of attacks before and after EVOO administration

Inflammatory markers

Analysis of acute-phase reactants and cytokine levels, which indicate the presence of inflammation, before and after the administration of extra virgin olive oil (EVOO), demonstrated a significant reduction in all inflammatory markers.

Before receiving EVOO, the mean ESR was 78.6 mm/h, which decreased to 27.8 mm/h. after treatment with a *P* value less than 0.001. The mean SAA level decreased from 123.82 to 59.78 mg/L after EVOO administration, also with a *P* value less than 0.001. The mean CRP level decreased from 34.22 to 7.84 mg/dl after EVOO treatment, with a *P* value of 0.007. Furthermore, the mean concentrations of NLRP3 dropped from 134.92 to 64.23 pg/ml with a *P* value less than 0.001 (Fig. 4A–D).

Fig. 4 [Images not available. See PDF.]

A–D Comparison between levels of acute-phase reactants (A: ESR; B: SAA; C: CRP and D: NLRP3 before and after EVOO administration in the study group. ESR: Erythrocyte sedimentation rate; SAA: serum amyloid A; CRP: C-reactive protein; NLRP3: nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (n = 30). # # (*p* value: <0.001) Significantly different as compared to the patient in the group before EVOO administration, using a paired t test for normally distributed quantitative variables, whereas using Wilcoxon signed-rank test for the nonparametric data

Additionally, the mean caspase-1 level decreased from 7.8 to 4.98 ng/ml with a *P* value less than 0.001. The mean levels of IL-6, IL-1β, and TNF-α were also reduced from 9.8, 18.14, and 52.7 pg/ml, respectively, to 5.95, 12.51, and 29.39 pg/ml, respectively, with a *P* value less than 0.001. These findings indicate a significant difference after the administration of extra virgin olive oil (EVOO), (Fig. 5A–D).

Fig. 5 [Images not available. See PDF.]

A–D Comparison between inflammatory markers (**A**: Caspase-1; **B**: IL-6; **C**: IL-1β; &**D**: TNF-α) before and after EVOO receiving by the study group. IL-6: Interleukin-6; IL-1 ß: Interleukin-1 beta; TNF-α: Tumor Necrosis Factor Alpha. (n = 30). ** (*p* value: <0.001) Significantly different as compared to the patient in the group before EVOO administration, using a paired t test for normally distributed quantitative variables, whereas using Wilcoxon signed-rank test for the nonparametric data

Quality of life (QOL)

The subjects were assessed using the Arabic version of the PedsQL 4.0 Generic Core Scales before and after receiving olive oil. The administration of EVOO led to a significant enhancement in the overall quality of life, as evidenced by significantly higher mean scores in school, emotional, social, and physical domains (*p* value: <0.001) (Table 1).

Table 1. Comparison between PedsQL domains of functioning before and after receiving EVOO

Score Item	Before	After
School	8.33 ± 5.45	89.79 ± 6.83 **
Emotional	10.00 ± 4.89	83.12 ± 13.97 **
Social	7.08 ± 5.50	84.17 ± 9.74 **
Physical	7.16 ± 3.96	96.48 ± 10.80 **

Data are expressed as mean ± SD (n = 30)

** Significantly different as compared to mean value in the group before EVOO administration using a paired t test was used for normally distributed quantitative variables, whereas the nonparametric Wilcoxon signed-rank test was employed for non-normally distributed quantitative variables (*p* value: <0.001)

Discussion

Colchicine resistance is characterized by a higher frequency or increased severity of attacks or elevated levels of

acute-phase proteins such as CRP and/or SAA (greater than 1.5 times the upper limit between attacks) [33]. Clinically, colchicine resistance is defined as at least one attack per month for six months at the maximal tolerable dose of colchicine in totally compliant individuals [40, 41].

In the present study, FMF patient-reported manifestations were considered attack only if they met the specific criteria identifying an attack and were confirmed by a study team member. In summary, an attack was defined as having the following characteristics: a fever equal to or greater than 38 °C, lasting from 6 h up to 7 days, and along with painful symptoms in the abdomen with signs consistent with peritonitis, chest symptoms consistent with pleuritis, joints symptoms consistent with monoarthritic involvement of large lower extremity joints, or skin symptoms. For evaluation of improvement in those patients, we used a modified FMF50 score. According to this scoring system, the fulfillment of five out of six criteria was considered an improvement [42]. This score required improvement by≥50 in the following: attack frequency, attack duration, global patient assessment, global physician assessment, frequency of attacks with arthritis, and levels of acute-phase reactants or the normalization of these markers. In our study, quality-of-life values (substituting patient global assessment in the original score). The measurements taken at the beginning of the study were compared to those obtained at the end to assess changes according to these criteria.

There is abundant evidence encouraging the significant role of EVOO in safeguarding against amyloidosis, a condition that can result in renal failure among patients with FMF. This protective effect is attributed to EVOO's capability to reduce the levels of SAA. Marina Aparicio et al. conducted a study demonstrating that a diet abundant in extra virgin olive oil can alleviate kidney injury in a pristane-induced model of systemic lupus erythematosus model [43]. Our own results align with these previous findings, as we observed a significant drop in SAA levels after three months of EVOO treatment.

Research has demonstrated that the underlying cause of FMF, an autoinflammatory illness, is mutations in the genes that produce pyrin [12]. This autoinflammation is mediated by IL-1β. Many studies have revealed that pyrin forms an inflammasome, which is a complex formed by the oligomerization of a nucleotide-binding domain-like receptor (NLR) in response to various pathogenic or sterile danger signals. This inflammasome facilitates the maturation of IL-1β by activating caspase-1 and triggers the pro-inflammatory cytokines release, including IL-1β [13, 44]. Another study carried out by Omenetti et al. also showed that those with FMF have increased NLRP3-dependent IL-1β release [45, 46]. It was discovered that OC inhibits both canonical and non-canonical inflammasome signaling pathways. Therefore, EVOO with oleocanthal properties may be a viable natural remedy for immune-inflammatory disorders in the future [47, 48]. In the current study, after three months of daily EVOO consumption, a significant reduction in the number of FMF attacks was observed. The levels of NLRP3 showed a significant reduction. Additionally, the mean caspase-1, IL-6, IL-1β, and TNF-α levels also showed marked reduction and according to much scientific evidence this improvement may be due to the oleocanthal content of EVOO.

Several studies, both clinical and experimental, revealed novel, relevant pharmacological properties of OC in a range of inflammatory disorders. Mice fed an oleocanthal-rich diet in an animal model of collagen-induced arthritis demonstrated significantly lower levels of matrix metalloproteinase-3, IL-17, TNF-α, IL-1β, IFN-γ, and IL-6 than mice fed on diet lacking in oleocanthal [49]. The observation by Rosillo et al. [50] of a significant reduction in IL-1β-induced TNF-α and IL-6 release in human synovial SW982 cells after exposure to a polyphenolic extract primarily composed of oleocanthal and oleacein from EVOO lends credence to these results. These findings have been confirmed also by Scotece et al., who found that OC has an inhibitory effect on particular cellular pathways engaged during inflammation in human osteoarthritis. As a result, genes linked to inflammation, such as COX-2, MIP-1α, TNF-α, IL-6, IL-8, and lipocalin-2, were expressed less frequently. The study also showed that OC could stop the action of an inflammatory protein called macrophage inflammatory protein-1 alpha [48].

These results were consistent with earlier studies [51] that showed OC can suppress the upregulation of pro-inflammatory signaling molecules mediated by lipopolysaccharides, such as IL-6, IL-1β, TNF-α, macrophage inflammatory protein-1α, and granulocyte macrophage-colony stimulating factor (GM-CSF).

Furthermore, previous study suggested that phenolic components in EVOO have an anti-inflammatory and

immunomodulatory role in systemic lupus erythematosus patients and may therefore be taken into consideration as a dietary component in the management of systemic lupus erythematosus [52]. Another study found that EVOO experienced synergistic analgesic, antipyretic, and anti-inflammatory properties when combined with ibuprofen in various albino mouse experimental models [53].

The current study findings indicate a significant improvement in the symptoms of abdominal aseptic peritonitis, which could be attributed to the capacity of oleocanthal present in EVOO to modulate peritoneal macrophages activation. This finding was supported by Montoya et al., who noticed that OC was able to attenuate macrophage activation induced by lipopolysaccharide via regulation of inflammasome: nuclear factor erythroid 2-related factor 2, and mitogen-activated protein-kinase signaling pathways.[51].

Furthermore, our findings also indicate a significant improvement in the school performance of the patients under study after three months of EVOO administration. This aligns with the research undertaken by Al Rihani et al., who demonstrated that EVOO rich in oleocanthal can restore the function of the blood–brain barrier and reduce Alzheimer's-associated pathology by inhibiting NLRP3 inflammasome, thereby reducing neuroinflammation [54].

The benefits of EVOO and its phenolic components are currently well understood. These benefits include their biological characteristics and antioxidant potential in thwarting immune-mediated inflammatory reactions. These include inflammatory bowel disease, cancer, diabetes, obesity, rheumatoid arthritis, atherosclerosis, and neurodegenerative disorders. The growing body of research conducted on this subject provides strong evidence, suggesting that olive polyphenols have the potential to be effective in addressing chronic inflammatory conditions. Furthermore, their potential clinical applications make them promising candidates in the fight against such inflammatory states.

Conclusion

Our study revealed that combining extra virgin olive oil, which is abundant in polyphenols, with the maximum tolerated dose of colchicine effectively reduced both the intensity and frequency of FMF attacks. Additionally, this treatment approach showed promising results in improving the overall quality of life for the patient. These findings provide hope for individuals with FMF, as it offers the potential for a better quality of life with reduced complications from the disease itself and the use of various biological therapies typically employed in FMF cases resistant to conventional treatments.

Limitations

It is a relatively short duration study, and it did not involve random assignment of participants to different groups (as it was pre- and post-study design). Furthermore, because all patients were recruited from a single hospital's Pediatric Rheumatology Outpatient Clinic, the findings of our investigation require confirmation in large multicenter prospective randomized future trials lasting considerably longer.

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Author contributions

WO designed the work. WO, HT, HA, and HD all contributed to data gathering and analysis. WO, HA, DL, HT, HD, MA, and OS wrote the original draft; supervision, material procurement, and facility support were supplied by all authors, as was the final text.

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Availability of data and materials

The corresponding author can provide data supporting the study's conclusions upon reasonable request.

Declarations

Ethics approval and consent to participate

The study has been approved by the Clinical Research Ethics Committee (N.320.2023) of Cairo University. All participants provided informed written permission after explaining the study aims and before the blood sample. The confidentiality of patient data was assured.

Consent for publication

Not applicable.

Competing interests

No competing interest to disclose.

Abbreviations

FMF

Familial Mediterranean fever

EVOO

Extra virgin olive oil

CRFMF

Colchicine-resistant familial Mediterranean fever.

ESR

Erythrocyte sedimentation rate

SAA

Serum amyloid A

CRP

C-reactive protein

NLRP3

Nucleotide-binding domain, Leucine-Rich-containing family, Pyrin domain-containing-3

IL-6

Interleukin-6

IL-1 ß

Interleukin-1 beta

TNF-α

Tumor Necrosis Factor Alpha

MEFV

Familial Mediterranean fever gene

PRRs

Cytosolic pattern recognition receptors

IL-18

Interleukin-18

EULAR

European Alliance of Associations for Rheumatology

OC

Oleocanthal

ADAMTS-5

ADAM Metallopeptidase with Thrombospondin Type 1 Motif

NF-kB

Nuclear Factor Kappa Beta

DMARDS

Disease Modifying Antirheumatic Drug

QOL

Evaluation of quality of life

GM-CSF

Granulocyte–macrophage–colony-stimulating factor

Nrf-2

Nuclear factor erythroid 2-related factor 2

MAPKs

Mitogen-activated protein kinase

Publisher's Note

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Pharmaceutical significance of Schiff bases: an overview

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ABSTRACT (ENGLISH)

Schiff bases are a diverse group of organic compounds with great pharmaceutical importance due to the presence of carbon–nitrogen double bonds (–C=N–). These compounds are synthesized by the condensation reaction between a primary amine and an aldehyde or ketone in a suitable solvent such as methanol. These compounds have shown antibacterial, antifungal, antiviral, anti-inflammatory, and antioxidant activities, which have garnered the attention of organic chemists in synthesizing these compounds. Recent advances have been summarized in this review paper mainly including compounds with potential antibacterial, antifungal, and antiviral activities. Synthetic schemes are included to provide a better understanding of the Schiff base synthesis mechanism. This review paper will provide a way forward for the pharmaceutical chemist to synthesize new compounds with potential biological activities.

FULL TEXT

Background

Hugo Schiff initially described in 1864, the synthesis of Schiff bases by a condensation reaction between a primary amine and multiple carbonyl compounds [1–6]. These Schiff bases are mostly referred to as the azomethine group represented in organic chemistry as $RHC=N–R^1$ [7, 8]. The corresponding alkyl, aryl, or heterocyclic groups can be the R or R^1 in Schiff bases. The Schiff bases are mostly referred to as the imines or the azomethine groups in synthetic chemistry [9, 10]. The presence of lone pairs is attributed to its sp^2 hybridization as the compound is highly reactive due to carbon and nitrogen atoms. Due to the presence of strong properties like adaptability, simplicity, and functionality, these compounds are of high importance [11, 12]. The significance is of high importance in biological assays that the Schiff bases can form diverse functional groups. An advanced approach to the existing nature of Schiff bases can be helpful nature to tackling the biological gaps and increasing the efficacy of Schiff base drugs [13–17] (Fig. 1).

Fig. 1 [Images not available. See PDF.]

General structure of Schiff bases

The discovery of Schiff bases dates back to the nineteenth century during which a chemist named “Hugo Schiff” first documented a reaction showing condensation between amines and carbonyl functional groups [13, 18–21]. In contemporary times, this domain of scientific inquiry about Schiff base coordination chemistry has seen significant growth and expansion [22, 23]. The significance of Schiff base complexes in the fields of material science, biomedical applications, bioinorganic chemistry, encapsulation processes, supramolecular chemistry, catalysis, and separation, and the generation of molecules with exceptional characteristics and structures has been widely acknowledged and extensively examined in the existing literature [24–27]. The literature has documented the use of Schiff bases derived from salicylaldehydes as agents for regulating plant development, as well as exhibiting antibacterial or antimycotic properties [28–30]. Schiff bases have been shown to possess analytical uses as well. The significance of these bases helps explain the difficult mechanisms inside biological systems and gives proper meaning to the imine group activity. These also provide broad-spectrum activity against several species, including *Candida*, *Plasmopora viticola*, *Trichophyton gypsum*, *Staphylococcus aureus*, *Erysiphe graminis*, *Mycobacterium*, *Albicans*, *Bacillus polymyxa*, and *Escherichia coli* [31–34].

Furthermore, the Schiff bases are significant in metal complexes due to their unique ability to form a structure holding transition central metals with the reactants of the condensation reaction as illustrated in the literature for metals like Hg(II), Gd(III), Cu(II), Co(II), Ni(II), Zn(II), Y(III), Al(III), Pb(II), and Ag(II) [35–44]. Extensive research has been conducted on Schiff bases due to their significant catalytic characteristics. These compounds exhibit catalytic properties when used in the process of hydrogenating olefins. Biomimetic catalytic processes are applicable in several contexts. One intriguing use of Schiff bases is in their utilization as a very efficient corrosion inhibitor, owing to their inherent capability to autonomously generate a monolayer on the targeted surface for protection. Numerous

commercial inhibitors often include aldehydes or amines; nevertheless, it is hypothesized that the enhanced efficacy seen in some instances is attributed to the presence of Schiff bases, likely owing to the presence of the C=N link [45–48]. Chemisorption is the primary mode of combination and the combinatorial mechanism of metals with inhibitors. The inhibitor molecule needs to possess active sites that are capable of establishing chemical connections with the metal surface via the process of electron transfer. In instances of this kind, the metal functions as an electrophile, while the inhibitor assumes the role of a Lewis base. The protective chemical has nucleophilic centers, namely oxygen and nitrogen atoms, that possess unshared electron pairs that are easily accessible for electron sharing. In conjunction with the atoms of the benzene rings, these entities provide several absorption sites for the inhibitor, facilitating the establishment of a stable monolayer [49–51]. The biological features of Schiff bases, including their antibacterial and antifungal activity, have been documented in the literature. Metal complexes have garnered significant attention in the scientific community due to their extensive investigation and exploration, primarily owing to their potential use in anticancer and herbicidal contexts. These organisms function as exemplars for species that have significant biological importance [52–58].

Main text

Pharmaceutical significance of Schiff bases

Antibacterial

Antimicrobials are well recognized as a very effective therapeutic approach in the field of medicine [59]. However, the efficacy of antimicrobials is significantly hindered by the emergence of antibiotic-resistant bacteria. The formation of Schiff bases of amino acids was achieved by the condensation reaction between isatin and several amino acids, including phenylalanine, leucine, cysteine, alanine, valine, and glycine. These amino acids exhibited notable antibacterial efficacy [60, 61]. Schiff bases derived from cellulose are produced by the condensation reaction between p-aminophenol and aldehyde moieties and have shown antibacterial efficacy against, *Staphylococcus aureus*, *Escherichia coli*, and *Enterococcus faecalis* [62].

A new collection of aromatic Schiff bases was prepared using a reflux (condensation) reaction between 5-aminopyrazoles and aromatic aldehydes [63]. The synthesis process yielded large quantities of the desired compounds. These Schiff bases were then assessed for their antibacterial efficacy through in vitro experiments against “multi-drug resistant bacteria (MDRB).” In normal circumstances, the majority of the compounds of Schiff bases exhibited superior biological efficacy. Furthermore, the molecular docking investigation revealed that kinase inhibition had a beneficial impact on the activity of dihydrofolate reductase enzymes and *Staphylococcus aureus* DNA gyrase. In addition, the data for drug-likeness indicated that these compounds under investigation satisfy the criteria outlined by Lipinski's rule and exhibit favorable biological drug scores for various activities [64]. The first findings about the Schiff base's effectiveness in combating MDRB have significant potential as a framework for the identification of novel medicines by derivatization or modification (Scheme 1).

Scheme 1 [Images not available. See PDF.]

Synthesis of Schiff Bases **7–18** [63]

The reaction included the utilization of heteroaromatic aldehydes, which were separately reacted with aminoanthracene and aminopyrene. As a result, new Schiff base derivatives 19a–19f and 20a–20f were produced with high yields [65]. The novel synthesized compounds were subjected to several tests to evaluate their reducing power, metal chelating, DNA binding abilities, antibacterial, and free radical scavenging [66]. The radical scavenging activity of the series of compounds was much superior to that of the 19 series compounds. The compound 19c exhibited the greatest metal chelating activity. In three antioxidant test methods, the standards demonstrated greater antioxidant activity compared to the DMSO solution of chemicals. Furthermore, it was shown that both compounds exhibited potential bacterial resistance activity against the employed microorganisms. Additionally, both compounds had a strong affinity for binding to CT-DNA. The findings of this research suggest that the recently examined compounds possess the ability to attach to DNA, making them promising candidates for cancer therapy. However, it is recommended that their structural composition be further modified in further investigations [67, 68] (Scheme 2).

Scheme 2 [Images not available. See PDF.]

Synthesis of anthracene- and pyrene-based Schiff base derivatives [65]

A total of twelve Schiff bases (21a–27a, 21b–25b) were synthesized by the reaction of isatin and 5-bromoisatin with various anilines as shown in Scheme 3. The synthesis process used green chemistry principles, specifically using microwave (MW) and ultrasonic (US) assistance. The antimicrobial efficacy of each chemical was assessed against a total of nine bacterial strains, including both standard and clinical isolates, using the Agar-well diffusion technique. The minimum inhibitory concentration against *Pseudomonas aeruginosa* for 21a and 21b was found to be 78 µg/mL, which is the lowest value obtained. The identification of all synthesized substances was accomplished by the use of multiple characterization techniques including ultra-violet spectroscopy, proton nuclear magnetic resonance, infrared spectroscopy, caron-13 nuclear magnetic resonance, and microanalysis. The antibacterial activity for newly synthesized Schiff bases was rationalized by using the “Molecular Electrostatic Potential Surface (MEPS) analysis” and “Quantitative Structure–Activity Relationship (QSAR).” The findings of the QSAR analysis, which included density functional theory (DFT)-based, steric, and hydrophobic descriptors, indicate that compounds exhibiting higher hydrophobicity and lower dipole moment have antibacterial properties against “*Klebsiella pneumoniae* ATCC700603” [69].

Scheme 3 [Images not available. See PDF.]

Synthetic route of Schiff bases of isatin [69]

According to the processes shown in Scheme 4, chlorobenzene aldehydes were reacted with amino derivatives like L-cysteine by condensing the reactants in a solvent methanol to produce Schiff base **28**, and methoxybenzene aldehydes were reacted with the amino mercapto acids by condensing in a solvent methanol to produce Schiff base **29**. The yields for compounds 1 and 2 were outstanding (77% and 85%, respectively) [70]. TLC was used to evaluate the reaction's progression and the purity of the synthesized compounds. The stationary phase used was of silica gel and the mobile phase was of methanol which ascended higher in the chromatogram. FT-IR, 13C, and 1H, NMR spectroscopy were used to characterize the synthesized compounds 28 and 29. With compound **28** having more antibacterial activity than compound **29**, the newly synthesized Schiff bases demonstrated action against the aforementioned microbes. The Schiff base 1 is a little reactive and more stable in a biological environment according to the estimated global chemical reactivity indices. However, further research will be needed in the future to fully understand the mechanism of these chemicals' antibacterial effects [71].

Scheme 4 [Images not available. See PDF.]

Synthesis of Schiff compounds [70]

Schiff base compounds have broad use across several domains, including organic, inorganic, analytical, and biological disciplines. In the contemporary period, pharmacology applications have exceptional potential and are extensively used within the pharmaceutical sector. A recent study based on the antibacterial activity of Schiff bases was carried out by reacting the compounds in a way to produce two main compounds named DmChDp (2,2′-(5,5-dimethylcyclohexane-1,3-diylidene)bis(azan-1-yl-1-ylidene)diphenol) and the DmChDa (N,N′-(5,5-dimethylcyclohexane-1,3-diylidene)dianiline). These newly synthesized Schiff bases have gained a lot of attention due to their diverse and significant antibiological activities against the strains of *Staphylococcus aureus*. The ligand interactions were also studied to understand the pro-drug-like features that aided in the efficacy of the final products. The characterization techniques included the docking process and the analysis using traditional spectroscopic techniques. The biological activities were evaluated against the six given proteins derived from *S. aureus*. The newly formed Schiff bases showed a great inhibitory effect for bacteria and helped in increasing the efficiency of drugs [17] (Scheme 5).

Scheme 5 [Images not available. See PDF.]

Structure of 30) DmChDp 31) DmChDa Schiff bases [17]

In brief, a series of new lanthanide complexes were synthesized by the use of Schiff base ligands, together with a “benzimidazole moiety.” These complexes were thoroughly characterized by utilizing several analytical methodologies, ensuring their unambiguous identification and understanding. Initial experiments for the compounds were performed to assess various pharmacological uses of the substances under investigation. These assessments were undertaken using a range of bioassays, including tests for antiproliferative activity, antiparasitic activity, and antibacterial activity. The findings indicate that the biological activities of the compounds are influenced by structural modifications. Specifically, ligands L1 and L2, along with their metal complexes, demonstrated minimum inhibitory concentration (MIC) values in comparison with ligands L3 and L4 and their respective complexes. This disparity in MIC values may be attributed to substitutions on the “aminophenol ring.” In a similar vein, it was shown that the compounds had an impact on the fluidity of the cell membrane by modifying the hydrophobic region inside the lipid bilayer. This observation suggests a possible correlation between such alterations and the potential therapeutic uses of these compounds. The aforementioned result has the potential to serve as a valuable point of reference in future endeavors aimed at the advancement of novel pharmacological medicines. Ongoing investigations in the laboratory are now exploring the impact of the alterations on the molecular architecture. This research aimed to provide deeper insights into the mechanism of action associated with these particular molecules [72] (Scheme 6).

Scheme 6 [Images not available. See PDF.]

Synthesis of Lanthanide complex Schiff bases [72]

Antifungal

The recent work included the chemical modification using diabetic insulin structure by the Schiff bases introduction onto the main chain of the reactant. Approximately six different derivatives of the insulin were produced by a simple method, and structures were characterized using FT-IR, proton NMR, and carbon-13 NMR spectroscopic techniques [73]. The structures exhibited variations in the quantity and positional substitution benzene ring using the phenoxide ions or phenolic groups. Following this, further research was conducted to investigate their biological properties, specifically focusing on their antioxidant and antifungal actions. The assessment of antioxidant activity included the determination of scavenging capacities toward superoxide radicals, hydroxyl radicals, and DPPH radicals in addition to antioxidant activities. These activities were of inulin which has shown a considerable enhancement in comparison with that of inulin. In addition, the in vitro evaluation of antifungal activity against three types of plant pathogenic fungus was conducted using the mycelium growth rate technique [15, 74]. The antifungal activity of the inulin derivatives was found to be much higher when compared to that of pure inulin. The biological activity of the inulin derivatives was influenced by many parameters, such as the degree of substitution (DS), as well as the quantity and location of phenolic hydroxyl groups. The products elucidated in this manuscript show significant promise as biomaterials characterized by favorable bioactivity and biocompatibility. Further investigation of the structure–activity link is warranted in future research endeavors [75, 76] (Scheme 7).

Scheme 7 [Images not available. See PDF.]

Synthesis of Schiff bases [73]

The synthesis of Schiff base derivatives of sulfa drugs involved the condensation of commercially available sulfa drugs, namely sulfamethoxazole, sulfamethazine, and Sulfamethoxypyridazine, with suitable “substituted aromatic aldehydes.” A variety of solvents with different levels of polarity were used to optimize the reaction conditions. Ultimately, a solvent combination consisting of ethanol and a small amount of acetic acid was determined to be the most suitable for the condensation processes, as shown in Scheme 8. In all instances, a stoichiometric amount of “sulfonamides and substituted aromatic aldehydes” was used, resulting in reaction yields ranging from 35 to 92%. The purification of the compounds was conducted using either recrystallization or liquid column chromatography before their characterization. It was ensured that all compounds had a minimum purity of 95% before they were deemed suitable for microbiological examination [77].

Scheme 8 [Images not available. See PDF.]

Reaction condition for the synthesis of Schiff base derivatives (**32a-32h**) of aminobenzenesulfonamied [77]
The present work effectively synthesized a range of chitosan derivatives containing active halogenated aromatic imines by the formation of Schiff bases, resulting in high degrees of substitution. The structural characterization of the sample was conducted via the use of elemental analysis, solid-state 13C nuclear magnetic resonance (NMR) spectroscopy, and Fourier transforms infrared (FT-IR) spectroscopy. Additionally, an examination was conducted to assess the antifungal efficacy against three prevalent plant pathogenic fungi, namely *Botrytis cinerea*, *Fusarium oxysporum* f. sp. *cucumerinum*, and *Fusarium oxysporum* f. sp. *niveum*, by in vitro hyphal measurements. The findings of the study indicate that the antifungal activity of double Schiff bases of chitosan derivatives was much higher than that of chitosan, particularly at a concentration of 1.0 mg/mL. The chitosan derivatives with dual Schiff bases, including halogenated benzene moieties, exhibited inhibitory indices of 95% at a concentration of 1.0 mg/mL against *Botrytis cinerea*. This high inhibitory activity may be attributed to the higher electron-withdrawing nature of the halogen substituents. The increased degree of substitution was shown to have a good impact on enhancing the antifungal activity. This work presents a pragmatic approach for the synthesis of novel double Schiff bases of chitosan derivatives including halogeno-benzenes, with the potential for further development as potent antifungal drugs [78] (Scheme 9).
Scheme 9 [Images not available. See PDF.]

Synthesis pathway for double Schiff bases of Chitosan [78]
Antiviral
In this study, a series of novel Schiff base ligands were synthesized by reacting 5-amino-4-phenyl-4H-1,2,4-triazole-3-thiol **33** with various substituted benzaldehydes (**34a-34d**). Additionally, metal complexes of these ligands with Cu(II), Fe(II), Au(III), and Mn(II) were also prepared. The synthesis of a novel benzothiazole derivative (**37**) was achieved by the reaction between the reactant compound and N-(benzothiazol-2-yl)-2-chloroacetamide through coupling. The spectral qualities of the subject were examined. The anti-HIV-1 and HIV-2 activity of the recently developed and synthesized Schiff base ligands and their corresponding metal complexes were evaluated by the analysis of their ability to suppress “HIV-induced cytopathogenicity in MT-4 cells.” Compounds **37** exhibited significant inhibitory activity in cell culture against HIV1, with EC50 values of 12.2 μg/mL (selectivity index (SI) = 4) and >2.11 μg/mL (SI = >1), respectively. Compound 11 also demonstrated inhibition against HIV-2, with an EC50 value above 10.2 μg/mL and a selectivity index of 9. This finding suggests that compound **37** has promise as a potential candidate for further refinement and enhancement [79] (Scheme 10).
Scheme 10 [Images not available. See PDF.]

Reagents and conditions i) EtOH, reflux, 2h; ii) **36**, K_2CO_3, acetone, 20 °C, then flux [79]
A novel series comprising 3-(benzylideneamino) compounds has been developed. The synthesis of 2-phenylquinazoline-4(3H)-ones included the production of Schiff bases from 3-amino compounds. The reaction of -2-phenyl quinazoline-4(3)H-one with several carbonyl compounds that have been replaced. The chemical structures of the compounds were determined by the use of spectrum analysis. The cytotoxicity and antiviral activity of the tested compounds were assessed against a range of viruses, including influenza B virus, influenza A H3N2 subtype, influenza A H1N1 subtype, vaccinia virus, feline herpes virus, herpes simplex virus-1 TK-KOS ACVr, Punta Toro virus, herpes simplex virus-2 (G), para influenza-3 virus, reovirus-1, Sindbis virus, Coxsackie virus B4, vesicular stomatitis virus, respiratory syncytial virus herpes simplex virus-1 (KOS), and feline coronavirus (FIPV). A compound formed exhibited superior antiviral efficacy against all evaluated viral strains [80] (Scheme 11).
Scheme 11 [Images not available. See PDF.]

Synthetic route for the title compounds [80]

This study presents a unique way to synthesize novel pro-drugs such as abacavir using nitrogen substitution using different ketone and substituted benzaldehyde derivatives. The results of the in vitro experiments demonstrated that compound (3-(2-(4-methylaminobenzylideneamino)-6-(cyclopropylamino)-9H-purin-9-yl)cyclopentyl)methanol (**38c**) exhibited the highest level of effectiveness against HIV, as shown by its EC50 value of 0.05 IM. Furthermore, the compound had an EC50 value above 100 IM, resulting in a selectivity index greater than 2000. Compound **38c** exhibited a much higher potency compared to the original medication, with a 32-fold increase in activity as shown by its EC50 value of 1.6 IM. At a pH of 7.4 and a temperature of 37 °C, the hydrolytic half-life (t1/2) exhibited a range of 120 to 240 min [81] (Scheme 12).

Scheme 12 [Images not available. See PDF.]

Synthesis of Schiff bases derivatives of Abacavir [81]

Conclusion

The pharmaceutical significance of Schiff bases has gained a lot of attention and this review focuses on giving an insight into the antibacterial, antifungal, and antiviral activities. The Schiff base-derived antibacterial drugs showed significant activities against bacteria by structural modifications while antifungal drugs proved to treat skin diseases mainly. The antiviral Schiff base drugs are currently being used against viral diseases such as influenza, herpes simplex, and HIV. The given literature also explains the mechanism by which the different products are synthesized and their potential activating groups. Furthermore, activities detail for various microorganisms is given which will help chemists to evaluate further compounds. The inhibitory effects of given compounds are also discussed. Overall, this review is a thoughtful and promising contribution to the field of Schiff bases that will bring positive outcomes in the future.

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Author contributions

IM contributed to the design of the study and drafted the paper; AA contributed to drafting the paper and critical revision of the article; all authors have read and approved the final manuscript.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

The authors declare no conflict of interest.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

MDRB

Multi-drug resistant bacteria

DMSO

Dimethyl sulfoxide

MW

Microwave

IR

Infrared

NMR
Nuclear magnetic resonance
MEPS
Molecular electrostatic potential surface
QSAR
Quantitative structure–activity relationship
DmChDp
2,2′-(5,5-Dimethylcyclohexane-1,3-diylidene)bis(azan-1-yl-1-ylidene)diphenol
DmChDa
N,N′-(5,5-dimethylcyclohexane-1,3-diylidene)dianiline
EC50
Half maximal effective concentration
CT-DNA
Circulating tumor DNA

Publisher's Note

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Brentuximab vedotin resistance in classic Hodgkin's lymphoma and its therapeutic strategies: a review

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ABSTRACT (ENGLISH)

Background

Bone marrow cancer has been at the forefront of cancer research. The propensity of cancers to extravasate to the bone makes it a very relevant topic in the topology of this heterogeneous disease. Our narrative review article addresses Brentuximab vedotin (BV) resistance in classic Hodgkin's lymphoma patients and discusses the current trends in the therapeutic process. The data have been collected from the works of well-established researchers, and the scientific evidence was abundantly supplemented with clinical and pre-clinical trial data. Although the findings cited are the latest, this review might not be very accurate for every population as the data from which this was derived have a population bias in several instances. The analysis has mostly been qualitative and interpretive, and quantitative evidence has only been used to explain the clinical trial results. We have divided our paper into the mode of action of BV, its probable and proven causes of resistance, and the therapeutic strategies employed to reverse them to ensure a systemic flow of information throughout the text.

Main body

Brentuximab vedotin is an antibody–drug conjugate with antineoplastic activity, used to target a novel immunophenotype tumor necrosis factor CD30. This factor is specific to the tumor-causing Reed-Sternberg cells in the inflammatory infiltrate. Though the drug had shown promise initially, the cancer was quick to develop resistance against the drug. We have analyzed and represented abundant statistical evidence to back this claim. The paper further discusses the role of the CD30 receptor, MDR1 gene, valine–citrulline linker, and tumor microenvironment in drug resistance. Lastly, we have discussed the possible therapeutics that can be used to overcome this resistance, discussing the well-established and trial-stage approaches taken in the endeavor.

Conclusion

The treatment is much better after the pursuit of reversing the drug resistance phenomenon. However, no therapeutic approach has been entirely successful in restricting the neoplastic property of cancer cells once and for all. This paper describes why that is so and how the heterogeneity of the disease complicates troubleshooting. We have tried to approach such problems through this specific example.

FULL TEXT

Background

Natural selection turns moot when normal human cells in vivo are brought into question. Though free-living unicellular organisms such as bacteria survive based on reproductive fitness, multicellular organisms thrive on collaboration. Somatic cells have a limit to the number of generations they can produce, thus capping their reproductive fitness, and will perish to keep the germ progeny alive and active. If in such a setting, a cell assumes a mutation that renders it superior reproductive fitness, it can soon choose to compete, rather than collaborate, thus developing cancer [1]. Extensive research has been performed which has backed seemingly random observations of metastatic behavior (for instance, prostate cancer often spreads to bones) [2]. The bone is a nutritionally enriched organ having an intricate vascular network and different types of cells. When the tumor affects the stem cells of the bone marrow, different forms of blood cancers develop [3].

Bone marrow cancer can broadly be classified under the following categories: myeloma, leukemia, lymphoma, myeloproliferative disorders, and myelodysplasia [4, 5].

Hodgkin's and non-Hodgkin's are both lymphomas but there are several differences between the two. Hodgkin's lymphoma is localized in a single group of axial lymph nodes and is spread contiguously by the lymph. Though the incidence rate might appear nominal at 3 per 1,00,000 person-years, it is important to note that about 50% of patients are young, within the age bracket of 15–35 years. The disease follows a bimodal age distribution with a second incidence peak in late adulthood (above 55) [6]. Why this is so has not yet been studied in-depth.

The following treatments are available for classic Hodgkin's lymphoma:

•High-dose chemotherapy and radiotherapy cause complete remission of the disease.

•Brentuximab vedotin drug treatment, often supplemented with allo-SCT (allogeneic stem cell treatment) or not.

•Programmed cell death-1 checkpoint inhibitor [7].

On the contrary, non-Hodgkin's lymphoma is localized in multiple peripheral lymph nodes because it spreads through the more extensive blood vessels. Because of this, the homing of the infection often occurs at non-contiguous lymph nodes and thus the disease requires a considerable time before the inflammation is externally perceivable. Diagnosis is thus relatively poor.

Histopathological studies have shown the presence of a special class of cells called the Reed-Sternberg cells which have a very strong correlation with the occurrence of Hodgkin's lymphoma. These cells are transformed B cells from the pre-apoptotic germinal center (GC) (in lymphoid organs) which have undergone extensive genetic reprogramming leading to epigenetic changes that have suppressed the expression of a large fraction (almost all) of the B-cell receptors (BCRs) [8].

Alongside suppressing the native receptor production, the neoplastic Hodgkin's Reed-Sternberg (HRS) cells have a profuse expression of a type I membrane protein, CD30, which is a member of the tumor necrosis factor (TNF) receptor superfamily. This rare expression profile of CD30 makes it a good immunophenotypic disease marker for Hodgkin's lymphoma. Also, HRS cells constitute a mere 1–2% of the inflammatory cell population and thus CD30 is a highly exclusive target for exterminating the tumor cells. Research has also proposed CD30 involvement in the genesis and proliferation of the HRS cells [9]. This makes CD30 a good target for antibody-directed therapies [10]. Though risk remains of cross-cellular CD30 targeting, the population of CD30 is so minimal in normal immune cells that this risk is often ignored [7].

Brentuximab Vedotin as shown in Fig. 1 is an antibody–drug conjugate (ADC). The ADC is synthesized by the chimeric mouse-human IgG1 anti-CD30 monoclonal antibody (SGN—30) conjugated to monomethyl auristatin E (MMAE) via a dipeptide linker, and it is used in the treatment of Hodgkin's lymphoma [10–12].

Fig. 1 [Images not available. See PDF.]

Chemical structure of Brentuximab vedotin showing the mAb (cAC10), dipeptide linker, spacer &drug (MMAE). The mAb is used to target the CD30 expressed as the unique immunophenotype on the HRS cells of the BV infiltrate. The dipeptide linker is cathepsin specific, though specificity to a large number of cathepsins can increase cytotoxicity by promoting non-specific payload release. Scientists are on the lookout for cathepsin B-specific linkers such that the linker is cleaved only in the cathepsin B-producing tumor cells. The spacer is usually a para-amino benzyl carbamate spacer, which helps maintain stability in the bloodstream and undergoes self-immolated disassembly to release the MMAE

Phase I studies showed a significant result with a positive response in patients with relapsed or refractory (R/R) Hodgkin lymphoma upon administration of BV. Phase II trials showed a complete response (CR) rate of 34% and an overall response rate (ORR) of 75% in CD30-positive lymphomas [13]. This outcome resulted in the FDA approving the usage of BV to treat patients with Hodgkin's after the failure of autologous hematopoietic cell transplantation [14]. BV was then started being used as part of initial therapy along with chemotherapy for advanced-stage Hodgkin lymphoma [15].

But soon resistance against the administered drug started registering. Several pharmacokinetic and pharmacodynamic challenges contributed to the gradual resistance to ADCs, due to failure in either antibody, linker, payload, or the poor ADMET properties of the drug [16, 17].

As mentioned above, in the phase II trials, BV demonstrated a CR rate of 34% and an ORR of 75% but patients with Partial Response PR had a very short remission time of medians of 3.5 months [13], therefore patients without a CR will gradually develop a progressive disease even with a continuous BV. Considering BV is the only therapeutic drug prescribed by the FDA in the last 20 years [14], understanding the mechanism of BV resistance and potential solutions are of great importance.

Main text

Brentuximab vedotin: mode of action

Brentuximab vedotin (BV) (ADCETRIS®; Aptuit [Glasgow] Ltd., Glasgow, United Kingdom) is composed of the chimeric monoclonal IgG1 antibody (cAC10, SGN-30) (derived from mouse xenograft model) that is specific to human CD30. It is conjugated to monomethyl auristatin E (MMAE), which is the payload. The payload is linked to the main body of BV by a protease-cleavable peptide linker containing a valine–citrulline combination [18]. MMAE is a synthetic antineoplastic agent and a very potent anti-mitotic drug (because of tubulin-disrupting properties) against Hodgkin's lymphoma [19]. Each of the antibodies is linked to an average of 4 MMAE groups [18].

As seen in Fig. 2 [20], the ADC is endocytosed upon binding to the extracellular CD30 antigen on the tumor cells. Inside the cells, the proteolytic lysosomal enzymes cleave the dipeptide linker thereby releasing the MMAE drug, which then goes and binds to tubulin leading to the collapse of the microtubular network. As a result, the G2/M-phase cell cycle gets arrested and apoptosis occurs (Francisco et al. [10]). The valine–citrulline peptide linker is highly stable in plasma, which results in comparatively low in vivo toxicity of the drugs when compared to other linkers [21]. BV also works by mAb-dependent cellular phagocytosis, affecting cell signaling in $CD30^+$ cells, or sometimes even due to free MMAE leaking out of the tumor cells and killing neighboring $CD30^+$ cells [18, 20, 22].

Fig. 2 [Images not available. See PDF.]

Mechanism pathway of BV, displaying its internalization into the cell, and subsequently disrupting the microtubular network. This figure shows how BV is internalized into the cell. The drug, through its CD30-specific mAb, binds to

CD30 outside the cell, on the cell surface. It is then internalized by endocytosis. Next, the endosome reacts with lysosomes, leading to the release of lysosomal proteases—the cathepsins in the endosome. Cathepsin-specific cleavage of the dipeptide linker occurs, leading to MMAE release. The MMAE exits the endosome and reaches the nucleus, where it binds to microtubules and triggers its anti-mitotic activity

Brentuximab vedotin: efficacy demonstration studies

In 102 Hodgkin lymphoma patients who had relapsed following high-dose chemotherapy and autologous hematopoietic stem cell transplantation, intravenous BV had been linked with an overall ORR (primary endpoint) of 75%. In 94% of patients with cHL, tumor reductions were seen, and the majority of these tumors shrank by almost >65%. The corresponding predicted 12-month survival rates were 89% [23]. The median duration of response for patients in CR was 20.5 months, and the median progression-free survival time was 5.6 months for all patients. After a median observation period of over 1.5 years, 31 individuals were still alive and had no signs of worsening disease that had been medically verified [13]. These trials showed that BV was generally well tolerated [23].

BV demonstrated cell death induction in CD30-positive cells with IC_{50} <10 ng/ml, but showed around 300-fold inactivity on CD30-negative cells, in patients with Hodgkin's lymphoma [10].

In a study conducted by Chen R., two subsets included a group of six patients who received consolidative allo-SCT (allogeneic stem cell transplantation) and another of 28 patients who did not [24]. This study analyzes overall survival (OS) and progression-free survival (PFS) based on the Kaplan–Meier methodology. The survival rates help analyze the efficacy of the allo-SCT treatment [24]. From the study, it is seen that stem cell transplantation often prolongs life or at least delays relapse as compared to only BV treatment [25].

CD30 characterization

The CD30 receptor was first identified from a monoclonal antibody (mAb) derived from an HL cell line [26]. Structural analysis has shown that the protein has intracellular, extracellular, and transmembrane domains [27], and sequence similarity has found CD30 to be similar to other TNF (Tumor necrosis factor) receptors in its extracellular sites, thus raising questions regarding the exclusivity of the target of BV. BV might also be useful in treating non-hematopoietic cancers, such as germ cell tumors (testicular embryonal carcinomas) [28].

These are the normal immune functions of the CD30 found as of now:

- Expressed on the surface of CD4+ and CD8+ T lymphocytes which are activated because of an infection and show a propensity to secrete Th2 cytokines (Il-4, Il-5) predominantly, although Th0 and Th1 cytokine production has also been reported [29].

- Negative selection of partially mature T lymphocytes having double positive CD4+CD8+ antigens is thought to be guided by a transient expression of CD30 in a relatively smaller population of thymic cells that can eliminate T cells with high affinity toward self-peptides via apoptosis, thus preventing potential autoimmune disorders [30, 31].

When analyzing CD30 as a potential cause for BV resistance in cHL patients, it is important to note that several researchers have found CD30 to be an active perpetrator of lymphomagenesis. It is established that CD30 is not downregulated upon and after BV treatment [32]. This rules out the possibility of the lack of a target for BV as a potential cause of drug resistance. However, the involvement of CD30 in continued lymphomagenesis as a potential cause of relapse in BV-treated patients is a glaring possibility. Downstream activation of nuclear factor kappa B (NFkB) and mitogen-activated protein kinase (or extracellular signal-related kinase) pathways both ultimately lead to the activation of anti-apoptotic and pro-proliferative genes. Contrasting views on the role of CD30 in the normal immune system have led to poor functional characterization [33]. The downstream effects of CD30 stimulation might be exploited when searching for a potential reversal of BV resistance.

Brentuximab vedotin resistance: potential causes

Surface antigen level downregulation

BV utilizes a mAb against the antigen CD30 on the cell surface of tumor cells as a potential drug target. It is thus possible that the downregulation of CD30 levels in the tumor cells causes BV resistance.

Chen et al. [32] conducted MTS assays to determine the IC50 (Half-maximal inhibitory concentration) of parental cell lines of L428 (HL). The BV-resistant cell models were selected using persistent exposure to sub-IC50 concentrations of BV and cell numbers in culture were kept on track for 3 months. However, the team was unable to obtain resistant L428 cells through constant exposure to the drug. Then a supra-IC50 concentration of BV exposure was applied and cell numbers were tracked and assessed twice a week until cessation of cell proliferation was seen. In the end, it was seen that BV-resistant cell lines were able to grow at supra-IC50 parental line BV concentrations. This was confirmed by cell proliferation assays. At the same concentrations, the parental cell lines quickly died. However, there was absolutely no significant decrease in the percentage of CD30 + cells [32].

In another research conducted by Nathwani et al. [34], two patients, a 27-year-old woman (with relapsed cHL in IVA stage following prior 6 cycles of ABVD, 2 cycles of ICE, and a cycle of ACT) and a 19-year-old man (with relapsed cHL in IIIA stage following ABVD, ICE and additional treatments including rituximab, gemcitabine, vinorelbine, liposomal doxorubicin, MOPP, and palliative radiation), were enrolled in a study to find out more about the role that CD30 plays in BV resistance. In both cases, the patients achieved a significant reduction in tumor sizes following the BV treatment through 8 and 10 cycles, respectively. Here too, a persistent level of CD30 throughout the treatment demonstrated that a reduction in levels of CD30 does not appear to be a potential cause of BV resistance [34].

Drug transporter protein overexpression

The same study conducted by Chen et al. [32] with L428-R (resistant) and L428-P (parental) cells showed a decrease in the amount of intracellular MMAE in L428-R as compared to L428-P cells. The R cells accumulated 6.7-fold (± 3.4-fold) more MMAE compared to the L428-P cells within 48 h when the cells were incubated with 20 μg/ml of BV [32]. To confirm this, they performed an additional test with rhodamine-123 dye for two days (Rhodamine is a substrate for the transporter responsible for MMAE efflux), and L428-R cells showed tenfold lesser fluorescence than L428-P cells. Subsequent qRT-PCR was done to see mRNA levels for *MRP1*, *MDR1,* and *MRP3*, in sets of resistant and parental cell lines [32]. Results showed increased MDR1 levels (ATP-dependent translocase ABCB1) and protein levels of P-glycoprotein (who preferentially exports hydrophobic cargo like MMAE out of cells) in L428-R cells relative to L428-P cells, though *MRP1* or *MRP3* mRNA levels in both cells lines were same [32].

In another study conducted by Chen et al. [11] they used another BV-resistant HL cell model KMH2-R. They again showed that CD30 expression was unchanged in KMH2-R compared with KMH2-P. And gradually re-confirmed the overexpression of the gene *MDR1* by qPCR in KMH2 cells, where *MDR1* presence was threefold higher in KMH2-R cells compared with KMH2-P cells [11]. *MDR1* RNA expression was threefold greater in KMH2-R cells and sevenfold greater in L428-R cells compared to respective KMH2-P and L428-P cells [11]. This displayed how drug transporters can play such a crucial role in BV drug resistance in HL.

Defective linker-payload processing

There are two types of linkers, cleavable and non-cleavable. An appropriate linker is not only required to prevent degradation during systemic circulation, but also to facilitate the quick and efficient release of the drug inside the tumor cells [35]. Non-cleavable linkers release their conjugated drug only after antibody degradation. For example, for Kadcyla which has a non-cleavable thioether linker, lysosomal membrane proteins are required to first transport the drug catabolite out of the lysosomal compartment and then exert a therapeutic effect [36]. Barok et al. [37] established that non-cleavable linkers are more susceptible to ADC resistance in tumor cells because any faulty

linker degradation will not lead to functional drug release.

BV is composed of protease-cleavable valine–citrulline peptide, where the MMAE does not depend on the degradation of the antibody backbone. Thus, it leads to much faster payload release after ADC internalization compared to a non-cleavable linker [38]. However, a faulty linker cleaving or non-cleaving can lead to an ineffective payload discharge. As per a study conducted by Caculitan et al. [39], valine–citrulline (Val-Cit) linker showed broad-spectrum specificity to different types of cathepsins, including cathepsin B, cathepsin L, cathepsin K, etc. [39]. Since only cathepsin B is postulated to be highly expressed in HL cells, and normal cells have other kinds of cathepsins, this phenomenon could be very deleterious as it would induce toxic side effects on other normal cells [40].

A defective linker thereby affects the cytotoxic drug delivery in many ways from the drug not reaching the target, to off-target toxicity and the drug not being able to dissociate from the mAb and the linker, thereby overall promoting resistance.

Tumor microenvironment

In cHL tumor, the entire cellular infiltrate contains only infrequent neoplastic HRS cells (about 1%) and is surrounded mostly by a characteristic tumor microenvironment (TME) composed of several benign immune and extracellular matrix stromal cells, including different types of T and B cells, eosinophils, fibroblasts, macrophages (M1 and M2). By contrast, NLPHL (nodular lymphocyte-predominant HL) differs from cHL1 based on specific histopathological characteristics [41].

Extensive crosstalk mediated by a large network of cytokines and chemokines between tumor cells and immune cells, acting in an autocrine and paracrine manner, suggesting the existence of an entire pro-malignant cancerous ecosystem present around the tumor cells has been established [41]. As Fig. 3 shows, the CD30 ligand (CD30L), as well as neutrophils and eosinophils are commonly mixed with HRS cells [42].

Fig. 3 [Images not available. See PDF.]

Different interactions of cell surface receptors of HRS cells with the tumor microenvironment describe the way the HRS cells influence the microenvironment. Such influence is the driving cause behind sustained tumor growth and unchecked proliferation. In the figure, we can see the CD30 binding eosinophils and mast cells and also interacting with mast cell-produced interleukins

TME-mediated development of drug resistance occurs by multiple mechanisms quite different from one another [43–45]:

- Metabolic reprogramming, leading to altered drug delivery and various tumor proliferation strategies.
- ECM remodeling through changes in the matrix-forming heterogeneous class of stromal cells.
- Development of cancer stem cell phenotype (CSC) through expression of various immunophenotypic markers such as CD44, CD24, and CD133 and the development of the conserved Nodge and Hedgehog pathways involved in cellular differentiation.
- Angiogenesis plays a major role by determining the level of development of the surrounding vasculature which provides the tumor with oxygen, nutrients, etc., and removal of metabolic wastes.
- Immune suppression mechanisms.
- Exosome-mediated trapping of therapeutic antibodies.

Therapeutic strategies to overcome BV resistance

Usage of different linker-payload combinations

MMAE is uncharged, and hence non-polar. This often leads to the death of cells near cancerous tumor cells known as the “bystander effect”. As seen in Fig. 4 MMAE internalization leads to the destruction of cancer cells and when these cells lyse, they release the MMAE [46]. Because of its hydrophobicity, it can easily pass through the phospholipid bilayer of the surrounding cells, causing anti-mitotic effects in them [46]. While it can have a positive effect if the surrounding cells are cancerous too, on the flip side, the cells develop BV resistance through MDR1 upregulation very quickly. To overcome this resistance, often the MMAE is replaced by a charged payload/linker combination, which is impermeable to the nearby cells upon lysis of the initially targeted cell. This keeps the bystander effect in check, helping in minimizing resistance [46].

Fig. 4 [Images not available. See PDF.]

Schematic of the bystander effect shown by non-polar conjugates showing cytoplasmic leakage shows the cytotoxicity difference between a polar and a non-polar payload because of the permeability exhibited by the non-polar payload in crossing the cell membrane into the nearby cell causing non-specific toxicity. This phenomenon can be positive if the nearby cell is toxic too but that is seldom the case and this kind of effect mostly kills healthy cells

A linker that used a cyclobutane-1,1-dicarboxamide (cBu) structure was designed by Wei et al. which was specific to cathepsin B cleavage. This was proven by intracellular cleavage studies in which a cathepsin B inhibitor stopped drug release from cBu-Cit-containing linkers by over 75%, while a cathepsin K inhibitor did not have an appreciable effect [40].

Peptide linkers have been seen to be easily optimizable by minimal structural changes, including the types and stereochemistry of the amino acids. For instance, valine–alanine (Val-Ala) has better hydrophilicity and stability than Val-Cit [47].

Ward and his coworkers [48] from Texas University have developed an innovative approach to solving drug resistance. They developed such a targeting moiety of the ADC (antibody–drug conjugate) such that its binding affinity was two orders lower in the endolysosomal lumen (pH < 6.5; $[Ca^{2+}] \sim 2$ μM) than it was in the extracellular space (pH > 6.8; $[Ca^{2+}] \sim 2$ mM) [48]. Since the drug is more easily accessible to its intracellular substrate because of faster dissociation from the antibody target, it gives a twofold advantage: recycling of the target to the cell surface to sequester more ADC, and faster downstream signaling from the drug binding to its substrate, which in the case of the anti-tubulin activity of MMAE is cessation of cell proliferation. This diminishes the cytosolic payload of the drug [48].

“Component switch” mechanism

The “component switch” mechanism is difficult to implement because the prognosis will change radically based on the stage of cancer and/or the treatment [46]. Age, ethnicity, and other variable factors will also bear a sensitive relationship to the treatment because targeting multiple pathways or events in an already heterogeneous disease can trigger severe physiological imbalances, resistant phenotypes, and immune weakening events. Think about the different variables involved with the single treatment pathway of ADC delivery: antibody target identification, internalization, payload release, binding of MMAE to tubulin, and the fate of tubulin after cell lysis—problems at a single step can jeopardize the entire treatment [46].

There are many problems with medical and healthcare procedures too. Analysis of resistant mechanisms is limited by the want of systematic and routine pre-and post-treatment biopsies and the problem of setting up standardized clinical assays for quantifying protein levels of clinical biomarkers [46].

Combination of ADCs with immune checkpoint blockade

Immune suppression of cancer is bypassed by the resuscitation of effector T cells which helps in infection response and memory. Antibodies designed to inhibit immune checkpoints help in this. Immune checkpoint blockade has proven promise in many long-lasting cures [46].

The use of such antibodies however has disadvantages. In tumors that have not developed any anti-tumor T cell response, usually comprised of “immune desert” or “immune excluded” types (the former means that the immune system is not recognizing the tumor as an infection, resulting in no T cell production against it, whereas the latter means that T cells are formed against the tumor and are present in the extracellular stromal matrix but are unable to penetrate the core tumor mass), the ICI (immune checkpoint inhibitor) antibodies do not work [49].

Anti-tumor immunity is conferred by ADCs and chemotherapeutics through the following mechanisms:

- release of tumor antigens from dying cells allows these antigens to be taken up by the dendritic cells, macrophages, or B cells, which phagocytose the antigens and present them to the T cells via the MHCs (major histocompatibility complexes) for immune activation [46].

- Maturation and activation of the antigen-presenting dendritic cells are largely influenced by the free payload that comes conjugated with the ADC. If the payload is PAMPs (pattern-associated molecular patterns) and DAMPs (damage-associated molecular patterns) (like Toll receptors), it can directly be presented by the APCs (antigen-presenting cells, here dendritic cells) for immune activation. Free payload can also lead to co-stimulatory molecule release (like CD40, and CD80) or cytokine release [46].

- Triggering cell death, often with the aid of anti-mitotic factors [46].

Combining ADCs with ICI antibody treatment can help immunologically “cold” tumors to get converted to tumors with an active T cell pool by the methods described above. Since T cell activation leads to an overall increase in adaptive immune response, a global mechanism to eliminate the tumors is undertaken without the dependency on surface antigen gene regulation [46].

Ongoing clinical trials involving BV and ICI are given below [46]:

- BV and Nivolumab block PD1

- BV and Pembrolizumab block PD1 too

- BV and Nivolumab +/− Ipilimumab blocks PD1 and CTLA-4

Overcoming MDR1-mediated resistance by using a modified linker

MDR1 transporters use maytansinoids as substrates to be transported across the cell membrane against their gradients. DM1 (Mertansine) is a thiol-containing maytansinoid that is actively pumped out by overexpressed MDR1 receptors as a form of response to DM1 treatment in HER-2-positive breast cancer. Kovtun and colleagues designed an ADC in which the DM1 was linked to an antibody using a hydrophilic linker, PEG4Mal, which was a replacement for the initial SMCC linker. This resulted in the release of lysine-PEG4Mal-DM1 instead of lysine-MCC-DM1 upon cathepsin cleavage. MDR1 does not recognize lysine-PEG4Mal-DM1 as a substrate for transmembrane transport, thus solving the problem of efflux of the payload drug. This method prevented MDR1-mediated resistance in both in vivo xenografts and in vitro cells expressing MDR1 [37]. This can be extended to cHL treatment by BV administration by modifying the dipeptide linker.

Using PD1 inhibitors

Cancer often leads to immunosuppression by inactivating the activated B and T cells. PD1 discovery, which was awarded the Nobel in 2018, led to the dawn of a new direction in cancer therapy. PD1 acts as a negative regulator on T cells. PD1 inhibitors such as pembrolizumab and nivolumab have led to promising clinical results because of the reactivation of immunosuppressed T cells [42]. This has led to the abatement of refractory cancers such as the HL249, 250 cell lines. PD1 inhibitors are now often administered with ADC, as forms of combination therapy. Though some have shown severe autoimmune complications, most patients have been able to accept the treatment, some even being fully cured. Preliminary results of patients diagnosed with advanced-stage Hodgkin's lymphoma administered with a combination therapy of nivolumab and AVD have shown good results [42].

Brentuximab vedotin plus Ibrutinib

BTK (Bruton's tyrosine kinase), an important oncogenic non-receptor tyrosine kinase is active in various subtypes of non-Hodgkin lymphoma and is also expressed in malignant Reed-Sternberg cells. Ibrutinib (Ibr) is a Bruton's tyrosine kinase (BTK) inhibitor which can also use a Th1-based response to inhibit IL-2-inducible kinase (ITK). This can promote immunogenic cell death in combination with BV [50].

According to the research conducted in a phase II trial of Ibr plus BV in patients with r/r HL, 39 patients were enrolled; 67% were male and the median age was 33. Of 36 evaluable patients, the CR rate was 33%, ORR 64%, and the median DOR (Diagnostic Odds Ratio) was 25.5 months (range) [50].

Results showed that Ibr imparted additional toxicity in comparison to BV monotherapy, and even showed no signs of increased efficacy in patients treated with both BV and Ibr, thus ruling Ibr out as a potential treatment [50].

MDR1 inhibition with CsA and VrP

Effects of CsA (cyclosporine) and VrP (vorinostat, a histone deacetylase inhibitor) were studied on the IC_{50} (measures potency of inhibiting tumor growth) of BV in L428-R (missense mutation involving resistant colon cancer cell) and KMH2-R cells [11]. Neither of the drugs had any effect on the viability of the parental and BV-resistant types of the two cell lines mentioned above in the absence of BV. PgP protein expression and *MDR1* mRNA expression were also unchanged on CsA treatment [11]. It was then seen that competitive MDR1 inhibition increased intracellular MMAE levels and resensitized the two-BV-resistant cHL cell lines. It was also seen that overexpressing exogenous MDR1 in the L428-P led to BV resistance, which was nullified on treatment with CsA [11]. These results conclusively support the hypothesis that resistance to BV in Hodgkin's lymphoma is brought about because of the cytosolic loss of MMAE by the ABC drug transporter MDR1/PgP, whose overexpression is triggered by rising MMAE levels [11].

It was previously reported that the addition of VrP led to a 3.9-fold reduction in BV IC_{50} [32] into L428-R cells and a sixfold reduction in that of KMH2 $IC_{50.}$ Compared to this, this reduction was 10,000-fold for L428-R cells and 600-fold in KMH2-R cells [11].

CRISPR-Cas9 system to reverse drug resistance targeting MDR1

It was reported that the expression of P-glycoprotein, which helps the ATP-mediated transfer of anti-mitotic drug against its gradient with the help of the transmembrane MDR1 protein, could be efficiently blocked using the CRISPR-Cas9 system. Inhibiting ABCB1 (another name for P-glycoprotein) in osteosarcoma MDR cell lines (U-2OSR2 and KHOSR2) helped in combating MDR against doxorubicin [51].

CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats and it uses Cas9, which can form a complex with single guide RNA (which shows Cas9 its point of activity on the DNA). The sgRNA-Cas9 complex then cleaves the DNA 3–4 base pairs upstream of a protospacer adjacent motif (PAM). Cas9 then helps generate DSBs (double-strand breaks) [52], which when repaired by NHEJ (non-homologous end joining) generates small insertions or deletions at the point of Cas9 activity. This is because NHEJ is very erroneous. These point mutations can

inactivate genes or genomic elements [52].

Similar CRISPR-based editing can be used for classic Hodgkin's lymphoma to target a lot of proteins: ABCB1 can be targeted to prevent efflux pump overexpression, so cytosolic MMAE levels are maintained. ADAM10 and ADAM17 inhibitors can help keep the cell surface concentration of CD30 nearly constant so that BV gets a docking site and no drug loss happens over the lack of a target molecule [53].

Nanoparticle-based MMAE targeting

MMAE is an anti-tubulin agent and can be detrimental to the normal cells of the body. Though BV is quite a specific drug because of its target being the distinct cancer immunophenotype CD30, cross-reactivities because of partial similarity with other F_{ab} segments (hypervariable regions can be assumed to be unique, but similarities in the variable region are possible) of other CD markers is not only possible but also expected [53]. Also, activated B and T cells often express CD30 as a normal phenotype (as previously discussed), so BV can often be led astray. This raises a two-fold possibility:

- Effective drug delivery to the Reed-Sternberg cells decreases.
- Normal cells are prevented from proliferating (mitosis prevented). This decreases the population of the already strained normal cells of the body, thus helping tumor proliferation (The citrulline-valine linker of BV is specific for a lot of cathepsins, including the ones with abundant expression in normal cells) [53].

Another factor causes reduced drug delivery. CD30 is often cleaved in active Hodgkin's lymphoma patients, which leads to a high concentration of sCD30 (soluble CD30) in the plasma. This ectodomain cleavage is caused by members of the ADAM group of proteins (ADAM 10 and ADAM 17), appropriately called sheddases [53].

Nanoparticles containing FRRG (Phenylalanine- Arginine- Arginine- Glycine) conjugated with MMAE (MMAE conjugated to C-terminus of FRRG) can be obtained by EDC-NHS coupling at 37 °C for 24 h. The self-assembled nanoparticle stabilized with the intermolecular hydrophobic interactions and needed no further carrier materials. These nanoparticles (prodrugs) showed vigorous uptake in in vitro breast cancer cells (4T1) [54]. FRRG has proven to be the minimal chain of peptides required to selectively trigger cathepsin B without requiring additional lipids or polymers for nanoparticle assembly [54].

Such nanoparticles can be used as a potential replacement for BV because of the specificity of its cleavable peptide linker and its independence of CD30 presence on the cell surface. The FRRG peptide is a much more specific linker than the citrulline-valine linker in BV. FRRG is specific to cathepsin B, the cathepsin with the most overexpression in tumor cells. Normal cells will not have high enough levels of cathepsin B to trigger the release of MMAE from the prodrug and hence the anti-mitotic action of MMAE will not be active in normal cells, minimizing collateral damage. Cleavage of CD30 ectodomains will also not cause inefficacy in drug delivery [55].

Epigenetic modifications to modulate resistance

Epigenetic modifications often lead the cancer cells to survive and proliferate in the face of subsequent rounds of chemotherapy and since epigenetic changes are defined by their transience, changes call for a lesser strain on the cellular machinery than a genetic change would. Nucleic acid methylation has been the best-characterized epigenetic process contributing to chemoresistance [56]. Methylation rates were studied in FL (Follicular lymphoma) and DLBCL (diffuse large cell B-lymphoma) and compared to that of normal B cells [56]. It was seen that increased dissimilarity in methylation patterns led to faster death of the cells, a fact held even between FL grades. Abnormal methylation patterns had a propensity to be targeted toward promoters of key regulatory factors such as *MYC, BCL6,* and *EZH2.* Upon investigating for similar methylation in DLBCL patients who showed different fates to treatment (durable vs. relapsed) [57], enriched promoter sites with differently hypermethylated regions were found.

For instance, *CTCF*, a transfactor involved with DNA methylation (through interactions with histone acetylases and deacetylases) was differentially hypermethylated at its promoter.

Chemoresistance is much attributed to epigenetic modifications since DNA methylation status can affect a broad range of housekeeping cellular activities like cell cycle, autophagy, protein degradation, immune response, apoptosis, and DNA damage. It also affects signaling pathways involving small molecular targets. For instance, Bruton's tyrosine kinase (BTK), which is used as an immuno-target to curtail mantle-cell lymphoma has undergone epigenetic modification to gain resistance to the inhibitor (of BTK) ibrutinib, which was a general treatment for the disease [58]. Among the major drugs involved with epigenetic changes, post-translational modifications (PTM) in many are assumed to be the major cause of activating chemo-resistant pathways. Among these, an important one is the impaired p53 activity, in which p53 acetylation activates the tumor suppressor and protects it from degradation. To induce the acetylation back, often class III HDAC inhibitors are used. Scientists are also looking at ways in which the epigenetic regulation of the tumor microenvironment immune surveillance cells can help in circumventing chemoresistance. To exemplify this, it has been seen that programmed cell death-1 (PD1) and programmed death ligand 1 (PD-L1) are involved in chemoresistance, both of which are under tight epigenetic control [59, 60]. Epigenetic modulating drugs could be supplemented with essential tumor shrinkage agents with the aim of rewiring pathways causing drug resistance, especially tightening checkpoint inhibitors. Toxicity problems limit the use of epigenetic drugs to minute concentrations. If proper precision medicine data can be obtained to classify patterns of epigenetic derangements, specific epigenetic drugs can be coupled with chemotherapy and immunotherapy to overcome drug resistance.

Conclusions

This narrative review discusses the various ways in which drug resistance can occur in cHL, analyses the existing methods to reverse it, and proposes new ones.

HRS cells are responsible for tumor proliferation in cHL. CD30 is backed by statistical evidence to be proven as the predominant cause of tumor spread and many subsets of patients were shown to have grown resistant against the ADC that was employed to tackle cHL. The potential causes of resistance were hypothesized, some of which were incorrect. For instance, it was seen that BV had an antibody derived from a mouse xenograft which was specific to the CD30 marker on the HRS cells. BV treatment might have led to the downregulation of CD30, thus robbing BV of docking sites to gain entry into the cell. However, clinical data reveals no such happening. This effect was later explained by the action of ADAM proteins, which led to the discovery of the shedding of ectodomains of CD30 in advanced cHL patients. That shedding caused the loss of docking sites and non-specific docking with the sCD30, both aiding in improper drug delivery.

Another hypothesis, the overexpression of MDR1 which pumped out the active payload of BV, and MMAE out of the cells was proved to be correct. Numerous alterations in the ADC were done to prevent the efflux (such as modifying the payload so that it was no more a substrate for MDR1). Changing MMAE to a charged substrate has often been thought to limit the bystander effect. The linker is often changed to ensure its stability under the treatment of non-tumor-specific cathepsins. This prevents collateral damage and reduces tumor proliferation by not killing normal cells and freeing up space and nutrients for the tumor. The antibody part is also engineered to make it more specific to CD30, as CD30 belongs to the class of TNF receptors, which have quite similar ectodomains, which can result in cross-reactivity. Immune checkpoint blockades such as PD1 inhibitors are thought to be more effective than ADCs because this leads to the global activation of the immune response of the body to attack the tumor. This review has therefore been successful in identifying the causes of BV resistance but the remedies for such resistance are under debate.

The use of CRISPR is being hypothesized to mutate MDR1-producing genes so that cytosolic loss of MMAE decreases or ADAM-producing genes to preserve BV docking sites. Nanobiotechnology has made nanoparticles very important drug delivery molecules. FRAG-MMAE-containing nanoparticles are employed to deliver the MMAE, instead of the use of an antibody interaction. This increases efficacy by removing the loss of drug due to lack of docking and also limits bystander effect because of the specificity of FRAG to cleavage only by cathepsin B (the one specific to cHL tumor). Finally, epigenetic modifications are being studied, employing the component switch strategy on various levels. These treatment options are relatively new and many of these are still under thorough investigative research or clinical trial phases. However, BV remains the most promising drug in public use until better solutions are discovered.

Further research should focus on a systems biology approach to investigate the individual causes of resistance, so we have an idea of the effect of each of the causes in the broader in vivo context.

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The authors declare that they have no competing interests.

Abbreviations

ABVD

Adriamycin-bleomycin-vinblastine-dacarbazine regimen

ADC

Antibody drug conjugate

ADME

Absorption, digestion, metabolism, excretion

Allo-SCT

Allogeneic stem cell transplantation

APC

Antigen-presenting cells

BCR

B-cell receptor

BTK

Bruton’s tyrosine kinase

BV

Brentuximab vedotin

cBu

Cyclobutane-1,1-dicarboxamide

cHL

Classic Hodgkin’s lymphoma

CD

Cluster of differentiation

CR

Controlled response

CRISPR

Clustered regularly interspaced short palindromic repeats

CSC

Cancer stem cells

CsA

Cyclosporine

DAMP

Damage-associated molecular patterns

DOR

Diagnostic odds ratio

DSB

Double strand break

EDC

Ethyl(dimethyl aminopropyl) carbodiimide

FDA

Food and Drug Administration

FRRG

Phenylalanine-Arginine-Arginine-Glycine

GC

Germinal Centre

HL

Hodgkin’s lymphoma

HRS cells

Hodgkin’s and Reed-Sternberg cells

Ibr

Ibrutinib

ICI

Immune checkpoint inhibitor

IC50

Half-maximal inhibitory concentration

Il

Interleukin

MMAE

Monomethyl auristatin E

MDR1

Multidrug resistance 1

MHC

Major histocompatibility complex

NFkB

Nuclear factor kappa B

NLPHL

Nodular lymphocyte-predominant Hodgkin lymphoma

ORR

Objective response rate

OS

Overall survival

PAMP

Pathogen-associated molecular patterns

PD1

Programmed cell death protein 1

PR

Partial response

PFS

Progression-free survival

qRT-PCR

Quantitative reverse transcription-polymerase chain reaction

RR

Relapsed/refractory

sCD

Soluble CD

Th

T-helper cells

TME

Tudor microenvironment

TNF

Tumor necrosis factor

VrP

Vorinostat

Publisher's Note

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DETAILS

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Nanotechnology-based strategies overcoming the challenges of retinoblastoma: a comprehensive overview and future perspectives

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ABSTRACT (ENGLISH)

Background

Retinoblastoma (RB) is a rare type of pediatric ocular cancer with difficulty in treatment and detection owing to alterations in tumor suppressor genes and the lack of focused, efficient, and cost-effective treatments.

Main body of the abstract

The current review presents different approaches adopted for the treatment of RB. Recently, nanodrug delivery-based systems have shown significant reported advancements in RB treatment owing to their effectiveness in delivering their cargo to the site of tumor growth, where they may induce programmed tumor cell death. Among various nanoparticulate systems employed in RB treatment are organic nanoparticles, lipid-based nanocarriers, polymeric nanoparticles, inorganic (metallic) nanocarriers (cerium oxide, iron oxide, gold and silver), and surface-tailored multifunctionalized nanocarriers.

Short conclusion

The current review article aims at demonstrating the superiority of nanotechnology-based formulations to traditional therapies for treatment of RB in order to enhance the bioavailability and targeting of drugs to posterior eye segment specifically, thus improving patient compliance and adherence to treatment by minimizing the number of dosing intervals and hence the likelihood of side effects.

FULL TEXT

Background

Anatomy of the eye

Briefly, the eye is composed of two segments: anterior and posterior segments. The anterior segment comprises (a) Iris which controls the amount of light entering the eye. In front of the lens, the pigmented region of the eye is visible [1], (b) Eye pupil which is the opening in the center of the iris via which light enters the eye lens. The iris controls the pupil's dilation and constriction [2], (c) Cornea, the transparent round anterior segment of the eyeball refracting incoming light onto the lens, which then directs the light to the retina. The cornea lacks blood vessels and has a high threshold for pain [3], and (d) Lens, a translucent structure behind the pupil. It is contained in a thin, transparent capsule and aids in the refraction and concentration of incoming light on the retina [4]. The posterior segment comprises (a) Choroid which separates the sclera and the retina. Additionally, it has a pigment that absorbs excess light to prevent blurred vision [5], (b) Ciliary body which connects the choroid to the iris [6], (c) Retina, a light-sensitive layer lining the interior of the eye, which is composed of rod and cone cells that are sensitive to light [7–9], (g) Optic nerve includes all visual information which is transmitted to the brain [10, 11] and (h) Sclera, the white portion of the eye, representing the protective outer layer of the eye formed by the cornea [12].

Etiology of RB

RB (Fig. 1) is a rare and aggressive, can be hereditary ophthalmological pediatric cancer [13]. Its incidence worldwide is 1/16000–1/18000 live births [14], which translates to around 8000–9000 new cases on annual basis, or 3–4% of all malignancies in pediatrics [15]. It is widespread among children under the age of five, with a prevalence of 1 in 15,000 [16, 17].

Fig. 1 [Images not available. See PDF.]

Comparison between **a** healthy eye and **b** retinoblastoma eye [15]

The prognosis for this condition is poor in around 80% of patients in low- and middle-income countries [18], due to poor health regimens and a lack of early detection; however, the survival rate is close to 100% in high-income countries [19]. Mutations in chromosome 13q14.2 result in the manifestation of RB in the first years of life [20]. The tumor suppressor gene RB1, located on chromosome 13q14.2 with aberrant phenotypic expression, is used to genetically characterize RB [21]. RB only occurs when both copies of the RB1 gene are abnormal functioning or absent [22].

The "two hit" hypothesis postulates that mutations at the germinal stage, which impact all retinal cells, resulting in cell cycle abnormalities and erroneous entrance into S phase of the cell cycle, may be the reason of RB [23]. RB may be hereditary or develop spontaneously. Bilateral RB (inherited RB) is caused by two mutations that occur before fertilization, causing malignancy to manifest at a younger age and often proceeds to bilateral RB [24].

In the spontaneous type of RB (unilateral RB), somatic mutations predominate, and both mutations develop in a single retinal cell following fertilization. This type of cancer usually occurs in the later years, and is not usually transmitted to the off springs [25].

The growth of RB tumors, developing from immature retinal cells, depends on the existence of different types of vasculatures. The tumor spreads as seeds (white mass) toward the vitreous and/or sub-retinal area. Although the seeds are avascular, the primary tumor is vascularized [22].

The retina's inner layer is made up of neurons that are sensitive to light [26]. These cells are linked to the brain through the optic nerve. Without prompt treatment, devastating consequences may occur, including loss of vision, secondary non-ocular cancers, and even death. In severe cases, changes in iris color and eye enlargement caused by high intraocular pressure may occur. The eye's unique anatomical and physiological structures offer an extensive barrier to drugs' delivery to diseased regions of the eye [27].

The blood retinal barrier (BRB), involved in the maintenance of ocular homeostasis, is another barrier to the transport of drug molecules into the eye [28]. The main parts of the BRB that allow solutes to cross from the blood to the retina comprise retinal pigment, retinal capillaries, sclera, and choroid. Retina is a part of the CNS, like the brain. As a result, tight junctions maintain retina's normal function in addition to the crucial oxygen consumption rate and

glycolysis [29–31].

Diagnosis and prognosis

Symptoms of RB include leukocoria (white pupil), strabismus (misaligned eyes) and reflection in the eye of the child. Changes in the iris color and larger eyes owing to increased intraocular pressure are common in advanced stages [32]. Detection, early medical intervention, involving a multidisciplinary team of radiologists, oncologists, geneticists, ophthalmologists, and suitable radiation, chemotherapy, and surgical treatments are necessary for the long-term improvement of patient life [33].

The diagnosis may usually be made using indirect ophthalmoscopy and pharmacologically dilated pupils. Ocular ultrasonography (β-scan) may be utilized to identify RB calcifications; however, MRI is required to assess trilateral RB and optic nerve invasion. Computed tomography should not be used on patients with RB1 mutations as radiation causes secondary malignancies [34, 35].

Alternatively, biopsies might provide crucial details on the histological type of ocular malignancy; however, sampling errors may lead to false negative results [36]. The development of ocular molecular imaging, which permits the early diagnosis of this eye disorder before the formation of any morphological abnormalities, is urgently needed despite the availability of numerous ophthalmic imaging techniques [37]. Fluorescein angiography, needle biopsy, ultrasonography, MRI, and CT are often used by physicians to identify RB based on the presence of retinal tumors [14].

Different treatments are used depending on how the tumor is progressing. However, in more severe cases of the disease, the whole globe and its intraocular contents must be surgically removed (enucleation), while in other cases; conventional treatments are employed [38].

For attaining best therapeutic efficacy and prognostication, RB must be classified. The original categorization was proposed by Reese–Ellsworth in 1963 [39]. Thereafter, in 2005, Murphree's International (IIRC) categorization [40], divides this ocular tumor into five groups (from A to E, with E being the most severe) [41]. Finally, this categorization was somewhat amended by the Children's Oncology Group (COG) [42].

The need for primary enucleation or conservative treatment may rely on the clinical status of the patient. All patients who have unilateral or bilateral RB of groups A, B, C, and D are eligible for specific therapy, however individuals who have bilateral RB of group E are only eligible for conservative treatment [43]. A direct biopsy cannot be used to diagnose RB in order to stop the development of metastases and the spread of disease outside the eye [35].

Main text

Different modalities for treatment of RB

To treat RB, anticancer drugs have been injected via the intravitreal, subconjunctival, topical, systemic or subtenon routes. Although these delivery systems are successful in managing the eye's anterior area, they have been ineffectual in treating the disorders of the eye's posterior segment where RB originates [44].

Different therapeutic procedures may be employed **(**Fig. 2**)**, depending on the stage of the disease. Systemic chemotherapy is frequently used in conjunction with local treatments (for example, cryotherapy, photocoagulation, brachytherapy, laser, radiotherapy, hormonal, and thermotherapy). In severe cases, surgery is frequently required to remove the whole globe and its intraocular components (enucleation) [45].

Fig. 2 [Images not available. See PDF.]

Different modalities used to treat RB and their drawbacks

The absence of a relationship between the outcomes of in-vivo research and in-vitro test findings is one of the obstacles to the development of cancer treatments.

Conventional treatment of RB

For the treatment of RB, several approaches have been presented. In the past, RB treatment was based on the administration of aggressive focused treatments in addition to systemic chemotherapy and beam radiation (EBRT) [46, 47]. The use of EBRT in particular has been reduced lately due to a number of adverse side effects that occurred including ototoxicity, leukemia, and future primary neoplasms. EBRT was widely utilized up until the

beginning of the twenty-first century [48, 49].

For these reasons, selective ocular delivery systems capable of augmenting the drug's effectiveness while decreasing the likelihood of side effects have been developed over the last decade. RB treatment is now tailored to the tumor's site (intraocular and/or extraocular disorders) and is meant to preserve vision. Moreover, patients with intraocular diseases, especially those related to bilateral ocular disorders, may have a high incidence of ocular protection when applying conservative tumor reduction using intravitreous chemotherapy (IVi) or ophthalmic artery chemosurgery (OAC) combined with extensive local treatment. Nowadays, radiation therapy is only used in cases of extraocular or intraocular disease progression [50, 51].

The conservative treatment is often associated with intense and early focused treatments, using conventional chemotherapeutic agents (i.e., Etoposide, Carboplatin, Palbociclib, Cisplatin, Cyclophosphamide, Doxorubicin, Melphalan, Vincristine, and Topotecan) at different durations and doses depending on the intraocular stage [22].

A physiological barrier, blood retinal barrier (BRB), regulates the flow of proteins, ions, and water inside and outside the retina [52]. The use of conventional anticancer drugs is accompanied by a number of adverse effects such as sores in the mouth and on other mucous membranes, hair loss, bone marrow toxicity, cardiac anomalies, and severe nausea and vomiting, besides, their efficacy is hampered by their inability to penetrate the BRB. Intra-arterial chemotherapy has often been employed to manage this problem [4, 53]. The ophthalmic artery and the femoral artery are both inserted with a micro-catheter as part of this method, and chemotherapeutic agents are then infused in a pulsatile manner [54].

Complications accompanying conventional treatment modalities include ophthalmic artery blockage, partial choroidal ischemia, branch retinal artery obstruction, visual neuropathy, ophthalmic artery spasm with reperfusion, vitreous hemorrhage, and in certain circumstances, patient death [55–57].

Drug discovery for RB treatment

Despite advances in treatment of RB, this disease may still be challenging to treat in certain refractory cases. As a result, researchers are now working to identify novel drugs that can cure both RB and intraocular malignancies utilizing two essential methods [58]:

1. Large-scale chemical high-throughput screening (HTS) employing cells.

2. Repurposing of approved drugs for other types of cancers.

Pharmacokinetic features (such as solubility, metabolism, and the capacity to cross the BRB) are regarded as crucial for the targeting of drug molecules and the design of drug delivery systems. With a focus on evaluating drugs on primary cell lines generated from cerebrospinal and intraocular fluids in which RB metastases, HTS has been used. Additionally, animal models with tumors xeno-grafted from intraocular or metastatic RB may be helpful for developing an effective treatment for humans [59].

Nutraceutical agents

In recent years, various naturally occurring agents have been demonstrated for RB treatment in addition to conventional anticancer drugs such as catechol derivatives (as curcumin), sterol derivatives (as ursolic and oleanolic acid), and naphthoquinones (as β-lapachone) [60]. Among these nutraceuticals is ARQ-501 (β-lapachone), an ortho naphthoquinone derivative, isolated from a tree whose extract has been used in medicine for generations, has been utilized for treatment of ocular tumors [61]. Although its exact mechanism of action is still unknown, several studies have shown its efficacy to block topoisomerase enzymes, resulting in DNA damage, cell cycling arrest, or even cell death. According to the latest studies, it may be advantageous in treating a variety of disorders, including cancer [62]. β-lapachone is now being assessed in fourteen clinical trials, primarily for solid tumors (such as pancreatic cancer, adenocarcinoma, and head and neck neoplasms), and for lymphoma [63].

Moreover, Celastrol, a Chinese herbal drug, showed its effectiveness against several tumor cell lines for treatment of

RB. A previous study reported that effectiveness of Celastrol in promoting dose-dependent apoptosis in SO-Rb50 human RB cells [64].

Routes of drugs' administration to posterior eye segment

The efficacy and safety of the drug are highly influenced by the route of administration. Modifying the administration route may boost and extend the therapeutic results. Delivery systems must be tailored to each administration route since each has its own pros and cons to transport drugs effectively and correctly [65, 66]. Different routes and modes of drugs' administration to posterior area of the eyes are discussed thoroughly in Table 1.

Table 1. Routes of drug delivery to the posterior segment of the eye

Delivery route		Advantages	Disadvantages	Present FDA and research approved examples	Ref.
Systemic	Oral	Simplicity of use	Systemic adverse effects		[8]
IV	Low cost Good Patient compliance Minimal systemic adverse effects	Low ocular bioavailability (1-2%)	Intravenous administration of PLA-PEG chains with cell-penetrating peptide nanoparticles enhanced light-triggered local delivery of drug to the infected choroid * Intravenous nanoparticles loaded with doxorubicin dramatically reduced the size of neovascular lesions *	[12]	Topical
Ointment	Low cost	Therapeutic doses can't be delivered to the retina owing to the existence of dynamic and static barriers	A dexamethasone-eluting contact lens achieved sustained therapeutic levels of dexamethasone to the retina 200-fold greater than hourly administered dexamethasone drops *	[127]	Eyedrops

Simplicity of use	Low therapeutic efficacy with amount less than 5% of the drugs diffusing through the eye following administration	An *in situ* thermosensitive hydrogel decreased laser-induced choroidal neovascularization in the posterior segment of the eyes of rats and pigs*	[100]	Gels	Low systemic side effects
	DEXTENZA®, a dexamethasone intra-canalicular inserted into the lower lacrimal punctum, showed good effectiveness in treating pain and ocular inflammation during eye surgery along with ocular itching accompanied by allergic conjunctivitis **	[126]	Eye Drops with nano/micro systems	Easily fabricated Good patient compliance	

	[103]	Peri-ocular	Injections or implants inserted within the orbital rim of the eyeball (ex: sclera)	Less invasive, with less risks and retinal impairment, compared to intravitreal injections.	Lower concentrations are tained as compared to intravit

					r e a l i n j e c t i o n s / i m p l a n t s

An iontophoretic hydrogel device improved the transport of intraocular nanoparticles and macromolecules to posterior eye segments *via* trans-scleral channels up to 300 times [*]	[127]	Intra-cameral	Implants or injections administered to the anterior segment of the eyeball	Cost-effective and efficient The optimal drug's dosage is administered. Reduced systemic and ocular surface adverse effects in comparison with topical administration Circumvents the challenging barrier of the cornea.	Possibility of triggering toxicant entering segments syndrom

					e. Endothelial cells of the cornea may be harmed. Invasive

					method.
DURYSTA™, an FDA approved biodegradable intracameral implant, containing bimatoprost for the management of ocular hypertension patients [**] DEXYCU™, the first FDA-approved anti-inflammatory drug for use after cataract surgery using dexamethasone intracameral injectable suspension [**]	[128]	Suprachoroidal	Microneedles	Higher bioavailability than periocular routes	Potential tissue damage

XIPERE®, an injectable suspension of triamcinolone acetonide administered into the suprachoroidal space (SCS) ** Microinjector®, an FDA-approved delivery system, given to SCS®, and used for the management of uveitis macular edema **	[13]		Suprachoroidal injected in situ gel	Targeting with high bioavailability in the retinal pigment epithelium and choroid.	Fabrication difficulty

In rabbit eyes, a microneedle-based device aided by iontophoresis boosted posterior targeting with efficiency more than 30% compared to nanoparticles in the SCS *	[77]	Intravitreal	Injection	Increased drug concentrations in vitreous and retinal regions.	Painful

Ozurdex®, an intravitreal FDA approved dexamethasone biodegradable implant used for the management of uveitis and diabetic macular edema **	[124]	Implant	Minimal systemic side effects	-Frequent injections can cause complications -Difficulty in implant's application and removal -Implant may result in acidity in the microenvironment	ILUVIEN®, an approved non-biodegradable, injectable cor

					t i c o s t e r o i d i m p l a n t c o m p r i s i n g F l u o c i n o l o n e a c e t o n i d e u

					sedforthemanagementofmacularedema*

*The described study is a pre-clinical trial

**The described study is an approved market product

Nanotechnology-based approaches for treatment of RB

Ocular cancer treatment has employed topical, systemic, intravitreal, and sub-conjunctival administration techniques. These delivery systems are beneficial for the anterior segments of the eye; however, they have not shown significance in treating disorders (such as RB) at the posterior segments of the eye [44, 67].

Nanodrug delivery systems (Fig. 3) can provide sustained drug release to maintain therapeutically effective concentrations over time, efficient drug targeting and augmentation of pharmacokinetics, pharmacodynamics, toxic, and immunogenic features, thus ensuring increased efficacy for treatment of ocular disorders [68, 69].

Fig. 3 [Images not available. See PDF.]

A schematic diagram showing nanotechnology-based drug delivery systems for the treatment of ocular disorders

[70]

The beneficial properties of nanoparticles (NPs), or particles with sizes between 1 and 1000 nm, have led to their widespread use in medicine. Since NPs are so tiny, they can penetrate cells, having a high surface area-to-volume ratio that amplifies all surface phenomena, thus causing little harm to cell membranes and surrounding cells [71]. Increasing penetration into the retinal pigment endothelium layer, that limits the transport of drugs into the tumor site, is a significant criterion for RB's nanodrug delivery systems. Nanodrug delivery improves the efficiency of cytotoxic anti-cancer drugs by decreasing their toxicity and non-specific interactions and increasing the solubility of weakly water-soluble therapeutic molecules [72]. As indicated in Table 2, many nanoparticulate systems have been utilized for the treatment of RB such as organic, inorganic, multifunctionalized nanocarriers and others. A summary of nanotechnology-based systems used for the management of RB is discussed in the upcoming lines.

Table 2. Summary of the nanocarriers employed in the treatment of RB

Nanodelivery system	Studies	Ref
Organic nanoparticles	Mudigunda et al. reported the effectiveness of hybrid PLGA/PCL NPs loading Palbociclib as well as a photothermal dye on Y79 RB cells with higher drug bioavailability in these cells compared to drug control	[78]

Sims et al. demonstrated the effectiveness of intra-arterial surface-tailored PLGA-NPs carrying melphalan for RB management paving the way for *in-vivo* application	[79]	Silva et al. demonstrated that PLGA-ursolic acid/oleanolic acid nanoparticles showed strong cytotoxic activity against RB cell line
[80]	Narayana et al. documented the efficacy of clinical-grade carboplatin and etoposide-loaded lactoferrin nanoparticles on RB y79 in terms of increasing drug retention, uptake and cytotoxicity compared to their standard drugs	[81]

Ahmed et al. demonstrated the potential of carboplatin loaded lactoferrin nanoparticles with high anti-proliferative activity into the RB cells compared to the drug alone	[82]	Lipid nanoparticles (LNPs)

Xu et al. reported the efficacy of switchable LNPs for the co-delivery of miR-181 and melphalan with good efficacy against RB	[83]	N'Diaye et al. demonstrated the efficaciousness of LNPs incorporating beta-lapachone and temoporfin against RBY79 cells, in which they could be supplied in a

		single intravitreal injection for treatment of RB

[84]	Solid lipid nanoparticles (SLNs)	Ahmad et al. showed the effectiveness of injectable etoposide SLNs for achieving safe and targeted drug against RB

[87]	Nanostructured lipid carriers (NLCs)	Almedia et al. reported the effectiveness of an eyedrop containing ibuprofen dispersion encompassing a combination of NLCs and a thermo-responsive polymer

		with considerable cytotoxicity, improved bioavailability, and therapeutic effectiveness, besides sustained-release drug profiles

[89]	Doxorubicin (DOX)-loaded poly-B-hydroxybutyrate microspheres	Hu et al. showed the functionality of these microspheres to extend DOX release to the posterior eyes' segment

[96]	Carboplatin hyper-branched PAMAM dendritic nanoparticles	Kang et al. proved the potential of carboplatin loaded on dendrimer type PAMAM to increase carboplatin's bioavailability which inhibited toxicity and tumor mass

		in RB compared to free carboplatin
[97]	Makky et al. proved an enhanced targeting and reduced toxicity outcomes when loading concanavalin in dendritic nanoparticles for treatment of intraocular tumors and RB	[98]
Polymeric nanoparticles	Arshad et al. proved that the successfulness of chitosan nanoparticles for delivering DOX to the Y79 RB cell line with increased folate receptor concentration	[100]

Delrish et al. demonstrated the augmented efficacy of thiolated chitosan nanoparticles comprising topotecan relative to free topotecan in Y79 RB cells	[101]	Delrish et al. demonstrated increased ocular bioavailability of thiolated chitosan carboxymethyl dextran nanoparticles in retinoblastoma induced rat eyes

[102]	Godse et al. revealed that galactose conjugated chitosan nanoparticles loaded with etoposide exhibited greater cytotoxicity and resulted in higher apoptosis in RB Y-79 cells relative to pure etoposide	[103]

Mohseni et al. demonstrated that lauric acid-grafted chitosan-alginate nanoparticles incorporating melphalan enhanced its penetrability to the vitreous cavity with augmented efficacy, delineating their potential for RB treatment	[104]	Boddu et al. reported that a micellar system comprising DOX exhibited a two-week continuous release of the drug and a four fold increase in cell absorption over

		the free drug
[105]	Das and Sahoo emphasized that folate-tagged PLGA nanoparticles containing curcumin and nutlin-3a were able to reverse multidrug resistance (MDR) pathways and increase cancer cell apoptosis, expanding therapeutic efficacy for RB treatment	[106]
Rebibo et al. revealed the superior efficacy of tacrolimus-loaded PLGA nanocapsules for RB treatment in augmenting drug's retention and enhancing penetration to posterior eye compartments	[107]	Silver NPs (AgNPs)

Remya et al. reported the cytotoxic efficiency of AgNPs derived from natural sources of brown seaweed Turbinaria ornata against RB cells	[110]	Rajanahalli et al. revealed that AgNPs resulted in cell cycle arrest in G1, and S phases mediated by repression of RB protein phosphorylation using stem mouse embry

		onic stem cells

[111]	Gold NPs (AuNPs)	Wang et al. proved the cytotoxicity of gold nanocages linked with iron oxide NPs in retinal pigment epithelium cells and RBY79 cells, indicating that the system was

		physiologically safe and potential for further use

[113]	Iron oxide NPs	Demirci et al. revealed that magnetic hyperthermia in the Y79 RB cell line utilizing dextran-coated iron nanoparticles resulted in the apoptosis of 46% to 73% of

		Y79 RB cells
[114]	Mesoporous silica NPs (MSNPs)	Gallud et al. reported the efficacy of functionalized mesoporous nanoparticles loaded with camptothecin in treating RB using Y79 cells

[117]	Qu et al. demonstrated that carboplatin loaded in MSNPs increased cancer cell death in RB cells compared to free carboplatin	[118]
Gary-Bobo et al. showed the effectiveness of camptothecin, mannose, or galactose in MSNPs against Y-79 RB cells	[119]	Warther et al. reported the efficacy of mannose-functionalized MSNPs for targeting and imaging RB cells

[120]	Cerium oxide NPs (CeONPs)	Stephen and Chen displayed the efficacy of CeONPs in inhibiting the apoptotic signaling pathway of RBY78 cell lines, increasing the expression of genes associate

		d wit h neu rop rote ctio n
[122]	Gao et al. demonstrated the efficacy of glycolic chitosan-coated cerium nanoparticles loaded with DOX in the significant inhibition of tumor growth as well as the biocompatibility of the proposed NPs with normal retinal cells *in-vivo*	[12 4]
Kartha et al. revealed the superiority of cerium-doped titanium dioxide nanoparticles in augmenting anticancer cytotoxicity compared to titanium dioxide nanoparticles	[125]	Sur fac e- mo difi ed Mel pha lan nan opa rticl es
Farhat et al. revealed that surface-modified melphalan nanoparticles exhibited superior association and effectiveness against RB cells for RB intravitreal chemotherapy	[128]	Gal act ose - fun ctio nali zed nan oca rrie rs

Godse et al. reported that etoposide loaded PLGA nanoparticles coated with chitosan and galactose improved the drug's cellular internalization, promoting superior anti-cancer activity	[100]	Hyaluronic acid (HA)-functionalized nanocarriers
Martens et al. reported that the formulation of electrostatically coated nanoparticles incorporating nonverbal polymeric gene DNA complexed with HA provided increased intravitreal mobility in RB cells	[126]	Folic acid (FA)-functionalized nanocarriers

Organic NPs

Organic nanoparticles are assemblages of organic molecules with an almost infinite number of distinct configurations. They are frequently produced by non-covalent intermolecular interactions, rendering them more malleable in nature and providing a route for elimination from the body. Owing to their flexibility, these nanoparticles can alter shape or conformation when exposed to external stimuli [73]. In RB treatment, the most common type of organic NPs are lipid-based nanoparticles (LNPs), lactoferrin nanoparticles and polymer-based (e.g., poly-caprolactone (PCL), chitosan (CH), polylactic-*co*-glycolic acid (PLGA), and polymethylmethacrylate (PMMA) NPs), showing biocompatibility, high bioavailability in RB cells, with no discernible toxicity besides excellent photothermal and photoacoustic imaging characteristics. Their photothermal properties allow them to absorb light energy and transform it into heat, raising the temperature of the surroundings and causing the death of ocular cancer cells. They can also enable the selective targeting of tumor cells, minimizing the damage to adjacent healthy tissues. The photothermal effect, in addition to destroying cancer cells, can generate acoustic waves that can be detected and turned into imaging signals, a process known as photoacoustic imaging. The method not only gives an additional imaging technique for RB diagnosis, but it also allows for the identification of various biologically relevant signals in a tumor microenvironment, such as reactive oxygen species (ROS), acidic pH, and certain enzymes [74].

A recent study by Mudigunda et al. [75] demonstrated the effectiveness of hybrid PLGA/PCL NPs encapsulating Palbociclib (PCB), as anticancer agent, together with a photothermal dye on Y79 RB cells with higher drug bioavailability in these cells compared to drug control. Furthermore, Sims et al. [76] demonstrated the effectiveness of surface-tailored PLGA-NPs carrying melphalan for intra-arterial treatment of RB, paving the way for *in-vivo* application. Additionally, Silva et al. [77] investigated PLGA nanoparticles sequestering ursolic acid (UA) and oleanolic acid (OA) as a single-dose combination therapy for the management of RB. PLGA-OA/UA nanoparticles showed potent cytotoxic potential against the Y-79 cell line delineating these NPs as a promising approach for treating RB. Moreover, another study documented the efficacy of clinical-grade carboplatin and etoposide-loaded lactoferrin nanoparticles on RB y79 in terms of increasing drug retention, uptake and cytotoxicity compared to their standard drugs [78]. Furthermore, Ahmed et al. [79] demonstrated the potential of carboplatin loaded lactoferrin nanoparticles to enhance ocular drug's retention and intracellular uptake and accordingly, resulting in high anti-proliferative activity into the RB cells compared to the drug alone.

Lipid NPs (LNPs)

Lipid nanoparticles have shown to be promising ocular drug delivery systems since they comprise natural excipients and can incorporate lipophilic drugs, in addition to their distinctive characteristics such as good biocompatibility, safety, and adhesion, which allow for increased bioavailability, compliance, and prolonged drug release. LNPs are useful nanotechnology-based systems used in drug delivery to manage different types of ocular disorders including RB. LNPs gained more attention in the treatment of infectious diseases and cancers, besides the absorption of heavy metals [72].

A previous study reported the effectiveness of switchable LNPs for the co-delivery of melphalan and miR-181 with good efficacy against RB [80]. Furthermore, N'Diaye et al. [81] created LNPs composed of a poly(D, L)-lactide (PDLLA) nanoparticle grafted with a phospholipid (1-palmitoyl-2-oleoyl-sn-glycero3-phosphocholine/1,2-dioleoyl-trimethylammonium propane) bilayer incorporating beta-lapachone as an anticancer agent and photosensitizer, temoporfin, for combined photodynamic and chemotherapy therapy against RB Y79 cells. The investigated system was shown to be efficacious in both chemotherapy and photodynamic treatment and could be supplied in a single intravitreal injection for treatment of RB.

Solid lipid NPs (SLNs)

SLNs represent lipid-based nanocarriers that combine the advantages of emulsions, liposomes, and polymeric particles. To create SLNs, solid lipid matrixes that combine crystalline, highly structured lipid droplets containing bioactive agents, are employed. It is possible to regulate the entrapment of bioactive compounds by changing the SLN lipid matrix's physical state. SLNs provide targeted ocular drug delivery, regulated drug release, and drug stability [82, 83]. A previous study conducted by Ahmad et al. [84] reported the effectiveness of SLNs for the targeted and safe etoposide's injection against RB.

Nanostructured lipid carriers (NLCs)

NLCs are the second generation of SLNs, designed by substituting liquid lipids for the fractional solid lipid components of SLNs, causing an expanded drug corporation space. NLCs are superior to traditional carriers for ocular drug delivery in a number of ways, including improved solubility, the ability to increase storage stability, enhanced bioavailability and permeability, longer half-life, fewer side effects, and tissue-specific delivery [13]. Table 3 provides a comparison between the pros and cons of NLCs and SLNs for ocular drug delivery [85].

Table 3. Advantages of NLCs compared to SLNs limitations regarding ocular drug delivery

Advantages of NLCs	Disadvantages of SLNs
Superior encapsulation efficiency Excellent ocular permeability Proper pharmacokinetic characteristics Extended and controlled drug release Maintaining adequate drugs' concentrations within the aqueous humor, vitreous humor, and retina -Increasing pre-corneal drug retention Increasing drugs' corneal permeation Increasing ocular bioavailability and distribution Positively-charged NLCs have shown to promote a prolonged ocular residence time, circumventing ocular toxicity owing to their close contact with negatively charged mucus membrane Superior biocompatibility and stability	Initial burst release of SLNs Low drug loading efficiency SLNs for ocular drug delivery have not recently assessed through extensive clinical trials, and most investigations have only undergone *in-vitro* evaluations Retinal cell toxicity of SLNs has not been thoroughly studied till current date

Almedia et al. [86] designed an eye drop dispersion of ibuprofen that comprised a combination of NLCs and a thermo-responsive polymer possessing muco-mimetic properties. The cytotoxicity of the formed dispersion was then tested on Y-70 human RB cells, and considerable cytotoxic potential was found. The cytotoxicity of NLCs was then tested using the Alamar Blue reduction assay, which revealed that they were harmless. Later, the results showed that ibuprofen exhibited enhanced bioavailability and therapeutic effectiveness, together with sustained-release drug profiles when loaded in these nanoparticles.

Nanovesicular delivery systems

Nanovesicles comprise an aqueous core enclosed by a lipidic bilayer membrane. Many pharmaceutical enterprises, including those in cancer therapy, employ these systems to encapsulate therapeutic compounds [87].

Liposomes

One of the most popular vesicular systems employed for the management of posterior eye segment disorders is liposomes. Amphipathic phospholipid-based vesicular structures known as liposomes may encapsulate both hydrophobic and hydrophilic molecules [88].

Despite their vulnerability to surface alterations, liposomes have been frequently employed to carry chemotherapeutic agents. Till the current date, no studies have been performed using liposomal systems for the management of RB. However, other studies reported the efficiency of liposomes for the delivery of fluorescein isothiocyanate (FITC) tagged polystyrene or fluorescent probes coumarin-6 to the retina owing to liposomal small particle size, facilitating its permeation across the BRB [89].

Polymeric nanoparticles (PNPs)

Polymeric nanoparticles are particles ranging in size from 1 to 1000 nm that can be laden with biologically active substances which are surface adsorbed onto or entrapped within the polymeric core. They comprise various kinds of polymers, used to produce nanocapsules or nanosphere (Fig. 4). Nanocapsules are reservoir-type systems where

the drug is encapsulated inside a cavity surrounded by a distinct polymeric membrane, whereas nanospheres are matrix-type systems where the drug is uniformly distributed throughout the polymer matrix [90]. The ability to protect drugs and other molecules exhibiting biological activity from the external environment and that to improve their therapeutic index and bioavailability are all advantages of employing PNPs as drug delivery systems [80]. In comparison with other types of nanoparticles, PNPs have received more attention for the management of RB [91].
Fig. 4 [Images not available. See PDF.]

Representation of polymeric-drug delivery systems; nanocapsules and nanospheres

Doxorubicin (DOX)-loaded poly-B-hydroxybutyrate microspheres

Microspheres are monolithic particles with a biodegradable polymer matrix that can be porous or solid. Owing to their pore interconnectivity, low mass density, and large surface area, they are of biotechnological interest where they can provide good particle size control. Polymeric microspheres produced from natural and synthetic polymers may be suitable as monolith templates. They provide a vital role as scaffolds for targeted distribution of bioactive chemicals in a controlled way to improve ocular drug delivery [92]. Regarding RB, DOX-loaded poly-B-hydroxybutyrate microspheres showed extended DOX diffusion to the posterior segment of the eyes, enhancing the drug's penetration into retinal tissues as compared to DOX suspension drops [93].

Carboplatin hyper-branched PAMAM dendritic nanoparticles

Hyper-branched poly(amidoamine) (PAMAM) dendrimers are a novel three-dimensional architecture with nanoscale size and cationic surface charge that could be used as siRNA condensing agents in addition to sturdy nanovectors for targeted ocular drug delivery [71]. Kang et al. [94] proved that carboplatin loaded on dendrimer type PAMAM resulted in an increase in carboplatin's bioavailability which lessened tumor mass in RB. Carboplatin-loaded dendrimers were retained in the tumor vasculature for a longer duration of time and penetrated the sclera until reaching the contralateral eye via the local vasculature, resulting in a prolonged therapeutic effect compared to free carboplatin. Meanwhile, Makky et al. [95] proved an enhanced targeting and reduced toxicity outcomes when loading concanavalin on porphyrin glycodendrimers used in photodynamic therapy for the management of intraocular cancers and RB.

Chitosan nanoparticles

Chitosan is a natural biodegradable polymer that has been extensively researched due to its significant mucoadhesive properties. The ionic interactions provided by chitosan's positively charged nature with the anionic ocular mucosa improve the drug's mucoadhesion, permeability, and retention time on the ocular surface. Consequently, chitosan-based nanoparticulate systems can reduce the number of ocular injections needed while increasing long-term patient compliance [96]. In a previous study, chitosan nanoparticles were fabricated with the goal of delivering DOX to the Y79 RB cell line with increased folate receptor concentration, in which they proved their superior cytotoxicity compared with their unmodified counterparts [97]. Another study demonstrated the augmented efficacy of thiolated chitosan nanoparticles comprising topotecan relative to free topotecan in Y79 RB cells [98]. Moreover, Delrish et al. [99] demonstrated increased ocular bioavailability of thiolated chitosan carboxymethyl dextran nanoparticles in retinoblastoma induced rat eyes. Additionally, Godse et al. [100] revealed that galactose conjugated chitosan nanoparticles loaded with etoposide exhibited greater cytotoxicity and resulted in higher apoptosis in RB Y-79 cells relative to pure etoposide. Furthermore, a previously performed *in-vivo* study showed that lauric acid-grafted chitosan-alginate nanoparticles incorporating melphalan enhanced its penetrability to the vitreous cavity with augmented efficacy, delineating their potential for RB treatment [101].
Another study reported the formulation of a micellar system based on the hydrophilic poly(ethylene glycol) (PEG)

and the biodegradable polymer PLGA comprising DOX, in which folic acid was added to the outer surface of PLGA-PEG-PLGA micelles in order to target the highly expressed folate receptor in Y79 RB cells. This delivery system showed a two-week prolonged release of DOX and a four-fold increase in cell absorption relative to the free drug [102]. A recent study reported the design of curcumin and nutlin-3a loaded folate-tagged PLGA nanoparticles to antagonize multidrug resistance (MDR) pathways and augment tumor cell death. The combined action of curcumin and nutlin-3a expanded therapeutic efficacy for RB treatment [103]. Also, Rebibo et al. [104] fabricated stable and non-irritant PLGA nanocapsules loaded with tacrolimus (TAC) for RB treatment. These nanocapsules showed superior enhancement in augmenting drug retention and diffusion to posterior eye compartments.

Inorganic NPs

Inorganic nanoparticles, comprising non-carbon-based molecules, have attracted significant attention in ocular drug delivery owing to their capacity to be altered in size, form, and crystallinity, besides their large surface area, high density of surface ligand attachment, and simplicity of functionalization. They are divided into metallic and non-metallic NPs [59]. Mesoporous silica, iron oxide, silver, gold, and cerium oxide NPs are the most common types of these nanoparticles used for the delivery of anticancer drugs in treatment of RB disease [7].

Metallic NPs

Metallic nanoparticles are flexible single-element nanomaterials. Some of the most common nanoparticles include Au, Ag, Pt, Cu, Pd, Re, Zn, Ru, Co, Cd, Al, Ni, and Fe nanoparticles. Owing to their flexibility, they can alter composition, shape, size, assembly, structure, and optical properties [72]. They've received much more attention due to their advanced characteristics, such as high surface energy, optical properties, quantum confinement and plasmon excitation, rendering them potential for ocular drug delivery [7].

Silver NPs (AgNPs)

Silver nanoparticles have been widely used in ocular administration of drugs owing to their distinct physical and chemical properties, large surface area-to-volume ratio, biocompatibility, and low production cost, which render them suitable candidates as drug delivery carriers [105]. Silver NPs were employed in RB treatment owing to their affordability, stability, environmentally friendly manufacturing process, and optical properties. Advanced plasma mass spectroscopic techniques, X-ray diffraction (XRD), high-resolution transmission electron microscopy (HR-TEM), Fourier transform infrared spectrum (FTIR), and UV–visible spectroscopy were employed to investigate the synthesized AgNPs [106]. A previous study reported the cytotoxic efficiency of AgNPs derived from natural sources of brown seaweed Turbinaria ornata against RB cells [107]. Meanwhile, another study reported that AgNPs resulted in cell cycle arrest in G1, and S phases mediated by repression of RB protein phosphorylation using stem mouse embryonic stem cells (mESCs) [108].

Gold NPs (AuNPs)

Gold is a noble metal noted for its peculiar optical characteristics, caused by the well-known phenomena of localized surface plasmon resonance. This effect is greatly affected by its shape and is the primary cause for its capability to penetrate biological tissues [85]. The effectiveness of AuNPs as therapeutic agents has been studied in RB treatment on the account of their large surface areas, which allow the adsorption of various functional agents [86]. Accordingly, gold NPs have been studied as drug carriers in RB owing to their ability to [109]:

1. Enhance permeability and retention (EPR) of drugs into the tumor's leaky neovessels, promoting their passive targeting capacity to the tumor site.

2. Sustain drugs' release in response to internal or external triggering factors.

3. Alter the surface with targeting ligands enhancing tumor-selective accumulation as compared to free drugs.

4. Increase the solubility and stability of the drug while also providing a high drug loading capacity by the virtue of their large surface area.

Wang et al. [110] proposed using mesoporous gold nanocages (AuNCs) linked with iron oxide (Fe_3O_4) nanoparticles loaded with muramyl dipeptide (MDP), an immunomodulator and perfluoropentane (PFP), a diagnostic imaging for RB diagnostic imaging and treatment. The cytotoxicity of AuNCs-Fe3O4/MDP/PFP in retinal pigment epithelium ARPE-19 cells and RB Y79 cells was verified, indicating that the delivery system was physiologically safe *in-vitro* and *in-vivo*, accelerating its implementation clinically.

Iron oxide NPs

Iron oxide NPs are magnetic nanoparticles composed of magnetic elements such as iron, cobalt, chromium, and manganese [103]. Since their reactive surface can be modified with biocompatible coatings or bioactive chemicals, they can form a robust drug delivery system that increases their selectivity toward biological targets while avoiding interaction with healthy cells [104]. Iron-containing magnetic nanoparticles combined with heat were employed for the treatment of RB. Hyperthermia is a powerful cancer treatment method because tumor cells are more heat sensitive compared to healthy cells. The temperature may be raised using a variety of techniques, such as microwaves, radio frequency, and focused ultrasound. Iron nanoparticles have been employed as nanoheaters that can target tumor cells without harming healthy tissues [111].

Demirci et al. [111] evaluated magnetic hyperthermia in the Y79 RB cell line utilizing dextran-coated iron nanoparticles. The results indicated that following 24 h of magnetic hyperthermia therapy, apoptosis in 46% to 73% of Y79 RB cells was denoted, suggesting the functionality of magnetic hyperthermia employing dextran-coated iron nanoparticles as an effective therapeutic approach for RB.

Mesoporous silica NPs (MSNPs)

Mesoporous silica nanoparticles are among the most extensively researched inorganic nanoparticles in ocular drug delivery. MSNPs are biodegradable nanomaterials that have the potential to break down into silica or silicic acid. Owing to their known biocompatible nature, they represent one of the most propitious substrates for biological applications including drug administration [7]. It has been determined that MSNPs may increase the solubility and bioavailability of lipophilic molecules due to the subsequent merits [112, 113]:

1. MSNPs' surface is hydrophilic, which improves their wettability. Additionally, the hollowness, surface chemistry, and pore size of microspheres may change the rate of drugs release from them.

2. Enhancement of the amount of entrapped drugs owing to MSNPs’ lack of crystallinity.

3. Large surface area and high dispersibility.

It was reported that functionalized mesoporous nanoparticles loaded with camptothecin (CPT), an anti-cancer agent, as well as one or two photon excitation photosensitizers for photodynamic therapy (OPE-PDT and TPE-PDT) showed their effectiveness using Y79 cells in treating RB [114]. Furthermore, Qu et al. [115] demonstrated that carboplatin (CRB) loaded MSNPs increased cancer cell death in RB cells relative to free CRB. Additionally, Gary-Bobo et al. [116] developed a one-photon excitation photodynamic therapy agent (OPEPDT) employing CPT, mannose, or galactose in MSNPs to target Y-79 RB cells which showed a propitious therapeutic synergy for destroying RB cells. Meanwhile, Warther et al. [117] reported the efficacy of mannose-functionalized MSNPs for targeting and imaging RB cells. MSNPs were almost always located in lysosomes, suggesting that they invade cells

via an endocytic pathway.

Cerium oxide NPs (CeONPs)

Cerium represents the first element in the lanthanide group and appears in both the CeO_2 and $Ce2O_3$ oxidation states. Cerium oxide nanoparticles have cerium (III) and cerium (IV) on their surface, and the pharmacological activity of these nanoparticles depends on their capacity for oxygen absorption and release [118].

Cerium oxide NPs (CeONPs), which have anti-inflammatory and antioxidant characteristics, have attracted a lot of attention in nanotechnology [119]. CeONPs are a viable alternative therapy for a range of acute and chronic disorders since ROS-induced oxidative stress is linked to several disorders [120]. In a previous study, CeONPs showed inhibition in the apoptotic signaling pathway of RB Y78 cell lines, increasing genes expression accompanied by neuroprotection, and decreasing the ROS [119]. Furthermore, Gao et al. [121] reported a novel nanocarrier composed of glycolic chitosan-coated cerium nanoparticles (GCCNP) as a pH-sensitive controlled drug delivery system that can deliver doxorubicin (DOX) for pH-sensitive and tumor-targeted combination therapy. This study reported a synergistic approach for improving the therapeutic potential and lowering the adverse effects of DOX with significant reduction of tumor growth, in addition to improving the *in-vivo* biocompatibility of the proposed NPs with healthy retinal cells. Additionally, Kartha et al. [122] demonstrated the efficacy of cerium-doped titanium dioxide nanoparticles (Ce-doped TiO_2) for their anticancer effects against Y79 RB tumor cells compared to TiO_2 nanoparticles. Both nanoparticles were incubated in Y79 RB cancer cells and then treated with UV irradiation for various time periods varying from one to six hours. Ce-doped TiO_2 showed augmented anticancer cytotoxicity compared to TiO_2 nanoparticles owing to the ability of cerium element to retain the integrity of DNA, generally lost in cancerous cells, by acting on the intimate pathways governing the survival of cancerous cells.

Multifunctionalized nanocarriers

Multifunctional NPs are advanced nanoparticulate systems which can deliver one or more therapeutic compounds, enabling biomolecular targeting through one or more conjugated antibodies or other ligands, and magnifying imaging signals by encapsulating contrast agents [65, 123].

Surface-modified melphalan NPs for the intravitreal chemotherapy of RB

Compared to unmodified NPs, surface-modified melphalan NPs exploited superior effectiveness against RB cells, in which they demonstrated higher efficacy compared to other NPs [124]. Future studies are required to demonstrate the capacity of these nanoparticles to enhance drug's transport to the vitreous humor, where it is expected that surface modification will have a bigger influence on efficacy.

Galactose-functionalized nanocarriers

The sugar entities ligand-based mechanistic technique for attaining enhanced and customized RB treatment is in great demand. RB cells express considerably more sugar moieties in the form of lectins than healthy cells. Hence, targeting overexpressed lectins is an effective way for achieving successful results [125].

Human RB cells express sugar receptors (lectins) with a preference for galactose and mannose residues, according to a prior work by Godse et al. [125]. Sugar is therefore a desirable ligand that can be used to target and improve the endocytosis of drugs-loaded NPs. Additionally, unlike folic acid, sugars do not have photosensitivity or stability concerns. The authors observed that etoposide loaded PLGA nanoparticles coated with chitosan and galactose for treatment of RB slowed the drug release rate and helped in the active targeting of RB cells. Also, cytotoxicity and apoptosis experiments demonstrated that these NPs had improved the drug’s cellular internalization, promoting superior anti-cancer activity.

Hyaluronic acid (HA)-functionalized nanocarriers

HA is an FDA-approved marine polymer possessing exceptional biodegradability, flexibility, mobility and shielding, in

addition to an anticancer action on the HA receptor, the CD44 receptor. A previous study reported that the formulation of electrostatically coated nanoparticles incorporating nonverbal polymeric gene DNA complexed with HA provided increased intravitreal drug delivery in RB cells [126].

Folic acid (FA)-functionalized nanocarriers

Coupling nanocarriers with a targeting moiety can be more successful compared to systemic chemotherapy in the targeted eradication of tumor cells [103] Targeted molecules allow spatial delivery of antitumor agents [98]. Folate receptors are highly expressed in RB cells, so exploiting them in RB treatment to selectively uptake NPs and only kill cancer cells will be very effective [127]. In a study reported by Mitra et al. [128], CNPs and DOX conjugated with folic acid proved their efficacy for targeting RB cells.

Conclusions and future prospects

Retinoblastoma is a type of challenging pediatric ocular cancer that is difficult to treat by the conventional approaches owing to drug expulsion and non-targeted delivery, resulting in therapeutic inefficiency. Nanoparticles-mediated antitumor drug delivery proved to increase therapeutic potential, lower toxicity, customize site-specific delivery and ligand binding that may transport drug through several routes of administration, hence causing cost-effectiveness and cytotoxicity management of RB. These delivery systems have shown their effectiveness to lower the barriers to treating RB and prevent the loss of normal cells. Emerging advances in multifunctionalization and biocompatible ligands in anticancer therapy and diagnosis are ushering in a new era of surpassing conventional barriers by strategically enhancing RB treatment and diagnosis. With the revolutionary breakthrough of nanomedicine in cancer diagnosis, experimental research is designed to establish cell/tissue-specific nanosystems to suit the demanding criteria of intraocular chemotherapy and diagnostics. The last frontier in this study is employing "intelligent nanosystems with several functions" (i.e., systems capable of reaching the challenging anatomical eye components affected by RB). However, further pre-clinical research is required before evaluating the method in clinical trials to determine its benefit-to-risk ratio.

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SH was involved in the methodology and writing the original draft; DM contributed to reviewing and editing; NE collected the data. All authors have read and approved the manuscript.

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Declarations

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This article does not include any studies involving human or animal subjects.

Consent for publication

This article does not contain any studies involving human subjects.

Studies involving plants

This article does not contain any studies involving plants.

Competing interests

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Abbreviations

AgNPs

Silver nanoparticles

AuNPs

Gold nanoparticles

BRB

Blood retinal barriers

CD44

Cluster of differentiation 44

CeONPs

Cerium oxide nanoparticles

CH

Chitosan

CNPs

Chitosan NPs

COG

Children’s Oncology Group

CSF

Cerebrospinal fluid

DCM

Dichloromethane

DOX

Doxorubicin

EDTA

Ethylenediamine tetra-acetic acid

FA

Folic acid

Fe_3O_4

Iron oxide

FITC

Fluorescein isothiocyanate

HA

Hyaluronic acid

HTS

High-throughput screening

IIRC

International classification of intraocular retinoblastoma

IVi

Intravitreous chemotherapy

LNP

Lactoferrin nanoparticle

LNPs

Lipid nanoparticles

MDP

Muramyl dipeptide

MRI

Magnetic resonance imaging

MDR

Multidrug resistance

MSNPs

Mesoporous silica nanoparticles

NLCs

Nanostructured lipid carriers

NPs

Nanoparticles

OAC

Ophthalmic artery chemosurgery

PCL

Poly-caprolactone

PFP

Per fluoro pentane

PLGA

Poly-d, l-lactic-*co*-glycolic acid

PMMA

Polymethylmethacrylate

RB

Retinoblastoma

ROS

Reactive oxygen species

SCS

Suprachoroidal space

Publisher's Note

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Quisqualis indica Linn.: HRLCMS/MS profiling and anti-asthma activity of leaf extracts

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ABSTRACT (ENGLISH)

Background

Asthma is a chronic inflammatory disorder of the airways, involving various cells and cellular elements precisely eosinophils, mast cells, neutrophils, T lymphocytes, epithelial cells, and macrophages. Worldwide, about 300 million people are affected by asthma, and is expected that 100 million people may get affected, in 2025. *Quisqualis indica* is commonly planted as an ornamental plant in India; and has medicinal uses. Therefore, the petroleum ether (60–80 °C) LPE and methanolic extract LME of the *Quisqualis indica* leaves were analyzed for anti-asthmatic activity by mast cell degranulation and Milk induced eosinophilia and leukocytosis in mice.

Results

It was found that *Quisqualis indica* leaf extracts exhibited protection against the degranulation of mast cells and a reduction in the difference count of leucocytes and eosinophils. LPE and LME (400 mg/kg) have shown 33% and 63% of mast cell protection. LME has shown the most significant mast cell stabilizing action comparable with the standard drug. The extracts decreased the difference count of leucocytes and eosinophils. LME (400 mg/kg) has shown a difference in eosinophil count and a decrease in leukocyte count most comparable with the standard Dexamethasone. Methanolic extract analyzed for phytochemicals by High Resolution Liquid Chromatography Mass Spectroscopy /Mass Spectroscopy method showed the presence of various Phyto-compounds.

Conclusion

From the analysis of methanolic leaf extract of *Quisualis indica* revealed the presence of phytocompounds such as Apigenin7-glucoside, Gallic acid, Quercetin, Quercitrin, Kaempferol, etc. The significant decrease in eosinophil and leukocyte count in animals might be due to the higher content of Tannin and flavonoids. A reduction in leukocyte and

eosinophil is regulated by type 1 hypersensitivity and adaptogenic factors hence *Quisqualis indica* is effectively helpful in allergy conditions like asthma.

FULL TEXT

Background

Asthma is a chronic inflammatory disorder of the airways, involving various cells and cellular elements, precisely eosinophils, mast cells, neutrophils, T lymphocytes, epithelial cells, and macrophages. Asthma is characterized by hindrance of airflow due to inflammation, bronchospasm, and increased airway secretions. [1] About 300 million people worldwide suffer from asthma, and by 2025, it's predicted that 100 million more will likely develop the condition. The prevalence, severity, and mortality of asthma are greatly affected by geographical variation. Asthma incidence is greater in high revenue countries, and maximum death caused by asthma happens in low middle revenue countries [2].

The multifaceted condition known as asthma is brought on by complex relationships between the environment and heredity. The pathophysiology of this condition includes intermittent airflow restriction, bronchial hyperresponsiveness (BHR), inflammation of the airways and bronchial remodeling. Depending on the severity of the condition, bronchodilators and anti-inflammatory medications can be used to treat asthma. Then, additional treatments are still required to better control asthma, and herbal remedies may be a viable substitute for current synthetic medications [3]. Additionally, plant polyphenols are potent components of natural foods and herbal medicine that have anti-inflammatory, antioxidant, and anti-allergic properties. Previous studies have demonstrated the anti-inflammatory effects of resveratrol, genistin, luteolin, and quercetin, as well as their multiple targets, multiple links, and comprehensive coordination [4].

The plant *Quisqualis indica* Linn.*(Combretaceae)* is an evergreen plant planted in homes and gardens of countries like India, China, Australia, the Amazonian region in Peru, and Pakistan. It is a large sub-woody climber shrub with 3–8 m long branches. Leaves are simple, oppositely having an oblong-elliptic to elliptic shape. It bears the inflorescence with white to pink-colored flowers. Flowers appear in constant succession in sagging clusters with a sweet scent [5, 6].

The leaves of *Quisqualis indica* were used to isolate four crystalline components, which were later identified as nicotinic acid and methylbetaine (trigonelline), L -proline, L -asparagines and potassium quisqualate [7]. Triterpenoids, flavonoids and tannins were isolated from the petroleum ether and methanolic extract of leaf and flower, respectively [8]. Ethno-pharmacologically, the flowers, fruit, seeds, leaves, stem and roots of the plant are used. Leaves are used to relieve pain caused by fever. Leaves decoction is given for flatulent distension and pain in the abdomen. Leaf juice is used to heal boils and ulcers [9]. Fruits and seeds are used in folk medicine as anthelmintic, anti-emetic, and anti-diarrhoea [10].

Different parts of plant have been studied for various pharmacological actions such as anti-microbial [11], anthelmintic [12], anti-pyretic [13], anti-inflammatory [14], anti-oxidants [15], insecticidal [16], immunomodulatory [17], anti-diarrheal [18], etc. due to the existence of various phytoconstituents. The stem bark and flowers were evaluated for the total tannin content and anti-oxidant activity [19]. Roots are used to treat rheumatism, cough and hiccup by Philippines [3, 20, 21]. As the whole plant is used as cough cure [10]; in the current work, we have looked into the phytoconstituents and anti-asthma activity of the *Quisqualis indica* leaves. The leaf petroleum ether extract (LPE) and leaf methanolic extract (LME) of *Quisqualis indica* were screened for phytochemicals by High Resolution Liquid Chromatography Mass Spectroscopy /Mass Spectroscopy (HRLCMS/MS) and anti-asthma activity. In the case of *Quisqualis indica* extract, which is high in flavonoids and phenolic compounds, reduces inflammation [14] and act as an immunomodulatory. [17]

Methods

Collection and preparation of plant material

The fresh leaves of *Quisqualis indica* Linn. plant were collected from the residential region in the Indian state of Maharashtra near Nashik. The plant was verified by the India Botanical Survey, which included Pune and

Maharashtra [22, 23]. The allotted authentication No. was BSI/WRC/IDEN.CER/2016/403 (A). The pulverized and sieved dried leaf powder was processed. The petroleum ether (60–80 °C) and methanol solvents were used to extract the phytochemicals such as steroids, flavonoids, tannins; alkaloids, etc. The petroleum ether (60–80 °C) and methanol solvents were used in a continuous hot percolation process (Soxhlet extraction) to extract the coarse powder successively for 48 h. A rotating vacuum evaporator (Evator) was used to concentrate the liquid extracts to produce a semisolid extract [24, 25].

Animals

The healthy adult Wistar albino mice (20–25 g) of either sex were housed in polypropylene cages, under 12 h light: 12 h dark cycle and temperature 22±2 °C and humidity 55±5% maintained in the animal house. The animals had free access to food and water. All the animals were deprived of food but not of water 4 h before the experiment [26]. The Institutional Animal Ethical Committee of the institute (Registration No. 121/1999/CPCSEA) has approved all the protocols of the study (Registration No. IAEC/Jan 2020/09).

Acute oral toxicity study

The acute oral toxicity study OECD guideline (2001) [26, 27] was followed to carry out acute toxicity study for the extracts of *Quisqualis indica* plant.

Mast cell degranulation

There were six groups of mice in total, with six mice in each group. A three-day treatment for drugs program has been followed properly. Group I Control groupwas given the treatment with 1% tween 80 solutions (5 ml/kg, i.p.); Group II received standard drug Sodium chromoglycate (50 mg/kg, i.p) and all test groups Group III to Group VI received LPE and LME at doses of 200 and 400 mg/kg. On the fourth day, 10 mL/kg of 0.9% saline solution was gently massaged into the peritoneal cavity of each mouse before being injected. Five minutes later, the peritoneal fluid was collected. The peritoneal fluid was then put into a test tube containing 7–10 mL of RPMI-1640 buffer medium (pH 7.2–7.3), which is made up of L-glutamine and 25 mM HEPES buffer but not sodium bicarbonate. At 400–500 rpm, this solution was then centrifuged. Centrifugation was used to rinse the mast cells pellets twice with RPMI-1640 buffer media, and the supernatant was rejected. Egg albumin was used to challenge the cell suspension produced from both the treated and control groups of mice, which was then incubated at 37 °C for 10 min.After being stained with 1% toluidine blue, the cells were examined under a microscope. Mast cells that had degranulated were seen as rupture cells rather than whole. In total of 100 cells from various visual regions were counted, and the percentage of protection against degranulation was calculated [28, 29].%Protectionmastcell=[1-(T/C)]x100where, T-No. of degranulated cells of test

C-No. of degranulated cells of control

Milk induced eosinophilia and leukocytosis

There were six groups of mice, six animals in every group. Blood samples (0.5 ml) were collected from the retro-orbital plexus. Group I Control group received vehicle, tween 80 (1%) solution (5 ml/kg, i.p.); Group II received Standard drug Dexamethasone (50 mg/kg, i.p) and test groups Group III to Group VI received LPE and LME at doses 200 and 400 mg/kg. Amul's Fresh milk was procured from the local market. All animals were treated with an injection of freshly cooked and chilled milk (4 mL/kg, s.c.) for 30 min following treatments. Total leukocyte and eosinophil count were carried out in each group before drug administration and 24 h after the milk injection. Difference in total leucocytes count before and after 24 h of drug administration was calculated [30, 31].

Statistical analysis

All the results of various studies were expressed as mean±Standard Error of Mean. Data was analyzed using a one-way ANOVA, which was followed by Dunnet's Multiple Comparison Test. $P<0.05$ was considered statistically significant. The graphs were calculated with the GraphPad Prism 5.

HRLCMS/MS profiling

The phytochemicals of the bioactive extract (LME) were analysed by HRLCMS/MS method. High-resolution liquid chromatography combined with the Q-Exactive Plus Mass Spectrometer TOF/Q-TOF mass spectrometer with ion source dual AJS electrospray ionization (ESI) was used [32]. The plant extract (50 mg) was dissolved in 1 mL of

water: acetonitrile (1:1) solvent mixture, ultrasonically processed for 10 min, and then centrifuged at 10,000 rpm for 10 min. The ZORBX Eclipse Plus C18, narrow bore 2.1 150 mm with 5 microns, was used for the LC/ESI-QTOF-MS/MS analysis. An amount of 5µL was delivered in both positive as well as negative mode. The Mass Spectra scan range was in 120 to 1200, with 1.00 scans rate spectra/second and 1.00 as MS/MS scan rate spectra/second. The gradient elution procedure was done by changing the proportion of water and acetonitrile from 95 to 5% within 30 min, with flow rate of 0.3 mL/min as a constant flow rate. Then, this section was linked to the TOF/Q-TOF Mass Spectrometer for MS/MS fragmentation spectra. Phytochemicals were recognized by comparing their m/z and MS/MS transitions with those documented in reference databases. Further, the molecular formula, retention time, and adduct formula were detected.

Results

Acute oral toxicity study

Oral administration of plant extracts shows no significant body weight variation, neither any sign of toxicity nor mortality of mice at the dose of 2000 mg/kg, body weight.

Mast cell degranulation

In the pathogenesis of allergic asthma, mast cells also play a crucial role. Inhaled antigens enter the lower respiratory tract and produce localised mast cell degranulation and inflammation that trigger an inflammatory response in the airways. These circumstances cause edema, fluid build up, and increased vascular permeability, which can block the airways. Asthma-related airway obstruction can result from bronchial constriction, which can happen as a result of smooth muscle contraction [33]. Novel medications or treatments that help lessen asthma symptoms by lowering the production of these inflammatory mediators can be found by researching the inhibition of mast cell degranulation. In the present study, the effect of LPE and LME at doses of 200 and 400 mg/kg, body weight was examined on mast cell degranulation. The control group showed (83.4 ± 0.34) degranulation of mast cells while groups pre-treated with extracts and disodium chromoglycate significantly protect degranulation of mast cells. [34, 35] LME at dose (400 mg/kg) showed (31.6 ± 3.67) and disodium chromoglycate (26.86 ± 2.34) protection against degranulation as shown in Figs. 1 and 2.

Fig. 1 [Images not available. See PDF.]

Effect of Quisqualis indica leaf extracts on egg albumin induced degranulation of mast cell

Fig. 2 [Images not available. See PDF.]

Mast cell granulated and degranulated

Values are in Mean ± SEM, $^{*}P<0.05$ when compared against control, $n=6$. One Way ANOVA was used to analyze all the data, and then Dunnett's test was performed. The graph was calculated by using PRISM 5 Software.

Milk induced eosinophilia and leukocytosis

Airway hyperresponsiveness (AHR), reversible airflow restriction, and airway inflammation are all symptoms of bronchial asthma, a chronic condition. Eosinophilic asthma and non-eosinophilic asthma are two types of asthma's pathophysiological mechanisms. According to recent studies, eosinophils are crucial to the emergence of asthma exacerbations. Immune-modulatory reactions brought on by a high blood eosinophil count include airway inflammation, hyperresponsiveness, epithelial lining damage, and increased mucus secretion, Eosinophilic inflammation affects nearly fifty percent of asthma sufferers. Studies have proven that eosinophilia has been associated with higher disease severity, exacerbation frequency, and symptom burden, as well as decreased lung functions [36]. Therefore, eosinophilic inflammation must be suppressed and eosinophilic and non-eosinophilic asthma must be distinguished in order to treat or avoid asthma aggravation [37]. The maximum increase in difference of eosinophil (578.00 ± 11.10) and leucocytes (11,266.16 ± 149.63) counts was found in the control group which has been treated with milk (4 mL/kg, s.c.) before 24 h *Quisqualis indica* extracts at doses of 200 and 400 mg/kg significantly inhibited milk-induced eosinophilia and leucocytosis in a manner dependent on dose. Like Dexamethasone [38], the LME (400 mg/kg) exhibits substantial inhibition as shown Figs. 3 and 4.

Fig. 3 [Images not available. See PDF.]

Effect of Quisqualis indica leaf extracts on Milk induced eosinophilia

Fig. 4 [Images not available. See PDF.]

Effect of Quisqualis indica leaf extracts on milk induced leukocytosis

Values are in Mean ± SEM, $^{***}P<0.05$ when compared against control, $n=6$. One Way ANOVA was used to analyze all the data, and then Dunnett's test was performed.The graph was calculated by using PRISM 5 Software.

Values are in Mean ± SEM, $^{***}P<0.05$ when compared against control, $n=6$. One Way ANOVA was used to analyze all the data, and then Dunnett's test was performed. The graph was calculated by using PRISM 5 Software.

LCMS/MS study of the bioactive extract

The LC–MS chromatogram of the methanolic extract of *Quisqualis indica* is shown in Figs. 5 and 6, and the important identified compounds are given in Tables 1 And 2.

Fig. 5 [Images not available. See PDF.]

Chromatogram of LME of *Quisqualis indica* positive ESI

Fig. 6 [Images not available. See PDF.]

Chromatogram of LME of *Quisqualis indica* negative ESI

Table 1. Important compounds identified in LME extract of *Quisqualis indica* by HRLCMS/MS positive ESI

Peak No	Retention time	Abundance	Name of compound	Molecular formula
	1.289	150,556	L-Proline	C5H9NO2
	1.337	45,242	Retronecine	C8H13NO2
	1.873	237,178	Fenapanil	C16H19N3
	1.926	200,358	Neotussilagine	C10H17NO3
	3.149	325,229	Ketotifen	C19H19NOS
	3.439	348,266	N(alpha)-t-Butoxycarbonyl-L-leucine	C11H21NO4
	5.704	136,079	Crotanecine	C8H13NO3
	6.222	121,724	6-C-Galactosylluteolin	C21H20O11
	6.515	379,580	Apigenin7-glucoside	C21H20O10
	7.138	299,350	Maritimetin	C15H10O6
	9.335	80,034	N1,N5,N10- Tricoumaroylspermidine	C34H37N3O6

	11.25	122,878	Phenmedipham	C16H16N2O4
	11.561	83,930	Sulfadimidine	C12H14N4O2S
	12.666	99,466	Schleicherastatin 6	C28H46O3
	14.802	267,218	23-Acetoxysoladulcidine	C29H47NO4
	14.887	190,078	Citronellyl hexanoate	C16H30O2
	23.236	197,160	Goyaglycoside c	C38H62O9

Table 2. Important compounds identified in LME extract of *Quisqualis indica* by HRLCMS/MS negative ESI

Peak No	Retention time	Abundance	Name of compound	Molecular formula
	1.734	129,716	1-O-Caffeoyl-(b-D-glucose 6-O-sulfate)	C15H18O12S
	2.59	206,438	Gallicacid	C7H6O5
	4.445	69,208	Chorismicacid	C10H10O6
	4.839	46,662	Kurigalin	C27H24O18
	4.893	3176.86	PunicacorteinB	C27H22O18
	5.189	14,789	Salicylicacid	C7H6O3
	5.864	297,109	Quercetin3-(2-galloyl glucoside)	C28H24O16
	6.503	1,238,967	Genistein8-C-glucoside	C21H20O10
	6.653	10,369	Myricitrin	C21H20O12
	6.94	297,109	Clocortolonepivalate	C27H36ClFO5
	7.167	152,689	Quercitrin	C21H20O11
	8.966	39,705	Kaempferol	C15H10O6
	10.103	45,242	Luteolin	C15H10O6

	14.291	–	Geranylfarnesyl diphosphate	C25H44O7P2
	16.371	13,987	StigmatellinY	C29H40O6

Discussion

A persistent inflammatory illness of the airways is allergic asthma. Leukocyte infiltration into the lung and airway is the primary characteristic of asthma [39]. In the current study, mice with milk-induced eosinophilia and leukocytosis were used to test the anti-asthmatic activity of LPE and LME at doses of 200 and 400 mg/kg. According to reports, giving milk subcutaneously causes a noticeable rise in the number of leukocytes and eosinophils 24 h later. Inflammatory mediators such as cytokines, histamine, and major basic proteins are released by leucocytes during asthmatic inflammation, promoting the continued inflammation. A peripheral eosinophil count that has abnormally increased to over 4% of the total leukocyte count is referred to as eosinophilia. The eosinophil count rises in asthmatic patients [40, 41].

While mice treated with various doses of plant extracts show a decrease in the difference in leukocyte and eosinophil counts, mice treated with 1% Tween-80 in the control group exhibit an increase in these two cell types. *Quisqualis indica* leaf extracts may be helpful in allergy conditions because adaptogenic and type I hypersensitivity mediate the decrease in leucocytes and eosinophils.

Mast cells degranulate in response to immunological stimuli where antigen antibody responses are prevalent. In a dose-dependent way, *Quisqualis indica* leaf extracts at doses of 200 and 400 mg/kg effectively prevent egg albumin-induced mast cell degranulation. At 400 mg/kg, LPE and LME protect mast cells similarly to disodium chromoglycate. This demonstrates the effectiveness of *Quisqualis indica* leaf extracts in type I hypersensitivity reactions and in mast cell stabilization. The anaphylactic allergic reaction, which can be triggered by a variety of triggers, is a potentially fatal reaction that releases mediators like histamine and pro-inflammatory cytokines.

The methanolic extract was subjected to a phytochemical analysis using the HRLCMS/MS technique, which revealed the presence of a number of phytocompounds including apigenin 7-glucoside, 3-Oxo-12, 18-ursadien-28-oic acid, 1-O-Caffeoyl-(b-D-glucose 6-O-sulfate), gallic acid, kurigalin, punicacortein B, etc. Gallic acid has shown a good amount of abundance in LME extract and acts by inhibiting mast cell activation, preventing the release of histamine, and the production of pro-inflammatory cytokines [42]. The highly galloylated compounds inhibits secretion of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) in a concentration-dependent manner, acting on the transcriptional activity of NF-κB [43]. According to the pharmacological effects of flavonoids like kaempferol, quercetin, and rutin, they may be able to treat allergic inflammatory disorders by inhibiting mast cell activation [44]. Apigenin has been found to be effective in allergic asthma through the decreased activation of epithelial cells, T cells, and eosinophils [45]. As per HRLCMS data of LME extract the flavonoids like kaempferol, quercetin, apigenin, and luteolin and few types of tannin like gallic acid and punicacortein B are present in a notable amount. As a result, *Quisqualis indica*'s leaf methanolic extract has shown significant mast cell degranulation and Milk-induced eosinophilia and leukocytosis action. Hence, *Quisqualis indica* leaves may be helpful in allergic conditions like asthma.

Conclusion

The plant extract is found to comprise phytochemicals such as apigenin 7-glucoside, 3-Oxo-12, 18-ursadien-28-oic acid, 1-O-Caffeoyl-(b-D-glucose 6-O-sulfate), gallic acid, kurigalin, punicacortein B, apigenin, kaempferol, quercetin, rutin, nicotinic acid methylbetaine (trigonelline), L-proline, L-asparagine, potassium quisqualate, etc. Thus, this plant is a plentiful source of phytochemicals with therapeutic value, and the findings of this study offer some scientific support for the use of the plant in traditional medicine for anthelmintic, anti-emetic, analgesic, antiulcer, and anti-diarrheal purposes. The results of the current investigation showed that milk-induced leucocytosis and eosinophilia reduced significantly by *Quisqualis indica* leaf extracts at 400 mg/kg dose. In anaphylactic reactions, extracts

suppress the release of histamine and stabilize antigen-induced mast cells. They also have anti-allergic properties. As a result of its anti-allergic and mast cell-stabilizing properties, which might be due to the presence of flavonoids, tannins, and triterpenoids, *Quisqualis indica* leaf extracts are beneficial in treating asthma.

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Author contributions

CTN carried out the sample collection, conceptualisation, extraction and anti-asthma activity, HRLCMS/MS analysis, results interpretation and write-up. The other author read and approved the final manuscript.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Ethics approval and consent to participate

All the experimental procedures and protocols used in this study were reviewed and approved by the Institutional Animal Ethical Committee (IAEC) of MVP's College of Pharmacy, Nashik, Maharashtra, India (Registration No. 121/1999/CPCSEA) constituted under Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India (Annexure 1). The animal ethical committee of the institute has approved all the protocols of the study (Registration No. IAEC/Jan 2020/09).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

LPE
Leaf petroleum ether extract
LME
Leaf methanolic extract
OECD
The Organization for Economic Cooperation and Development
HRLMS/MS
High Resolution Liquid Chromatography Mass Spectroscopy /Mass Spectroscopy
AJS
Jet Stream Technology Ion Source
ESI
Electrospray ionization
TOF/Q-TOF
Tandem time-of-flight /quadrupole Tandem time-of-flight
RPMI-1640
Roswell Park Memorrial Institute
ANOVA
Analysis of Variance

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Seaweeds as a potential resource in diabetes management: a review

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ABSTRACT (ENGLISH)

Background

Seaweeds are the marine macroalgae predominantly found in the coastal regions. These species have unique chemical profiles which makes them stand different from terrestrial plants. They are found to be rich in secondary metabolites which have potential in treating various unhealthy conditions. Diabetes is a chronic condition where an individual suffers from high or low sugar levels in the blood.

Main text

This review article aims to review such seaweed species that are potent in treating and managing diabetes. It has become one of the rapidly growing diseases in the world with a high occurrence rate. This paper details the mechanism of glucose regulation in the human body. Seventy percent of the total earth’s surface is accomplished by marine ecosystem. It offers home for about 25,000 species. Among them, many possess health benefits hence these are utilized directly as food or in the form of medicine.

Conclusion

In this article, various such seaweed species which have antidiabetic property has been discussed. Including edible seaweeds in daily diet have numerous healing properties. Inhibition of alpha amylase and alpha glycosidase enzyme has been associated with lowering the postprandial glucose level. This review article attempts in exploring how seaweeds and managing diabetes are interrelated.

FULL TEXT

Background

Diabetes mellitus, commonly referred to as diabetes, is categorized as a group of metabolic diseases that arise when either the pancreas is unable to produce sufficient insulin to support the body's normal blood sugar mechanism or when the body is unable to efficiently utilize the insulin that is already produced. Pancreas has two main functions, out of which one is endocrine function that regulates blood sugar. β-islets of Langerhans cells in the pancreas produce insulin, a peptide hormone whose purpose is to maintain a normal blood glucose level. It is achieved by regulating carbohydrate, lipid, and protein metabolism, facilitating cellular glucose uptake. Insulin is encoded by INS gene. Hyperglycemia refers to elevated levels of glucose in the blood. This condition is commonly associated with diabetes, a chronic disease that affects the body's ability to regulate blood sugar. The lack of insulin's action on the target tissue or its secretion in pancreatic cells is the primary reason for increased glucose

±levels in blood [1]. One more condition is low blood sugar called hypoglycemia. Diabetes comprises of two major types, Type 1, in which the pancreas produces little or no insulin over time, is a chronic condition. Type 2 diabetes is characterized by elevated blood glucose levels because of either absolute or relative insulin deficiency. The decrease in beta-cell mass or impairment in beta-cell function is the cause of this drop-in insulin levels or insufficient insulin secretion [2]. About 90% of cases of diabetes are type 2 [3]. It is primarily the result of an unhealthy lifestyle, excess weight, and lack of physical activity. Initially, only adults were more prone to type 2 diabetes, now it is affecting among children as well.

Incidence and mortality rate

Diabetes has had the fastest incidence rate over a decade. Its prevalence has increased from 108 million in 1980 to 422 million in 2014. In 2015, the count rose to 451 million among the individuals aged between 18–99 [4]. In 2017, type 1 diabetes affected 9 million people; the majority of the cases were reported from high-income countries. By 2021, only children and adolescents (ages 0 to 19) will make up more than 1.2 million. Neither its cause nor the means to prevent is known. Polyuria, that is excessive urination, polydipsia (increased thirst), persistent hunger (polyphagia), weight loss, blurry vision, and fatigue are some of the symptoms in this instance.

Diabetes as a global health issue

According to the recent publication by IDF diabetes organization published in 2021, worldwide, approximately 537 million adults suffer from diabetes, and the number is expected to rise to 643 million by 2030 and 783 million by 2045. China currently has the highest number of adults aged 20 to 79 with diabetes, followed by India and Pakistan. Every 3 in 4 adults are living with diabetes now. About 240 million adults with diabetes are undiagnosed and has resulted in 6.7 million fatalities. Twenty-one million pregnant women are at risk of getting affected by diabetes during their pregnancy [4]. It is medically termed as gestational diabetes. They have the risk of developing type 2 diabetes later at any point of time of life. T2DM affects 541 million adults at an increased risk. The reason it is being categorized as global health issue is due its increased risk of effecting other parts of the organ system. Increase in blood sugar levels might risk the patient in leading way to other diseases, such as glaucoma, cataract, blindness, chronic kidney disease, neuropathy, hypertension, and other heart diseases.

Prevalence

Global diabetes prevalence is estimated to be 9.3% in 2019, or 463 million people. By 2030, it will rise to 10.2% (578 million) and by 2045, to 10.9% (700 million). Urban areas have a commonness rate of 10.8%, while rural areas have a commonness rate of 7.2%. In low-income nations, the prevalence rate is 4%, while it is 10.4% in high-income nations [5]. Half of the population—one in two, or 50.1%—is unaware that they have diabetes. In 2019, the global prevalence of impaired glucose tolerance is estimated to be 7.5 percent (340 million), rising to 8.6 percent (548 million) in 2030 and 2045 [5]. Obesity especially central obesity and increased visceral fat due to insufficient physical activity along with the consumption of high calorie diet, high sugar-based diets are the contributing factors.

Treatment

Till today, there is no exact medication or complete cure discovered for diabetes. Diabetes can be treated by monitoring the sugar levels, switching to healthy lifestyle, practising physical activities that could be yoga, cycling, running any activity of people’s choice. Most of the treatment for type 1 diabetes involves injecting exogenous insulin and by oral medications to sustain normal healthy life [6]. Regular screening from physicians in case of any complications is mandatory requirement. Modifications in lifestyle will be a selfcare treatment which includes proper and healthy diabetic diet, exercising regularly, weight management, nutritional counselling, keeping count on carbohydrate intake. For type 2 diabetes, metformin is typically prescribed [7]. It actions primarily by making the liver produce less glucose and enhancing the body’s sensitive to insulin, allowing it to use insulin way more effectively.

Management

Most people manage their diabetes themselves. No matter how advanced the treatment technology is, the outcome will be poor if the patient is unwilling or unable to manage it on a daily basis [8]. Management of diabetes includes following a proper healthy diet, physical exercise, and keeping their daily sugar and carb intake in control. Regular physician visits and check-ups also help. Now, technology has evolved so much, and we have many diabetes

testing kits through which we can monitor blood glucose level just in fraction of time at home. The American Dietetic Association introduced medical nutrition therapy (MNT) in 1994 to better explain the nutrition therapy process for diabetic patients. This program explains how to use specific nutrition services to treat a condition, injury, or illness. MNT for diabetes consists of a four-step procedure that, when followed correctly, can help reduce the risk of developing diabetes. They are.

i. Assessing the patient's diabetes self-management abilities and nutrition knowledge.

ii. These are designed and tailored for each person, identifying, and negotiating nutritional goals.

iii. Nutritional intervention that combines the patient's needs with a flexible approach to meal planning and education materials so that the patient can put the plan into action.

iv. Keeping tabs on all the activities and evaluating the outcomes.

These are essential for patients to acquire and maintain the knowledge, skills, mindset, behaviour, and commitment necessary to successfully face daily diabetes self-management challenges [9].

Indulging oneself in physical activity is another one such process which is known to have numerous health benefits, both general and specific to diabetes [10]. Practising yoga is found to be an effective way in managing diabetes. Lifestyle related modifications, ditching sedimentary living style and participating in any active form can make an individual lessen the risk of developing diabetes. People with obesity are much prone to diabetes. Weight management is an added advantage. Despite significant advances in diagnosis and treatment, the persistence of inadequate metabolic control continues. Patients fail to manage diabetes on their own and even clinicians' ineffective approaches to intervention which may contribute to poor glycaemia control [11].

Glucose enters our body through food. Absorption of this food, the breakdown of glycogen and gluconeogenesis are the three main ways where body cells meet the glucose. Glucose is stored in the form of glycogen in liver and the biochemical pathway of generating glucose from non-carbohydrate substrate in the body is called gluconeogenesis. Insulin plays a significant role in balancing glucose levels in body [12]. The pictorial representation of mechanism of glucose regulation in humans is explained in Fig. 1.

Fig. 1 [Images not available. See PDF.]

Mechanism of glucose regulation

Main text

Seaweeds

Seaweeds are naturally occurring marine macroalgal species which are found in marine environment. These are adapted to salt conditions provided by seas and oceans. During monsoons, these seaweeds are carried to the seashore along with the waves due to the increase intensity of waves. They are usually found attached to the rocky substrate or any other solid surface along the seaside [13]. They are macroscopic, multicellular, and completely different form of higher plants. Seaweeds do not possess true or definite roots, stem, or leaves [14]. Seaweeds are highly pigmented, based on their pigmentation, they are put into three main algal classification, Rhodophytes (red algae), Phaeophytes (brown algae), and Chlorophytes (green algae).

Brown algae constitute of 16 orders with approximately 285 genera and 1800 species. They are the largest and fastest growing of all the seaweeds. They exhibit a rich composition of pigments of different biosynthetic origins. Chlorophyll *a* and *c,* carotenes particularly α-carotene and β-carotene and xanthophylls. They also exhibit fucoxanthin, violaxanthin and diatoxanthin. Red seaweed is second largest phyla of algae containing over seven thousand species currently recognized. Chlorophyll *a,* phycocyanin, phycoerythrin and phycobilin are the pigments that give red colour to these species. Chloroplasts in green algae contain chlorophyll a and b, which gives them their bright green colour. They are also rich in carotene (red orange) and xanthophylls (yellow) in their stacked thylakoids (Fig. 2).

Fig. 2 [Images not available. See PDF.]

a Structure of phlorotannin, **b***Ecklonia stolonifera* rich in eckol, **c***Ecklonia cava* rich in dieckol, **d***Ishige okamurae* rich

in diphlorethohydroxycarmalol

Marine macroalgae are the promising source for bioactive secondary metabolites which has vast application in drug developments, pharmaceutics and other biotechnological applications [15]. There is an increasing demand for macroalgae and macroalgae derived food. Bioactive compounds are the chemical constituents that are present in the species which has an influence on a living organism, tissue or a cell. They have the capability of regulating or deregulating on specific disease or damage caused to the cell when used in correct manner. They are often referred to as secondary metabolites [16]. This property of theirs is demonstrated by conducting basic research which could be in-vivo (inside the organism's body) or in-vitro (in controlled environment outside the organism's body). Seaweed species are found to have potential phytochemical constituents which are capable of being antioxidant, anti-inflammatory, anticancer, antidiabetic agents. Hence, they play a key role in pharmaceutical industry. These are also used in cosmetics.

Seaweeds and diabetes

Much research works on seaweeds have found out the presence of phytochemical constituents that are responsible in treating and managing diabetes. Including edible seaweeds in daily diet and its regular consumption is associated with the reducing the risk of developing diabetes. A variety of polyphenolic compounds have been isolated from seaweed which are well known for their target against diabetes [17]. According to the studies, some of the seaweeds may be able to prevent enzymes from hydrolyzing carbohydrates in-vitro and lowering effect of blood glucose was noticed in-vivo after the consumption of meal [18]. Bioactive compounds derived from seaweed play a significant role in glucose-induced oxidative stress and the reserve of starch-digestive enzymes [19]. In diabetic patients, the levels of antioxidant parameters tend to decrease, so in this such case, many studies have proven that phytochemicals that can exert antioxidant and free radical scavenging activities can improve the body's sensitivity to insulin.

Phaeophytes

Phaeophytes possess number of active constituents like phlorotannins which is a unique secondary metabolite, that has got specified biological activities [20]. Studies on brown seaweeds have suggested that these species influence in glycemic management and could be benefit patients with type 2 diabetes. Fucales, dictyotales and laminariales are the majorly used orders of phaeophyceae that are extensively used for the extraction of secondary metabolites [21]. Species belonging to fucaceae are great with phytochemicals that have great interest from industrial point of view. Phlorotannins, a class of marine-only polyphenols, have received a lot of attention in recent years due to their enormous potential for therapeutic use [22]. Brown algal species contain significant number of phenolic compounds, a polysaccharide which has got extensive high biological activities. These phytochemicals are found to be more effective as antioxidants when compared to green and red algal species [23].

Phlorotannins are class of phenolic secondary metabolites which has a wide range of biological treatments. Research on this has proven phlorotannins to have antidiabetic, antioxidant, cancer, inflammation, adipogenesis, and numerous other biomedical uses [24]. They are synthesized by polyketide pathway (acetate-malonate pathway) produced by the polymerization of phloroglucinols [25]. These can be later extracted from various extraction methods such as chromatography [26]. α-amylase and α-glucosidase are the two digestive enzymes that are responsible for digestion of carbohydrates and increase the postprandial glucose level in diabetic patients. By inhibiting the enzymatic action of these two enzymes, postprandial hyperglycemia, and the risk of developing diabetes can be reduced.

Brown seaweeds are also rich in fucosterol and its derivatives [27]. This compound was first isolated from *Cystoseia foeniculaceae* and *Dictyota ciliolate* by RP-HPLC method. A fraction of the compound was analysed by NMR technique [28]. Polyphenols found in some species of brown algae support the algae's structural development and protect them from biotic and abiotic stress conditions [29, 30]. It has been reported that marine brown algal species with sulphated polysaccharides possess useful therapeutic properties. Fucoidan has antioxidant and antidiabetic potential. Fucoxanthin is an accessory pigment of some of the algal species. *Fucus*, *Dictyota* and *Laminaria* are rich in carotenoids and even used for extracting it from them. This active constituent has proved to exhibit antioxidant,

anticancer and antidiabetic activities [31].

Red seaweeds

Red seaweeds are major source of vitamins, minerals, calcium and magnesium. They are widely consumed as food in many continents. Bromophenols from red algae have been reported for antidiabetic activities. They are the source of dietary fibres promoting health benefits like lowering bad cholesterol and regulate blood sugar levels. Rhodophytes are rich in sulphated polysaccharides [32]. These SPs were associated in combating different oxidative stress induced diseases [33]. Experiments on red and green seaweeds have proven that they possess inhibiting properties of α-glucosidase enzyme [34]. *Rhodomela confervoides* (Fig. 3a), *Symphyocladia latiuscula* (Fig. 3b) and *Polysiphonia urceolata* (Fig. 3c) consists of bromophenols, 2-piperidione, benzene acetamide, n-hexadecanoic acid and polysaccharide derivatives that were found to exhibit hypoglycaemic potential by inhibiting α-glucosidase [34]. Red seaweeds are beautiful home for hydrocolloids specially, agar and carrageenans. Hydrocollloids are extracted from them and are widely used in diverse medicinal and food industries [35]. Polysaccharides have significant antidiabetic effects in multiple ways, most notably by preventing gastric emptying, inhibiting the activities of amylase and glucosidase, increasing insulin secretion, and improving insulin function [36]. As a result, polysaccharides derived from algae may be effective treatments for diabetes and its complications.

Fig. 3 [Images not available. See PDF.]

a*Rhodomela confervoides*, **b***Symphyocladia latiuscula*, **c***Polysiphonia urceolata*

Green seaweeds

Green seaweeds belong to the genus Ulva, they have been reported to possess hypoglycemic activity. Due to their high soluble fibre content, they are used for various food dishes in Asian countries. The aqueous extract of green seaweeds *Ulva lactuca* (Fig. 4a) (Inhibition-α-amylase: 83.4%; α-glucosidase: 61.81%) and *Ulva reticulate* (Fig. 4b) (Inhibition-α-amylase: 89.1%; α-glucosidase: 76.02%)were effective against the enzymes α-amylase and α-glucosidase at a concentration of 100 g/ml after an 8-h extraction period at 37 °C in a water bath, allowing the phytochemicals and colloids to be released into the extract more slowly. Similarly, the crude extract of *Ulva ohnoi* (Fig. 4c) exhibited α-amylase inhibition by 41.7% and complete α-glucosidase inhibition at 10 mg/mL [15, 37, 38].

Fig. 4 [Images not available. See PDF.]

a*Ulva lactuca*, **b***Ulva reticulate*, **c***Ulva ohnoi*

Reducing postprandial hyperglycemia is one of the healing methods in diabetes management. This can be achieved by reducing carbohydrate hydrolyzing enzymes in the digestive tract suspending glucose absorption. The breakdown of carbohydrates is mostly done by α-amylase and α-glucosidase. Delay in the release of glucose from dietary multiple carbohydrates sources results in the decreased postprandial plasma glucose and hyperglycaemia level [39]. By simultaneously absorbing glucose from the intestine and breaking down starch with pancreatic α-amylase, α-glucosidase and -amylase react to digest in the human body. By hydrolyzing inner 1,4-glycosidic linkages, pancreatic amylase determines the degree of starch digestion and produces linear and branched malto-oligosaccharides. These are then acted upon by α-glucosidase enzymes, which play a crucial role in the conversion of carbohydrates into glucose and may initiate postprandial hyperglycaemia [40].

Alpha-glucosidase is responsible for the ultimate step of hydrolysis of starch or disaccharides into simple glucose units, while alpha-amylase is involved in the digestion of long-chain carbohydrates. As a result, inhibitors of these enzymes delay the absorption of glucose, lowering the blood glucose level after eating [41]. A protease enzyme called dipeptide peptidase-IV participates in the breakdown of incretins, which are a group of metabolic hormones that cause the ß cells of the Langerhans islet to release insulin. Incretins are delivered after supplement admission, and they postponed gastric discharging and decline glucagon emission notwithstanding feeling of insulin discharge [42].

Alpha-amylase participates in the digestion of long-chain carbohydrates, while alpha-glucosidase is responsible for the final step in the hydrolysis of starch or disaccharides into simple glucose units. Therefore, inhibitors of these

enzymes delay the absorption of glucose, resulting in a drop in blood glucose levels following a meal [1]. Incretins, a class of metabolic hormones that trigger insulin release from the ß cells of the Langerhans islet, are broken down by a protease enzyme called dipeptide peptidase-IV. After taking a supplement, incretins are given. Despite the sensation of insulin being released, they delay gastric emptying and reduce glucagon release [43].

Marine macroalgae are found to have bioactive compounds which have DPPH-IV inhibiting potential. Dipeptidyl peptidase IV is also referred to as adenosine deaminase complexing protein 2 which is an enzyme present in human body [44]. It is a protein encoded by DPP-IV gene. This enzyme destroys incretin. Incretins are the group of hormones that stimulates a decrease in blood glucose levels [45]. Its main physiological role in human body is to regulate the amount of insulin that is secreted after the consumption of food. DPPH-IV inhibitors block the action of this enzyme, which destroys this hormone incretin. This hormone is responsible to produce insulin only when there is a need of it. Therefore, reducing the amount of glucose being produced by liver [46] (Table 1).

Table 1. Different seaweed species with their antidiabetic potential

Seaweed	Class	Bioactive compound used	Nature of study	Refs.
Sargassum kjellmanianum	Brown	Polysaccharide-alginate	In-vivo In-vitro	[42]
Ecklonia cava	Brown	Phlorotannin-Dieckol	In-vivo	[1]
Cystoseira compressa	Brown	phlorotannin	In-vivo	[43]
Laurencia dendroidea	Red	Bromophenols	α-glucosidase α-amylase	[44]
Sargassum confusum	Brown	oligosaccharides	In-vivo	[45]
Sargassum horneri	Brown	fucoidan	In-vivo	[46]
Macrocystis pyrifera	Brown	polysaccharide	In-vivo	[47]
Pelvetia siliquosa	Brown	Fucosterol	–	[48]
Grateloupia elliptica	Red	2,4,6-Tribromo phenol	α-Glucosidase inhibition	[49]
Polysiphonia morrowii	Red	3-Bromo-4,5-dihydroxy benzyl alcohol	α-Glucosidase inhibition	[50]

Gelidium amansii	Red	β-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose units	In-vivo	[51]
Gracilaria opuntia	Red	sulphated galactopyran	α-amylase and α-glucosidase inhibition	[32]
Hypnea spinella	Red	sulfated polysaccharide	In-vivo	[36]
Enteromorpha prolifera	Green	–	Inhibition of the JNK1/2 insulin pathway in liver of mice	[52]
Chaetomorpha aerea,	Green	–	Inhibition against alpha-amylase	[45]
Chlorodesmis	Green	z,z-6,2 8-heptatriactontadien-2-one	α-amylase inhibition	[45]

Seaweeds in food and nutrition

Seaweeds which are fit for human consumption are put under edible seaweed lists which are widely been consumed by Asian countries. Chinese consume seaweeds as traditional medicine. Koreans use seaweeds as a key ingredient in their soups. Women consume seaweed soup after childbirth which is known to boost their immunity and strengthen them along with infant's health. When consumed in the appropriate amounts, edible seaweeds are known to offer a rich and long-lasting source of macronutrients and micronutrients for human consumption [47]. *Porphyra tenera* and *Palmaria palmata* are the red seaweeds which contain elevated levels of proteins that is 47.5 and 30%, respectively [48]. Nori or purple laver, the edible seaweeds belonging to *Porphyra* species, a blackish-purple seaweed that enhances the flavour of sushi, which is a Japanese ethnic food usually wrapped with rice. It is mostly grown in China, Japan, and the Republic of Korea [49]. Nori is the most widely produced product from marine culture in Japan. Some of the edible seaweeds with their regional names are mentioned in Table 2.

Table 2. Edible seaweeds consumed in different part of the world with their regional names

Seaweed	Image	Common name
Ascophyllum nodosum		Egg wrack
Laminaria digitate		Kombu or konbu
Laminaria saccharina		Sweet kombu
Himanthalia elongate		Sea spaghetti
Undaria pinnatifida		Wakame

Porphyra umbilicalis *Porphyra vietnamensis*		Nori
Palmaria palmata		Dulse or Dillisk
Chondrus crispus		Irish moss
Ulva lactuca		Sea lettuce

In the recent article, possibilities of using seaweed capsules which function as a needle-free life for diabetic patients was published on The Economic Times, Tokyo. It further stated that researchers have created a novel capsule using seaweed extract to preserve insulin-producing pancreatic cells, giving diabetics hope for an injection-free treatment. Insulin injections are administered daily to patients with type 1 diabetes [50]. Pancreatic islet transplantation is a successful treatment that can eventually reduce or eliminate insulin dependence completely. Cryopreservation is the general method of preserving and transporting the cells [51]. This process also comes with a disadvantage of sharp ice crystals that can break and pierce into cell membrane and compromise with cell viability.

Although seaweeds being highly nutritious and carry benefits when consumed in proper manner, they are attached with some minor limitations. Marine species though available in huge mass, they are time and temperature restricted. Distinct species need different environmental condition for their active and optimum growth [52]. Their unavailability throughout the year becomes a limitation. As these are marine species, they need intense cleaning and specific storage conditions. Seaweeds possess elevated levels of iodine which when consumed might lead to high levels of iodine in some people which might lead conditions like hyperthyroidism.

Conclusion

Seaweeds offer 60% of marine species in the world. They have unique chemical profile which makes them withstand high salinity conditions. They are usually categorized as unwanted plants in the coastal regions. But these plants are macroalgae which possess various health benefits for humans when consumed as part of their daily diet. Seaweeds have all the potential to the overcome the diseases, such as diabetes, cancer, inflammation. They are distinct from terrestrial species due to the presence of distinct bioactive compounds in them. Diabetes is a disease where a person’s body either does not produce enough insulin required for normal metabolism or body’s inability to accept the insulin. The cause for the diabetes is numerous. As it is one of the lifestyle related disorder, making changes in the living style and switching to a healthy and organized lifestyle can help overcome it. Seaweeds serve enough macronutrients and micronutrients and other essential dietary fibres which when consumed in daily diet can reduce the risk of developing such lifestyle related disorders like diabetes, cancer, etc. Hence, studying these seaweeds species is equally important. In this review article, various research papers on treating diabetes with different seaweed species were studied. Alpha amylase and alpha glucosidase are two digestive enzymes which are responsible for digestion of sugars in the gut itself in postprandial cases. Many of the seaweeds have the potential to inhibit these enzymes which helps in curing diabetes. These studies revealed that, the seaweeds can be used as food, has significant role in medicine, pharmaceuticals, beauty and other industries. So, this study concludes that, such healthy species should be identified and can be used in treating diabetes. This article lays background to the seaweed research area giving an overview for the research scholars sharing equal interest in the area.

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Author contributions

VJ has done data collection and major contributor in review manuscript writing. PS has designed the concept, corrections, and SG has done drafting of the manuscript. All the authors have read and approved the manuscript.

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Abbreviations

MNT
Medical nutrition therapy
NMR
Nuclear magnetic resonance
RP-HPLC
Reverse phase high performance liquid chromatography
DPPH
2,2-Diphenyl-1-picrylhdrazyl

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Comprehensive preclinical studies on the bioactivity of Orbignya phalerata Mart. (Babassu) and its derived products: a systematic review

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ABSTRACT (ENGLISH)

Background

Babassu (*Orbignya phalerata* Mart.) is a palm tree well distributed in Latin America, whose fruit has a mesocarp and kernel used for human feeding, and empirically related to the treatment of gastritis, vaginitis, and wound healing.

Main body of the abstract

The activities attributed to babassu can guide new research on health applications and, for this reason, this study aimed to report in vitro and in vivo biological activities of *O. phalerata* constituents through a systematic review. Searches terms were applied in five world databases and the data from the publications were collected according to PICOS criteria, including the fruit component, concentration/dose, time of exposure, and comparative groups. All outcomes were reported and the most relevant outcomes were described by a narrative synthesis and a risk of bias assessment. A total of 28 in vitro ($n=15$) and in vivo ($n=11$) studies were included, and two showed both experimental designs. The studies were heterogeneous, with the predominance of metabolic analysis, wound and peptic ulcer healing, besides in vivo toxicity, among others. For in vitro analysis, antioxidant tests, cell viability and antimicrobial activity predominated. All in vivo ones used rodents. Meanwhile, tumor and non-tumor cell lines, bacteria strains, *Leishmania amazonensis*, *Artemia salina*, and antioxidant reactions were considered for in vitro protocols.

Short conclusion

The most frequent applications included mesocarp and kernel in a wide range of extracts, emulsions, and concentrations. Their low in vitro lethality and cytotoxicity, and no acute toxicity in vivo open possibilities for the development of long-term toxicity assays with repeated doses in rodents and interventions in clinical trials.

Graphic abstract

FULL TEXT

Background

Orbignya phalerata Mart. (syn. *Attalea speciosa*), belonging to the Arecaceae family, is a palm tree well distributed in different biomes in Latin America, such as Amazon rainforest, Atlantic forest, and especially in Cerrado and Caatinga, and popularly known as Babassu, uauaçu, and catolé [1]. The genus *Orbignya* has more than 20 species, but the binomial nomenclature *Orbignya phalerata* Mart. was adopted by this review because it is the most used and recent in the bibliography consulted (Fig. 1). Its fruit is generally completely used, but only its mesocarp and kernel are edible. The studies related to babassu describe about the mesocarp and/or the kernel, mainly. The first is used in cosmetics industry [2] and the mesocarp flour has been empirically consumed for the treatment of gastritis, vaginitis, and topically as wound healing [3].

Fig. 1 [Images not available. See PDF.]

Parts of *Orbignya phalerata* Mart. **a** palm; **b** whole fruit; **c** cross section.

Source: personal archives

Plant species are continuously studied about their potential developing new drugs and products based on

the diverse biological activities and pharmaceutical properties, mainly influenced by primary and secondary plant metabolites. Primary constituents, such as fatty acids, carbohydrates, and amino acids, are crucial macromolecules aiding plant survival and structural development. Meanwhile, secondary metabolites, derived from primary metabolism, play key roles in physiological processes and act as a defense mechanism against biological or chemical agents, such as polyphenols, which are one of the most common types of bioactive compounds, including the flavonoids catechin, epicatechin, proanthocyanidin, and others, all capable of scavenging or neutralizing reactive or radical species [4], which explains, at least in part, their antioxidant, anti-inflammatory, and antimicrobial properties, to name a few [5].

Among the edible parts of babassu, the kernel stands out for its elevated concentrations of fatty acids, primarily lauric acid (12:0; 46.89%), myristic acid (C14:0; 16.95%), and oleic acid (C18:1; 13.54%) [6–8]. Conversely, the mesocarp flour is a source of energy (1375 kJ/100 g), complex carbohydrates (79.19%), potassium (3.62%), magnesium (0.39%), phosphorus (0.35%), and a small content of protein (1.41%) [9].

In human metabolism, while carbohydrates are an energy source, flavonoids act as scavenger molecules. Simultaneously, fatty acids not only contribute to caloric supply but also play specific roles such as bactericidal [10]. Regarding the total phenolic compounds in the mesocarp (558.87 mg/100 g), the most important among them are the flavonoids extracted by organic solvents [9] (Table 1).

Table 1. Flavonoids from mesocarp extracted by organic solvents

Extract	Flavonoid	Content	References
Hydroethanolic	Catechin	8.02—8.22[a]	[62]
Epicatechin	14.17—15.26[a]	Aqueous	Catechin
0.28[b]; 0.88 µg/g[c]	[63]	Epicatechin	na[b]; 29.61 µg/g[c]
Ethyl acetate	Epicatechin	18.4[a]	[35]
Dimer	17.7[a]	Trimer	23.8[a]
Tetramer	18.0[a]	Hydroethanolic	Proanthocyanidin
421.7 mg CE/g	[35]	Ethyl acetate	Proanthocyanidin

[a]Retention time in minuts (HPLC or spectrophotometry)

[b]Before in vitro digestion (spectrophotometry)

[c]After in vitro digestion (spectrophotometry)

na not available, *CE* catechin equivalents

Preclinical trials have elucidated the biological effects of babassu crude components and its extracts. However, there is no evidence of compiled findings in a systematic review. Therefore, this study reported in vivo and in vitro biological activities of *O. phalerata* constituents in order to contribute to the development of original studies and applications for health care and research.

Main text

Methods

This systematic review was conducted according to PRISMA guidelines [11] (Preferred Reporting Items for Systematic Reviews and Meta-Analyzes) and registered in PROSPERO [12] (International Prospective Register of Systematic Reviews, registration n° CRD 42022302309).

Publications eligibility was established based on the guiding question “Do different parts of babassu contain compounds capable of exerting biological effects in vivo and/or in vitro?” and PICOS criteria [P: Population; I: Intervention; C: Comparator; O: Outcome; S: Study] (Table 2). Only original articles from indexed journals were eligible, with no limitation of period or language. All publications from gray literature or not fully published articles were excluded.

Table 2. Eligibility criteria according to PICOS

	In vivo	In vitro
P	Experimental strains of any sex, age, and weight	Tumor and non-tumor cell lines and/or microorganism strains, and/or chemical assays
I	Oral or gavage offering of crude or processed babassu components or isolated extracted compounds	Assays with crude or processed babassu components or isolated extracted compounds
C	Positive, negative, and/or parallel control	Positive, negative, and/or parallel control
O	Favorable or unfavorable biological effects, local or systemic, acute or chronic	Favorable or unfavorable biological effects for/against pathogenic or non-pathogenic microorganisms, cell lines, or chemical reactions
S	In vivo experimental studies	In vitro experimental studies

For animal model studies, topical, intraperitoneal, parenteral, or rectal interventions were excluded. To be considered, both in vivo and in vitro designs should clearly describe the type of substance, concentration, and intervention period, at least for the main outcome.

Data sources, searches and studies selection

Explorations in the LILACS [13], SciELO [14], Science Direct [15], Web of Science [16], and PubMed [17] databases were performed by search terms registered in MeSH, combined by AND and OR Boolean operators (Table 3) during April 1st to April 7th, 2022 and again on March 24th, 2022 to update indexes. The searches were exported to Rayyan for prior systematization and metrics [18].

Table 3. Search terms and Boolean operators

Search term 1		Search term 2
Orbignya phalerata OR *Attalea speciosa*	AND	*Polyphenols* OR *Phytochemicals*

Initially, the studies were screened using the title and abstract (by ND and IOC), independently. Those considered eligible were read in full (ND, IOC) and references were kept for possible inclusion if they met the criteria. Disagreements were solved by consensus and, when necessary, a third researcher (JMCS) was consulted.

Data extraction and description of results

Data extraction included authors, publication year, title, locality, and study design. For in vivo publications, characteristics of the population were collected and for in vitro assays, cell lines, strain, and/or chemical reactions

were described. Interventions included fruit part and its product, concentration, time of exposure, and comparative groups. Favorable or unfavorable outcomes were reported, as well as the most relevant assessment instruments and results. The compiled data were organized into tables, facilitating a comprehensive narrative synthesis and outcomes comparison, when applicable. Values were expressed as mean and standard deviation, when available, and compared with control and/or parallel groups. Results were considered significant when $p<0.05$.

Risk of bias assessment

The risk of bias assessment for in vivo experiments was carried out independently by ND and IOC using the SYRCLE Risk of Bias Tool and the individual results were compared between ten domains distributed in six biases (selection, performance, detection, attrition, reporting, and other bias). Each domain was defined as “yes” (low risk of bias), “no” (high risk of bias), or “unclear” (uncertain risk) [19].

For in vitro analysis, the tool developed by the World Cancer Research Fund/University of Bristol was used with adaptations [20]. Each of the six questions was answered with “yes” (low risk of bias), “no” (high risk of bias), “not clear” if details were not recorded properly, or “not applicable”. Risk of bias analysis was recorded individually by study and by domain. Inter-rater reliability was determined by Cohen’s kappa coefficient [21].

Results

Search results and study characteristics

Figure 2 highlights the study selection process. Initially, 424 publications were identified, of which 146 were duplicated ones and removed automatically. A total of 278 articles were eligible by the title and abstract, but only 23 were selected for full-text review and 15 of them met the inclusion criteria. On the other hand, 13 articles not included by unidentifiable reasons in the initial search were included.

Fig. 2 [Images not available. See PDF.]

Screening flow diagram based on The PRISMA 2020 Statement [11]

According to the included reports, 39.3% ($n=11$) refer exclusively to in vivo (Table 4) and 53.6% ($n=15$) to in vitro assays (Table 5). Moreover, two articles showed both experimental designs and, for this reason, were described in both tables [22, 23]. Regardless of the taxonomy mentioned in the publications (*Orbignya phalerata*, *O. martiana*, *O. speciosa*, or *Attalea speciosa*), only the articles of Gaitan et al. [22] and Hovorková et al. [8] are not from Brazil, suggesting great interest about the plant and fruits in the Brazilian scenario. In preclinical interventions with animals, metabolic analysis and toxicity predominated (Fig. 3A), while in vitro assays highlight antioxidant tests (Fig. 3B).

Table 4. Baseline characteristics, intervention, methods, and main results of in vivo studies

References	Sex; strain; age; weight; n/group	Intervention/methods	*O. phalerata* component; dose/volume; time of exposure	Main results
Azevedo et al. [41]	Male (C57BL/6); 8–12 w.o.; 20–25 g; $n=20$	Antithrombotic effect: carrageenan-induced thrombosis, evaluation of necrosis frequency and extension; Onco BCG injection for NO induction in peritoneal cells; platelets, PT, and aPTT analysis	Mesocarp aqueous solution; 500 mg/kg/day; 240 days	↓ induced thrombosis; ↑ PT, and aPTT, no changes in platelets number; ↑ NO production by peritoneal macrophages previously activated by Onco BCG

Barbosa et al. [26]	Male (*Mesocricetus auratus*); 7–10 w.o.; 122–146 g; *n* = 39	Macromolecular permeability and leukocyte adhesion in cheek pouch after ischemia induction. After reperfusion, histamine topical application. Less than 10 leaks/site accepted; IL-1, IL-6, and TNF-α analysis	Crude kernel oil; 0.02 ml, 0.06 ml, 0.18 ml twice/day; 14 days	↓ ischemia-induced leaks during reperfusion in higher doses; ↑ leaks in lowest dose; less pronounced microvascular permeability after histamine in higher doses; ↓ leukocyte adhesion in lowest dose; no significant changes in TNF-α, IL-1, and IL-6
Barroqueiro et al. [27]	Male/female (C3H/HePas); 8–12 w.o.; 25 g; *n* = 40	Acute toxicity: weight, macroscopic, and histological analysis of heart, liver, spleen, kidney, and brain; glucose, urea, creatinine, ALP, TC, TG analysis	MEE; 1000 mg, 3000 mg, 5000 mg/kg; single dose	No deaths after 14 days; no significant increase in body weight; no cutaneous, neurological or behavioral changes, glucose, TC, or creatinine; ↑ ALP in 3000 and 5000 mg/kg groups; ↑ urea in all groups; ↑ TG in 1000 mg/kg
Gaitan et al. [22]	Female (Sprague–Dawley); 7 w.o.; weight (NA); *n* = 25	Antithyroid effect: 14 mcg/day of iodine-rich diet; MAE and KPPS acute intake. After 1 h, i.p. Bq $Na^{125}I$; ^{125}I uptake and organification analysis by MIT + DIT coupling in thyroid tissue	MAE (16 g) and KPPS (2 g); single dose	Distinct antithyroid effects for MAE and KPPS; ↓ iodine uptake in 16 g MAE group; ↑ inorganic iodine % in MAE and KPPS; ↓ iodine % as MIT + DIT; ↑ ratio ^{125}I/MIT + DIT in MAE and KPPS

Maia and Rao [24]	Male/female (Wistar); age (NA); 180–220 g; *n*= 107. Male and female (Swiss); age (NA); 25–30 g; *n*=88	Anti-inflammatory effect: carrageenan-induced inflammation in paw and edema analysis; subcutaneous cotton pellet implant-induced granuloma, pellet removal and weighing; formaldehyde-induced arthritis in paw and edema analysis; carrageenan-induced inflammatory exudate by leukocyte migration. Ulcerogenesis: gastric lesion assessment. Coagulation: bleeding time after opening the abdomen, micropuncture in a mesenteric vein, and time of hemostasis; Antipyretic: *S. cerevisiae*-induced pyrexia; Analgesia: hot-plate positive response; writhing induced by acetic acid; acute toxicity after 72 h	Mesocarp chloroformic extract; 125 mg/kg, 250 mg/kg (carrageenan, hot-plate, writhing) in a single dose; 250 mg/kg (granuloma) for 7 days; (arthritis) for 10 days; (ulcer) for 21 days; (pyrexia) in a single dose; up to 4 g/kg (toxicity); dose (NA) (exudate) 3 times/24 h; dose (NA) (bleeding) for 3 days	↓ dose-dependent acute inflammatory edema after carrageenan induction; ↓ granuloma; ↓ edema in arthritis on 7th and 8th days; absence of peptic ulcer; no influence on leukocyte migration, no effects on bleeding time and pyrexia control; no significant effect on hot-plate response; ↓ writhing; no overt acute toxicity
Pinheiro et al. [39]	Male (BALB/c, DBA/2, CBA, C3H/HePas, C57BL/6); 8–12 w.o.; 20–30 g; *n*=10	Glycolipid profile: TC and urea analysis. Immunotoxicity: medullary and splenic cell analysis	MAE; 50 mg/kg/day; 30 days	↓ splenic cells in C3H/HePas and BALB/c; no changes in medullary cells; ↑ TC in CBA and ↓ in C3H/HePas; ↓ U in all strains, except in C57BL/6

Scheibe et al. [43]	Male (Wistar); age (NA); 271.3 g (average); *n*=54	Wound healing: laparotomy with cecum exteriorization. Monitoring and euthanasia on 7th, 14th, and 21st day; macroscopic wound evaluation (infection, dehiscence, haematoma,and fistulae); abdominal cavity evaluation (collection, infection, fistulae, and adherence); terminal ileum air insufflation test; vascular congestion analysis, edema, presence of mono and polymorphonuclear leukocytes, angiogenesis, fibrosis, and collagen deposition	MAE; 50 mg/kg/day; 7, 14, 21 days	Adequate healing in all animals; no evidence of infection, dehiscence, haematoma, abscess; widespread adhesion, without adhering to the abdominal wall (grade III) in 1 rat on 7th day, grade II adherence on the 21st day in 100% of the animals; ↓ burst pressure over time after insufflation test; ↓ polymorphonuclear leukocytes, congestion, angiogenesis, fibroblast proliferation, and collagen deposition on the 14th day
Silva et al. [23]	Male (Swiss); 8 w.o.; 25–30 g; *n*=28	Locomotor activity: total crossings in activity cage. Motor coordination: remaining time on rotarod test (180 s at 9 RPM)	MAE; 1, 2, 3 g/kg; single dose	No significant changes in the number of crossings or in remaining time on the rotarod test

Silva et al. [44]	Male (Wistar); 7–8.5 w.o.; 275.6 g (average); *n*=54	Colonic healing: cecorrhaphy by laparotomy an exteriorization of the colon. Euthanasia on 7th, 14th, and 21st days. Animal weighing, wound analysis (infection, dehiscence, haematoma, and fistulae); abdominal cavity analysis (collection, infection, fistulae, and adhesion); terminal ileum air insufflation test; analysis of congestion, edema, presence of mono and polymorphonuclear leukocytes, angiogenesis, and fibrosis	MAE; 50 mg/kg; single dose	No dehiscence, fistulae or other complications; ↑ grade II adherence on 21st day; moderate/severe mononuclear leukocytes, congestion, and angiogenesis on the 7th day; ↓ congestion and angiogenesis on 14th day
Silva et al. [25]	Non-obese diabetic male mice (strain NA); 12 w.o.; 29–33 g; *n*=25	Onset of diabetes: individual average of extract, water, and food intake for 6 days; weighing, blood glucose analysis on 0, 30th, 90th, and 120th day; anti-insulin IgM antibodies, and total antibodies (IgG, IgM) with anti-immunoglobulin antibodies	MAE; average 66 mg/day; 120 days	Daily average intake of MAE 3.3±0.9 ml; ↑ weight from 20 to 50th day and ↓ after 50th; ↓ glucose on 30th day; ↓ IgM; no significant changes in anti-insulin IgM and IgG
Silva and Parente [40]	Male (BALB/c); age (NA); 15–20 g; *n*=15	AIE and IME: Evans blue and i.p. acetic acid-induced vascular permeability. Leak analysis in the peritoneal cavity; phagocytic activity by colloidal carbon injection. Blood sample dissolved in Na_2CO_3 and absorbance determination at 660 nm	Mesocarp isolated α-glucan; 100 mg/Kg (AIE) single dose; 50 mg/kg/day (IME) for 5 days	↓ vascular permeability; ↑ phagocytic activity

Soares et al. [6]	Male (Swiss); 8.5 w.o.; 35–40 g; *n*=32	Lipid profile and IME: pre and post-euthanasia weighing; macroscopic and histological analysis of heart, liver, kidneys, spleen, gastrocnemius muscle, and retroperitoneal fat. AST, ALT, TC, LDL, HDL, TG, glucose, urea, creatinine, IL-6, and TNF-α analysis; immunophenotyping of splenic cells; RT: stair climbing with load adjustment in daily sessions, 5 days a week for 8 weeks	MAE; 5 mg/kg/day; 5 days/week for 8 weeks	MAE: ↓ total weight; ↓ fat tissue; ↓ TG; ↓ TC; ↑ AST; ↑ medullary cells; ↑ B lymphocytes; ↓ activated macrophages; ↓ IL-6; ↑ TNF-α MAE/RT: ↓ total weight; ↑ kidneys weight but no histological changes; ↓ fat tissue; ↓ TG; ↓ TC; ↑ AST/ALT; ↓ splenic or medullary cells; ↓ activated Th; ↑ activated B lymphocytes; ↑ macrophages; ↓ activated macrophages; ↓ IL-6; ↑ TNF-α
Torres et al. [42]	Male (Wistar); 6 w.o.; 180–240 g; *n*=40	Prevention and treatment of peptic ulcer: macroscopic analysis (ulceration, mucosal hyperemia, loss of fold, and/or bleeding; histopathological analysis (inflammation, necrosis, fibroblasts, fibrosis, reepithelialization, neocapillar generation)	MAE; 2 g/kg/day; 3 days	Prophylaxis: ↓ peptic ulcer, mucosal hyperemia, loss of fold, and bleeding; treatment: ↓ necrosis, fibrosis, reepithelialization, and neocapillar generation; marked inflammation and mucosal ulceration

↑ increase; ↓ decrease; *AIE* anti-inflammatory effect, *ALP* alkaline phosphatase, *ALT* alanine aminotransferase, *aPTT* activated partial thromboplastin time, *AST* aspartate aminotransferase, *BCG* bacillus Calmette–Guérin, *DIT* diiodotyrosine, *HDL* high-density lipoprotein, *IgG* immunoglobulin G, *IgM* immunoglobulin M, *IL* interleukin, *IME* immunomodulatory effects, *i.p.* intraperitoneal, *KPPS* kernel pressed paste with skin, *LDL* low-density lipoprotein, *MAE* mesocarp aqueous extract, *MAE/RT* mesocarp aqueous extract combined with resistance training, *MIT* monoiodotyrosine, *NA* data not available, *NO* nitric oxide, *PT* prothrombin time, *RPM* rotations per minute, *TC* total cholesterol, *TG* triglycerides, *Th* T helper lymphocytes, *TNF-α* tumoral necrosis factor, *w.o.* weeks old

Table 5. Baseline characteristics, intervention, methods, and main results of studies

References	Species/cell lines/strains/reactions	Intervention/methods	*O. phalerata* component; concentration	Main results

Barroqueiro et al. [46]	*E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *E. faecalis* (ATCC 29212), *S. aureus* (ATCC 25923), *S. aureus* (MRSA)	AMA: disk diffusion method by analyzing zones of inhibition; MIC: growth absence or presence after inoculation in tubes	MEE; 500 and 250 mg/mL (disk diffusion); 500 to 0.9 mg/mL (MIC)	Disk diffusion: inhibition >7 mm on *S. aureus*, MRSA, and *E. faecalis*; no inhibition on *E. coli* and *P. aeruginosa*. Complete inhibition on *S. aureus*, MRSA, and *E. faecalis* by the highest concentration. MIC: 7.8 mg/mL (*E. faecalis*)
Bezerra et al. [38]	Promastigote forms of *L. amazonensis*	Leishmanicidal activity: promastigotes incubated during 24 h with extract. Count of living protozoa after 24 h by flagellar motility. CL_{50}	MAE; serial concentrations 500 to 31.25 µg/mL	CL_{50} >500 µg/mL. Deaths about 20% in all concentrations
Caetano et al. [37]	*S. aureus* (ATCC6538, ATCC9144), *S. aureus* MRSA (2 nosocomial strains), *S. aureus* MSSA (3 nosocomial strains)	AMA: disk diffusion method by analyzing zones of inhibition	Mesocarp hydroalcoholic extract; 30 mg/mL	Growth inhibition on all strains (14 to 18 mm zones)
Ferreira et al. [45]	AMA: *S. epidermidis* (ATCC12228), *S. aureus* (ATCC25923), *E. coli* (ATTC11229), *P. aeruginosa* (ATTC27853); $DPPH^{\bullet}$; *A. salina*	AMA disk diffusion method by analyzing zones of inhibition; AA: $DPPH^{\bullet}$ scavenging. EC_{50}; Lethality: *A. salina* assay for 24 h. CL_{50}	Kernel oil; 10 mg/mL (AMA); 300, 250, 200, 150, and 100 mg/mL ($DPPH^{\bullet}$); 50 to 0.05 mg/mL (lethality)	AMA: no inhibition; $DPPH^{\bullet}$: EC_{50} 70.57 mg/mL; CL_{50} >1000 µg/mL
Gaitan et al. [22]	Porcine thyroid slices; TPO	Antithyroid effect: thyroid hormone synthesis assessed by measuring total accumulated iodine and iodine organification in MIT + DIT, and $T_3 + T_4$; TPO: spectrophotometry by oxidation of I^- to I^{3-}. Inhibition of TPO-catalyzed iodination, compared to PTU concentration. I_{50}	MAE, MME, kernel skin, KPPS, kernel oil; cellular assays: 1×10^5 µg/5 mL (MAE, skin); 5×10^4 µg/5 mL (KPPS, oil); TPO: 100 to 200 µg/mL	Cellular: antithyroid effect in all extracts by ↑ ^{125}I/MIT + DIT ratio, ↓ % ^{125}I demonstrated as $T_3 + T_4$ (NA). Kernel oil 1/5 from KPPS I/MIT + DIT ratio (I/MIT + DIT = 15). Higher ratio for mesocarp; TPO: ↑ inhibition % by MME and kernel skin

Hovorková et al. [8]	*E. cecorum* (CCM3659, CCM4285), *C. perfringens* (CIP105178, CNCTC5454, UGent 56), *Listeria monocytogenes* (ATCC7644), *S. aureus* (ATCC25923), *Bifidobacterium animalis* (CCM4988, MA5), *Bifidobacterium longum* (TP1, CCM4990), *Lactobacillus fermentum* (CCM91), *Lactobacillus acidophilus* (CCM4833)	AMA: previous kernel oil hydrolysis with porcine pancreatic lipase. Inoculation of each strain with emulsion. Growth assessment by the culture turbidity read at 405 nm. MIC_{80}	Kernel hydrolyzed lipid emulsion; 4.5 mg/mL	MIC_{80}: 0.56 mg/mL on *C. perfringens,* 1.13 mg/mL on *S. aureus*, and 2.25 mg/mL on *E. cecorum.* No effect on pathogenic strains before hydrolysis or on commensal strains
Nobre et al. [30]	TBARS, deoxyribose degradation, $DPPH^{\bullet}$, iron chelation, FRAP	AA: TBARS: phospholipids diluted in the extract with or without iron to induce peroxidation and absorbance with MDA; Deoxyribose degradation: induction of sugar decomposition by Fe/ H_2O_2 added to the extract to produce MDA; $DPPH^{\bullet}$: scavenging, read at 518 nm; Iron chelation: chelation potential by extract (plus Fe^{2+}, Tris–HCl, and phenanthroline), absorbance read at 510 nm; FRAP: ability to reduce the equivalent of 1 mM $FeSO_4 \cdot 7H_2O$. IC_{50}, EC_{50}, EC	Kernel methanolic extract; 1000 to 100 µg/mL (TBARS, deoxyribose, $DPPH^{\bullet}$, iron chelation); 50 to 5 µg/mL (FRAP)	No TBARS inhibition; deoxyribose IC_{50} >1000 µg/mL; $DPPH^{\bullet}$ EC_{50} 3517.01 ± 77.07 µg/mL; iron chelation: <20%, IC_{50} >1000 µg/mL; FRAP EC: 1560.2 ± 18.30 µmol.L^{-1}/g
Nobre et al. [10]	*S. aureus* (ATCC12692), *P. aeruginosa* (ATCC15442), *E. coli* (ATCC25922), *E. coli* (Ec27), *S. aureus* (Sa358)	Antibacterial activity: growth evidence after inoculating extract with resazurin solution into tubes. MIC	Fixed kernel oil: 512 to 8 µg/mL	Higher inhibition on *E. coli* 27 (MIC 23 µg/mL); ↓ MIC in the association between oil and amicacin on *S. aureus* 358 and *P. aeruginosa*, as well as neomycin on *S. aureus* 358, *P. aeruginosa*, and *E. coli* 27 (NA)

Pessoa et al. [31]	Enteropathogenic *E. coli*, mononuclear phagocytes	Cellular viability: slides fixed by acridine orange method; viability index by counting dead and alive cells in a total of 100. Functional activity by phagocytic index: number of cells that ingested at least 3 bacteria in a pool of 100 cells. Bactericidal index: dead/alive bacteria ratio by acridine orange	Kernel microemulsion and oil; 20 μL	↑ viability index and ↑ phagocytic index by microemulsion; ↑ bactericidal index by oil
Rennó et al. [28]	Leukaemic cell lines (HL-60, K562), K562-Lucena 1 MDR counterpart, lymphocytes, mouse fibroblast cell line (3T3-L1), human breast cancer cell line (MCF-7)	Cellular viability: erythroleukemic cells evaluated by permeability to trypan blue up to 24 h. Inhibition of cell proliferation calculated by comparing treated/untreated cells. Morphology: non-viable stained cells were separated in retained shape and lysed; lymphocytes evaluated with trypan blue after 24 h; MCF-7 and 3T3-L1 trypsinized and evaluated with trypan blue. Metabolism: 6-phosphofructo-1-kinase activity to convert into fructose-1,6-bisphosphate in HL-60. ID_{50}	Mesocarp with epicarp ethanolic extract; 2000, 1500, 1200, 600, 300, and 150 μg/mL	ID_{50} more effective on HL-60; moderate sensitivity on K562, K562-Lucena 1, and MCF-7; Resistance on 3T3-L1 and lymphocytes; morphological changes by 1200 μg/mL on HL-60; ↑ 6-phosphofructo-1-kinase on HL-60

Santos et al. [33]	Mouse fibroblasts (L929) and peritoneal macrophages	Cellular viability: MTT assay up to 72 h; absorbance read at 560 nm; macrophages stimulated by LPS from *E. coli* for 1 h and treated with kernel oil. NO and cytokines measured after 24 h; Scratch assay: fibroblasts migration in monolayers, production of 1.2001.500 μm width wounded area. Cellular migration measured each 6 h	Kernel oil; 100 to 1.56 μg/mL	No toxicity up to 100 μg/mL; cell proliferation with MTT metabolism ↑ above 25 μg/mL on L929; dose-related ↓ NO; ↑ IFN-γ, IL-6, and ↑TNF-α by 3.12 μg/mL; ↑ fibroblasts migration in scratch assay by 6.25 and 12.5 μg/mL
Santos et al. [32]	$DPPH^{\bullet}$, mouse fibroblasts (NIH/3T3, ATCCR, CRL-1658)	AA: $DPPH^{\bullet}$ scavenging, read by electron spin resonance; EC_{50}; Cellular viability: MTT assay with nanoemulsion up to 72 h, IC_{50}	Kernel oil, lipidic nanoemulsion; 4.0; 24.9; 49.9; 74.8, and 99.8 mM ($DPPH^{\bullet}$); 2500 to 39.06 $\mu g.mL^{-1}$ of nanoemulsion (MTT)	$DPPH^{\bullet}$ EC_{50}: nanoemulsion: 0.4329 mg mL^{-1}; oil: 0.5488 mg mL^{-1}; cytotoxicity by nanoemulsion ≥ 78.12 $\mu g.mL^{-1}$
Silva et al. [34]	$DPPH^{\bullet}$, *S. cerevisiae* (BY4741)	AA: $DPPH^{\bullet}$ scavenging, read at 518 nm; $IC_{50.}$ *S. cerevisiae*: incubation of 0.1 mg mL^{-1} cells with 3 mM TBH and 5 mg ml^{-1} extracts; microorganism viability assessed after 72 h	Endocarp, flowers, and leaves ethanolic extract; 1.0 mg ml^{-1}	$DPPH^{\bullet}$ IC_{50}: 4104.3 ± 6.7 $\mu g.ml^{-1}$ (endocarp); 427.4 ± 1.8 $\mu g.ml^{-1}$ (flowers), and 895.9 ± 2.3 $\mu g.ml^{-1}$ (leaves); no yeast survival

Silva et al. [23]	NO, TBARS, deoxyribose degradation	AA: nitrite measured by Griess reaction after NO generated in sodium nitroprusside decomposition. Absorbance read at 540 nm. TBARS: phospholipids diluted in the extract with AAPH as peroxidation inducing agent, absorbance read at 532 nm; $OH^{\bullet}$ production by MDA synthesis, a product of deoxyribose degradation induced by Fe/H_2O_2 added to the extract, absorbance read at 532 nm	MAE; 1000, 100, 10, and 1 μg/mL	No effect on NO and $OH^{\bullet}$ removal or over lipid peroxidation
Silva et al. [35]	$DPPH^{\bullet}$, $ABTS^{\bullet+}$, FRAP, tyrosinase	AA: $DPPH^{\bullet}$ scavenging, absorbance read at 517 nm; $ABTS^{\bullet+}$ scavenging absorbance read at 734 nm; FRAP: analysis of ability to reduce Fe^{3+} into Fe^{2+} with, read at 593 nm; Tyrosinase inhibition: L-tyrosine for monophenolase reaction and 3,4-dihydroxyphenylalanine for diphenolase reaction. Absorbance read at 492 nm	Mesocarp hydroalcoholic extract and fractions (hexane, chloroform, ethyl acetate, hydroalcoholic); 100 to 5 μg/mL ($DPPH^{\bullet}$), 6 to 1 μg/mL ($ABTS^{\bullet+}$), 100 to 1 μg/mL (FRAP), 200 to 50 μg/mL (tyrosinase)	↑ AA by ethyl acetate fraction in $DPPH^{\bullet}$ IC_{50}: 3.38 ± 0.05 μg/mL, $ABTS^{\bullet+}$ IC_{50}: 2.04 ± 0.03 μg/mL and FRAP: 15.41 ± 0.18 mmol Fe^{2+}/g; IC_{50} for tyrosinase: 48.43 ± 29.51 μg/mL (monophenolase) and 132.63 ± 5.71 (diphenolase)
Silva et al. [29]	Promastigote forms of *L. amazonensis* (IFLA/BR/67/PH8)	Leishmanicidal activity: promastigotes incubated with the isolated extract and mesocarp-loaded microparticles. Protozoa counting after 48 h by flagellar motility. CL_{50}	MAE; 500 to 62.5 μg/mL and mesocarp-loaded microparticles; 100 to 3.125 μg/mL	Lethality: pentamidine >microparticles>extract in solution. CL_{50} microparticles: 12 pg/mL

Souza et al. [36]	Benign prostate hyperplasic cells	Cellular viability: MTT assay, absorbance read at 570 nm up to 72 h; cytotoxicity by LDH release: damage estimated after from 4 up to 48 h, absorbance read at 490 nm; Immunohistochemistry: cultures treated for 24 h, PCNA immunostained; Apoptosis: TUNEL test by blocking endogenous peroxidase. DNA fragmentation assessment; Histomorphometry: nuclei counting with PCNA or TUNEL stained, distribution and intensity of staining assessment inside the glandular epithelium or stroma; Morphology: indirect assessment using phalloidin after 4, 6 and 12 h	Kernel crude oil, oily extract, nanocomposite with lipophilic extract; 300 to 100 µg/mL (MTT), 300 µg/mL (LDH, Immunohistochemistry, histomorphometry, apoptosis)	Viability: ↑ dose-related inhibition by nanocomposite; time-dependent disorganization of the actin cytoskeleton; ↑ time-dependent LDH release; immunohistochemistry: preserved glandular architecture; ↓ cell proliferation in 24 h; diffuse apoptosis and ↑ in apoptotic index after 24 h

↑ increase; ↓ decrease; *AA* antioxidant activity, *AMA* antimicrobial activity, *AAPH* 2,2′-azobis(2-methylpropionamidine) dihydrochloride, *ABTS*$^{\bullet+}$ 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), CL_{50} lethal concentration, *DIT* diiodotyrosine, *DPPH*$^{\bullet}$ 2,2-diphenyl-2-picryl-hydrazine, *EC* equivalent concentration, EC_{50} effective concentration, *FRAP* ferric-reducing antioxidant power, I_{50}/IC_{50} inhibitory concentration, *IFN-γ* interferon-γ, *IL-6* interleukin-6, *KPPS* kernel pressed paste with skin, *LDH* lactate dehydrogenase, *LPS* lipopolysaccharide, *MAE* mesocarp aqueous extract, *MDA* malondialdehyde, *MEE* mesocarp ethanolic extract, *MDR* multidrug resistant, MIC/MIC_{80} minimum inhibitory concentration, *MIT* monoiodotyrosine, *MME* mesocarp methanolic extract, *MRSA* methicillin-resistant *Staphylococcus aureus*, *MSSA* methicillin-sensitive *Staphylococcus aureus*, *MTT* (3-[4,5-dimethylthiazol-2y]-2,5-diphenyltertrazolium bromide), *NA* data not available, *NO* nitric oxide; $O_2^{\bullet}$ superoxide anion, $OH^{\bullet}$ hydroxyl radical, *PCNA* proliferation cell nuclear antigen, *PTU* propyl-2-thiouracil, *TBARS* thiobarbituric acid reactive substances, *TBHtert*-butylhydroperoxide, T_3 triiodothyronine, T_4 thyroxine, *TNF-α* tumor necrosis factor-α, *TPO* thyroperoxidase

Fig. 3 [Images not available. See PDF.]

Assessed outcomes from in vivo (**A**) and in vitro (**B**). Some publications have more than one outcome

Risk of bias assessment

Individual and for each domain in vivo

SYRCLE RoB Tool identified 130 entries distributed in ten domains for each one of the 13 studies (Table 6). It was found uncertain risk or inappropriate records in 80 entries (81.54%), low risk was present in 45 (34.61%) and high risk in five studies (3.85%). The average agreement by Cohen's kappa was 0.53 (68%), classified as intermediate to good [21].

Table 6. In vivo individual risk of bias

Domains: D1—sequence generation; D2—baseline characteristics; D3—allocation concealment; D4—random housing; D5—blinding (performance bias); D6—random outcome assessment; D7—blinding (detection bias); D8—incomplete outcome data; D9—selective outcome reporting; D10—other sources of bias. Total: 130 (100%); (+) Yes: 45 (34.61%); (−) No: 5 (3.85%); (?) Unclear: 80 (61.54%) [19]

Uncertain risk prevailed for the domains related to sequence generation, allocation concealment, random housing, and random outcome assessment (Fig. 4). There was no record of uncertain risk related to selective outcome reporting. In the domain related to other sources of bias, only one entry was registered due to the lack of clearness if the same animals or distinct animals were submitted to the different tests and if there was no contamination by the induction of some substance used in control groups [24].

Fig. 4 [Images not available. See PDF.]

In vivo risk of bias assessment by domain. (legend) Domains: D1—sequence generation; D2—baseline characteristics; D3—allocation concealment; D4—random housing; D5—blinding (performance bias); D6—random outcome assessment; D7—blinding (detection bias); D8—incomplete outcome data; D9—selective outcome reporting; D10—other sources of bias. Yes: low risk; No: high risk; Unclear: uncertain risk [19]

The analysis of the effective risk of bias revealed a higher frequency of low risk in baseline characteristics (92.3% of entries for the domain), selective outcome reporting, and other sources of bias (84.61% for both). Low risk was evidenced due to the appropriate description regarding the specificities of the animals. As well as for high risk of bias, specific analysis indicates that the circumstances in the intervention route were not similar in all groups [23], and there was animal loss with unknown reason, groups equalization, and randomization of the final sample [25]. Besides that, some primary outcomes were not showed [26, 27].

Individual and for each domain in vitro

The analysis of 17 studies checked 102 entries divided into six questions (Table 7). There was a predominance of low risk of bias by adequate methods reporting (65.69%), high risk in eight (7.84%), and uncertain risk in seven studies (6.86%). The average agreement by Cohen's kappa was 0.39 (51.96%), classified as low [21].

Table 7. In vitro individual risk of bias

Questions: Q1—cells/strains from validated repository or appropriately verified; Q2—technical repeats and controls inclusion; Q3—use of different cell lines/strains/reactions; Q4—comparable conditions between groups/assays; Q5—selective outcome reporting; Q6—comparison of different cell lines/strains/substances. Total: 102 (100%); (+) Yes: 67 (65.69%); (−) No: 8 (7.84%); (?) Unclear: 7 (6.86%); (∅) Not applicable: 20 (19.61%). Questions adapted from Lewis et al. [20]

The most detailed and complete descriptions were seen in antimicrobial evaluation of hydrolyzed kernel lipid emulsion in strains of pathogenic and commensal microorganisms [8], kernel oil in pathogenic strains [10], and mesocarp with epicarp methanolic extract in viability, changes in morphology, and metabolism in different cell lines [28].

The high risk of bias was highlighted by the use of a single organism and the mean values of primary outcomes have not been presented [29]. Nine studies (47.05%) demonstrated the non-application of two questions, most frequently for the impossibility of comparing cell lines/strains or culture conditions between groups, as they were assays with a single microorganism, strain or distinct chemical reactions (Q4, Q6) [23, 29–36].

When analyzing the methodological quality by domain (Fig. 5), the selective result was avoided in 94.11% of the studies and the low risk of bias was in 76.47%, regarding the validation of cells and strains repository and the use of a control group (Q1, Q2). Less frequently, there were some doubts about the cell line origin (Q1, 5.88%) and technical repeats (Q2, 17.65%) [22, 29, 30, 37].

Fig. 5 [Images not available. See PDF.]

In vitro risk of bias assessment by domain. (legend) Q1—cells/strains from a validated repository or appropriately

verified; Q2—technical repeats and controls inclusion; Q3—use of different cell lines/strains/reactions; Q4—comparable conditions between groups/assays; Q5—selective outcome reporting; Q6—comparison of different cell lines/strains/substances. Yes: low risk; Não: high risk; Unclear: uncertain risk. Questions adapted from Lewis et al. [20]

The most frequent classification for 'high risk' was based on the use of a single cell line/strain in 41.17% of the investigations [22, 29, 31–33, 38] and, less frequently, for the reason of the impossibility in comparing reactions due to different outcomes [23], and again, due to selective report when the result was presented by a single sample [29].

In vivo interventions and main results

Animals' interventions and related results are described in Table 4. The antithyroid effect was evaluated by offering acutely mesocarp aqueous extract (MAE) and kernel pressed paste with skin (KPPS) after a previous iodine-rich diet. This effect was observed by thyroid uptake reduction of ^{125}I and by the ratio between ^{125}I and monoiodotyrosine (MIT) coupled to diiodotyrosine (DIT). As a result, ^{125}I uptake was suppressed by MAE, but not significantly by KPPS. However, the ratio $^{125}I/MIT+DIT$ was high for both MAE and KPPS, suggesting a thionamide-like antithyroid effect [22].

Concerning the effect on lipid metabolism, urea and the development of type 1 diabetes, three studies offering MAE evaluated the intake of 50 mg/kg for 30 days in five mouse strains and found a significant increase in total cholesterol (TC) levels only in CBA strain (27 vs. 9 mg/dL) and a decrease only in C3H/HePas (18 vs. 47 mg/dL). There was a significant decrease in urea levels, except for C57BL/6 (49 vs. 67 mg/dL). Despite the glycolipid profile outcome mentioned, levels of lipoproteins, triglycerides (TG) or glucose were not demonstrated [39]. Another intervention offered a dose ten times lower than the previous study for 40 non-consecutive days, associated or not with resistance training (RT). They verified weight of loss after an 8-week supplementation period and, associated with RT, after 4 weeks. In addition, animals supplemented combined or not with RT had retroperitoneal fat reduction up to 73%, a decrease in TC levels (MAE: 79 mg/dL; MAE/RT: 70 vs. 97 mg/dL) and TG (MAE: 90 mg/dL; MAE/RT: 82 mg/dL vs. 166 mg/dL). There was a reduction in glucose only in RT animals (131 vs. 145 mg/dL). Animals trained without supplementation showed greater Delta force, suggesting a possible ergolytic effect of the substance [6].

On the other hand, Silva et al. [25] observed weight gain with intake of 3.3 mL/day of a suspension of MAE 20 mg/mL. In this study, the animals were not subjected to any type of physical labor and weight gain occurred between the 20th and 50th days, but with an abrupt drop afterwards. There was blood glucose fluctuation with lower levels on the 30th day and returning to baseline on the 60th. They did not observe significant changes in immunoglobulin IgG levels, possibly due to the low effect of MAE on T lymphocyte activation and cytokine production.

Acute toxicity effects were observed with high doses of mesocarp extracts and measured by organ relative weight and histological and biochemical analysis. No deaths were registered after a single dose up to 5000 mg/kg of mesocarp ethanolic extract (MEE) [27], as well as significant physical and or behavioral changes were not found. The same investigation also evidenced a reduction in urea levels at 5000 mg/kg (26 vs. 40 mg/dL) and increased TG at 1000 mg/kg (104 vs. 54 mg/dL) in a dose-dependent way. Alkaline phosphatase (ALP) increased after 3000 and 5000 mg/kg intake (23 and 21 U/L, respectively, vs. 6 U/L). No toxic effects were observed with gradual doses up to 4000 mg/kg [24]. MAE, mainly associated with RT, increased both aspartate (AST) and alanine aminotransferase (ALT) [6]. Alterations in locomotor activity and motor coordination can also be a sign of toxicity. Thus, a single dose of up to 3 g/kg of MAE did not affect these outcomes [23].

Low toxicity was observed since no changes in the number of medullary cells and a decrease of splenic cells only in BALB/c and C3H/HePas (both 3×10^7 vs. 5×10^7) was noted. Weight loss in organs was reported for HePas after 30 days exposure at 50 mg/kg of MAE [39]. On the other hand, MAE 5 mg/kg was able to increase the number of medullary cells in the absence of RT in Swiss mice (30×10^6 vs. 13×10^6), but in the scenario of splenocyte levels' preservation (41×10^6). When associated with RT, splenic cells were reduced (30×10^6) [6].

After different doses and periods, Maia and Rao [24] evaluated the effect of mesocarp chloroform extract intake on the inflammatory process. Analyzing the carrageenan-induced inflammation in edema paw, it decreased up to 32%

with progressive doses. The edema reduction caused by formaldehyde-induced arthritis was also observed on the 7th and 8th days of treatment. In this same study, seven-day intake at 250 mg/kg inhibited subcutaneous granuloma, measured by the weight of the cotton pellet introduced (112 vs. 192 mg) and it reduce leukocyte migration (24.19 vs. 23.62 cells/mm^3) and inflammatory exudate after a subcutaneous sponge implant impregnated with carrageenan.

In a stage before exudate induction, Silva and Parente [40] verified a significant inhibition in vascular permeability progression following a single dose of an isolated polysaccharide from mesocarp composed of alpha-(1 → 4) linked D-glucopyranose residues. Meanwhile, Barbosa et al. [26] observed the same effect in microvessels after volumes greater than 0.06 mL of the crude kernel oil twice a day. This study also reported attenuation in leukocyte adhesion with intake of 0.02 mL.

When analyzing proinflammatory cytokines, MAE intervention decreased interleukin-6 (IL-6) and increased tumoral necrosis factor-alpha (TNF-α) levels [6]. However, kernel oil was not able to cause significant changes in IL-1, IL-6, and TNF-α [26].

Leukocyte-specific activity is closely related to inflammatory stages. In this regard, five days of α-glucan from mesocarp increased zymosan-like phagocytic activity induced by colloidal carbon [40]. However, MAE associated with RT increased total splenic macrophages, but reduced activated macrophages, without changes in monocytes [6]. There was an activated T helper decrease and an increase of B cells, suggesting an immunomodulatory effect well [6].

Other outcomes, similar or antagonistic, were also evaluated. Although the antithrombotic effect of the aqueous mesocarp suspension was indicated by the prothrombin time increase (11.2 vs. 10 s) and activated partial thromboplastin time (33.6 vs. 29.5 s) [41], on the other hand, the chloroform extract of mesocarp given for three days did not extend the hemostasis time (98.0 vs. 90.3 min) [23].

Regarding peptic ulcer induction, prevention, or treatment, Maia and Rao induced ulceration with phenylbutazone and observed an average score of 1.03 for the lesion in five of six animals treated with phenylbutazone, on a 0–4 scale [24]. In opposition, animals treated with mesocarp chloroformic extract for 21 days did not exhibit lesions. In a different study, the prophylactic and therapeutic effect of MAE was compared to omeprazole, before or after ethanol-induced ulcers. In the preventive treatment, the effect was similar to omeprazole in the absence of lesions (60% of the animals), but superior to the drug in the absence of hyperemia, bleeding, and preservation of folds (100% vs. 60% on all outcomes). Despite the accentuated related-inflammatory lesions, such damage reached only the mucosa in MAE-treated animals, while there was deeper damage up to the submucosa in those ones receiving vehicle. Moreover, microscopic analysis showed MAE prevented necrosis in 80% of animals [42].

Scheibe et al. [43] evaluated 21-day MAE intake for healing. After this period, the animals underwent laparotomy with cecum exteriorization. Significantly, they identified a grade II adhesion (two adhesions between organs or between an organ and abdominal wall) in 100% of the animals on the 21st postoperative day compared to the negative control. Morphological evaluations did not identify polymorphonuclear leukocytes (vs. moderate presence), and mild congestion and angiogenesis (vs. severe) and moderate fibroblasts (vs. severe) were detected in MAE-exposed animals. Collagen production was also more intense than in the control group. Silva et al. [44] used the same procedures, but offered a MAE single dose. They found similar results, except for collagen production.

Finally, antipyretic and analgesic properties of mesocarp chloroform extract were analyzed [24]. After pyrexia induced by *Saccharomyces cerevisiae*, no time-related decrease in body temperature was observed (1 h: 39.18 vs. 39.18 °C; 2 h: 39.1 vs. 39.3 °C; 3 h: 39.18 vs. 39.31 °C). The analgesic effect was compared to morphine after exposure to a hot-plate at 55 °C. After 30 min of 250 mg/kg intake, the extract was not able to promote analgesia. Reaction time was similar to negative control and shorter than morphine (extract: 3.5 s; control: 2.83 s; morphine: 14.0 s). In contrast, the same dose was able to reduce acetic acid-induced writhing around 62% [24].

In vitro assays and main results

In vitro studies are useful for preliminary testing of substances in a controlled environment. The studies included are described in Table 5. Six studies evaluating antimicrobial and phagocytic activities on pathogenic or commensal

strains [8, 10, 27, 31, 37, 45] and two investigations against protozoa [29, 38] were identified. Cellular assays were carried out with tumor or non-tumor cell lines to study viability or cytotoxicity, morphological or metabolic changes [22, 28, 31–33, 36]. Lethality assays were carried out in microcrustaceans (*Artemia salina* Leach.) [45].

Assays using chemical reactions or yeasts can also be developed to measure preliminary effective concentrations (EC_{50}) for achieving half desirable effects. This review included six studies which evaluated the antioxidant activity of babassu [23, 30, 32, 34, 35, 45].

The most common component of *O. phalerata* was the kernel (52.9% of the studies) [8, 10, 22, 30–33, 36, 45]. MAE and mesocarp alcoholic extracts were tested in 47% and one publication associated mesocarp with epicarp [22, 23, 28, 29, 35, 37, 38, 46]. Only Silva et al. [34] used endocarp, flowers, and leaf extracts. The concentrations ranged a lot from 500 mg/mL of MEE [46] to assess the antimicrobial effect to 1 µg/mL of MAE for the antioxidant activity [23].

The disk diffusion assay was used to evaluate the effect of mesocarp on pathogenic bacteria strains. At 250 and 500 mg/mL, MEE promoted concentration-dependent inhibition zones on *Staphylococcus aureus* (18.5 mm), methicillin-resistant *S. aureus* (17.4 mm), and *Enterococcus faecalis* (14.4 mm). The most relevant minimum inhibitory concentration was observed for *E. faecalis*, while 500 mg/mL inhibited the growth of them completely. In contrast, there was no inhibition of *Escherichia coli* and *Pseudomonas aeruginosa* [46]. Similar levels of inhibition on methicillin-resistant or sensitive *S. aureus* were observed with lower concentrations of the hydroalcoholic extract [37].

Kernel oil also did not demonstrate efficacy on Gram-positive and negative strains [45], but after hydrolysis, it inhibited 80% of *Clostridium perfringens*, *S. aureus,* and *Enterococcus cecorum* [8]. Furthermore, the fixed oil increased the effectiveness of antibiotics over *S. aureus*, *P. aeruginosa,* and *E. coli* [10].

After evaluating mononuclear phagocyte activity and bactericidal potential, Pessoa et al. [31] found a higher rate of phagocytosis by lipid microemulsion (69.1 vs. 47%), and the bactericidal activity was higher by isolated oil (47.9%). When testing the leishmanicidal effect of MAE, it was observed a low activity when compared to Glucantime® (LC_{50} >500 vs. 440.3 µg/mL) [38]. On the other hand, microparticles loaded with the same extract demonstrated upper effectivess, but lower than pentamidine (IC_{50} 12 vs. 0.8 pg/mL) [29].

In a study developed by Rennó et al. [28] using the trypan blue method, leukemic promyelocytes (HL-60) were more sensitive to the crude ethanolic extract from mesocarp combined with epicarp when compared to negative control (ID_{50} 9.3 vs. 125 µg/mL), whose activity was time dependent (150 µg/mL: 8.6 h; 2 mg/mL: 0.4 h). The concentration of 1.2 mg/mL promoted changes in HL-60 morphology, with decrease in size and cytoplasmic/nuclear condensation. Interestingly, there was an increase in levels of 6-phosphofructo-1-kinase (PFK) enzyme at 300 µg/mL were tested, 6.6 times greater than the negative control. In this study, non-tumor cells showed greater resistance, such as L929 (127 vs. 88.7 µg/mL) and human lymphocytes (141.2 vs. 84.4 µg/mL). It is important to highlight that both erythroleukaemic sensitive to chemotherapy line and its multidrug-resistant counterpart were equally affected.

Regarding cytotoxicity, Santos et al. [32] tested a lipid nanoemulsion from kernel oil droplets in L929 cells and it was found a time and concentration-dependent IC_{50} after 24 h of exposure (396.1 µg mL^{-1}), 48 h (363.3 µg mL^{-1}) and 72 h (333.1 µg mL^{-1}). Santos et al. did not find kernel oil toxic effects on L929 after MTT (3–4,5-dimethyl-thiazol-2-yl-2,5-diphenyltetrazolium bromide) assay. Conversely, the same effect was not verified on peritoneal macrophages. Furthermore, they reported an increased migration of L929 cells in the scratch assay, concentration-dependent nitric oxide attenuation (0.31 to 0.29 vs. 1.15 µM), and higher levels of INF-γ (interferon-γ) production with 6.25 µg/mL of the nanoemulsion (2214.2 vs. 980.4 pg/mL), TNF-α (107.7 vs. <0.9 pg/mL) and IL-6 with 3.12 µg/mL (1286.1 vs. 584.4 pg/mL) by macrophages, suggesting a modulation of inflammatory response in wound healing situations.

Concerning phagocyte viability, a higher viability index similar to culture medium was found for lipid microemulsion when compared to isolated oil (98 vs. 94.3%) [31]. Another study using benign prostatic hyperplasic cells observed viability inhibition up to 75% after 24–72 h of exposure to a 300 µg/mL of nanocomposite with lipophilic extract. Even after the addition of fetal bovine serum, the inhibition was sustained. There was also induction of disorganization (disassembly) or disruption in the structure of actin microfilaments in a time-progressive manner, and a progressive lactate dehydrogenase (LDH) release. For this enzyme, there was a release of up to 75% after 48 h.

Immunoreactivity for proliferation cell nuclear antigen (PCNA) was also tested and proved to be 50% lower when compared to the negative control in association of apoptosis induction [36]. The single report about multicellular organisms observed no toxicity on *A. salina* (LC_{50}>1000 μg/mL) after testing concentrations up to 50 mg/mL of kernel oil [45].

The antithyroid effect in porcine thyroid slices was tested by Gaitan et al. [22] with different babassu components. The study verified that, although ^{125}I absorption by thyroid tissue was similar between the substances, the extracts showed a higher incorporation of ^{125}I/MIT + DIT, mostly after MME (mesocarp methanolic extract) exposure indicating iodine organification inhibition. Concerning thyroperoxidase activity, the MME and kernel peel evidenced an EC_{50} of 140 and 160 μg/mL, respectively, for thyroperoxidase-catalyzed iodination. Tyrosinase inhibition was also tested and a higher IC_{50} was found for the mesocarp ethyl acetate in comparison with extract or hydroalcoholic fraction for both monophenolase and diphenolase activity [35].

The antioxidant potentiality of babassu is detailed in Table 5. EC_{50} required for $DPPH^{\bullet}$ (2,2-diphenyl-1-picryl-hydrazyl) scavenging was described for kernel oil [37, 45] and for a nanoemulsion [32]. Nobre et al. [30] did not find thiobarbituric acid reactive substances (TBARS) inhibition and evidenced a low effect on deoxyribose degradation, $DPPH^{\bullet}$, and iron chelation by kernel methanolic extract. In this study, the expected effect occurred only for FRAP (ferric-reducing antioxidant power), but required higher concentrations than quercetin (1560 vs. 155.2 μmol $L^{-1}Fe^{2+}$). Likewise, no antioxidant effect of MAE up to 1000 μg/mL was detected. Endocarp, flower, and leaf alcoholic extracts also did not demonstrate antioxidant potential based on reaction with $DPPH^{\bullet}$. Furthermore, there was no yeast survival when the same extracts were associated with tert-butylhydroperoxide [23]. A positive result was verified only by Silva et al. [35] for mesocarp ethyl acetate fraction on $DPPH^{\bullet}$ (IC_{50} of 3.38 μg/mL), $ABTS^{\bullet+}$ (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) scavenging (2.04 μg/mL), and FRAP (15.41 mmol Fe^{2+}).

Discussion

In vivo studies

The fruit of babassu has shown favorable biological activities. However, the absence of results or undesirable outcomes were also observed, such as the antithyroid effects.

In animal models, the sex, age, weight, and metabolic condition are essential to optimize the relevance of the results [47]. Despite the analysis of beneficial effects, the in vivo trials seem to be useful for acute, subacute, or chronic toxicity tests, since even natural substances cannot be considered completely safe. Notably, widely studied nutrients also exhibit upper limits and restrictions [48]. In this context, investigations subjected animals to varied experimental conditions, including a single dose [23, 27], short or moderate durations throughout their lifetime [6, 39], or progressive dose regiments [24], but not always waiting for long latency periods [23], which limits the outcomes.

One of the alterations found that could indicate possible toxicity suggests that even after 14 days of latency, ALP increase was not followed by changes in liver and kidney functions. The hypothesis suggests that young animals are susceptible to alterations in this enzyme according to their diet [27]. In turn, increasing levels in aminotransferases should be evidence of oxidative stress due to lipid peroxidation, notably when there is a greater oxygen demand, as the rodents submitted to RT [6]. In terms of immunotoxicity, the decrease of splenic cells and spleen weight were both selective. The authors suggest sparse effects due to the divergence of results in strains with distinct haplotypes [39]. In short, despite the cited changes, all publications suggest low toxicity of the kernel and the mesocarp, not only with a single dose intervention up to 5 g/kg [23, 24, 27], but also with low doses up to 40 days [6, 39].

Results on metabolic effects show discrepant changes in serum TG. This marker increase could suggest an increment in lipogenesis due to the high carbohydrate content in the mesocarp (79.2%) [6], but it did not worse with doses above 1000 g/kg [27]. The presence of fibers in the mesocarp (17.9%) could also be associated with TC and LDL (low-density lipoprotein) decrease by reducing lipids absorption when they interact with lipase and/or colipase and limit the enzymatic activity [6, 49].

The reason for urea level reduction lies in the fact that foods with a high carbohydrate content, mostly resistant types, are not absorbed and remain available for gut microbiota fermentation. In this condition, endogenous proteins and plasma nitrogen would be recruited to ensure microorganism growth, reducing plasma urea levels, especially

when dietary protein intake is deficient [50].

Investigations have also displayed weight gain and glycemic fluctuation after intervention with MAE, which would contraindicate mesocarp intake, mainly for type II diabetes patients, in which weight loss is the most common aim, or when the risk of developing diabetes is increased [25]. However, the glycemic load of foods with a high content of digestible carbohydrates can be attenuated in association with proteins, lipids, and dietary fiber, constituting an essential strategy for maintaining adequate glycemic levels [51]. In opposition, the absence of weight gain and retroperitoneal adiposity reduction was demonstrated by Soares et al. [6] after MAE intake associated or not with RT, indicating a possible adjuvant effect of mesocarp in fat loss and weight control.

The anti-inflammatory activity of mesocarp chloroform extract suggests its application in subacute situations due to the inhibition of granuloma, a proliferative phase of inflammation. However, as the chloroform extract had no effect on pyrexia or leukocyte migration, arachidonate metabolism is not possibly involved in the mechanism [24].

Despite the high content of saturated fatty acids in crude kernel oil, the hypothesis is that vascular permeability decrease and leukocyte adhesion observed by Barbosa et al. [26] may have attributed to the anti-inflammatory action of oleic acid and the antioxidant effect of α-tocopherol.

Other positive effects on inflammation processes demonstrated by α-glucan propose that the residual chains of $1 \rightarrow 3$ bonds not hydrolyzed by amylase may be long enough to contribute to phagocytic amplification and vascular protection [40].

In vitro studies

The selective antibacterial effect of MEE, mainly on *E. faecalis* and *S. aureus*, has likely clinical effects because these strains are associated with nosocomial infections and usually resistant to antibiotics. Its mechanism of action probably involves the generation of complexes between phenolic acids, proteins, and polysaccharides capable of breaking cell wall and inhibiting microorganisms' enzymes [37, 46]. In turn, the synergistic effect between mesocarp and antibiotics may occur by different pathways, including changes in drug receptors [10, 52]. The microparticle encapsulation system can delay compound cytoplasm release, which improves the effect [29]. Applications with crude kernel oil on gram-positive or negative strains were not effective [45]. After its hydrolysis, it showed a selective effect against pathogenic bacteria, but not commensal strains [8]. This effectiveness is related to the free lauric acid, which can cross cell membranes, acidify the intracellular medium, and block bacterial growth, besides the oxidative effect after phagocyte activation [31, 53].

The results observed against tumor cells only demonstrated relative selectivity of mesocarp with epicarp ethanolic extract, including over chemoresistant cell lines, a phenomenon that represents the main failure of antineoplastic treatments. However, the absence of PFK inhibition on HL-60 suggests that the remaining tumor cells may have increased metabolic activity in face of extract toxicity [28, 36].

Fibroblasts have been employed because they are abundant cells in the human body and one of the first to come into contact with substances during absorptive processes. Thus, nanoemulsions based on kernel oil extracts showing low toxic effects against fibroblasts in vitro can predict in vivo studies, as well as the low toxicity against *A. salina* by a wide number of plant species [32, 45, 54].

Experimental evidence demonstrated the antithyroid effect of mesocarp flours, imputing its intake for the persistence of endemic goiter in Maranhão (Brazil) in the mid-1990s [22]. Nonetheless, since the sodium chloride iodination strategy was implemented in the 1950s, the prevalence of iodine deficiency disorders in Brazil has reduced from 20.7% in 1955 to 1.4% in 2000 [55].

The inhibition of tyrosinase activity promoted by the mesocarp ethyl acetate fraction was justified by the high concentration of proanthocyanidins (453.7 mg CE/g) in the inhibitory steps of monophenolase and diphenolase activity [35]. This enzymatic inhibition is one of the key mechanisms for alterations in melanogenesis, responsible for some characteristics of malignant melanoma [56]. The mechanism of this phytochemical consists of blocking L-tyrosine oxidation, depressing L-dopa oxidation products, and preventing pigment synthesis [57].

Given the importance of controlling oxidative stress and its pathological consequences, some tests identified the antioxidant potential of babassu to neutralize free radicals and prevent lipid peroxidation. After finding a total

phenolic content of 288 mg/g in kernel oil, the antioxidant activity did not overcome the ascorbic acid, there may not be a direct correlation between high levels of phytochemicals and better effects though [45]. It is also suggested that specific flavonoids may act in the intermediate process of lipid peroxidation, but they do not neutralize specific radicals [30]. Some concerns about the interference of kernel oil pigments in antioxidant assays have some grade of plausibility, such as DPPH$^{\bullet}$, because they can affect the optical density, which can lead to misinterpretations [58]. Therefore, the use of electronic spin resonance may be useful to bypass this interference [32].

The low affinity between MAE and lipids in TBARS can be answered by the lack of interaction with specific lipids, or hydrophilic portions of amphipathic lipids, as they are more sensitive to radical activity [23], even using polar solvents which can extract flavonoids that provide hydroxyl radicals to neutralize reactive species. However, different solvents can optimize the extraction of polyphenols [59, 60]. With the use of ethyl acetate to prepare samples from mesocarp, it was possible to identify monomers and tetramers of catechins. While the monomer is capable of reacting with a single free radical, its polymer can neutralize three radicals simultaneously [61]. On the other hand, the lack of effect by the endocarp, leaf, and flower ethanolic extracts on radicals and protection against tert-butylhydroperoxide, can be explained by low flavonoid content [34].

Conclusion

This review highlights the prevalent use of mesocarp and kernel in alcoholic extracts and emulsions, emphasizing their low lethality and weak cytotoxicity in vitro, along with the absence of acute toxicity in vivo. This opens perspectives for advancing to in vivo toxicity assays with repeated doses. The empirical intake of both mesocarp flour and kernel oil provides a basis for considering an extension to clinical trials to comprehensively understand the potential applications and effects of these substances.

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Author contributions

ND conceived the review idea, conducted the literature review, database searches, data extraction, risk of bias assessment and edited drafts as well as the final version. IOC performed the database searches, data extraction, risk of bias assessment and provided intellectual input into draft versions. ABSS contributed to the review, edited drafts, provided intellectual input into draft versions. VAO edited drafts, provided intellectual input into draft versions. HAN developed the graphic abstract and provided intellectual input into draft versions. PMPF provided intellectual input into draft and final versions. JMCS contributed to the review idea, conducted the risk of bias conflicts, edited drafts and provided intellectual input into draft versions as well as the final version. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. All the co-authors approved this submission.

Abbreviations

ABTS

2,2′-Azino-bis(3- ethylbenzothiazoline-6-sulfonic acid)

ALP

Alkaline phosphatase

ALT

Alanine aminotransferase

AST

Aspartate aminotransferase

DIT

Diiodotyrosine

DPPH

2,2-Diphenyl-1-picryl-hydrazyl

FRAP

Ferric-reducing antioxidant power

EC_{50}

Effective concentration

IC_{50}

Inhibitory concentration

ID_{50}

Inhibitory dose

IFN-γ

Interferon-γ

Ig

Immunoglobulin

IL

Interleukin

KPPS

Kernel pressed paste with skin

LC_{50}

Lethal concentration

LDH

Lactate dehydrogenase

LDL

Low-density lipoprotein

MAE

Mesocarp aqueous extract

MEE

Mesocarp ethanolic extract

MIT

Monoiodotyrosine

MME

Mesocarp methanolic extract

MTT

3-4,5-Dimethyl-thiazol-2-yl-2,5-diphenyltetrazolium bromide

PCNA

Proliferation cell nuclear antigen

PFK
6-Phosphofructo-1-kinase
PRISMA
Preferred Reporting Items for Systematic Reviews and Meta-Analyzes
PROSPERO
International Prospective Register of Systematic Reviews
RT
Resistance training
SYRCLE
SYstematic Review Center for Laboratory Animal Experimentation
TBARS
Thiobarbituric acid reactive substances
TC
Total cholesterol
TG
Triglycerides
TNF-α
Tumoral necrosis factor-alpha

Publisher's Note

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Screening and characterization of bioactive compounds from two epiphytic microlichen and evaluation of their in vitro antioxidant activity

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ABSTRACT (ENGLISH)

Background

Lichens in symbiosis produce a wide range of primary and secondary fine compounds in extreme environmental conditions that have a broad range of biological properties as well as antioxidant potential and can be used in future pharmaceuticals as a natural source of antioxidant molecules.

Results

The two microlichen species collected are identified based on morphological and molecular techniques; further studies are carried out by analyzing phytochemicals (FTIR, GC MS), and antioxidant assays are evaluated. The non-enzymatic antioxidant activity is evaluated by DPPH and FRAP assays. The methanol extract of both lichens showed virtuous DPPH scavenge with IC50 of *P. nitida* (125.76 ± 0.023 µg/ml) and *G. scripta* IC50 (176.90 ± 0.058 µg/ml). FRAP activity was prominent in the methalonic extract. The enzymatic antioxidant activity is observed by SOD and catalase activity. The cytosolic (Cu–Zn-SOD and Fe-SOD) and mitochondrial SOD (Mn-SOD) are

detected in lichens, though *P. nitida* shows mitochondrial Mn-SOD and cytosolic Cu–Zn-SOD and Fe-SOD, whereas *G. scripta* has a single cytosolic Cu–Zn-SOD; however, two isoforms of catalase were reported. GC–MS analysis screened bioactive metabolites such as phenols, Quinons, heterocyclic compounds, benzofurans, fatty acids, pyrans, carboxylic acid, aliphatic aldehydes, organic alcohol, fluorinated aliphatic substances, ketones, terpenes and fatty alcohols in *P. nitida*, whereas, in *G. scripta* screened fatty acids, alcohols, hydrocarbons, carbonyl compounds, polyols, terpenes, glycosides, phenols, and sugar alcohols detected in the chromatogram peak. FTIR analysis revealed functional groups like Alcohols, Amines, Amides, Alkanes, Aldehydes, Carboxylic acid, Alkynes, Esters, Ketones, Anhydrides, Acid chlorides, Alkenes, Aromatic compounds, Nitro compounds, Alkyl and Aryl Halides in both lichens.

Conclusions

The results obtained in the present study proved that *P. nitida* and *G. scripta* have promising antioxidant activity owing to the presence of polyphenols and terpenes, as evidenced by DPPH and FRAP assay along with enzymatic analysis (SOD and CAT). Thus both the lichens may be used as natural sources of new bioactive molecules having pharmaceutical interest.

FULL TEXT

Background

Lichens are poikilohydric symbiotic organisms with non-flowering living structure composed of an alga (phycobiont) and a fungus (mycobiont). They live in their natural habitats and can subsist in extreme environments. The water content of lichen thallus broadly depends upon environmental conditions. They have the potential to sustain long durations in a dry ecological condition with high adaptability to slower metabolic activity but can rapidly revive normal physiological activity upon rehydration [1].

Metabolic activities like respiration and photosynthesis are sensible for the generation of ROS (reactive oxygen species) causing damage to cell, thereby enhancing a number of degenerative diseases such as premature aging, inflammation, atherosclerosis, deoxygenation of ischemic tissues and cancer [2]. Free radicals attack unsaturated fatty acids, including PUFAs (polyunsaturated fatty acids), in plasma membranes, resulting in lipid peroxidation, decreased membrane fluidity and dissolution of membrane-associated proteins. Oxidative stress is influenced by environmental factors and aging, but some oxidative diseases and stresses can be treated with antioxidant agents. Antioxidants prevent oxidative chain reactions by eliminating free radical intermediates, which prevent cellular components from getting oxidized [3]. The carcinogenetic effect of synthetic antioxidants implicates the growing interest in natural antioxidants for human sustainability [4]. These are enhanced or triggered stress, such as dietary restriction, xenobiotic exposure, desiccation and/or rehydration. To overcome the latent damaging effects of reactive oxygen species (ROS) and maintain their limited consistency, cells have adopted protection mechanisms, including non-enzymatic antioxidants such as ascorbate, *α*-tocopherols, *β*-carotene and other minerals, and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidases (GTX), as well as low molecular weight antioxidants such as glutathione reductase (GRX) and glucose 6-phosphate dehydrogenase (G6PD) [5].

Graphis scripta and *Pyrenula nitida* are two epiphytic crustose lichens predominantly distributed to different phorophytes on the MSCBU campus. The lichenized thallus of both species consists of Ascomycetes fungus with associated *Trentepohlia* alga. This was associated with a burst of cellular ROS generation by both the mycobiont and the photobiont, as well as nitric oxide production that we only found in the fungus. These actions resulted in a transitory decrease in water-soluble low molecular weight antioxidant capacity but did not induce significant membrane damage. Imbibition of some lichens after desiccation stimulated extracellular antioxidant production [6]. Some recent evidence regarding the antioxidant response of lichens to rehydration reveals that this increases with thallus pre-treatment prior to testing species, drying and rehydration procedures, and experimental time periods [7, 8]

Several lichens have reported numbers of unique secondary metabolites such as terpenes, usnic acid, pulvinic acids, chromones, depsons, depsidones and xanthone derivatives that have been broadly labeled in the regulation

of lichen growth as well as protection along with series of biological activities [9] and the synthesis of these compounds increases their antioxidant capacities [10]. Recently, lichens have emerged as a valuable source of antioxidants, with implications for the development of pharmaceuticals and cosmetics [11]. Furthermore, this study was carried out to screen and identify the major bioactive constituents of both the lichens, *P. nitida* and *G. scripta*, and to evaluate in vitro, the enzymatic and non-enzymatic antioxidant activity by using different solvent extracts. This is the first report from this region on the isolation and characterization of compounds from both the lichen species having their antioxidant activity.

Methods

Sampling and study area

MSCBU campus positioned at 21.9303° N 86.7636° E, Odisha, India (Fig. 1). Seasonal field visits (Spring, Rainy and Winter) and collection (Dates: 07/03/2021, 22/08/2021 and 21/01/2022) of lichens were performed regularly, and all collected lichens were sampled by random sampling technique. Collected lichens are wrapped in white paper bags with zippers, with its microhabitat data (latitude, longitude, temperature and humidity) of each bisect.

Fig. 1 [Images not available. See PDF.]

Study area: MSCBU campus, shadow area showing coverage and distribution of lichens

Diversity index of collected samples

This study explored a number of epiphytic lichen species under 12 genera in 8 families from the MSCBU campus, which is situated near the Similipal Biosphere Reserve (SBR). The preceding study was carried out by studying the epiphytic lichen species on twenty dominant trees/phorophytes. The lichen family Graphidaceae has the maximum distribution and is represented by SWI (Shannon-Weiner Index) as $H=2.053$, with a SIV (species index value) of 206.72, and both the lichen species with SIV as in *Pyrenula nitida*- OQ146904 (55.96) and *Graphis scripta*- OP861477 (63.46) were distributed within vegetation.

Morphological identification of lichens

Morphological identification was done using a stereo-zoom microscope (Stemi-305, 40 × magnifications) and scanning electron microscope (Model-S3400N, magnification 10X to 300,000 X Max) observation. Morphological identifying characteristics are studied using the lichen identifying key book [12].

Molecular identification

DNA isolation from lichens

For the isolation of DNA from Lichen thallus, 10 mg of scraped lichen thallus was used and put into a micro-vial, deepen in liquid nitrogen with three or four 2.5-mm sterile glass beads for 30 s and disrupted with a Mini-Beadbeater-24. The sample was stirred vertically with 300 µL KCl extraction buffer, 300 µL chloroform and kept reversed. The sample mixture was centrifuged at 12,000 rpm for 1 min. The supernatant was transferred to a microcentrifuge vial with the addition of 180 µL of chilled isopropanol and centrifuge at 12,000 rpm for 1 min; the supernatant was descanted. The remaining pellet was poured out with 300 µL chilled ethanol (70%). The pellet was dried at 55 °C in oven, then 100 µL of TE buffer was added, and purified DNA was stored at 4 °C [13].

PCR and sequencing

A partial sequence of DNA for analysis (28s, and 18s rDNA) was isolated from the upper cortex of both lichens. Amplification of rDNA performed using PCR primers for the 28s larger sub-unit of *P. nitida* strain PNSB04 used forward primer used was PN1- 5′-aacaggggggtgagatgtcaga-3′ (25 nm STD, GC: 54.5%, Tm: 60 °C, ΔG: − 41.7 kcal/mol), and reverse primer PN2-5′-ctagtacgatacattcaaatgt-3′ (25 nm STD, GC: 31.8%, Tm: 47.8 °C, ΔG: - 34.33 kcal/mol) for *G. scripta strain* SPB25 rDNA, Forward primer-GS1: 5′-ttgtaatttggagaaggtgttt-3′ (25 nm STD, GC: 31.8%, Tm: 50.4 ΔG: − 38.42 kcal/mol), and reverse primer-GS2: 5′-catcctagcttttgcgcggacc-3′ was used, following the protocol of White et al. [14]. PCR products were visualized on a 1.5% agarose gel analysis for 25 min with content 120 V, to authenticate the presence of amplicons size, then exonuclease-I and recombinant shrimp alkaline phosphatase used for molecular purification and sequenced bidirectionally by genetic analyzer (ABI 3730). Proofreads (forward and reverse) were assembled in MEGA and Sequencher v. 5, with GenBank submission having

accession number for *G. scripta* (OP861477) and *P. nitida* (OQ146904).

Antioxidant activity of Lichens

Preparation of the lichen extracts for non-enzymatic antioxidant activity

Collected lichen samples were dried in a hot air oven and then crushed to a fine powder, and 10 g of dry powder was poured into a Borosil Soxhlet at 45–50 °C for 24 h using methanol, acetone, benzene and diethyl ether. After 6–8 cycles, the solutions are collected and further evaporated in oven at 42–45 °C. After evaporation, the dry extract of each solvent was taken for further analysis.

DPPH radicals scavenging assay

DPPH (1,1-diphenyl-2-picryl-hydrazil) is a complex radical mixture used in an H^+ Ion transformed-based scavenging non-enzymatic antioxidant assay [15]. An earlier prepared 1 ml DPPH solution (0.1 mM) was added to 3 ml of different progressive concentrations (100, 250, 500, 750 and 1000 µg/ml) of lichen extract and instantly incubated in dark conditions for 30 min at room temperature. After incubation, the absorbance was taken at 517 nm using a Janway-119 spectrophotometer. The radical scavenging activity (RSA) was measured in percentage (%) by using the formula Activity (%) = [(control Absorbance (A_0)—sample Absorbance (A_1))/control Absorbance (A_0)] × 100, where A_0 is the absorbance of the − ve control, and A_1 is the absorbance of the sample added reaction mixture or standards, i.e., butylated hydroxytoluene. Based on the percentage of radical scavenging activity, the IC-50 value was calculated according to the increase in percentage of radical scavenging. For comparative analysis, the natural antioxidant Ascorbate was used as a + ve control against the sample.

Ferric-reducing assay

ET (electron transfer)-based antioxidant assay determined the ferric ion-reducing activity of both lichens by the method of Oyaizu [16]. The concentrations of a standard range of lichen extracts (100–1000 µg/ml) were mixed with 2.5 ml of PO_4 buffer of pH 6.6 (0.2 M) with 1% potassium ferricyanide and incubated for 25 min at 50 °C and subsequently mixed with 10% trichloroacetic acid, followed by centrifugation at 3000 rpm for 20 min. The collected supernatant was vigorously mixed with 2.5 ml of distilled water while simultaneously adding 0.5 ml of 0.1% $FeCl_3$, and absorbance was measured at 700 nm. For the comparative observation, BHT (butylated hydroxytoluene) was taken as the positive control.

Determination of antioxidant enzymatic assay

Preparation of cellular extract

For analysis of superoxide dismutase and catalase activity, 0.5 g of lichen extract was prepared by using ethylene diamine-tetra acetate (50 mM), sodium phosphate buffer of pH 7.4 (50 mM), polyvinyl pyrrolidone 10% (*w*/*v*) and phenyl methyl sulfonyl fluoride (2 mM) in a frozen condition with a pestle in the dark. The well-gelatinous ground material was centrifuged at 14,000 rpm at 4 °C for 20 min in a collapsing centrifuge. After centrifugation, the supernatant was evaluated for SOD and CAT activities.

Superoxide dismutase (SOD) activity

The activity and quantity measures of SOD were evaluated by the standard procedure of Das et al. [17]. This activity measured the superoxide-driven nitrite formation inhibition from hydroxylamine hydrochloride. The reaction cocktail was prepared in dark conditions by adding 1.11 ml of phosphate buffer of 50 mM (pH 7.8), 0.075 ml of 10 mM hydroxylamine hydrochloride, 0.04 ml of 1% Triton X-100 (*v*/*v*), 0.075 ml of L-methionine (20 mM), 0.1 ml of 50 mM EDTA and 80 µl of 50 mM, riboflavin to the mixture. After preparation of the reaction mixture, 10 min of light exposure is required to produce the appearance of white fluorescence and absorbance measured at 543 nm with the addition of Griess reagent. A single unit of enzyme activity represented the amount of SOD, which prevented 50% of nitrite formation. The enzymatic activity was measured using the formula V_0/V_{-1}, where V_0 (control absorbance) and V (sample absorbance). The activity and total enzyme were expressed in units nkat (nanokatal per liter)/mg or U (Unit per liter)/ml.

Catalase (CAT) activities

The catalase activity of two tested lichens is evaluated by the method of Aebi [18]. The preparative chemical mixture for catalase analysis contained 2 ml of 0.1, potassium phosphate buffer (pH 6.8), an enzyme extract of 500 µL and H

$_2O_2$ of 500 µL that reached a final volume of 3 ml. The catalase activity was measured over a 3-min time interval at 240 nm against the blank by the rate of H_2O_2 consumption.

Native-PAGE analysis for SOD and CAT enzyme

For the analysis and estimation of total SOD and catalase, native-PAGE analysis was performed. The SOD and catalase staining was performed by Beauchamp and Fridovich [19]. Enzymatic analysis through native-PAGE is done by early preparation of 10% resolving and 5% stacking gels and then loaded the sample solution at 4 °C with a constant current of 40 V for 12 h. For gel support, 10% glycerol was added [20]. The gel was stained in dark for 30 min by using solutions (i.e., 50 mM sodium phosphate buffer of pH 7.8), tetramethylethylenediamine (28 mM), riboflavin (0.003 mM), NBT(0.25 mM) and EDTA (0.1 mM), and the gel after 30 min of light exposure, resulting in the visible protein bands. Similarly, the catalase staining was done in a dark condition. The gel was washed with water (ddH_2O) and stained with 0.003% H_2O_2 for 10 min. Thereafter, a mixture of 2% potassium ferric cyanide, 2% ferric chloride and 1% HCl was added for the catalase isoforms to appear dark green in the background of the gel. The activity and total enzyme were expressed in units nkat (nanokatal per liter)/mg or U (Unit per liter)/ml.

Fourier transform infrared spectroscopy (FTIR)

In FTIR analysis, the IR spectra are equipped with a Thermo Nicolet iS10 FTIR spectrometer (Thermo Scientific, USA) with the Smart iTR attenuated total refraction (ATR) accessory. Soxhlated dry lichen extract is placed on a horizontal ATR crystal of the spectrometer under constant pressure. The sample is 32 scans across the range of (v4000 to 400 cm^{-1}) with a resolution of 4.0 cm^{-1}, an absolute threshold of 96.395 and a sensitivity of 50. For analysis of spectral data, essential FTIR and prism software (version 8.0.1) was used, and all sample analysis was carried out in triplicate.

Gas chromatography–mass spectrometry analysis (GC–MS)

For GC–MS analysis, methanol is used as a reliable solvent for extraction. GC–MS analyses were performed on an Agilent Capillary-column 60.0 m × 250 µm × 25 µm. Injection in autosampler was used 1.5 µl of the sample split to 10:1. Oven: Initial temp 60 °C, ramp (temperature regulation) 7 C/min to 200 °C, (hold for 3 min), ramp 10 °C /min to 300 °C (hold 5 min), inject autosampler = 280 °C, Volume = 1.5 µl, split = 10:1, Helium as carrier gas, Solvent delay = 7.00 min, Transfer temp = 160 °C, Source temp = 150 °C, scan = 50 to 600 Da and column 60.0 m × 250 µm. The GC peak regions were used to calculate the percentage of extract composition. PubChem and the NIST Chemistry web-book used for analysis.

Determination of total phenolic

The estimation of total phenols includes total aromatic and polyphenols and is expressed against the gallic acid standard calibration curve as GAE mg/g of total DE (dry extract). The Folin–Ciocalteu reagent method is used for estimating total phenol [21]. Lichen extract of 100 µl was taken and mixed with 2 ml of 2% sodium carbonate, and in a 10-min interval, 500 µl of Folin's reagent was added and incubated for 20 min. Absorbance was measured by Jenway-119 spectrometer at 650 nm.

Estimation of total flavonoid

Total flavonoid estimation of both lichen species was carried out by method of Zhishen et al. [22] and expressed as quercetin equivalent, i.e., µg QE/g dry extract. One milliliter of Lichen extract was mixed with 10% of aluminum chloride (300 µl), 500 µl of sodium nitrite and 1 ml of 1 M hydroxide, and final volume was made up to 5 ml with dd H_2O. After a period of incubation absorbance was measured at 510 nm.

Estimation of total tannin

The total tannin content of two lichens was estimated, expressed as tannic acid equivalent (TAE/mg/g DE) of dry extract and calibrated in a tannic acid calibration curve using the method of Oyaizu [16]. The lichen extract of 200 µg/ml was taken and added to 7 ml of distilled water, followed by 0.5 ml of Folin phenol reagent and 1 ml of a 35% sodium carbonate solution and volume adjusted with added dd H_2O and up to 10 ml. The mixture was shaken and incubated for 30 min, and absorbance was taken at 725 nm.

Estimation of total terpene

The quantitative estimation of total terpene in both lichens was carried out by the standard procedure of Ghorai et al.

[23], and total terpene was plotted against the linalool equivalent (a monoterpene) calibration curve and expressed as LE mg/g DW. Lichen extract of 1 ml was mixed with conc. 1 ml, conc. H_2SO_4 and 1 ml chloroform and then shaken gently, to take absorbance at 538 nm.

Results

Thallus morphology and identification

Pyrenula nitida PNSB04

Substrate: Mostly on bark, but also on non-calcareous rock, the genus is in its current delimitation characterized by the combination of clypeate ascomata and brown, distoseptate ascospores (brown, ellipsoid to fusiform) and simple paraphyses. Thallus: crustose, submerged but sometimes exposed, areolate, possibly with pseudocyphellae or crystal pockets, ascomata (perithecial) are black masses that are habitually grouped in pseudostromata. Ascomatal wall: black, generally continuous under the hamathecium and frequently partially buried in the thallus with true exciple (brown to pale brown, entire or not developed below the perithecial cavity, colorless or orange) (Fig. 2A, 3A).

Fig. 2 [Images not available. See PDF.]

A*Pyrenula nitida* and fig **B***Graphis scripta* under stereo-zoom microscope (Stemi-305)

Fig. 3 [Images not available. See PDF.]

A Scanning electron microscope (Model-S3400N) observation showing: A colonies of perithecium in upper surface of thallus, B–D bunch of ascomata (perithecium), E two perithecium with ostiolar canal opening, F individual perithecia with ascospore and ostiole canal opening of *P. nitida*. **B** Scanning electron microscope (Model-S3400N) observation showing: A and B branching Apothecia on thallus surface with dark gray to whitish pruina margin with black and thin exciple, C–Edistally carbonized excipular lips (base and entire), F individual apothecia with carbonized excipular lips and epihymenium of *G. scripta*

Graphis scripta SPB25

Substrate: Found on the smooth bark of deciduous trees in humid and submontane zones. Thallus: crustose surface with a continuous to somewhat rugose surface: dull, cream-colored, white, pale gray or grayish green apothecia. Apothecia: oblong, flexuous and branching, 1–3 × 0.2–0.4 mm. Disc: narrow to broad and open, dark gray to brown with whitish pruina margin: strongly developed, covering the lateral half of the ascocarps excipular lips. Excipular lips: black, whole and occasionally thin Exciple: poorly formed and not carbonized at the base, and carbonized distally, entire excipular lips, the basal slice of which is typically less developed and less carbonized epihymenium. Epihymenium: brown, with distinctive brown or yellowish-brown tips ascospores (Fig. 2B, 3B).

Molecular identification

Evolutionary relationships of taxa

The evolutionary history analysis of both lichens using the Minimum Evolution method [24]. The bootstrap consensus tree inferred from 600 replicates that represent the evolutionary history of the taxa by analyzed Cluster-W preliminarily. Branches with lesser then 50% bootstrap are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test of 600 replicates are shown next to the branches [25]. The evolutionary distances were measured by Maximum Composite Likelihood method and the molecular evolutionary tree (MET) tree was searched using the CNI (Close-Neighbor-Interchange) algorithm at a search level of 1. [26, 27] Here, the neighbor-joining algorithm was also used to generate the phyla analysis [28]. This analysis involved 30 closer neighbors with high accuracy of nucleotide similarity. There were a total of 928 positions *for P. nitida* PNSB04and 472 positions for *G. scripta* SPB25 in the final dataset (Fig. 4 (A&B)). Evolutionary analyses were conducted in MEGA11 software [29].

Fig. 4 [Images not available. See PDF.]

A Phylogenetic analysis of *P. nitida* by using Minimum Evolution method of MEGA 11 software. **B** Phylogenetic analysis of *G. scripta* by using Minimum Evolution method of MEGA 11 software

Phylogenetic relationship of taxa

Data validation

All nucleotide is NCBI (National Centre for Biotechnology Information)/BLAST (http://www.ncbi.nlm.nih.gov/ Basic Local Alignment Search Tool) analysis and submitted to GenBank with accession number, *G. scripta* (OP861477) and *P. nitida* (OQ146904). Proofreads were edited and assembled in MEGA (Molecular Evolutionary Genetics Analysis-Version 11) and Sequencher v. 5.

Non-enzymatic antioxidant activity

DPPH scavenging activity

DPPH scavenging activities were analyzed in both lichens by using different solvent extractions such as acetone, methanol, diethyl ether and benzene in a particular concentration range (100, 250, 500, 750 and 1000 µg/ml). Both species highlighted significant radical scavenging in the methanol extracts i.e., *P. nitida* (83.09 ± 0.89%) (Fig. 5A) and *G. scripta* (80.65 ± 0.96%) (Fig. 5B). The acetone and diethyl ether extracts of both the lichens show moderate radical scavenging activity, whereas the benzene extracts have minimal scavenging activity. However, the methanolic extract of *P. nitida* showed comparatively higher scavenging activity with half the minimal concentration, IC-50 (165.76 ± 0.003 µg/ml) followed by *G. scripta* IC-50 (176.90 ± 0.025 µg/ml) in the final data set.

Fig. 5 [Images not available. See PDF.]

A DPPH radical scavenging activity of *Pyrenula nitida* was carried out by taking 100–1000 µg/ml concentration of lichen extract in X-axis and scavenging activity (% of inhibition) in Y-axis, butylated hydroxy toluene (BHT) represented as standard. **B** DPPH radical scavenging activity of *Graphis scripta* was carried out by taking 100–1000 µg/ml concentration of lichen extract in X-axis and scavenging activity (% of inhibition) in Y-axis, butylated hydroxy toluene (BHT) represented as standard

Ferric-reducing activity

The ferric-reducing activity of both the lichens was analyzed using four organic solvents such as acetone, methanol, diethyl ether and benzene. The reducing activity was significant in methanolic extract of test lichen species followed by acetone, diethyl ether and benzene. However, the methanol extract of *P. nitida* and *G. scripta* exhibits comparatively maximal ferric ion reducing activity (0.35 ± 0.051 and 0.33 ± 0.036 nm) (Fig. 6A and B) in the final data set against standard butylated hydroxytoluene (BHT).

Fig. 6 [Images not available. See PDF.]

A Ferric-reducing antioxidant power assay of *P. nitida* was carried out taking 100–1000 µg/ml concentration of lichen extract in X-axis and reducing activity (absorbance at 700 nm) in Y-axis. The experiment was carried out in triplicate, while the error bars indicate standard deviation among the replicates. **B** Ferric-reducing antioxidant power assay of *G. scripta* was carried out taking 100–1000 µg/ml concentration of lichen extract in X-axis and reducing activity (absorbance at 700 nm) in Y-axis. The experiment was carried out in triplicate, while the error bars indicate standard deviation among the replicates

Enzymatic antioxidant activities (SOD and CAT)

The cellular extracts of two microlichens were evaluated for SOD and CAT activity. The better SOD and CAT activities were obtained in *P. nitida* (34 U/ml or 566. 667 nkat/mg and 3.120 U/ml or 52 nkat/mg), compared to *G. scripta* (27 U/ml or 450 nkat/mg and 2.280 U/ml or 38 nkat/mg). Further, in 2D native-PAGE analysis, *P. nitida* showed two significant isoforms of SOD, such as mitochondrial Mn-SOD and cytosolic Cu–Zn- Fe-SOD, whereas *G. scripta* had purely a single cytosolic Cu–Zn-SOD. On the contrary, both lichens from the same habitat contained two isoforms of CAT in native gel analysis (Fig. 7 i, ii).

Fig. 7 [Images not available. See PDF.]

i and **ii** Native-PAGE analysis of SOD and catalase enzyme in two lichens, arrow indicates SOD and catalase isoforms. A- *P. nitida* and B- *G. scripta*

Quantitative estimation of total phenol, flavonoid, tannin and terpene

Quantitative phytochemical estimation of lichens was carried out by estimating major phytochemicals, i.e., phenols, flavonoids, tannins and terpenes. Maximum phytochemical content was recorded in methanol extract of both the lichens i.e., *Pyrenula nitida* and *Graphis scripta* (Table 1).

Table 1. Quantitative phytochemical estimation of *P. nitida* and *G. scripta*

Solvents	*Pyrenula nitida*				*Graphis scripta*			
Phenolic (mg/g) GAE	Flavonoid (mg/g) QE	Tannin (mg/g) TAE	Terpenoid (mg/g) LE	Phenolics (mg/g) GAE	Flavonoid (mg/g) QE	Tannin (mg/g) TAE	Terpenoid (mg/g) LE	Acetone
0.378 ± 0.013	0.031 ± 0.007	0.21 ± 0.0012	0.16 ± 0.041	0.297 ± 0.033	0.029 ± 0.01	0.184 ± 0.002	0.180 ± 0.0018	Methanol
0.511 ± 0.07	0.049 ± 0.002	0.47 ± 0.003	0.192 ± 0.003	0.682 ± 0.008	0.041 ± 0.03	0.32 ± 0.007	0.252 ± 0.0012	Diethyl ether
0.2 ± 0.0014	0.21 ± 0.008	0.134 ± 0.001	0.144 ± 0.003	0.197 ± 0.035	0.018 ± 0.002	0.11 ± 0.003	0.162 ± 0.003	Benzene

Phytochemicals were estimated in triplicate and expressed as mg/g of dry extract, phenol (GAE—gallic acid equivalent), flavonoid (QE—quercetin equivalent), tannin (TAE—tannic acid equivalent) and terpenoid (LE—linalool equivalent), and data were interpreted as mean and standard deviation.

FTIR analysis

For FTIR analysis for *P. nitida* (L_1), here, IR spectrum shows bands with following frequencies (cm − 1) (Fig. 8A), v Amines, Amides (N–H stretch) and Alcohols (O–H stretch), Alkanes (C–H stretch), (3332–2932 cm^{-1}), vAlkanes, Aldehydes (C–H stretch), and Carboxylic acid (O–H stretch) (2932–2853 cm^{-1}), vAlkanes, Aldehydes (C–H) and Alkynes (C≡C) and (2853–2192 cm^{-1}), vAlkynes (C≡C stretch), Carboxylic acid, Esters, Ketones, Aldehydes, Amides, Anhydrides and Acid chlorides (C=O stretch) (3192–1744 cm^{-1}), vAlkenes (C=C stretch), Aromatic compounds (C=C stretch and C–H bend), Amides (C=O stretch), and Nitro compounds (NO_2 stretch) (1744–1452 cm^{-1}), vAlkyl and Aryl Halides (C–F stretch, C–Cl stretch and C–Br stretch) and (1394–857 cm^{-1}).

Fig. 8 [Images not available. See PDF.]

A FTIR analysis *P. nitida*, wavelength in ranges between (500 and 4000 Cm^2), wavelength in plotted in X-axis and transmittance (%) in Y-axis, graph with numerical order showing presence of functional group in IR spectrum range (v). **B** FTIR analysis *G. scripta*, wavelength in ranges between (500 and 4000 Cm^2), wavelength in plotted in X-axis and transmittance (%) in Y-axis, graph with numerical order showing presence of functional group in IR spectrum range (v)

For FTIR analysis for *G. scripta* (L2), Here, IR spectrum showing bands with following frequencies (cm^{-1}) (Fig. 8B), vAlkynes (≡C–H stretch), Alcohols (O–H stretch), Amines (N–H stretch), Amides (N–H stretch), Alkanes, Aldehyde (C–H stretch) and Carboxylic acid (O–H stretch) (3320–2920 cm^{-1}), vAlkanes, Aldehydes (C–H stretch), and Carboxylic acid (O–H stretch) (2920–2847 cm^{-1}), vAlkynes (C≡C stretch) (2178–2059 cm^{-1}), vAlkenes, Aromatic compounds (C=C stretch), Amides (C=O stretch), Alkyl &Aryl Halides (C–F stretch), and Nitro compounds (NO2 stretch) (1647–1048 cm^{-1}), vAlkyl and Aryls (C–F stretch), Alkenes (=C-H bend) and Aromatic compounds (C-H bend) (1048–568 cm^{-1}).

GC–MS analysis

Gas chromatography–mass spectrometry screened for lichens and some compounds are analyzed through gas chromatography and other known and unknown class of compounds are analyzed throughout mass spectrometry (Fig. 9A–D). Compounds classes like phenols, Quinons, benzofurans, heterocyclic compounds, pyrans, hydrocarbons, carboxylic acid, aliphatic aldehydes, alkanes and fatty acids characterized through gas chromatography, while mass spectrometry detected some classes of compounds like phenolics, organic alcohol, fatty acids, fluorinated aliphatics, ketones, terpenes and fatty alcohols in *P. nitida* (Table 2). However, in *G. scripta*, GC detected fatty acids, alcohols, hydrocarbons, carbonyl compounds and fatty alcohols, and MS detected terpenes, glycosides, phenols and sugar alcohols in highlighted pick areas of chromatogram (Table 3).

Fig. 9 [Images not available. See PDF.]

A Gas chromatogram in *P. nitida* showing retention time in the X-axis and % of peak area in the Y-axis, autosampler ejection and running carried out thrice for retention peak conformation. **B** Major compounds in chromatogram of *P. nitida*. **C** GC–MS chromatogram of *G. scripta* showing retention time in the X-axis and % of peak area in the Y-axis, autosampler ejection and running carried out thrice for retention peak conformation. **D** Major compounds in chromatogram of *G. scripta*

Table 2. Bioactive compounds obtain from *P. nitida* through GC–MS analysis with their molecular formula, compound class, retention time, percentage (%) of area and retention index

Compound	Compound structure	Compound class	Retention time	% of area	Retention index
3-Hydroxy-5-methoxytoluene ($C_8H_{10}O_2$)		Phenol	21.25	1.27	1342
1,4-BENZOQUINONE ($C_6H_4O_2$)		Quinone	21.25	1.27	143
3-methoxy-5-pentyl-phenol ($C_{12}H_{18}O_2$)		Phenolics	25.06	2.08	–
Furan-2(5H)-one ($C_4H_4O_2$)		Heterocyclic compound	25.06	2.08	915
2,3,5-trimethylbenzene-1,4-diol ($C_9H_{12}O_2$)		Phenols	25.06	2.08	–
[4-(hydroxymethyl) phenyl] methanol ($C_8H_{10}O_2$)		Alcohol	25.06	2.08	–
Anethofuran ($C_{10}H_{16}O$)		Benzofurans	25.06	2.08	1178
11, 14-icosadienoic acid ($C_{20}H_{36}O_2$)		Fatty acid	33.90	11.38	–
(5E,9E)-hexacosa-5,9-dienoic acid ($C_{26}H_{48}O_2$)		Fatty acid	33.90	11.38	–
4-Pentyl-5-propyl-2,2-bis(trifluoromethyl)-1,3-dioxolane ($C_{13}H_{20}F_6O_2$)		Fluorinated substances	38.22	0.77	–

2-trimethylsiloxy-6-hexadecenoic acid ($C_{20}H_{40}O_3Si$)		Fatty acid	38.22	0.77	–
1-(2,4,6-trihydroxyphenyl) propan-1-one ($C_9H_{10}O_4$)		Ketones	22.14	0.62	–
Non-8-ynoic acid ($C_9H_{14}O_2$)		Fatty acids	22.14	0.62	–
Undec-10-ynoic acid ($C_{11}H_{18}O_2$)		Fatty acids	22.14	0.62	–
Ethylformic acid ($C_3H_6O_2$)		Fatty acids	22.14	0.62	745
Methanecarboxamide (C_2H_5NO)		Fatty acids	25.55	0.42	1763
Tetrahydrobenzene (C_6H_{10})		Hydrocarbons, cyclic	25.55	0.42	674
4-Methyl-5,7-dihydroxycoumarin ($C_{10}H_8O_4$)		Pyrans	25.55	0.42	2451
Benzenecarboxylic acid ($C_7H_6O_2$)		Carboxylic acid	26.16	0.76	2380
1,3-Benzenedicarboxaldehyde, 2-hydroxy-5-methyl- ($C_9H_8O_3$)		Phenols	26.16	0.76	–
Chloratranorin ($C_{19}H_{17}ClO_8$)		Phenols	26.16	0.76	–
(Z)-Octadec-9-enoic acid ($C_{18}H_{34}O_2$)		Fatty acid	31.36	0.96	–
7,11,15-trimethyl-3-methylidenehexadec-1-ene ($C_{20}H_{38}$)		Terpene	31.36	0.96	–
2-Tetradecyloxirane ($C_{16}H_{32}O$)		Alkanes	31.36	0.96	1708
2-Hexadecen-1-ol, 3,7,11,15-tetramethyl- ($C_{20}H_{40}O$)		Terpene	31.36	0.96	–
(Z)-7-Hexadecenal ($C_{16}H_{30}O$)		Aliphatic aldehyde	31.36	0.96	2144
1,10-hexadecanediol ($C_{16}H_{34}O_2$)		Fatty alcohols	33.14	0.73	–

Table 3. Bioactive compounds obtain from *G. scripta* through GC–MS analysis with their molecular formula, compound class, retention time, percentage (%) of area and retention index

Compound	Compound structure	Compound type	Retention time	% of area	Retention index
Deoxynivalenol 3-acetate ($C_{17}H_{22}O_7$)		Terpenes	21.27	1.27	-
2,3-Dihydroxanthosine ($C_{10}H_{12}N_4O_6$)		Glycosides	13.12	1.18	-
Pentadecylic acid ($C_{15}H_{30}O_2$)		Fatty acids	34.54	4.46	2822
Linolenelaidic acid ($C_{20}H_{34}O_2$)		Fatty acids	34.54	4.46	1671
2,3-dimethylbenzene-1,4-diol ($C_8H_{10}O_2$)		Phenol	34.54	4.46	-
Benzenemethanol, 2-methoxy- ($C_8H_{10}O_2$)		Alcohol	34.54	4.46	2083
4-propan-2-ylcyclohex-3-ene-1-carbaldehyde ($C_{10}H_{16}O$)		Hydrocarbons	34.54	4.46	1195
Cyclopentanepropanoic acid ($C_8H_{14}O_2$)		Carbonyl compound	34.54	4.46	2168
D-mannitol ($C_6H_{14}O_6$)		Sugar alcohols	36.26	4.46	-
Docosen-(13)-ol-(1) ($C_{22}H_{44}O$)		Fatty alcohols	33.15	0.73	2468
Perfluorocaprylic acid ($C_8HF_{15}O_2$)		Fatty acids	33.15	0.73	1046

Statistical analysis

Statistical analyses were performed with the EXCEL, IBM-SPSS and GraphPad Prism software version 8.0.1. Data were presented as mean ± standard deviation (SD) of three replicates. The *P*-values less than 0.05 were considered significant.

Discussion

In the current study, both the tested lichens have marked antioxidant properties; through the intensity of antioxidant activity in *in-vitro* oxidative systems depends upon the solvents used for extraction. The variations in the antioxidant potential of solvents could be related to their ability to extract bioactive molecules [30], whereas the aqueous extracts had the least amount of antioxidant activity because lichen metabolites are partially soluble or insoluble in aquas [31]. Thus, organic solvents were used which withstand significant extraction of bioactive metabolites. Several findings proved that environmental factors such as air pollution, desiccation, high temperatures, rehydration and high light have a great influence on the antioxidant activity of lichens, affecting the increasing as well as decreasing and synthesis of antioxidants [32, 33]. Formerly, it was concluded that natural compounds, including tocopherols, phenolics, flavonoids, terpenes and carotenoids, have strong antioxidant activity. Due to their ability to scavenge free radicals such as singlet oxygen, superoxide and hydroxyl radicals, phenolic ingredients such as flavonoids, tannins and lichen phenolics such as depsides, depsidones, diphenyl ethers, polycyclic and monocyclic aromatic classes are classified as high-level antioxidants [34].

The present study was aimed at exploring the antioxidant potential, screening and characterization of the total phytochemicals of *Graphis scripta* and *Pyrenula nitida*. For this purpose, enzymatic (SOD and catalase) and non-

enzymatic antioxidant activities (DPPH and FRAP), reducing power, and total phenolic, flavonoid, tannin and terpene content of the extracts of both lichens were determined, in vitro. Tatipamula and Kukavica [35] examined *D. consimilis* with acetone (DA) and methanol (DM) extracts against DPPH assays. From their findings acetone extract of *D. consimilis* showed better IC50 values of 80.90 ± 6.42 mg/mL against DPPH compared to DM with 95.04 ± 6.36 mg/mL. As indicated in our findings, relatively maximal radical scavenging was measured in the methanol extract of *P. nitida* at 83.99% of RSA with an IC-50 62.90 ± 0.003 mg/ml and it was also rich in terms of total phytochemicals. From our previous findings indicated methanol extracts of *D. applanata* and *P. andium* have strong DPPH scavenging activity with IC50 i.e., 471.16 ± 0.85 µg/ml and 534.77 ± 0.75 µg/ml with acetone extract showing comparatively lower scavenging in both species [36]. Thus, present findings are in agreement with the experimental results of previous workers Manojlović et al.; Hawrył et al. [37, 38].

According to some researchers, complex ion-reducing power is usually a better indicator of the antioxidant capacity of extracts and purified compounds. Thus, the extracts, which had metal ion-reducing power, were considered a potential antioxidant source [15]. While Gordan [39] established antioxidant effect of reaction chain termination of the free radicals by the donation of electrons, the reduction of ferrous ion (Fe-3) to ferric ion (Fe-2) is measured qualitatively by the density of visibility (green–blue outline of solution) that measured by spectroscopically (absorbance at 700 nm). The reducing power of the methanol extract of *Cetraria islandica* may also indicate its potential antioxidant activity [40]. However, the reducing power of the methanol extract of *P. nitida* was higher as compared to other solvents (0.33 ± 0.054 nm). Earlier study reported methalonic extracts of *P. nitida* had strong ferric-reducing activity compared to *G. scripta*, which related to [36]. The reducing features are mainly linked to the presence of ionic reduction reactions. The outcomes provided here suggest that the extract's high ferric-reducing power activity is related to the presence of polyphenols (phenol and its derivatives), which may operate similarly to reductions in that they react with free radicals to convert them into stable products and terminate free radical chain reactions [41]. SOD and catalase estimation by native-PAGE analysis of *Pyrenula nitida* and *Graphis scripta* was used for the first time to perform qualitative enzymatic antioxidant analysis.

Multiple studies have found a link between lichen antioxidative activity and the quantity of phenolic compounds content [42]. Kosanić and Ranković [43] estimated the total phenolic and flavonoid constituents in *C. furcata* and *U. polyphylla* and *H. physodes* at 52.76 µg/ml of pyrocatechol equivalents (PE); however, higher phenolic contents were also found in methanol extracts of *C. furcata* and *U. polyphylla* with 52.67 and 52.65 µg/ml PE. But in the present study of total phenol estimated against gallic acid equivalent (GAE mg/g) using acetone, methanol, diethyl ether and benzene extract, maximal phenol content was reported in methalonic extracts of both lichens, *P. nitida* (0.511 ± 0.07 mg/g GAE) and *G. scripta* (0.682 ± 0.008 mg/g GAE), whereas benzene extract showed relatively lower content in both lichen species. Total flavonoid content was found in the acetone and methanol extracts of *U. polyphylla* (30.31 µg/ml of Rutin equivalents (RE) for acetone and 47.24 µg/ml for methanol extracts). Good flavonoid content was also found in the acetone and methanol extracts of the lichen *H. physodes* (30.09 µg/ml of RE for acetone and 32 µg/ml of RE for methanol extracts) as reported by [44]. The methalonic extract of both lichens reported a higher content of flavonoid *P. nitida* (0.049 ± 0.002 mg/g QE) and *G. scripta* (0.041 ± 0.03 mg/g QE), among other solvents. However, the quantification of total tannin against tannic acid equivalent (TAE mg/g) and total terpene against linalool equivalent (LE mg/g) was carried out for the first time and the relatively higher content of tannin and terpene recorded was methanolic extract of both lichens, i.e., *P. nitida* (0.47 ± 0.03 mg/g TAE and 0.252 ± 0.012 mg/ml LE) and *G. scripta* (0.32 ± 0.007 mg/g TAE and 0.192 ± 0.006 mg/ml LE); these estimations support the previous findings that methanol extract of *Trypethellium virens* SPTV02 and *Phaeographis dendritica* SPB041 quantified relatively maximum and acetone and diethyl ether contains moderate and benzene extract quantify lowest in all tested lichens [45].

Conclusions

In vitro antioxidant and phytochemical screening of both lichens showed high bioactive potential. GC–MS and FTIR analysis confirm the presence of active metabolites, including primary and secondary metabolites such as phenols, quinons, benzofurans, carboxylic acid, alkanes, aliphatic aldehydes, organic alcohol, fluorinated aliphatic

compounds, ketones, terpenes, and fatty alcohols, fatty acids, alcohols, hydrocarbons, fatty alcohols, terpenes, glycosides and sugar alcohols. As a result, this investigation deals with the use of lichen extracts as natural antioxidants. These lichens appear to be promising and alternate supply of synthetic antioxidant chemicals as well as a momentous source of polyphenols. Upcoming research should essence on isolating different bioactive phenolic compounds at the next step to assess their natural qualities in vitro and in vivo having a wider application in pharmaceuticals and cosmeceuticals.

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Author contributions

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Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

The authors declare no conflict of interest.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

$AlCl_3 \cdot H_2O$
Aluminum chloride
ANOVA
Analysis of variance
ATR
Attenuated total refraction
BHT
Butylated hydroxyl toluene
CAT
Catalase
OD
Optical density
DMSO
Dimethyl sulfoxide
DNA
Deoxyribonucleic acid
DPPH
1,1-Diphenyl-2-picryl-hydrazil
$FeCl_3$
Ferric chloride
FRAP
Ferric-reducing antioxidant power assay

FTIR

Fourier transform infrared spectroscopy

G. scripta

Graphis scripta

GAE

Gallic acid equivalent

GC–MS

Gas chromatography–mass spectrometry

H_2O_2

Hydrogen peroxide

IC_{50}

Half-maximal inhibitory concentration

$K_2S_2O_8$

Potassium persulfate

$K_3Fe(CN)_6$

Potassium ferricyanide

KCl

Potassium chloride

LE

Linalool equivalent

Na_2CO_3

Sodium carbonate

$NaNO_2$

Sodium nitrite

NaOH

Sodium hydroxide

NIST

National Institute of Standards and Technology

P. nitida

Pyrenula nitida

PCR

Polymerase chain reaction

PO_4 buffer

Phosphate buffer

PUFAs

Polyunsaturated fatty acids

QE

Quercetin equivalents

ROS

Reactive oxygen species

SD

Standard deviation

SOD

Superoxide dismutase

TAE

Tannic acid equivalent

TCA

Trichloro acetic acid
TE buffer
Tris–EDTA buffer
TFC
Total flavonoid contents
TPC
Total phenolic content

Publisher's Note

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Assessment of potential drug–drug interaction knowledge, attitude, and practice among Egyptian hospital and community pharmacists: a cross-sectional multicenter study

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ABSTRACT (ENGLISH)

Background

Pharmacists have an important role in preventing prescribing errors and providing appropriate information. They can detect potential drug–drug interactions (DDIs), which are associated with a more extended hospital stay and higher medical costs that lead to substantial financial burdens on healthcare systems. This study aimed to evaluate and assess the knowledge of community and hospital pharmacists toward drug–drug interaction and their attitude and motivation to find DDI information, in addition to identifying the pharmacist factors affecting this knowledge. A cross-sectional multicenter study was conducted using a self-administered questionnaire. Nineteen drug pairs, that are common in clinical practice, were evaluated. This study aimed to evaluate and assess the knowledge of community and hospital pharmacists toward drug–drug interaction and their attitude and motivation to find DDI information, in addition to identifying the pharmacist factors affecting this knowledge.

Results

A total of 4363 pharmacists (2260 community pharmacists and 2103 hospital pharmacists) have completed the survey. The participants' knowledge of DDIs was 58.25%, and there was no significant difference in pharmacist knowledge between community and hospital pharmacists ($p=0.834$). The highest correct answer was for sildenafil and isosorbide mononitrate pair 78.8%. The most used source of information was the internet or mobile applications, 47.1%. Participants who always considered PDDIs while prescribing detected more drug interactions than those who did not ($p=0.001$).

Conclusion

According to the findings of this study, community and hospital pharmacists had comparable knowledge of DDIs. However, before dispensing uncommon prescriptions, they should consult evidence-based drug information resources and DDI software to identify potential drug interactions.

FULL TEXT

Background

Drug–drug interactions (DDIs) can be defined as a clinical response to drug administration of a combination of two or more drugs that is different from the expected effects of the individual drugs when given alone [1]. DDIs are one of the medication errors that threaten patient safety as a result of pharmacodynamic or pharmacokinetic interaction between the administered drugs, which can lead to failure of treatment strategy or adverse effects or specific toxicity [2, 3]. The consequences of DDIs vary from minor to severe impacts that can be lethal to patients [4, 5]. The recrudescence of DDIs was found to be about 15–45% of hospitalized patients, with many studies linking DDIs with the increase in the length of hospital stay and healthcare costs [6, 7].

Chronic disease prevalence, polypharmacy, elderly, and cancer patients are all associated with an increased incidence of drug interactions [8–11]. The management of DDIs is a complex process that requires understanding the risk rating of DDIs mechanism, severity, and reliability and includes risk–benefit assessment [12, 13]

Computerized provider order entry (CPOE) combined with clinical decision support systems (CDSS) are being widely implemented to prevent adverse drug events (ADEs). Still, the effectiveness of these systems remains unclear and does not appear to avoid clinical ADEs reliably. In Egypt, most works identifying potential DDI depend on pharmacists' knowledge or the usage of online applications for drug–drug interactions. Methods for decreasing the possibility of drug interactions include the improvement of the knowledge of healthcare providers, developing systems for checking DDIs, and improvement of patient education regarding drug use [14].

Pharmacists play essential roles in preventing prescribing errors, providing appropriate information, and detecting potential DDIs. For example, community pharmacists can detect and prevent DDI in their pharmacies by detecting potential interactions and giving advice to patients [15]; in addition, published studies have reported that the rates of potential DDIs in hospitalized patients vary from 2.2% to 30%, so the high knowledge of DDIs among hospital pharmacists is essential in the reduction of DDIs complications that are associated with longer hospital stay [16]. In addition, exposure to potential DDIs can result in unnecessary healthcare costs; for example, a study revealed that the costs due to preventable ADRs in the USA and European countries range from €2,851 to €9,015 [17], and these medical costs represent a financial burden on healthcare systems [18, 19].

Despite drug information resources and online websites that are used to identify potential DDIs, the occurrence of possible DDIs is still high, which is usually due to different causes such as availability of pharmacist time, trustiness of patient to pharmacist, and cooperation between patient and pharmacist [20]. The studies of DDIs and their consequences in Egyptian community pharmacies [21] and hospitals [22, 23] are limited, and these interactions may be associated with severe adverse events. Pharmacists in community settings or hospitals play essential roles in DDI detection; however, their knowledge and attitude toward drug interactions do not appear to be studied sufficiently [24]. Most published studies evaluated the knowledge among prescribers [25].Our study aimed to evaluate and assess the knowledge of community and hospital pharmacists toward drug–drug interaction and their attitude and motivation to find DDI information, in addition to identifying the pharmacist factors affecting this knowledge.

Methods

Study design

This cross-sectional multicenter study was conducted using a self-administered questionnaire distributed with pharmacy students' aid between February 1, 2021, and June 30, 2021. Pharmacists working in a private or chain community pharmacy and Ministry of Health or university or private hospital pharmacy were included in the study; those in other sectors, such as industry and academia, were excluded.

Survey questionnaire and data collection

The DDI questionnaire was designed and developed from previous studies that assessed the knowledge of healthcare professionals about DDIs [5, 26, 27]. The structured questionnaire consisted of two sections. The first one was demographic data of the participant pharmacists, including pharmacists' educational level, age, gender, setting, and experience years. The second one contains 19 pairs of common drug interactions in clinical practice [24]. Pharmacists' knowledge was assessed as “No interaction, Contraindication, May be used together with monitoring, and Not sure.”

After finishing the questionnaire, participants were supplied with the correct answers to raise their awareness and knowledge about these potential drug–drug interactions. Participants were informed that their responses and the information was kept securely.

Outcome measures

The primary objectives were to assess the knowledge of community and hospital pharmacists toward potential drug–drug interactions (PDDIs) that reflect the quality of health systems in pharmacies, while the secondary outcomes were to determine the predictor factors impacting pharmacist knowledge of drug interactions.

Sample size calculations

According to Egyptian pharmacists' syndicate records, there are approximately 216,072 registered pharmacists in Egypt, with a confidence level of 97% and a margin of error of 5%. If 50% of the pharmacists would have good knowledge, the minimum acceptable sample size is 470 participants.

Statistical analysis

The data were analyzed using SPSS software version 24 for Analysis. For descriptive Analysis, results were presented as frequencies and percentages. Mann–Whitney test was used for independent nonparametric data. A linear regression model was used to determine the potential predictors of (potential drug–drug interactions) PDDIs knowledge, including participant's age, education, settings, years of practice, and attitude toward PDDIs. For all tests, $p<0.05$ was considered statistically significant.

Results

Characteristics of the participants

A total of 4363 pharmacists (2260 community pharmacists and 2103 hospital pharmacists) have completed the survey. As shown in Table 1, the majority (58.4%) aged from 20 to 29 years old. Most (61.1%) had bachelor's degrees, and 69.1% of the participants were from urban regions. About 79.6% of the participants had practiced up to 10 years. There is no significant difference in age and years of practice between community and hospital pharmacists as shown in Table 2.

Table 1. Frequencies and percentage of the demographic characteristics of the study participants ($n=4363$)

Character	Number of participants (%)
Age	
20–29	2550 (58.4)
30–39	1287 (29.5)

40–49	405 (9.3)
≥50	121 (2.8)
Gender	
Male	2198 (50.4)
Female	2165 (49.6)
Education	
Bachelor	2666 (61.1)
Postgraduate certificate holders	453 (10.4)
Master	370 (8.5)
Pharm D	426 (9.8)
Board-certified pharmacists	91 (2.1)
PhD	357 (8.2)
Geographic region	
Rural	1347 (30.9)
Urban	3016 (69.1)
Years of practice	
5<	2178 (49.9)
05-Oct	1296 (29.7)
Oct-15	542 (12.4)
15–20	199 (4.6)
>20	148 (3.4)
Settings	
Community pharmacists	2260 (51.8)

Hospital pharmacists	2103 (48.2)

Table 2. Comparison of the demographic characteristics of community and hospital pharmacists

Character	Mean rank (community pharmacist)	Mean rank (hospital pharmacist)	*p* value
Age	2165.35	2199.89	0.304
Gender	2096.62	2273.75	0.00 (<0.05)*
Education	1925.94	2457.17	0.00 (<0.05)*
Geographic region	2097.77	2272.52	0.00 (<0.05)*
Years of practice	2178.99	2185.23	0.859

Mann–Whitney test was used

*For this test, $p<0.05$ was considered statistically significant

Knowledge of pharmacists of DDIs

The participant's knowledge of DDIs was 58.25% (average of correct answers about DDIs). Table 3 summarizes participants' responses to the DDI questions, presenting the frequencies (percentages) of respondent answers for each of the 19 drug pairs. The lowest correct answer was between alprazolam and itraconazole ($n=778$, 17.8%). In contrast, most participants answered the remaining 18 drug pairs questions correctly, and the most correct answer was for sildenafil and isosorbide mononitrate pair ($n=3438$, 78.8%).

Table 3. Frequencies and percentages of participants' response to potential DDIs

Drug–drug interaction pairs	No interaction	Used with monitoring	Contraindication	Not sure
n (%)	*n* (%)	*n* (%)	*n* (%)	Acetaminophen/codeine and amoxicillin
3395 (77.8)	580 (13.3)	167 (3.8)	22 1 (5.1)	Warfarin and sulfamethoxazole/trimethoprim
403 (9.2)	**2432 (55.7)**	1241 (28.4)	287 (66)	Warfarin and digoxin
2431 (55.7)	887 (20.3)	794 (18.2)	251 (5.8)	Digoxin and amiodarone

353 (8.1)	**2240 (51.3)**	1434 (32.9)	336 (7.7)	Cyclosporine and rifampicin
718 (16.5)	**2147 (49.2)**	1014 (23.2)	484 (11.1)	Digoxin and itraconazole
447 (10.2)	**2552 (58.5)**	942 (21.6)	422 (9.7)	Digoxin and sildenafil
2884 (66.1)	457 (10.5)	768 (17.6)	254 (5.8)	Simvastatin and itraconazole
359 (8.2)	716 (16.4)	**2906 (66.6)**	382 (8.8)	Sildenafil and isosorbide mononitrate
262 (6)	363 (8.3)	**3438 (78.8)**	300 (6.9)	Conjugated estrogens and raloxifene
2224 (51)	681 (15.6)	760 (17.4)	698 (16)	Theophylline and ciprofloxacin
522 (12)	**2119 (48.6)**	1339 (30.7)	383 (8.8)	Pimozide and ketoconazole
371 (8.5)	594 (13.6)	**2876 (65.9)**	522 (12)	Warfarin and Fluconazole
412 (9.4)	**2529 (58)**	1054 (24.2)	368 (8.4)	Alprazolam and itraconazole
413 (9.5)	326 (7.5)	**778 (17.8)**	2846 (65.2)	Digoxin and clarithromycin

404 (9.3)	**2502 (57.3)**	1087 (24.9)	370 (8.5)	warfarin and sulfinpyrazone
443 (10.2)	**2519 (57.7)**	795 (18.2)	606 (13.9)	Dopamine and phenytoin
567 (13)	**2388 (54.7)**	995 (22.8)	413 (9.5)	Fexofenadine HCL and metoprolol
3025 (69.3)	453 (10.4)	349 (8)	536 (12.3)	Itraconazole and quinidine

The boldness indicates the correct ones

As shown in Table 4, the highest frequency of correct answers among the community pharmacists was 17 questions, and 0.8% of the community pharmacists answered all the questions incorrectly. However, the highest frequency of correct answers among the hospital pharmacists was 13 questions, and 0.9% answered all 19 questions incorrectly. None of the pharmacists knew all the 19 questions correctly in both groups. The mean rank of the sum of correct answers for the community and hospital pharmacists was 2178.14 and 2186.14, respectively, with a *p* value of 0.834 (the difference in knowledge between community pharmacists and hospital pharmacists was nonsignificant).

Table 4. Frequencies and percentages of the community pharmacists and hospital pharmacists correct answers

Number of correct answers	Frequency (%) (community pharmacist)	Frequency (%) (hospital pharmacy)
0	18 (0.8)	19 (0.9
1	25 (1.1)	13 (0.6)
2	54 (2.4)	36 (1.7)
3	79 (3.5)	57 (2.7)
4	84 (3.7)	85 (4)
5	102 (4.5)	108 (5.1)
6	139 (6.2)	123 (5.8)
7	134 (5.9)	127 (6)
8	142 (6.3)	153 (7.3)

9	151 (6.7)	143 (6.8)
10	182 (8.1)	176 (8.4)
11	194 (8.6)	137 (6.5)
12	164 (7.3)	166 (7.9)
13	145 (6.4)	180 (8.6)
14	140 (6.2)	124 (5.9)
15	138 (6.1)	126 (6)
16	143 (6.3)	173 (8.2)
17	221 (9.8)	153 (7.3)
18	5 (2)	4 (2)
19	0 (0)	0 (0)
Total	2260 (100)	2103 (100)

Source of potential DDI information

The sources of DDI information are shown in Fig. 1. The internet or mobile applications were the most used source of information ($n=2057$, 47.1%). The least commonly used sources were knowledge bases in Arabic and package inserts (2.2% and 1.2%, respectively).

Fig. 1 [Images not available. See PDF.]

Source of PDDIs information

Attitude toward potential DDIs

As shown in Fig. 2, about 86.98% of the participants consider DDIs when prescribing, with 48.38% agreeing with the statements and 38.60% strongly agreeing, respectively, and only about 1.5% do not consider it when prescribing. More than 88% of participants said that DDI information is essential for their practice. In addition, more than 85% always check DDI when unsure about it, and about 80% are willing to learn about it.

Fig. 2 [Images not available. See PDF.]

Participants attitude toward PDDIs

Predictors of PDDIs knowledge

As shown in Table 5, the linear regression model indicates that significant predictors of a higher number of recognized drug pairs were age, education, and attitude toward PDDIs. Participants who always considered PDDIs while prescribing detected more drug interactions than those who did not ($p=0.001$). In addition, those who did not check PDDIs when not sure about it had lower scores for PDDIs than those who did ($p<0.001$).

Table 5. Predictors of the knowledge level of the study participants for PDDIs

Character	Unstandardized β coefficient	*p* value
Constant	6.252	<0.001
Age	0.297	0.034*
Geographic region	0.145	0.308
Education	−0.121	0.004*
Setting	−0.146	0.28
Years of practice	0.052	0.618
Attitude I always consider PDDIs while prescribing	0.351	0.001*
I think PDDIs information is important for my practice	0.145	0.204
I always check PDDIs when I am not sure about it	0.379	<0.001*
I am willingness to learn more about PDDIs	0.09	0.356

Linear regression test was used

*For this test, $p<0.05$ was considered statistically significant

Discussion

The recognition of interacting drugs is critical for any healthcare providers, including pharmacists, to decrease the DDIs and, consequently, reduce the drug-related morbidity and mortality that may occur as a result of these interactions [5, 28]. Although thousands of articles on drug interactions have been published and numerous computerized screening systems have been developed, patients continue to suffer from adverse drug interactions. It was found that about twenty percent of the adverse drug effects in the developed countries, which are responsible for more than 700,000 deaths, are due to drug–drug interactions [14]. Possible methods for reducing the risk of drug interactions include improving healthcare providers' knowledge, improving computerized screening systems, providing information on patient risk factors, increasing pharmacogenetic information, more attention to drug administration risk factors, and improving patient education on drug interactions.

In this survey, we assessed the ability of pharmacists to recognize clinically significant drug combinations. It was found that among DDI information sources, internet or mobile applications and medical textbooks were the most used sources of information. The majority of the participants (47.1%) tended to receive information regarding DDIs from electronic sources, which is consistent with a previous study conducted in Iran [29]. The possible explanation for this finding may be the high percentages of young participants in the present study, and people in this age group are usually interested in technology and use it in many fields. A small percentage of the participants in our survey (1.2%) reported that they use package inserts, which is a risk factor for incorrect use of drugs [30].

Our study showed no significant differences in the proportion of community pharmacists and hospital pharmacists who correctly answered the same number of questions about DDIs. The level of the participants' knowledge of DDIs was 58.25% (average of correct answers about DDIs), and this finding is comparable to another study (53.3%) [31]. However, our results are not consistent with another study [24] that revealed a level of knowledge among

pharmacists of about 37.3%, but the later study included 26 drug pairs; however, our study included 19 pairs, and these differences in drug pairs may be the cause of the differences in the level of the participant's knowledge on DDIs.

Among the drug pairs selected to assess DDIs knowledge, sildenafil, and isosorbide mononitrate were the most highly recognized drug pairs (78.8%), which is consistent with another study [32]. The lowest recognized pairs were alprazolam and itraconazole (17.8%), which are contraindicated with each other. In accordance with another study [5], even if one justified that the drug combinations classified as contraindicated could be used with close monitoring and considered both choices to be correct, up to 65% of the participants remained unsure if there was a potential interaction or not. In our study, we tried to investigate the predictors of DDI knowledge of participants, including age, education, setting, years of practice, and the participants' attitudes toward DDIs. It was found that a significant correlation exists between participants' age and their knowledge level in DDIs. The older participants answered more DDI questions than younger ones, which is consistent with another study that used identical drug pairs as our study [33]. Another study, in contrast to ours, found no connection between age and DDI knowledge level [34], but this may be explained as the later study involved participants of relatively the same age.

Another predictor of DDI knowledge is the education level, which was found to have a significant correlation with the level of knowledge of DDIs. Unexpectedly, participants with bachelor's and postgraduate certificate holders recognized a higher number of interactions than did those with Ph.D. and board-certified pharmacists, and this finding was in accordance with a study carried out in Khartoum state and showed that pharmacists with bachelor's recognized higher number of DDIs than those with master [31]. The results of our study were in contrary to a previous study [33] that reported that participants' education level did not affect the knowledge level of DDIs. These findings could be due to several factors, including the fact that Ph.D. holders are not recent graduates and may not have as good recall of DDIs, as well as the fact that the majority of pharmacists have bachelor's or postgraduate diploma degrees rather than PhDs and board certifications.

Interestingly, the number of years of experience of the participants was not a significant predictor of DDI knowledge level, and this is consistent with other studies that used different drug pairs for DDI knowledge assessment [5, 31].

Regarding attitudes toward DDIs and their relation to the level of DDI knowledge, it was found that participants who always consider DDIs while prescribing and checking about DDIs when not sure about them recognized a more significant number of DDIs than those who did not, and these results are consistent with previous study conducted in China [33]. This indicates the strong association between the participants' tendency to check references and their knowledge of PDDIs, as proved by the correct recognition of the drug pair interactions.

Astonishingly, this study revealed no significant association between setting (whether community or hospital pharmacists) and the level of their knowledge of DDIs.

The findings of the present study have several implications for practice. This article highlights an important issue that requires urgent attention in Egypt which is the improvement of drug–drug interaction knowledge among community and hospital pharmacists. It is crucial for improving patient safety and healthcare outcomes in the country to ensure the rational and optimal use of drugs. By raising awareness of this issue and identifying potential solutions, this study makes an important contribution to the field of healthcare in Egypt and beyond. Our study has identified some recommendations to improve the knowledge and practice of hospital and community pharmacists regarding DDIs. Based on the study's findings, it is recommended that continuing education and training programs should be developed for hospital and community pharmacists in Egypt to improve their knowledge of DDIs. The Egyptian Ministry of Health should develop guidelines and protocols for the management of DDIs in hospitals and community pharmacies to ensure consistency in practice. Additionally, these pharmacies should have access to electronic databases that provide up-to-date information on DDIs to support their practice. This study highlights the need for ongoing education and training programs, updated guidelines, and increased resources to support pharmacists in their efforts to provide safe and effective care to patients. Finally, future studies should be conducted to assess the impact of education and training programs on hospital and pharmacists' knowledge and practice regarding DDIs.

The limitation of our study is that the 19-drug pairs might not be adequate to reflect the extent of knowledge

applicable to the vast number of PDDIs. In addition, the study's sample size may limit the generalizability of the findings. The sample size is small or not representative of the entire population of hospital and community pharmacists in Egypt, so, the results may not accurately reflect the overall knowledge level of pharmacists in the country. Additionally, the study's reliance on self-reported data from pharmacists introduces the possibility of response bias. Participants may overestimate their knowledge to present themselves in a more favorable light or may underreport their knowledge about DDIs due to various reasons, such as social desirability bias. Furthermore, the study focuses solely on assessing the pharmacists' knowledge without considering other factors that may influence their ability to apply that knowledge in practice, such as time constraints, workload, or access to resources. In addition, future studies of larger sample sizes of pharmacists and more drug pairs are required to face the challenges and limitations of this study taking into consideration other factors such as time constraints, workload, or access to resources that may affect their ability to apply that knowledge in practice,

Conclusion

According to our study findings, community and hospital pharmacists had comparable knowledge of DDIs. Pharmacists should improve their knowledge of drug–drug interactions (DDIs) to ensure patient safety. Also, they should consult evidence-based drug information resources and DDI software to identify potential drug interactions before dispensing prescriptions. Developing a system for checking DDIs is necessary.

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Author contributions

Study conception and design were done by EAW, MA, and AE. Data analysis and interpretation were performed by AEAW, AE, YSAD, and EMS. EAW, AEAW, MA, AE, and EMS helped in drafting, revision of the paper, and final approval of the published version.

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Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on a reasonable request.

Declarations

Ethics approval and consent to participate

All procedures performed in the study involving human participants were in accordance with the ethical standards of Faculty of pharmacy October 6 University ethics committee and with the 1964 Declaration of Helsinki and its later amendments. The study was received approval from the Ethics Committee and institutional review boards of October 6 University (Approval Number: PR-Ph-2112005). Pharmacist's participation in this survey was voluntary. Informed consent was obtained from all participants. They were asked before questionnaire if they participate in this study, and they able to refuse take part in the research or exit the survey at any time without penalty. Pharmacists were only required to complete the questionnaire once. Participants informed that their personal details were not linked to their responses and the information were kept securely.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

PDDIs

Potential drug–drug interactions

DDIs

Drug–drug interactions

CPOE
Computerized provider order entry
CDSS
Clinical decision support systems
ADEs
Adverse drug events

Publisher's Note

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DETAILS

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A unique revolutionary eco-friendly spectrophotometric technique for solving the spectral overlap in the determination of carvedilol and ivabradine in their binary combination: stability study

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ABSTRACT (ENGLISH)

Background

Carivalan® dosage form containing carvedilol and ivabradine is widely indicated for patients with stable angina. Three precise, eco-friendly and reproducible spectrophotometric techniques were created for resolving the researched drugs in their mixtures using zero and/or ratio spectra. Technique I is a factorized dual wavelength coupled with spectrum subtraction (FDW-SS), technique II is a ratio difference, and technique III is a constant center coupled with spectrum subtraction (CC-SS). Moreover, CAR and IVA were simultaneously determined in the existence of their oxidative degradation products exploiting the newly developed induced tripartite amplitude difference coupled with ratio subtraction (ITAD-RS) technique.

Results

The calibration curves for CAR and IVA showed linearity within 3.0–30.0 µg/ml, each. Techniques' precision, accuracy, and linearity ranges were resolved and validated in harmony with ICH guidelines. Additionally, the specificity was examined by examining created combinations of the proposed drugs with LOD of 0.258 and 0.290 for CAR, while for IVA 0.272 and 0.204.

Conclusion

These techniques were used to determine the presence of the provided drugs in Carivalan® tablets. There is statistical comparison between the found results of the offered spectrophotometric techniques and the previously reported ones with no discernible variance in the acquired results.

FULL TEXT

Background

Carvedilol (Fig. 1a) is chemically [1-(9H-carbazol-4-yloxy)-3-[[2-(2methoxyphenoxy) ethyl] amino] propan-2-01]. It is a non-selective beta-blocker which works by reducing heart beats and facilitating smoother blood circulation. Additionally, it widens some blood arteries, acting similarly to an alpha blocker, which in turns helps in lowering the

blood pressure [1, 2].

Fig. 1 [Images not available. See PDF.]

Chemical structures of **a** carvedilol and **b** ivabradine

Ivabradine (Fig. 1b) is chemically (3-[3-[[(7S)-3,4-dimethoxy-7-bicyclo[4.2.0]octa-1,3,5-trienyl]methyl-methylamino]propyl]-7,8-dimethoxy-2,5-dihydro-1H-3-benzazepin-4-one). It is a drug that lowers heart rate by precisely and selectively blocking the cardiac pacemaker current; (I_f) and hence regulates the heart rate via direct sinus node inhibition without affecting myocardial contractility or blood pressure [3, 4].

Carivalan® containing CAR and IVA has received a widespread indication for treating the symptoms of stable angina, where few analytical HPTLC [5] and HPLC [6, 7] techniques for analyzing CAR and IVA have been stated. In the meantime, their simultaneous determination was achieved spectrophotometrically by only one reported research [8], while no spectrophotometric techniques have been previously conveyed for the declared drugs' resolving in the occurrence of their degradation products.

It is well known that the chromatographic analytical technique, which is distinguished by its superior accuracy and reproducibility in the process of samples' separation and analysis in parts per million (PPM) level as well as its speed and automation [9], is one of the most well-liked and frequently used techniques in the field of drug analysis. However, and particularly in light of the current conditions faced by the majority of the world's nations, especially developing nations, many analysts and researchers now turn to the usage of less expensive analytical techniques while keeping accuracy and precision.

The UV spectrophotometric approach is at the forefront of the methodologies that achieve most researchers' objectives because of its ease of use, low implementation cost besides widespread accessibility in laboratories for quality control [10].

This work focuses on the creating and validation of three green UV spectrophotometric techniques for the selective resolution of CAR and IVA in their pure forms and Carivalan® tablets by utilizing zero and/or ratio spectra; FDW-SS, RD [11–13] and CC-SS techniques [13–15]. Furthermore, induced tripartite amplitude difference (ITAD), a newly developed spectrophotometric technique, was able to solve the issue of resolving and analyzing CAR and IVA in their quaternary mixture with their oxidative degradates and succeeded in determining them without even requiring any preliminary separation steps. Green assessment of the offered, official and the reported techniques was achieved by using Analytical Eco-Scale [16], National Environmental Methods Index (NEMI) [17], Green Analytical Procedure Index (GAPI) [18] and Analytical Greenness Metric (AGREE) [19]. The proposed techniques' suitability for the intended usage has been validated in accordance with ICH criteria [20].

Factorized dual wavelength coupled with spectrum subtraction technique (FDW-SS)

This technique depicts coupling between dual wavelength [21–24] and spectrum subtraction [24–26] for analyzing CAR and IVA in their mixture, where one drug X shows a noteworthy absorbance difference at two selected wavelengths which is directly proportional to its concentrations. While the other interfering drug *Y* shows equal absorbance reading with an insignificant absorbance difference at the same selected wavelengths (ΔA=zero), thus its effect is negligible. Recovering the parent D^0 of *X* is accomplished by firstly preparing *X*'s factorized $\triangle A$ spectrum using computer software via dividing (D^0) *X* at any concentration within Beer's law by the ΔA between the chosen wavelengths; X(D◦)ΔA.

Secondly, $\triangle A$ is recorded in the laboratory mixtures between the chosen wavelengths then multiplied by *X*'s factorized $\triangle A$ spectrum.

1

ΔA.X(D◦)ΔA=RecoveredD◦ofX

While D^0 of the interfering drug *Y* is obtained via spectrum subtraction [25–27] technique; $D^0\ (X+Y)-D^0\ (X)$.

Induced tripartite amplitude difference coupled with ratio subtraction technique (ITAD-RS)

An advanced technique that can be used on a ratio spectrum of quaternary mixtures made up of X+Y+Z+WW′ at selected wavelengths at which component X has a ΔP noteworthy value and the amplitude readings of *Y* are not

equal ($\Delta P \neq 0$) at the same chosen wavelengths.

Meanwhile, component *Z* exhibits an equal amplitude value with a zero difference ($\Delta P = 0$); thus, the effect of *Z* is canceled. It is a moderation of both induced dual-amplitude difference [27] and dual-amplitude difference [28] techniques.

The effect of *Y* was canceled via calculating the equality factor [13, 29] of *Y* by getting average Py1/Py2 at the suggested wavelengths (FY). Component *Y* will be canceled by calculating the difference. As a result, *P* (FyPm1–Pm2) is only connected to *X*.

A factorized induced ratio spectrum of X ;X/W′ΔP.Fy is set via division of the ratio spectrum of X by the calculated induced amplitude difference.

For laboratory mixtures, the induced amplitude difference values (ΔP. Fy) at the suggested wavelengths are noted and multiplied by X's factorized induced ratio spectrum to acquire X's ratio spectrum.ΔP.Fy.XW′ΔP.Fy=XW′

Multiplying the recovered ratio spectrum of *X* by the divisor, *W*′, will attain the parent D^0 of X; XW′×W′=X(D0)

Finally, subtracting the regained D^0 of *X* from the quaternary mixture's D^0 spectrum and applying the same previously mentioned steps, the other components in the mixtures will be determined.

Exploiting the factorized induced ratio spectrum and the divisor's spectrum multiplication in this newly developed method allows the mentioned component's determination at its λ_{max} through minimal signal output analysis of data. In addition, random error is highly minimized since the preparation of factorized spectrum is founded on the response only in contrast to normalized spectrum which is based on concentration.

Methods

Instrumentation

Shimadzu double-beam spectrophotometer (UV, Japan), Sonicator (Model 3510, UK), Analytical Balance, Italy, Digital Stirring Hot Plate, China, Pure laboratory flex to obtain distilled water

Silica gel F254 (20 × 20 cm) pre-coated TLC plates (Sigma-Aldrich, Germany). The degradation tracing and plate visualization were carried out using a UV lamp with a wavelength of 254.0 nm.

Reagents and chemicals

Pure samples of CAR and IVA were kindly obtained from Global Napi, 6th of October, Egypt. The pureness was certified to be 99.88 ± 0.95 and 99.60 ± 1.06, respectively, depending on their official [1] and reported [8] techniques. The market-available Carivalan® tablet dosage form, made in France by Les Laboratories Servier Industrie, was purchased. According to the product label, Carivalan ® batch number (29,044) contains 12.5 mg of CAR and 5.0 mg of IVA. 30% H_2O_2 (ADWIC, Egypt).

Standard solutions

To prepare stock solutions of carvedilol and ivabradine (100.0 µg/ml), in volumetric flasks, 100 ml, 10 mg of each of CAR and IVA was dissolved separately in distilled water.

In two rounded flasks, 10 mg of each of CAR and IVA was refluxed with 10 ml of 30% H_2O_2 for 8 h at 80 °C. After refluxing, hydrogen peroxide was evaporated using hot plate at 50 °C, transfer oxidative degraded product to volumetric flask, 100 ml, which is completed with distilled water to prepare standard solutions of 100.0 µg/ml for each.

Laboratory mixtures

Exact amounts of the stated drugs were conveyed into different volumetric flasks, 10 ml, with varied ratios and topped off with distilled water to produce variable sets as follows:

Set A: Binary mixture containing CAR and IVA.

Set B: Quaternary mixture containing CAR and IVA along with their oxidative degradation products.

Procedures

Stress stability studies

Studied drugs' stress studies were conducted as per ICH guidelines [30] by applying different stress acidic, basic, photolytic, thermal and oxidative surroundings. Different molar concentrations of HCl and NaOH were tried out where, in each trial, 10 ml of each of the investigated medications was combined individually with either HCl or

NaOH. Reflux was applied at 100 °C for 3 h.

To conduct oxidative trials, 10 mg of each medication was refluxed individually in 10 ml of 10, 20 and 30% H_2O_2 for 8 h at 80 °C.

Drugs' photostability was tested on their solid powdered form by the exposing them to UV light, 254.0 nm for 10 h.

Finally, drugs' thermal stress testing was performed in glass ampoules by heating them in a thermostatic oven at 10 °C boosts (50–100 °C) for 10 h.

Spectral characteristics

D^0 spectra of each examined drugs were measured at 200.0–400.0 nm.

Construction of calibration graphs

To prepare calibration standards across concentrations of 3.0–30.0 µg/ml for CAR and IVA, from each standard stock solution (100.0 µg/ml), an accurately transferred amounts were conveyed to separate series of volumetric flasks, 10 ml, and finalized with distilled water. The D^0 spectra acquired after scanning solutions between 200.0 and 400.0 nm were recorded. The calibration graph for each suggested technique was performed via the mean of three experiments, as shown below:

Factorized dual wavelength coupled with spectrum subtraction technique (FDW-SS)

The regression equations were created by plotting the stored D^0 maximum absorbance of CAR and IVA at 285.6 and 287.0 nm, respectively, against the associated concentrations. A factorized ∆A spectrum for IVA was constructed using spectrophotometric software by division of D^0 of a certain concentration of IVA by (∆A) at 285.2 nm and 255.0 nm.

Ratio difference technique

The ratio spectra were produced via division of the scanning spectra of the CAR and IVA solutions by the absorption spectra of the IVA (15.0 µg/ml) and CAR (15.0 µg/ml) standard solutions, respectively. CAR's and IVA's concentrations were plotted against their respective amplitude differences at 252.6 and 275.0 nm and 294.0 and 320.0 nm, respectively.

Constant center coupled with spectrum subtraction technique (CC-SS)

The regression equations have been calculated by setting two calibration curves. The first one is between the maximum absorbance of D^0 of CAR and IVA at 285.6 nm and 287.0 nm and their related concentrations, respectively. The second one is between the ΔA at 294.0 nm and 320.0 nm versus amplitudes at 294.0 nm for IVA after generating a ratio spectrum by dividing of the stored absorption spectra of IVA by the absorption spectrum of CAR; 15.0 µg/ml.

Induced tripartite amplitude difference coupled with ratio subtraction technique (ITAD-RS)

The regression equations were computed by plotting the maximum absorbance of the stored D^0 of CAR and IVA at 285.6 nm and 287.0 nm versus their corresponding concentrations, respectively.

The equality factor of IVA ratio spectra was calculated by using amplitude ratios' average at 296.0 nm ($P_{296.0}$) and 242.7 nm ($P_{242.7}$) using CAR's oxidative degradate spectrum as a divisor.

Via dividing the ratio spectrum of a certain concentration of CAR using CAR's oxidative degradate's spectrum as a divisor by ΔP at 242.7 nm and 296.0 nm after multiplying the former by F, ($FP_{242.7}$–$P_{296.0}$), factorized induced ratio spectrum for CAR was created.

Through the division of the ratio spectrum of IVA using CAR's oxidative degradate's spectrum as a divisor by the value of the ∆P at 242.7 nm and 296.0 nm, factorized ratio spectrum for IVA was created.

Laboratory mixtures' analysis

Different mixtures in different sets, set A (binary mixture of CAR and IVA) and set B (quaternary mixture of CAR and IVA with their oxidative degradates), were analyzed by carrying out the following manipulation actions for each method:

Analysis of set A containing CAR and IVA in their binary mixtures

Factorized dual wavelength coupled with spectrum subtraction technique (FDW-SS)

The D^0 of IVA was obtained by noting ΔA at 285.2 nm and 255.0 nm and multiplying by IVA's factorized ΔA

spectrum in the prepared laboratory mixtures of CAR and IVA. CAR's D^0 spectrum was resulted from the subtraction of IVA's D^0 spectrum from the spectra of laboratory mixture.

Ratio difference technique

The D^0 of binary laboratory mixture in set A was divided separately by the absorption spectrum of standard IVA' and CAR' (15.0 µg/ml, each). The amplitudes of the obtained ratio spectra were noted at 252.6 and 275.0 nm for CAR and 294.0 and 320.0 nm for IVA. The concentrations of CAR and IVA were resolved from their related regression equations.

Constant center coupled with spectrum subtraction technique (CC-SS):

Via employing the formerly manipulated ratio spectra in RD method, the following steps were employed: Firstly, IVA's ratio spectrum in each mixture was recorded at 294.0 nm and 320.0 nm using CAR's D^0 as a divisor. Secondly, the postulated amplitude at 294.0 was acquired from the correspondent related regression equation followed by subtracting the postulated amplitude from the recorded one to obtain a constant value; CAR/CAR'. Finally, via multiplying the constant value by the spectra of 15.0 µg/ml standard CAR', the parent D^0 of CAR was obtained in which its concentration might be determined using the matching regression equation built at its maxima. As a result, the D^0 of IVA could be calculated via subtraction of the acquired spectra of CAR from the spectra of its related mixtures' spectra. The concentration of IVA was estimated using the relevant regression equation.

Analysis of set B containing CAR and IVA with their oxidative degradates in their quaternary mixtures

Induced tripartite amplitude difference coupled with ratio subtraction technique (ITAD-RS)

CAR/CAR's degradate ratio spectrum in mixtures of set B was acquired after multiplication of CAR's factorized induced ratio spectrum by ($FP_{242.7}-P_{296.0}$) of each mixture's ratio spectrum. After multiplication of the former ratio spectrum, CAR/CAR's degradate by the D^0 spectrum of the CAR oxidative degradates, CAR's D^0 was calculated. The ratio spectrum comprising, IVA + IVA's degradate + CAR's degradate/CAR's degradate was acquired by subtraction of the obtained ratio spectrum; CAR/CAR's degradate from that of quaternary mixture.

For IVA, its ratio spectrum in the combination can be acquired by multiplication of ΔP at 242.7 nm and 296.0 nm by the formerly set IVA's factorized ratio spectrum. IVA's parent D^0 was acquired after multiplication of the acquired ratio spectrum by the spectrum of the CAR oxidative degradate.

CAR and IVA concentrations were figured using their respective regression equations generated at their maxima.

Application to pharmaceutical formulation

Five Carivalan® tablets were crushed and blended together. Amounts equivalent to 25.0 mg CAR and 10.0 mg IVA were precisely moved to beaker, 100 ml, dissolved in 30 ml distilled water, sonicated and filtered into a volumetric flask, 100 ml, then accomplished with distilled water.

Further dilutions were proceeded to produce concentrations of 10.0 µg/ml for CAR and 4.0 µg/ml for IVA. The proposed techniques were used for the analysis of the examined Carivalan® following the described measures under laboratory-prepared mixtures analysis to compute their concentrations utilizing the related regression equation for each proposed drug.

Results

As a result of the current global conditions that most countries are going through either economically or practically, the main movement has become toward using analytical techniques at the lowest costs and in the easiest and most accurate ways at all once.

This was the main reason behind the resort to the use of spectrophotometric technique in drugs' analysis, as it proved accuracy, reliability and simplicity among the rest of the other analytical techniques as well as its availability in most quality control laboratories.

Unfortunately, the presence of extremely overlapped spectra made several challenges to use direct absorbance measurements in zero-order spectra, which led to numerous obstacles during spectrophotometric analysis, particularly for multicomponent mixtures.

Therefore, the primary objective is to concentrate on creating highly advanced eco-friendly spectrophotometric techniques that enable both the researcher and the analyst to explore complex mixtures and to overcome any

challenges that may arise.

Carvedilol and ivabradine were chosen as a model for demonstrating the successful application of new spectrophotometric techniques for their analysis, either in their binary or in their quaternary mixture with their oxidative degradation products without the need for pre-separation procedures.

Complete CAR's and IVA's oxidative degradation was observed. Quite the reverse, they conveyed relative acidic, basic, photolytic and thermal stability. Samples were frequently taken out to track the drugs' degradation; for the drugs in question, total oxidation was verified by the disappearance of the TLC spot using TLC aluminum plates. CAR and IVA along with their oxidative degradation products were UV scanned and found to be highly overlapped in the UV region 200.0–340.0 nm, Fig. 2. Direct measurement of the studied drugs was not possible. Thus, two sets were constructed, one for determining CAR and IVA in their binary mixture (set A) exploiting zero or ratio spectra through the application of FDW-SS, RD and CC-SS methods [15], while the other set (set B) was created for the proposed drugs' determination in the presence of their oxidative degradates exploiting the innovative induced tripartite amplitude difference together with ratio subtraction (ITAD-RS) spectrophotometric technique.

Fig. 2 [Images not available. See PDF.]

Zero-order spectra of carvedilol, ivabradine and their oxidative degradation products (10.0 µg/ml, each)

Set A: CAR and IVA in their binary mixture:

An overlapped (D^0) spectrum was observed for CAR and IVA. Using suitable recommended techniques, simultaneous determination of the considered drugs was performed.

Factorized dual wavelength coupled with spectrum subtraction technique (FDW-SS)

This suggested technique was used to analyze CAR and IVA in their binary mixture via picking two wavelengths (285.2 nm and 255.0 nm) where the ΔA is proportional to IVA concentration versus a zero ΔA for CAR at the selected wavelengths, as shown in Fig. 3a.

Fig. 3 [Images not available. See PDF.]

Spectral scheme of resolution of 10.0 µg/ml of each CAR (red line) and IVA (black line) using DW-SS in set A

Figure 3b shows how the original (D^0) of IVA was acquired from each combination after noting (ΔA) value at 285.2 nm and 255.0 nm and multiplying this value by IVA's factorized ΔA spectrum.

Subtraction of IVA's (D^0) spectrum from (D^0) spectrum of the mixture yielded CAR's parent (D^0) spectrum, Fig. 3**c**.

Via relieving the recorded absorbance values at the maxima of each of CAR and IVA in their corresponding regression equations, each drug's concentration was calculated, Table 1**.**

Table 1. Assay parameters and results of determination of pure samples of CAR and IVA by the proposed methods

	CAR				IVA		
RD	FDW-SS	CC-SS	ITAD-RS	RD	FDW-SS	CC-SS	ITAD-RS
Linearity (µg/ml)	3.0–30.0				3.0–30.0		
Slope	0.6057	0.0408			0.43	0.0379	
Intercept	0.0044	0.0017			0.025	0.0173	
Correlation coefficient (r)	0.9998	0.9998			0.9999	0.9999	

Accuracy(Mean ± SD)	99.38 ± 0.92	98.45 ± 0.851	99.47 ± 1.41	99.15 ± 0.874
*RSD%[a]	0.791	1.244	0.823	1.202
**RSD%[b]	0.839	1.357	1.229	1.323
LOD	0.258	0.290	0.272	0.204
LOQ	0.783	0.878	0.823	0.618

*RSD%[a], **RSD%[b]: the intra-day AND inter-day, respectively, (n = 3) relative standard deviation of concentrations (6.0, 20.0, 25.0 µg/ml for CAR and 6.0, 18.0, 25.0 µg/ml for IVA)

Ratio difference technique

The paramount principle of this technique is that D^0 of the mixture is divided by D^0 of the interfering element obtaining a ratio spectrum. At this moment, the studied drug has a marked (ΔP) at two wavelengths opposing a canceled contribution of the interfering component for being a constant all over the curve. Thus, the interfering component's determination is possible.

This proposed technique was utilized to determine CAR and IVA simultaneously in binary set A mixtures. Dividing the mixture's absorption spectra by the absorption spectrum of IVA' (15.0 µg/ml) was performed to achieve a ratio spectrum. CAR in the mixture was determined using the amplitudes at 252.6 and 275.0 nm, Fig. 4.

Fig. 4 [Images not available. See PDF.]

Ratio spectra of 10.0 µg/ml of CAR (red line), IVA (black line) and their binary mixture (red dotted line), separately in distilled water, using spectrum of IVA (15.0 µg/ml) as a devisor showing the two selected wavelengths (252.6 and 275.0 nm)

Similarly, for the estimation of IVA in the prepared mixture utilizing CAR' (15.0 µg/ml) as a divisor, the amplitudes at 294.0 and 320.0 nm were used, Fig. 5.

Fig. 5 [Images not available. See PDF.]

Ratio spectra of 10.0 µg/ml of IVA (black line), CAR (red line) and their binary mixture (black dotted line), separately in distilled water, using spectrum of CAR (15.0 µg/ml) as a devisor showing the two selected wavelengths (294.0 and 320.0 nm)

The estimated ΔP of the ratio spectra at 252.6 and 275.0 nm and 294.0 and 320.0 nm, respectively, was shown to have a linear relationship with the corresponding concentrations of CAR and IVA, respectively, Table 1.

RD is advantageous over FDW method which requires the specific choice of two wavelengths for the studied drug opposing an equal absorbance value for the other interfering component, while in the RD approach, the wavelength selection is considerably more flexible because the interfering element is a straight line for being a constant.

Constant center coupled with spectrum subtraction technique (CC-SS)

This proposed technique is regarded as a complement to the previously applied RD method, where a ratio spectrum for the binary mixture in set A was obtained with a marked amplitude difference at 294.0 and 320.0 for IVA using CAR' as a divisor. Meanwhile, the practical ratio amplitude of each mixture was noted at 294.0 nm {(IVA/CAR') + (CAR/CAR')}. The supposed ratio amplitude value of (IVA/CAR') was obtained by putting the recorded ratio amplitude in the equation expressing the linear relationship between the ΔP at 294.0 and 320.0 nm with the associated ratio amplitudes at 294.0 nm.P1-P2ΔP=0.8801P1-0.0862r=0.9999, where $P1$, $P2$ are the ratio amplitudes of the ratio spectra of different concentrations of IVA (3.0–30.0 µg/ml) by utilizing CAR; 15.0 µg/ml as a divisor at 294.0 and 320.0 nm.

The constant value (C.V.);CAR/CAR' was figured as the deviation among the measured amplitudes of the mixture's ratio spectra at 294.0 nm and the postulated amplitudes obtained from the established regression equation.; [$P_{recorded}$]−[$P_{postulated}$].

The recovered CAR's D^0 in the mixture, Fig. 3c, was obtained by multiplication of the laboratory mixture's obtained constant (CAR/CAR') by the CAR's divisor.

For IVA, its recovered D^0, Fig. 3b, can be calculated by subtraction of the acquired spectra of CAR from the corresponding spectra of their laboratory-generated mixtures.

By substituting the recorded absorbance values at the maxima of each of CAR and IVA in their corresponding regression equations, each drug's concentration was calculated, Table 1

Set B: CAR and IVA in their quaternary mixture with their oxidative degradates:

The determination of CAR and IVA existed with their degradates was not that possible by the commonly used spectrophotometric techniques. Thus, a novel technique, namely, induced tripartite amplitude difference was devised to assign the targeted drugs by overcoming their completely overlapped spectra.

Induced tripartite amplitude difference coupled with ratio subtraction technique (ITAD-RS)

On the ratio spectrum of CAR, two wavelengths, 242.7 nm and 296.0 nm, were chosen using its oxidative degradate as a divisor.

The ratio spectrum of IVA degradate demonstrates equal amplitude values at these specified wavelengths. The ratio spectrum of IVA, on the other hand, revealed un-equalized values. As a result, an equality factor (F) of 2.497 [$P_{296.0}$/$P_{242.7}$] was calculated by obtaining the average of ΔP of diverse concentrations of IVA's ratio spectra using CAR degradate's spectrum as a divisor at (296.0 nm and 242.7 nm). This factor prospered to match the amplitudes of IVA at the proposed wavelengths, Fig. 6a.

Fig. 6 [Images not available. See PDF.]

Spectra scheme of resolution of 10.0 µg/ml of each of CAR (red line), IVA (black line), CAR's degradate (red dotted line) and IVA's degradate (black dotted line) using ITAD-RS in set B

After calculating the ΔP at 242.7 nm and 296.0 nm along with the equality factor for each set B quaternary mixture, multiplication of the previous value with the previously prepared CAR's factorized induced ratio spectrum resulted in an additional spectrum reflecting the CAR/CAR's degradate ratio spectrum in the mixture, Fig. 6b. By multiplication of the generated ratio spectrum by CAR oxidative degradate's D^0 spectrum, CAR's D^0 was successfully obtained, Fig. 6c.

Subtracting the acquired CAR's ratio spectrum from that of the quaternary mixture resulted in a ratio spectrum comprising IVA + IVA's degradate + CAR's degradate/CAR's degradate. The ratio spectrum of IVA/CAR's degradate in the combination was obtained by recording ΔP at 242.7 nm and 296.0 nm and then multiplying by IVA's factorized ratio spectrum, Fig. 6d. The parent D^0 of IVA was revealed afterward by multiplication of the acquired ratio spectrum by the spectrum of CAR oxidative degradate, Figure 6e

CAR and IVA concentrations were obtained using the respective regression equations generated at their maxima, Table 1.

Privileges proposed by the developed methods

From the achieved results, it was found that determination of the cited drugs through grabbing the beneficial impact of using spectral factorization gave the proposed methods, FDW-SS and the newly developed ITAD-RS a huge privilege. This was attributed to the great ability of factorized spectrum to recover the parent profile of each drug and their determination at each corresponding maxima. Additionally, manipulation using factorized spectra allows the resolution of the analytes via minimal arithmetic operation and spectrophotometer software's manipulation steps. Moreover, CC-SS method occupied a high capability in analyzing the cited drugs through obtaining their D^0 spectra in addition to highest accuracy in their quantification at their λ_{max}. Finally, and through using a spectral divisor, RD method was able to cancel the interfering component in the form of constant, which is a straight line across the curve; as a result, there would not be any crucial measurements at any particular wavelength, and the difference

between any two wavelengths will always equal to zero.

Discussion

Method validation

The suggested spectrophotometric techniques were validated in accordance with the ICH guidelines [20]. Accurate linear results were obtained by analyzing 6 concentrations ranging from 3.0 to 30.0 µg/ml of each cited drug conferring to obedience to Beer's law and their concentration in the pharmaceutical dosage form, Table 1. Accuracy of the results was also checked through analyzing different CAR's and IVA's blind samples where their concentrations were obtained from the corresponding regression equations for the percentage recoveries to be calculated, Table 1. Moreover, three concentration levels were prepared and analyzed in triplicates within and between days for each of CAR and IVA to assess the precision of the employed methods obtaining percentage relative standard deviation (RSD %) not exceeding 2%, Table 1.

Table 2 displays the outcomes of laboratory-prepared mixtures: sets A and B having various ratios of the drugs with or without their oxidative degradates, verifying the specificity of the specified techniques when results were within the calibration range and acceptable. The suggested techniques were additionally successful in distinguishing the drugs contained in Carivalan® tablets. The standard addition approach was used to further evaluate the suggested methods' validity, Table 3.

Table 2. Determination of CAR and IVA in their laboratory-prepared mixtures by the proposed methods

CAR and IVA in absence of their degradation products (set A)								
CAR: IVA	Concentration (µg/ml)		Recovery%					
CAR			IVA			CAR	IVA	RD
FDW-SS	CC-SS	RD	FDW-SS	CC-SS	1:2.5	6	15	101.67
101.84	98.65	100.20	98.10	100.92	1:3	8	24	101.00
101.20	98.19	100.75	98.14	101.00	1:1	10	10	101.00
99.58	98.41	101.60	98.60	101.77	2.5:1*	15	6	100.27
98.74	98.77	98.33	98.81	101.89	3:1	24	8	100.13
99.30	98.09	101.88	98.85	100.83	Mean ± SD			100.81 ± 0.63

CAR and IVA in presence of their oxidative degradation products (set B)			
% Degradation	CAR: IVA: CAR':IVA'	Recovery%	
CAR':IVA'	CAR	IVA	ITAD-RS
ITAD-RS	10	6:15:5.4:13.5	99.80
98.80	30	8:24:5.6:16.8	98.44
101.46	50	10:10:5:5	99.34
101.20	70	15:6:4.5:1.8	98.09
99.67	90	24:8:2.4:0.8	98.27
100.13	Mean ± SD		98.78 ± 0.74

*Ratio found in pharmaceutical dosage form

Table 3. Determination of CAR &IVA in tablet dosage form by the proposed methods and application of standard addition technique

Sample	CAR (mean% ± SD)			
Method	RD	FDW-SS	CC-SS	ITAD-RS
Carivalan®	90.40 ± 0.69	97.95 ± 0.99	97.75 ± 0.51	101.63 ± 1.56
Batch No.29044	99.83 ± 0.40	99.83 ± 1.19	99.48 ± 0.81	98.93 ± 1.37
Sample	IVA (mean ± SD %)			
Method	RD	FDW-SS	CC-SS	ITAD-RS
Carivalan®	98.17 ± 0.58	98.75 ± 1.14	100.07 ± 1.98	97.87 ± 0.38
Batch No.29044	*Standard addition*	100.50 ± 1.39	98.75 ± 0.43	100.71 ± 0.26

Statistical analysis

Tables 4 shows a statistical comparison of the found results acquired for the identified drugs using the proposed techniques, official [1] and the reported spectrophotometric technique [8] in pure forms. The computed *t* and F values were fewer than the theoretical ones, indicating no statistically significant variance in obtained results between the suggested and reported approaches.

Table 4. Statistical analysis of the proposed, official and the reported methods of CAR and IVA in their pure forms

Parameter	CAR			
RD	FDW-SS	CC-SS	ITAD-RS	Official method*
Mean	99.38	98.45		
99.88	SD	0.92	0.85	
0.95	N	5	5	
4	Variance	0.85	0.72	
0.9	Student's *t* test*** (2.365)	0.796	2.352	
	*F***	1.059 (9.120)	1.250 (6.590)	

Parameter	IVA			
RD	FDW-SS	CC-SS	ITAD-RS	Reported method**
Mean	99.47	99.15		
99.6	SD	1.41	0.87	
1.06	N	5	5	
5	Variance	1.99	0.76	
1.12	Student's *t* test*** (2.306)	0.165	0.734	
	*F*** (6.390)	1.777	1.474	

*BP determination of carvedilol by official potentiometric method [1]

**Derivative ratio spectrophotometric method [8] for determination of ivabradine

***The values in the parenthesis are the corresponding theoretical values of *t* and *F* at ($p = 0.05$). No significant difference by using one-way ANOVA with *F* equals 0.637 ($F_{crit} = 3.239$) and *p* equals (0.602)

Greenness profile assessment

The goal of the green analytical chemistry is to boost the environmental friendliness without compromising the performance of analytical methods by recommending the reduction of hazardous substances' usage. Applying various assessment techniques to compare the proposed methods' levels of greenness allowed for the method's greenness to be evaluated.

Analytical Eco-Scale [31] is a semi-quantitative approach that operates on the method's penalty points (PP) minus 100. National Environmental Methods Index (NEMI) [32] is a circular NEMI pictogram, consisting of four quadrants coded with green if they meet the requirements. Green Analytical Procedure Index (GAPI) [32] is a color-coding representation. The output representation will provide insight into the method's greenness and the list of chemicals that must be verified in this GAPI in accordance with the National Fire Protection Act (NFPA) by applying the necessary data in the 11 quadrants of the GAPI. Analytical Greenness Metric (AGREE) [32, 33] is a software-based quantitative method. The necessary information was entered into the software to obtain the pictogram.
As a result, greenness assessment of the proposed techniques, official [1], and the reported spectrophotometric technique [8] was performed for obtaining a more fruitful and purposeful comparison. The obtained results showed that the proposed spectrophotometric technique is greener than the official and the reported methods as shown in Table 5. Regarding Analytical Eco-Scale, the developed method shows a 97 green Eco-score in comparison with both official and the reported methods where they possess an Eco-score of 79 and 93 for CAR and IVA, respectively.

Table 5. Greenness assessment of the proposed, official and the reported methods according to Eco-Scale, NEMI, GAPI and AGREE tools

Tool	Proposed	Official [1]	Reported [8]
Analytical Eco-scale			
Water	0	–	–
Acetic acid	–	8	4
Perchloric acid	–	8	–
Energy Consumption	0	0	0
Occupational Hazard	0	0	0
Waste	3	5	3
Total Penalty Point	3	13	7
Eco-score	97	79	93
NEMI			
GAPI Tool			
AGREE Tool			

Additionally, the developed method's greenness assessment profile by applying national environmental method index tool (NEMI) obtained four full green quadrants as the used solvent; water is completely safe regarding persistence, bioaccumulation and corrosiveness, while only 1 and 2 green quadrants for both the official and reported methods of CAR and IVA, respectively, were obtained.

Moreover, method’s complete framework greenness profile was acquired using green analytical procedure index tool (GAPI) where ten green and five yellow zones with a complete absence of any red zones were possessed in comparison with official and reported methods.

Finally, a score value of 0.84 was obtained which is greener than that obtained by official, 0.73, and reported method, 0.76, after applying the analytical greenness metric tool.

Briefly, the usage of distilled water and minimizing instrumental's energy consumption in the proposed methods was reflected by gaining high analytical greenness scores besides having full green NEMI pictogram and a complete absence of any red zones in GAPI pictogram.

Conclusion

UV spectrophotometric techniques have several advantages over other analytical techniques, including their simplicity, low solvent usage and low cost. The suggested spectrophotometric techniques demonstrated great sensitivity and accuracy for determining CAR and IVA in dosage form, laboratory-generated combinations, and in presence of their oxidative degradates with no interference and excellent outcomes. Additionally, the greenness profile was argued green by applying various green assessment tools as analytical eco-scale, national environmental method index, green analytical procedure index and analytical greenness metrics. Therefore, the proposed method is capable of routine analysis of the cited drugs in quality control laboratories.

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Author contributions

HAEF helped in conceptualization, methodology, software data curation, software, validation, writing—original draft preparation, writing—reviewing and editing. DAA was involved in conceptualization, methodology, software data curation, software, validation, writing—reviewing and editing. NVF, AMB and MFAEG contributed to validation, supervision, writing—reviewing and editing.

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Availability of data and materials

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Declarations

Ethics approval and consent to participate

Not concerned.

Consent for publication

We certify this manuscript has not been published elsewhere and is not submitted to another journal. All authors have approved the manuscript and agreed to submit it to Future Journal of Pharmaceutical Sciences.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

CAR

Carvedilol

IVA

Ivabradine

FDW-SS

Factorized dual wavelength coupled with spectrum subtraction

RD

Ratio difference technique

CC-SS

Constant center coupled with spectrum subtraction technique

ITAD-RS

Induced tripartite amplitude difference coupled with ratio subtraction technique

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In vitro and in vivo evaluation of nanoliposomes loading quercetin and 3-bromopyruvate against glioma

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ABSTRACT (ENGLISH)

Background

Diffuse astrocytoma (a type of glioma) and its prevalence are matters of concern worldwide. Patients with this type of tumour have a poor prognosis because after surgical treatment, radiotherapy and/or chemotherapy, these tumours eventually regrow or progress. To date, there is no effective treatment that can cure affected patients. Quercetin and 3-bromopyruvate are chemical compounds that have been proven to have antitumour effects alone or in combination with other compounds. Nevertheless, combination treatments including these agents are not used for treating diffuse astrocytoma.

Methods

The use of nanoliposomes loaded with quercetin and 3-bromopyruvate as combination therapy was evaluated by treating C6 cells in vitro and in vivo (in Sprague–Dawley rat brain).

Results

The 0.5 mg/mL quercetin + 0.75 mg/mL 3-bromopyruvate combination treatment decreased the expression of the biomarkers Annexin V and Caspase-3 and inhibited tumour growth; this was consistent with the in vivo results that revealed the administration of this treatment resulted in improved animal survival.

Conclusions

The observations in the present study support the further exploration of this combination of active agents in the treatment of high-grade diffuse astrocytoma, especially in cases for which wide resection is possible.

FULL TEXT

Background

The incidence rates of all primary malignant brain tumours range from 6.10 to 8.65 per 100,000 person-years; among these tumours, 80% are diffuse gliomas and 76% are high-grade astrocytomas and glioblastomas [1]. These tumours have become issues of concern worldwide since the population will increase to 2 billion people in the next 30 years [2]. The reason for the appearance of diffuse gliomas is unclear, but the process seems to be multifactorial. Factors that contribute to the development of diffuse gliomas include population ageing, overdiagnosis, ionizing radiation, air pollution, virus infection, etc. [3, 4]. The degree of malignancy depends on location, patient age, growth rate, infiltration of healthy tissues, and the presence of established and specific molecular markers [5]. Nevertheless, in most patients with grades 3 and 4 gliomas, aggressive evolution results in poor prognosis, and risk of mortality increases one year after diagnosis [6, 7]. Despite increasing technological advances to achieve more significant tumour surgical resection, effective treatment is lacking [5, 8, 9]. Consequently, the recurrence of these tumours is frequent [10, 11]. Therefore, searching for new treatments and using more effective drugs to target tumour progression or regrowth is a significant issue in neuro-oncology [12, 13].

In vitro and in vivo studies make it possible to evaluate the efficacy of new cancer treatments. The direct administration of chemical compounds and drugs to C6 cell cultures and the intrathecal or intraperitoneal administration of these agents to tumour cell transplantation rat models are both widely used to study gliomas [14]. These models have histopathological and molecular features that are similar to those of developing adult-type

diffuse gliomas in humans [15]. The pharmaceutical preparations that have been proposed for treating high-grade gliomas include formulations with nanoparticles, particularly formulations with nanoliposomes (liposomes with a radius smaller than 100 nm), which have more attractive characteristics such as enhanced bioavailability of carried substances and increased efficacy due to the active substances and liposome components [16–18].

Moreover, nanoliposome formulations containing quercetin (Quer, a flavonoid with anti-inflammatory, antioxidant, and antineoplastic properties) have been shown to exert an antitumour effect against high-grade glioma [19, 20]. In recent reports, Ersoz et al. found that quercetin-loaded nanoparticles improve cytotoxic effects and antioxidant activity in C6 glioma cells [21]. Wang et al. also showed that PEGylated Quer-containing nanoparticles exert similar effects [22]. Zang et al. reviewed several Quer-containing formulations that are characterized by high encapsulation efficiency, stability, sustained release, prolonged circulation time, improved accumulation at tumour sites, and therapeutic efficiency. In addition, the authors suggested that combining quercetin with specific agents enhances the ability to detect or treat tumours [23].

One active compound that been used to treat glioblastoma is 3-bromopyruvate (3BP), which is a pyruvate-like alkylating compound that inhibits hexokinase II and glyceraldehyde-3-phosphate dehydrogenase [24]. 3BP exerts effects against several tumour cells, and its cytotoxicity is associated with the induction of autophagy; however, at the doses required for 3BP to be effective against glioblastoma cells, 3BP exerts toxic effects against healthy cells, which indicates a need to administer lower doses to limit systemic adverse effects [25]. In this sense, combinations of valproate, antimycin, menadione, and other antineoplastic agents with low doses of 3BP have also been suggested as effective and safe combinations for targeting some neoplastic processes [26, 27].

Hence, in this work, a nanoliposome formulation loaded with Quer and 3BP was evaluated as a combination therapy for treating C6 glioblastoma cells in vitro and in vivo. The current observations support further exploration of this combination of active agents for the treatment of diffuse astrocytoma.

Methods

Cellular culturing and selection of cells

The rat cell-line C6 (ATCC, USA) was cultured in culture recipients of 75 cm^2 with D-MEM FK12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, ATCC, USA) media, supplemented with 20% horse foetal serum (HSF, ATCC, USA); 10% bovine foetal serum (BFS, ATCC, USA) and 2% antibiotic/antimycotic media (A.A., GIBCO, USA). The culture plates were kept in an incubator (Water Jacketed, Nuaire, USA) at stable condition, 5% CO_2 at 35 °C for growth and propagation.

The sub-cell-cluster (Sub-C6) was obtained with serial dilutions 1:10 from the starting C6-cells. The last dilution was seeded in a 96 wells-plate/100 µL with supplemented D-MEM FK12 (20% HSF, 10% BSF, 10% A.A.). The wells with one cell were selected, then, growth to confluence. After that, sub-clusters were recovered with trypsin (Trypsin-EDTA 0.05%, cat 25,300,054, ThermoFisher) and spread into 6 well-plates and bottles of 75 cm^3. The cluster with highest FOXM1 (K19 clone SC500, SantaCruz biotechnology, USA) and VEGF (C20 clone- SC152, SantaCruz biotechnology, USA) protein expression was selected.

Apoptosis-markers detection

Cells, each for treatment from C6 cluster and sub-clusters, were seeded in 24 well-plates with 5×10^4 cells each well in supplemented D-MEM FK12 (20% HSF, 10% BSF, 10% A.A.) and treated with 5.7 ng, 17.1 ng y 34.2 ng of 3BP (376,817-M, Millipore) and/or 3.8 ng, 11.4 ng and 22.8 ng of Quer (Q4951, Sigma-Aldrich) during 3, 6, 12, 24, 48 and 72 h. Next, cells were washed with PBS and fixed by using paraformaldehyde 2%. Treated cells were incubated with the primary antibody against Annexin V (1:500, Santa Cruz Biotechnology, mouse sc-74438) by 48 h at 4 °C and Caspase 3 (1:500, Santa Cruz Biotechnology, mouse sc-56053), then with the secondary antibody Alexa 546 (anti-mouse 1:1000, cat A-11030, ThermoFisher) during 48 h a 4 °C, finally contrasted with Hoechst 33,342 (cat H3570, Invitrogen) by 20 min.

Samples from cultures were analysed with an inverted confocal microscope (Nikon Ti Eclipse with A1 through the NIS Elements v.4.5.0 software). Three photography were acquired to different fields at 20× in each treatment. The quantification and analysis were determined by a binary mask (black and white) to discard noise or artefacts by

using the Image-Fiji software (London SW7 2AZ, UK). Values considered as Caspase 3 activation and translocation by Annexin V were calculated as mean density by each cell.

Liposomal formulations preparation and characterization

10 mL of each unilamellar liposome preparation was made with a mixture of Quer and 3BP. Three different classes of liposomes were prepared: (1) control liposomes or empty liposomes, (2) low-dose liposomes (0.5 mg/ml Quer + 0.75 mg/mL 3BP) and (3) high-dose liposomes (0.75 mg/mL of Quer + 1.125 mg/mL of 3BP), based on the reverse phase evaporation method [28]. Briefly, for the formation of the lipid bilayer, 10 mg of cholesterol were dissolved in 2–3 mL of chloroform and added, together with 440 µL of phosphatidylcholine, in a conical flask with two necks (one with vacuum connection and the other with ability to place a removable filter) from 13 to 100 mm in diameter, and dried under vacuum so that the lipids remain uniformly distributed at the bottom of the flask.

Simultaneously the active ingredient was dissolved in milli-Q® water. As far as the Quer is concerned, it was dissolved in chloroform and a (1:1) proportional amount of water was added to them. Subsequently, the lipid bilayer was dissolved with 3 mL of diethyl ether and the previously dissolved active ingredient was added to this solution. The flask was then vortexed for 1 min and through immersion in a sonicator for an additional 1 min (5 s pulses). To carry out the control of liposomes, water was added instead of the active principle. Returning the flask to the vortex, a vacuum was applied for approximately 1 min, during which the ether was evaporated [29]. Maintaining erasure, 6 mL of saline solution containing 0.13% spermine was added. At this point, the preparation was evaluated by visualization (a cloudy suspension is considered adequate; the formation of lumps, inadequate). The liposome preparations obtained were filtered by extrusion, with Millipore Swinnex® membranes with 0.22 µm pore diameter (pressure 100–100 psi), to homogenize the size of the vesicles and to preserve them under sterile conditions. This suspension of liposomes was titrated with saline to a volume of 10 mL, pH 7.0.

Morphological characterization was done by means of atomic force microscopy, using a Nanos-Senterra (Bruker Optiks, Ettlingen, Germany) in the non-contact / tapping-mode; analysis was done on a plate with a maximum xy scan range of 40 × 40 µm and a z range of 8 µm. Cantilevers were standard microfabricated (POINTPROBE-PLUS® Silicon-SPM-Sensor, Nanosensors® Wetzlar-Blankenfeld, Germany). The length of the AFM tip was 200 mm, and the resonance frequency was 165 kHz. The average height and roughness of the cell surface were analysed, and images were processed by using SPIP® software (Image Metrology, Hørsholm, Denmark) as previously [30].

Animal model

Animals

Forty-two male *Sprague–Dawley* rats, ageing 10 weeks, and weighting 210–255 g at the start of assays, were used. They were contained in acrylic boxes (50 × 40 × 40 cm), maintained under 12:12 h light/dark cycles, with FormuLab Diet #5008 food and water ad libitum.

This protocol was evaluated by the local committee for research and ethics in health research 3601 (Registering Number: R-2012–3601-106). The surgical procedures were done in the vivarium by using all the aseptic and antiseptic protocols and materials. The project followed the local laws for animal care and the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (available at https://arriveguidelines.org/arrive-guidelines) for avoiding suffering to involved animals.

Stereotaxic approach was done in anaesthetized animals (90 mg/kg ketamine + 10 mg/kg xylazin). Once the head was fixed, the skull was exposed a trephine was done at anteroposterior 2 mm, lateral 2 mm and deep 2 mm from bregma [31]; at this site, 2.5×10^5 or 1×10^6 cells suspended in D-MEM FK12 medium (ATCC, USA) were injected and the skin was sutured. Then, a 5-day recovery period was permitted, being at the first three days treated with antibiotics (Gentamicin 80 mg + Benzathine penicillin 1,200,000 IU) and analgesic (Tramadol dose, 10 U).

Individual and combined treatments

The animals were divided into four groups (N = 24, n = 6 per group): Control) Treated with empty liposomes; Experimental 1) Treated with liposomes + Quer (1 mg/kg of Quer); Experimental 2) Treated with liposomes + 3BP (1.5 mg/kg of 3BP) and Experimental 3) Treated with liposomes + Quer + 3BP (1 mg/kg of Quer + 1.5 mg/kg of 3BP).

Low and high combined treatments

The animals were divided into three groups (N = 18, n = 6 per group): Control) Treated with saline solution; Experimental 1) Treated with low-dose liposomes (1 mg/kg of Quer + 1.5 mg/kg of 3BP) and Experimental 2) Treated with high-dose liposomes (1.5 mg/kg of Quer + 2.25 mg/kg of 3BP).

Administration of treatments and obtaining samples

The liposomal formulations were administered 3 times, at 72 h intervals, via i.p. being the first administration on the sixth day of the implant. Finally, the animals were sacrificed three weeks after implantation, due to an overdose of pentobarbital (100 mg/kg), to obtain and dissect the brains.

Samples processing

The brains were perfused and fixed in 4% paraformaldehyde for posterior paraffin embedding. After that, 5 µm slices were obtained every 250 microns for tumour identification. Briefly, the slices were mounted on slides covered with poly-L-lysine (10%, P4832, Sigma), the excess paraffin was removed (55ºC, oven), and they were rehydrated (xylol, 100% alcohol, 96% and 70%, and water). Subsequently, the sections were stained with Harris haematoxylin for 5 min, rinsed with water, treated with lithium bicarbonate, and counterstained with eosin. Before mounting the slides with Entellan resin (107,960, Merck-Millipore), they were dehydrated (70%, alcohol, 96% and 100%, and xylol) [32]. The images were photographed with a NI5_Elements D (5.110064 bit, Nikon, Japan) microscope 40 × and the calculation of the tumour areas were calculated with the Image Pro7 (Media Cybernetics, Rockville, MD 20852 USA) software. Tumour volumes were calculated with the formula:vµm3=∑VnVn+113∗250∗(An+An+1+An∗An+1) Subsequently, the values were converted to mm^3 and 75% of the volume of water was added.

Statistic analysis

The differences in the medians of Caspase 3 activation and translocation detected by Annexin V in C6-cells culture by exposure to Quer, 3BP or the combination were analysed using the nonparametric Kruskal–Wallis test. Differences in tumour size were analysed using the Mann–Whitney U nonparametric median comparison statistical test. All performed by using the Prima STAT v 12.0 software, a p-value < 0.05 was considered significant.

Results

Liposomal formulations were obtained as described for similar liposome formulations that contain nanoparticles (with radii ranging from 20 to 200 nm, Fig. 1). The Quer and 3BP encapsulation efficiency was not determined, but it was estimated to be > 90%, as described in multiple previous reports of similar systems [28, 33, 34]. When in solution, the rest of the described compounds were administered as original formulations and were used in our in vivo evaluations.

Fig. 1 [Images not available. See PDF.]

Morphological approach of nanoliposome formulation containing Quer and 3BP. **A** Three-dimensional topographic image was obtained by atomic force microscopy and shows liposomal nanoparticles. **B** Topographic mode view on the left. Length values are presented in the centre and are marked in cross-sectional lines of amplitude view on the right

Effects of Quer and/or 3BP on C6 cell culture

Annexin V detection.

Decreased cell viability in the initial stage of apoptosis induction was reflected by Annexin V-stained Alexa 546-positive cells [35]. The administration of Quer to cell cultures induced significant differences in proportion of apoptotic cells, which differed with both Quer concentration and treatment time (p < 0.0001). The highest proportions of Annexin V-positive cells were observed at 12 and 72 h, and the lowest proportion of Annexin V-positive cells was observed at 6 h after treatment with all Quer concentrations (Fig. 2A).

Fig. 2 [Images not available. See PDF.]

Annexin V (as a marker of initial phase of cellular death involving cell membrane dysfunction) staining of C6-cells treated with Quer and/or 3BP. C6 cells were treated with different concentrations of Quer or 3BP, and then, the mean cell density was measured at 3, 6, 12, 24, 48 and 72 h. **A** Cells treated with 3.8, 11.4 or 22.8 ng Quer. **B** Cells

treated with 5.7, 17.1 and 34.1 ng 3BP. **C** Cells treated with 3.8 ng Quer + 5.7 ng 3BP, 11.4 ng Quer + 17.1 ng 3BP and 22.8 ng Quer + 34.1 ng 3BP. Significant differences were determined by the Kruskal–Wallis test and are indicated by &p<0.01, †p<0.001, #p<0.005 and *p<0.0001

The administration of 3BP to cell cultures induced significant differences in the proportion of Annexin 5-positive cells at all the concentrations that were tested and at all the time points that were studied (p<0.0001). The highest proportion of Annexin V-positive cells was observed at 72 h, and the lowest proportions of Annexin V-positive cells were observed at 3, 6 and 24 h (Fig. 2B).

When both compounds were added to cell cultures, significant differences were observed in the proportions of Annexin V-positive cells at all the concentrations that were tested and at all the time intervals that were studied (p <0.0001), except for 6 and 48 h. The highest proportions of Annexin V-positive cells were observed at 12 and 72 h, and the lowest proportion of Annexin V-positive cells was observed at 6 h (Fig. 2C).

Finally, when individual treatments were compared with the combination treatment, a higher proportion of Annexin V-positive cells was observed in the group that received the combination treatment.

Caspase 3 activation.

The apoptosis execution pathway was studied by measuring Caspase 3 activation in cultured cells as described elsewhere [36]. The administration of Quer to cell cultures induced significant differences in Caspase 3 activation, which differed with both Quer concentration and treatment time (p<0.0001). In fact, the highest degree of Caspase 3 activation was observed at 12 h, followed by 48 and 72 h. In contrast, the lowest degree of Caspase 3 activation was observed at 6 h (Fig. 3A).

Fig. 3 [Images not available. See PDF.]

Caspase 3 (marker of apoptosis pathway) expression in C6-cells treated with Quer and/or 3BP. C6 cells were treated with different concentrations of Quer or 3BP, and then, the mean cell density was measured at 3, 6, 12, 24, 48 and 72 h. **A** Cells treated with 3.8, 11.4 or 22.8 ng Quer. **B** Cells treated with 5.7, 17.1 and 34.1 ng 3BP. **C** Cells treated with 3.8 ng Quer + 5.7 ng 3BP, 11.4 ng Quer + 17.1 ng 3BP and 22.8 ng Quer + 34.1 ng 3BP. Significant differences were determined by the Kruskal–Wallis test and are indicated by an asterisk, *p<0.0001

Additionally, 3BP administration to cell cultures induced significant differences in Caspase 3 activation, which differed with both Quer concentration and treatment time (p <0.0001); these results similar to those after Quer treatment (Fig. 3B).

When both compounds were administered, significant differences were observed in the activation of Caspase 3, which differed with both concentration and treatment time (p<0.0001), except for the 3-h timepoint. In this case, the highest degree of Caspase 3 activation was observed at 3 and 12 h, and the lowest degree of Caspase 3 activation was observed at 6 h and 12 h (Fig. 3C).

Finally, when the effects of the single treatments were compared with those of the combination treatment, the highest degree of Caspase 3 activation was observed after treatment with 3BP.

In vivo effects of liposomal formulations with combined treatment on tumour growth

A subclone of the C6 cell line was used, and the number of implanted cells that allowed the longest model survival was determined; this number of cells was used for subsequent experiments. For this experiment, intracranial tumour growth was measured in rats (n = 20) that received 1×10^5 cells (n = 10) or 2×10^5 (n = 10) cells. A placebo treatment of empty liposomes (n = 5) or saline solution (n = 5) was administered, and the experiment was continued for three weeks. One hundred per cent of the animals in the 1×10^5 cell implant group survived at 21 days; however, only 80% of the animals in the 2×10^5 cell implant group survived at 21 days (data not shown).

Subsequently, the Quer and/or 3BP individual and combination treatments that had been tested in cell cultures and had elicited the best apoptotic pathway response (11.4 ng Quer, 17.1 ng 3BP and 11.4 ng Quer + 17.1 ng 3BP) were administered.

Figure 4 shows representative tumour growth in the section with the largest area that was identified in each case. A tumour region was observed mainly in the control groups, and similar regions were observed in the groups treated

with either Quer or 3BP alone; in contrast, no areas of necrosis and less angiogenesis were observed in the combination treatment group (liposomes with Quer + 3BP), and only the inoculated cells, without obvious tumour formation, was observed.

Fig. 4 [Images not available. See PDF.]

Sections of the brain from rats implanted with 2.5×10^4 C6 cells and treated with liposomes containing Quer or 3BP were stained with H&E. **A** Treated with empty liposomes, **B** treated with liposomes loaded with 0.5 mg/mL Quer, **C** treated with liposomes loaded with 0.75 mg/mL 3BP and **D** treated with liposomes loaded with 0.5 mg/mL Quer + 0.75 mg/mL 3BP. These sections were obtained from rats 3 weeks after C6 cell implantation. The arrows indicate histopathological alterations due to the tumour. Scale bar 100 μm

Consequently, it was decided to test the combination treatment at a higher dose and to increase the cellular inoculum to further test the efficacy of the treatments.

After the administration of low and high doses of the liposomal formulations of the combination treatment, the animals were reactive, and obvious tumour formation was observed. Figure 5 shows representative tumour growth after the administration of low and high concentrations of liposomes. Smaller tumours were observed in the low-dose treatment group, but an apparent reversal of the therapeutic effect was observed in the high-dose treatment group.

Fig. 5 [Images not available. See PDF.]

Comparison of brain sections with free development of implanted C6 cells and brain sections from rats implanted with 1×10^5 C6 cells and treated with liposomes containing Quer or 3BP. Above are the entire brains, below are brain sections. All sections were stained with Haematoxylin &Eosin. **A** Treated with saline solution, **B** treated with liposomes loaded with 0.5 mg/mL Quer + 0.75 mg/mL 3BP and **C** treated with liposomes loaded with 0.75 mg/mL Quer + 1.125 mg/mL 3BP. These sections were obtained from rats 3 weeks after C6 cell implantation. The arrows indicate histopathological alterations due to the tumour. Scale bar 100 μm

Figure 6 shows a plot of the mean tumour volumes in the three groups of liposome-treated animals; there was a significant decrease between the control group and the group treated with low-dose combination treatment of Quer + 3BP ($p<0.05$).

Fig. 6 [Images not available. See PDF.]

Tumour volumes and effect of liposomal formulation treatment. Tumour volume from rats 3 weeks after implantation of 1×10^5 C6-cells treated with **A** empty liposomes, **B** liposomes loaded with 0.5 mg/mL Quer + 0.75 mg/mL 3BP and **C** liposomes loaded with 0.75 mg/mL Quer + 1.125 mg/mL 3BP. Significant differences were determined by the Kruskal–Wallis test and are indicated by #$p<0.05$

Discussion

The increasing incidence of glioblastoma and the regrowth of tumours after gross total resection followed by adjuvant treatment with temozolomide (gold standard in chemotherapy) and radiation therapy require the development of pharmacological tools for glioblastoma treatment [37, 38].

The implantation of glial cells into rat brains has been used to model human glioma for more than four decades; effective models have been used to test different treatments that control the growth and development of these cells [39]. Auer et al. (1981) determined the number of cells necessary to obtain a reliable model by studying an implant concentration gradient; they determined that the implantation of 1×10^4 cells results in a 100% glioma formation rate [40]. In this work, we implanted 2.5 and 10 times more cells than the number originally recommended by Auer et al. because preliminary experiments in our laboratory (not shown) revealed no tumour development after implantation of the original cell number.

The aim of this study was to test a liposomal formulation of Quer and 3BP combination treatment. These two components have been separately tested as neuroprotectors and glioblastoma cell regulators, although not in a combined formulation, and they have been demonstrated to exert effects in animal glioma models [21, 26, 41–44].

Notably, the material in the liposomal formulation used to improve drug availability to neoplastic cells is a third component of this formulation. In this work, the methodology used in the formulation of liposomes assumes a high encapsulation efficiency as has been reported [28]. Originally, the encapsulation efficiency was reported >65%, but currently is near 90% (84.7 ± 5%) [45].

Quercetin is insoluble in water due to its lipophilic property, so it has poor absorption, low bioavailability, and a limited ability to cross the BBB, therefore could not be used for the treatment of gliomas. The use of this liposome formulation increased the solubility of quercetin and guaranteed the probability of acting in the brain as well as limiting peripheral effects and reduction of drug-related toxicity [20, 33, 34, 46, 47]. In fact, several concentrations were evaluated for treating rats acting as a murine model of grade 4 astrocytoma, with the purpose of evaluating the possible therapeutic effects of adjuvant treatment after wide (implantation of 2.5×10^4 cells) or partial resection (implantation of 1.0×10^5 cells).

Anticancer properties of Quer are a consequence of different mechanisms that favour the progression of cancer cells. The antioxidant property is reflected in being an effective reactive oxygen species (ROS) scavenger and inhibiting lipid peroxidation; also, regulating signal transduction pathways, such as NRFB, MAPK and AMPK, as demonstrated by in vitro studies [48–50]. The anti-inflammatory properties are related to the inhibition of pro-inflammatory cytokines (TLR4 pathway) and a decrease in the production of cyclooxygenase (COX) and lipoxygenase (LOX) [51, 52]. Cell cycle progression of different cancer cells is affected by the arrest of the G0/G1 and G2/M phases, because of the inhibition of cyclins, release of p53 and caspase activation [53–55]. The synergistic effect of Quer with different chemotherapeutic agents and with radiotherapy has been reported; these studies are complemented by the cytotoxicity of Quer in glioma cells when the late stage of autophagy is inhibited [56, 57].

The suggested mechanisms of 3BP action include decreasing ATP by disrupting the function of several cysteine-rich proteins [58, 59]. Additionally, 3BP-mediated inhibition of hexokinase II, which is involved in the survival of glioblastoma cells, has been demonstrated, probably providing additional sources of ATP in neoplastic cells [60, 61]. In addition, several reports showed increased intracellular ROS production in diverse malignancies after 3BP exposure as well as the specific pyruvilation of glyceraldehyde 3-phosphate dehydrogenase, which is a major intracellular biochemical mechanism, resulting in the metabolic disruption of cells and inducing apoptosis [62–64]. Thus, considering that tumour growth and progression are favoured by acidic microenvironments and the reactive oxygen and nitrogen species that are produced during anaerobic glycolysis (Warburg effect), in which hexokinase II (HK II) degrades glucose into pyruvate, producing two molecules of ATP and various glycolytic intermediates that are fed into multiple biosynthetic pathways, it is suggested that the combined effect of this formulation is additive, as differences were observed with formulations that included only one of these compounds. 3BP can also act directly because pyruvate is converted into lactate in the cytoplasm by the enzyme lactate dehydrogenase (LDH). In contrast, normal astrocytes use the combination of acute aerobic glycolysis (Crabtree effect) and slow aerobic glycolysis, favouring high glucose contents and ATP generation without affecting the integrity of the mitochondrial membrane and maintaining a balance between glycolysis and respiration [65, 66].

Annexin V, which is specifically related to the processes that are associated with the initial phases of cell death, is considered a marker of membrane dysfunction [35]. Quer administration modulated the changes in the cell cycle that are associated with the initial phase of apoptosis, as shown by the increased proportion of Annexin V-positive cells described in prostate, colorectal and other neoplastic cells [67–69]. 3BP administration also induced high expression of Annexin V or high proportions of Annexin V-positive cancer cells, such as was observed in melanoma and lung neoplastic cells [70, 71].

Complementarily, in this study, caspases (cysteine proteases) are considered key proteins in apoptotic processes, specifically in the execution phase. Caspase 3 expression and activation have been shown to be modulated by Quer administration in different cells [72–74]. Similarly, 3BP induced an increase in the Caspase 3 staining intensity in potentially different types of neoplastic (liver, lung, colorectal) cells, including glioma cells [64, 71, 75].

Regarding the formulation used (simple bilayer liposomes with charges neutralized by spermidine addition), it should

be noted that the main advantages of the use of liposomes is efficient transport to the target site and evasion of natural barriers in the organism; however, other functional properties have been described in the treatment of gliomas; among these the facilitation of drug transport across the blood–brain barrier, the improvement of cellular uptake and the reduction of P-glycoprotein (P-gp) excretion of drugs, reversing of multidrug resistance, regulation of autophagocytosis and induction of apoptosis [34].

Additionally, formulations could include active protein members of the ATP-binding cassette (ABC) transporter superfamily. In the brain, these proteins are found in the blood–brain barrier, the blood-cerebrospinal fluid barrier, and the blood-arachnoid barrier, while in tumour cells, the overexpression of these ABC transporters is associated with drug resistance and regulated by metabolites that are generated during aerobic glycolysis; thus, 3BP could act indirectly by inhibiting the expression of these transporters [76–78]. On the other hand, there are reports that Quer favours the overexpression of P-gp, which is considered a substrate of these transporters [79, 80].

It should be noted that in vitro (Annexin V and Caspase 3 expression) and in vivo (limitation of growth) results are congruent and in line with the involvement of the mechanism(s) of action linked to regulation of death cells, mainly by apoptosis. However, other mechanisms such as metabolism disorders, avoiding of drugs expulsion of neoplastic cells and limitation of vascular development could be key in the observed effect. In this sense, an interesting finding of our in vivo experiments is that the combination treatment strategy seems to inhibit vascular formation. An antiangiogenic effect of Quer (at different doses and in different formulations) has been reported in multiple in vitro and in vivo studies, including those using liposomes as carriers [41, 81–85]. In contrast, scarce data suggest an effect of 3BP on vascularization in tumours; truly, its effects are linked to metabolism disruption, as briefly described above. In this work, the inhibition of neovascularization by the combination treatment could be an effect of both disrupted metabolism (particularly aerobic glycolysis) and direct inhibition of the production and actions of stimulating factors, such as VEGF (Vascular endothelial growth factor) [86, 87]. Further studies are required to support or refute these hypotheses. The determination of efficient doses of each component, the exploration of additional nanoliposome formulations as well as the comparison of effects from diverse administration pathways are desirable for testing this formulation in human cells, since this would increase the potential outcomes of this study and the possible clinical application. Also, an intentioned toxicity evaluation in higher or prolonged administration (studies of posology) compared with those used in this study should be done.

Conclusions

The tested combination liposomal formulation (Quer + 3BP) inhibited the expression of the biomarkers caspase-3 and Annexin V and tumour growth, but in vivo, its administration resulted in higher animal survival and lower volumes of developed tumours than in control groups. These observations support the further exploration of these active agents in the treatment of high-grade diffuse astrocytoma. Specifically, this exploratory study suggests that postsurgical treatment with liposomes loaded with Quer 1 mg/kg + 3BP 1.5 mg/kg allows glioma inhibition in cases where wide resection is possible or slows tumour growth when partial resection is possible. Further studies are required to support these findings.

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Author contributions

The author’s contributions are follows: Conceptualization was done by MASU and IAFR; methodology was done by AVG, VBA, ALON and JMM; formal analysis was done by MASU and IAFR; investigation was done by MASU and IAFR; resources were done by IAFR; writing—original draft preparation was done by MASU and IAFR; supervision was done by IAFR; funding acquisition was done by IAFR. All authors have read and agreed to the published version of the manuscript.

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Availability of data and material

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The local ethics committee evaluated this protocol (Registering Number: R-2012-3601-106). The project followed the local laws for animal care NOM-062-ZOO-1999 and the ARRIVE guidelines for avoiding suffering to involved animals.

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interests.

Abbreviations

AA
Antibiotic/antimycotic solution
BFS
Bovine foetal serum
D-MEM FK12
Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
FOXM1
Forkhead Box M1
H&E
Haematoxylin–eosin
P-gp
P-glycoprotein
Quer
Quercetin
3BP
3-Bromopyruvate
Sub-C6
Sub-cell-cluster from rat cell-line C6
VEGF
Vascular endothelial growth factor

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Antioxidant and acetylcholinesterase inhibitory activities, in silico analyses, and anti-Alzheimer's disease potential of leaf extracts of three Nigerian

endemic medicinal plants (Spondias mombin , Carica pa paya and Kalanchoe crenata)

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ABSTRACT (ENGLISH)

Background

The evaluation of the correlations between antioxidant and anti-acetylcholinesterase activities of methanol leaf extracts of three Nigerian endemic plants, *Spondias mombin*, *Carica papaya* and *Kalanchoe crenata*, was carried out. Their constituent phytochemicals were identified by HPLC–DAD fingerprinting. The antioxidant activity as typified by 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$), 2,2′-azino-bis-(3-ethylbenthiazoline-6-sulfonic acid ($ABTS^{\cdot+}$) and nitric oxide (NO) scavenging activities were evaluated. The acetylcholinesterase (AChE) inhibitory activity of the extracts was also determined.

Results

The extracts contained appreciable amounts of the flavonoids, quercetin and kaempferol. The extracts of *Spondias mombin*, *Carica papaya* and *Kalanchoe crenata* showed concentration-dependent inhibitory activities against $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ with IC_{50} of 43.29 ± 0.443 μg/mL, 59.27 ± 0.644 μg/mL and 80.20 ± 0.414 μg/mL; 25.43 ± 0.325 (μg/mL), 39.84 ± 0.163 μg/mL and 59.02 ± 0.376 (μg/mL), respectively. The IC_{50} for the NO scavenging activities of the *Spondias mombin*, *Carica papaya* and *Kalanchoe crenata* extracts were 41.99 ± 0.217 μg/mL, 50.44 ± 0.281 μg/mL and 60.12 ± 0.512 μg/mL, respectively. The IC_{50} for the inhibitory effects on AChE was 53.24 ± 0.327 μg/mL, 60.95 ± 0.290 μg/m and 70.5 ± 0.426 μg/mL, respectively. The effectiveness of the plant in all the experimental tests was in the following order: *S. mombin>C. papaya>K. crenata.* The total flavonoid and total phenolic contents have extremely significant positive correlations with the antioxidant activities and AChE inhibitory activity. The correlation coefficients (r^2) of DPPH scavenging activity and NO scavenging activity with the AChE inhibitory activity were 0.8295 μg/mL and 0.7337 μg/mL, respectively (P<0.0001). The molecular docking and pharmacokinetic analyses on some constituent phytochemicals showed that quercetin, kaempferol, ferulic acid, leucocyanidin, gallic acid and isorhamnetin fulfilled the requirements for an anti-Alzheimer drug.

Conclusions

The results suggest that the plant species provide a significant source of secondary metabolites that can act as natural antioxidants and acetylcholinesterase inhibitors, which will be helpful in the treatment of Alzheimer's disease.

FULL TEXT

Background

Discovering improved disease modifying therapies against dementias remains a major challenge. Dementias are characterized by the gradual onset and continuing decline of higher cognitive functioning as exemplified by Alzheimer's disease [1, 25]. Alzheimer's disease (AD) patients present a gradual decrease of acetylcholine levels, which arises from loss of the cholinergic neurons in the hippocampus and cortex of the brain. Other defects that occur include accumulation of decrepit plaques and neurofibrillary tangles [31]. Consequently, inhibition of

acetylcholinesterase, that enhances the build-up of acetylcholine at the synapse, will improve the cholinergic shortage, which is a remedial target for the development of drug for AD ("cholinergic hypothesis"). Galantamine, rivastigmine and donepezil are drugs for AD, all are acetylcholinesterase inhibitors [14].

Synthetic drugs show unwanted serious side effects accompanied by insufficient response rates. They mainly get rid of symptoms of AD rather than curbing the progression of the disease so the problem of how to treat the disease still persists [19]. Therefore, there remains an urgent need for new, safe and effective drugs. This opens an avenue for the exploration of medicinal plants. Medicinal plant products have proved to be favorable sources of acetylcholinesterase inhibitors [48]. AChE inhibition is also considered as a promising remedial strategy for other types of dementia, myasthenia gravis, glaucoma and Parkinson's disease in addition to AD [32].

The formation of reactive oxygen species, which leads to oxidative stress, is another significant neurotoxic pathway in AD. Oxidative stress is produced by the disturbance of equilibrium between free radicals and antioxidants. Damage of biomolecules such as lipids and proteins in relation to increased free radical levels leads to oxidative damage of cells and consequently, to overexpression of oncogenes, formation of mutagens, induction of atherogenic activity, or inflammation [47]. Oxidative stress is suggested to play a crucial role in the pathogenesis of numerous neurodegenerative diseases like AD, myasthenia gravis, glaucoma and Parkinson's disease [50].

As of late, there has been an upsurge of interest in plant-derived antioxidants because of their ability to break the chain reactions of free radicals [56]. Numerous constituents of herbal extracts have inherent antioxidant properties. Along these lines, reestablishing oxidative equilibrium might be one of the fundamental mechanisms by which therapeutic plants can protect against ageing and cognitive decline. The antioxidant activity of plants might be because of the presence of polyphenolic compounds, for example, phenolic acids and flavonoids [17, 39]. Medicinal plants with remarkable antioxidant and AChE inhibitory properties could therefore offer benefits in the therapy of neurodegenerative diseases.

Nigeria, one of the most important countries in West Africa, is richly blessed with an incredible variety of medicinal plants. Notable among them are *Spondias mombin, Carica papaya and Kalanchoe crenata.* The plants are known by various names but among the Yorubas, *Spondias mombin* is known as Iyeye, *Carica papaya* is known as ibepe and *Kalanchoe crenata* is known as Odundun. They are used independently or in combination with other herbs for the management of neurodegenerative diseases in Nigeria [41, 55]. Industrially, *Spondias mombin* fruit is commercialized as frozen pulp in Brazil where it is utilized for the production of juices, popsicles, ice creams, yogurts and jams [6, 58]. By-products of *Carica papaya* such as pectin and papain are used in the food industry [15]. *Kalanchoe* is a popular genus, typically produced for the floriculture industry. The new variety is suitable for both indoor and outdoor ornamental uses [13, 46].

Spondias mombin has antioxidant, antimicrobial, cardioprotective, antiepileptic and antipsychotic properties [2, 4, 5, 9, 42]. On the other hand, *Carica papaya* leaves have anticancer and muscle relaxant activities [11, 51]. *Kalanchoe crenata* leaves have been reported to demonstrate antioxidant and anticonvulsant effects [8, 40]. However, there is a dearth of information in the literature on the acetylcholinesterase inhibitory and anti-Alzheimer's disease activities of these plants, which could shed more light on their therapeutic potentials against neurodegenerative diseases. Therefore, this study evaluated the antioxidant and anticholinesterase properties of methanol extracts of *Spondias mombin*, *Carica papaya* and *Kalanchoe crenata* and ascertained the strength and direction of the correlation between these properties.

Methods

Chemicals

Thiobarbituric acid (TBA), trichloroacetic acid (TCA), Ellman's reagent (DTNB), N-(1-Naphthyl)ethylenediamine dihydrochloride, neocuproine, 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), 2,2-diphenyl-1-picryl-hydrazyl (DPPH), acetylthiocholine iodide and quercetin were obtained from Sigma-Aldrich, USA. Methanol was obtained from Merck (Darmstadt, Germany). The remaining chemicals and reagents used for this study were obtained from other standard sources.

Extraction of plant leaves

Spondias mombin, *Carica papaya* and *Kalanchoe crenata* leaves were obtained from farmlands (Latitude 7° 18′ 15.372″ N and longitude 5° 8′ 13.247″ E; Latitude 7° 18′ 37.076″ N and longitude 5° 15′ 28.789″ E; Latitude 7° 16′ 57.698″ N and longitude 5° 13′ 39.065″ E, respectively) in Akure, Southwest Nigeria, in July 2019. Authentication was carried out at The Federal University of Technology, Akure, Nigeria, and voucher specimens were deposited at the University's herbarium. The leaves were air-dried at 25–30 °C for 2 weeks with relative humidity ranging between 56 and 57%. The dried plant materials were pulverized, and 200 g of each powdered sample was extracted by maceration in 800 mL of 80% methanol for 48 h. The mixtures were filtered, using Whatman (No. 1) filter paper, concentrated and lyophilized to obtain the dry extracts of the plants. The percentage yields were *Spondias mombin* 10.5%, *Carica papaya* 8.5% and *Kalanchoe crenata* 7.0%.

Qualitative and quantitative phytochemical screening

Qualitative and quantitative phytochemical screening were carried out to detect and quantify phytochemicals present in the plant extracts.

Qualitative phytochemical

The preliminary phytochemical studies were performed to identify diverse classes of chemical compounds present in the plant extracts using standard procedures. Test for tannins, alkaloids, anthraquinones, saponins [59], test for flavonoids [54] and test for steroids [23] were performed as previously described.

Determination of total phenolic content (TPC)

Deionized water (0.5 mL) and 125 µL of Folin–Coicalteu reagent were added to 125 µL of extract (1 mg/mL), mixed and then allowed to stand for 6 min before 1.25 mL of a 7% (w/v) Na_2CO_3 solution was added. The reaction mixture was then allowed to stand for an additional 90 min before the absorbance was taken at 760 nm. Various concentrations of gallic acid solutions (6.25, 12.5, 25, 50, 75, 100, 200 µg/mL) were prepared and used to create a standard curve. The amount of total phenolics was expressed as gallic acid equivalents (GAE, mg gallic acid/g sample).

Determination of total flavonoid content (TFC)

The total flavonoid content was determined using a colorimetric method described by [16]. Extracts (1.0 mg/mL), 75 µL of 5% (w/v) $NaNO_2$ solution, 0.150 mL of freshly prepared 10% (w/v) $AlCl_3$ and 0.5 mL of 1 M NaOH solution were added. The final volume was then adjusted to 2.5 mL with deionized water. The mixture was allowed to stand for 5 min, and the absorbance was measured at 510 nm. Various concentrations of quercetin solutions (6.25, 12.5, 25, 50, 75, 100, 200 µg/mL) were prepared and used to create the standard curve. The amount of total flavonoids was expressed as quercetin equivalents (QE, mg quercetin/g sample).

Determination of tannin content

Tannin content of extracts was determined by the Folin–Ciocalteu method [28]. Sample (0.1 mL) was added to a 10-mL volumetric flask containing 7.5 mL of distilled water, 0.5 mL of Folin–Ciocalteu phenol reagent, and 1 mL of 35% sodium carbonate solution and diluted to 10 mL with distilled water. The mixture was thoroughly shaken and kept at room temperature for 30 min. A standard curve was prepared with graded concentrations of tannic acid (6.25, 12.5, 25, 50, 75, 100, 200 µg/mL). The absorbance was measured at 700 nm and tannin content was expressed in terms of mg of tannic acid equivalent/ g of dried sample.

HPLC–DAD fingerprinting

High-performance liquid chromatography (HPLC) was used to identify the presence of phytocompounds in methanolic leaf extracts of *Spondias mombin*, *Carica papaya* and *Kalanchoe crenata.* The samples were dissolved in aqueous acetonitrile (10 mg/20 mL) and mixed vigorously for 30 min. After mixing, the aqueous end was run off while the organic solvent end was collected into a 25-mL standard flask. The analysis was performed on a Shimadzu (NexeraMX) HPLC system fitted with uBONDAPAK C18 column (length 100 mm, diameter 4.6 mm, and thickness 7 µm). The mobile phase consisted of a mixture of an aqueous acetonitrile (acetonitrile/water, 80:20). The flow rate of the sample was 2 mL/min. Compounds were detected by a UV detector (Diode Array Detector, DAD) at 254 nm. The retention times of the identified compounds of interest were measured by standard solution at a concentration of 15.69 mg/g. The extract was injected into the high-performance liquid chromatographic machine to obtain a curve

providing peak area and retention time in a chromatogram. The peak area of the sample was compared with that of the standard relative to the concentration of the standard to obtain the concentration of the sample.

Evaluation of antioxidant and radical scavenging potentials

DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging activity

The ability of the extracts to scavenge DPPH radical was determined according to the method described by [33]. One mL of 0.3 mM DPPH methanol solution was added to individual extracts and quercetin (6.25–200 µg/mL, 2.5 mL) and allowed to react at room temperature for 30 min in the dark. The absorbance of the resulting mixture was measured at 517 nm and converted to percentage antioxidant activity.

Superoxide radical scavenging activity

The superoxide radical scavenging capacity was determined according to the method of [26]. Tris–HCl buffer (50 mM, pH 8.2, 4.5 mL), 25 mM pyrogallol solution (0.4 mL), sample (1 mL) were mixed together and incubated at 25 °C for 5 min. Then, 1 mL of 8 mM HCl solution was dripped into the mixture promptly to terminate the reaction. The absorbance was measured at 420 nm. Quercetin was used as the reference standard. The superoxide radical scavenging capacity was calculated using the formula:Scavenging capacity%=A0-A1A0∗100where A_0 is the absorbance of the control, A_1 is the absorbance of the sample.

Nitric oxide (NO) scavenging activity

NO scavenging activity was determined as previously described [10]. The reaction mixture (3 mL) containing sodium nitroprusside (10 mM) in phosphate-buffered saline and the extract were incubated at 25 °C for 150 min. Then, 0.5 mL of the reaction mixture was removed, and 0.5 mL of Griess reagent was added. The absorbance of the chromophore formed was measured at 546 nm. The results were expressed as percentage radical scavenging activity.

Hydroxyl radical scavenging activity

A mixture containing $FeCl_3$ (10 mM), ascorbic acid (1 mM), H_2O_2 (10 mM), deoxyribose (28 mM), EDTA (1 mM) and different concentrations of test samples in 500 µL phosphate-buffered saline (PBS, 20 mM, pH 7.4) was incubated for 30 min at 37 °C. After adding 1 mL of trichloroacetic acid (10%, w/v) and 1 mL thiobarbituric acid (2.8% w/v; in 25 mM NaOH), the reaction mixture was boiled for 15 min. After cooling, the extent of oxidation was measured at 532 nm and the scavenging activity of test sample was expressed as the percentage inhibition of the deoxyribose degradation to malondialdehyde [22].

2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity

The $ABTS^{\cdot+}$ stock solution was prepared by mixing the two stock solutions (7 mM ABTS solution and 2.45 mM $K_2S_2O_8$ solution) in equal quantities and allowing them to react for 16 h at room temperature in the dark. The working solution was then prepared by mixing 5 mL $ABTS^{\cdot+}$ solution with 145 mL of distilled water to obtain an absorbance of 0.076 ± 0.001 units at 734 nm. Extracts (1 mL) at various concentrations (6.25–200 µg/mL) were allowed to react with 1 mL of $ABTS^{+}$ solution, and the absorbance was measured at 734 nm after 30 min using a spectrophotometer [45]. The percentage scavenging activity was calculated using the formula: scavengingActivity%=Ac-AsAc∗100where A_c is the absorbance of control and A_s the absorbance of the extract.

Fe^{2+} chelating ability

The principle of the assay is based on disruption of *O*-phenanthroline-Fe^{2+} complex in the presence of a chelating agent. The Fe^{2+} chelating ability of the extracts was assayed according to a previously described method [36]. $FeSO_4$ (500 µL, 500 µM) and 200 µL of extract were incubated for 5 min at room temperature, and 500 µL of 1,10-phenanthroline (0.5 mM) was added. The absorbance of the orange-colored solution was read at 510 nm with a spectrophotometer. The in vitro Fe^{2+} chelating ability of the sample is calculated using the formula:Chelating ability% =Ac-AsAc∗100where A_c is the absorbance of control and A_s the absorbance of the extract.

Cupric ion-reducing antioxidant capacity (CUPRAC)

Determination of the cupric ion (Cu^{2+})-reducing ability of the individual extracts was based on a previously described method [7]. $CuCl_2$ solution (0.01 M), 1.0 M ammonium acetate buffer solution and 7.5 mM of ethanol neocuproine solution were added to each test tube containing different concentrations of standard antioxidant (Trolox) or extracts.

Finally, the total volume was adjusted to 2 mL with distilled water and incubated for 30 min at room temperature. Absorbance was measured at 450 nm against a reagent blank.

Ferric-reducing antioxidant power (FRAP)

The assay involved the rapid reduction of ferric-tripyridyltriazine (Fe^{3+}-TPTZ) to ferrous-tripyridyltriazine (Fe^{2+}-TPTZ), a blue-colored product by antioxidants present in sample [12]. FRAP reagent comprising 300 mM acetate buffer (pH 3.6), 100 mM TPTZ in 40 mM HCL solution, and 20 mM ferric chloride (10:1:1) was prepared, and 0.2 mL of each sample was mixed with 1.3 mL of the FRAP stock solution. Absorbance was measured at 620 nm, and FRAP value was extrapolated from a standard curve of Fe^{2+} solution.

Lipid peroxidation inhibitory activity

Brains were obtained from albino rats and homogenized in ice-cold Tris–HCl buffer (20 mM, pH 7.4). The resulting homogenate was centrifuged at 3000 rpm for 10 min to obtain the supernatant. Aliquot (0.5 mL) of the supernatant was added to 0.2 mL extracts of various concentrations (6.25–200 µg/mL), and the volume was made up to 1 mL with distilled water. Then, 0.05 mL of 0.07 mM $FeSO_4$ was added, and the mixture was incubated at 37 °C for 30 min and 1.5 mL of acetic acid (pH 3.5, 20%) was added. Thereafter, 1.5 mL of 0.8% (w/v) TBA in sodium dodecyl sulfate 1.1% (w/v) was added. The mixture was heated at 95 °C for 60 min. Then, the samples were cooled and centrifuged at 3000 rpm for 10 min. The intensity of the pink-colored complex was measured at 532 nm and converted to percentage inhibition of lipid peroxidation [49].

Acetylcholinesterase (AChE) inhibitory activity

AChE inhibitory activity was measured by the colorimetric method of [18]. Rats were decapitated; the brains quickly removed and placed on an ice-cold plate. The brain was weighed and homogenized in cold 10 mM Tris–HCl buffer, pH 7.2, containing 160 mM sucrose. The homogenates were centrifuged at 10,000×*g* for 10 min at 4 °C, and the resulting clear supernatants were used as enzyme sources. Briefly, enzyme in 20 mM phosphate buffer (pH 7.4) was incubated in the presence of 10 mM DTNB solution with different concentrations of each extract. The enzyme reaction was initiated by the addition of 75 mM acetylthiocholine iodide after the pre-incubation times of 0, 1, 2 and 3 min. Substrate hydrolysis was monitored by the formation of a yellow anion of 5-thio-2-nitrobenzoic acid at 415 nm. Enzyme activity was estimated through differences in absorbance/min and the percentage inhibition of AChE.

Prediction of pharmacokinetic properties

Pharmacokinetic properties of natural compounds such as MW (molecular weight), LogP, HBD (number of hydrogen bond donors), HBA (number of hydrogen bond acceptors), TPSA (topological polar surface area), nrtB (number of rotatable bonds), nViolation (violations of Lipinski's rule of five) were predicted using SwissDock Online server (http://www.swissadme.ch/) and Molinspiration Online tool (http://www.molinspiration.com/). The percentage of absorption (% ABS) was calculated using the Zhao et al. formula:%ABS=109-0.3345×TPSA

Molecular docking

The molecular docking study of compounds was performed to evaluate the binding interaction mode in the active site of the AChE enzyme (4EY5) that was obtained from the Protein Data Bank. The binding pocket of the receptor was predicted using DogSite platform of the protein-plus webserver (http://proteinsplus.zbh.unihamburg.de). The protein was prepared by removing co-crystallized ligands and additional water molecules using Pymol 2.5.1. The 3D sdf file of the compounds (Quercetin (CID: 5280343), Kaempferol (CID: 5280863), Ferulic acid (CID: 445858), Lycopene (CID: 446925), Leucocyanidin (CID: 3705436), Gallic acid (CID: 370), Isorhamnetin (CID: 5281654) were obtained from PubChem database and OpenBabel 2.4.1 was used to convert to the pdb format. AutoDock Vina version 1.1.2 was used for molecular docking process. Docking analysis was carried out with the grid size set as 60 ×60×60 with 1.0 Angstrom spacing and Centres *x*, *y* and *z* to be −2.857, −40.075 and 30.865, respectively. The exhaustiveness that determines how comprehensive the software search for the best binding mode was set to the default value of 8 Angstrom. Biovia Discovery Studio 2021 was used for visualization and analyzing of the docking results.

Inhibition constant (Ki)

The inhibition constant (Ki) of all the compounds against AChE was calculated from docking energy using the following equation:KinM=exp$\Delta G \times 1000/RT$

where $T = 298.15$ K, $R = 1.987$.

Correlation analyses

The strength and direction of the relationship between the antioxidant properties and AChE inhibitory activities of the extracts were evaluated statistically.

Statistical analyses

All statistical analyses were performed using the GraphPad version 6 software. Results were expressed as mean ± SEM ($n = 3$). One-way analysis of variance was used for data analysis. Significant differences between groups were detected in the analysis of variance using Duncan's multiple range test at $P < 0.05$. Statistical differences between mean values of individual tests were detected using independent-sample t test. The correlation analyses the GraphPad software.

Results

The phytochemical screening of *Spondias mombin, Carica papaya* and *Kalanchoe crenata* leaves methanolic extract showed the presence of alkaloids, flavonoids, and tannins (Table 1).

Table 1. Phytochemicals detected in methanol extracts of the plants

Test	*Spondias mombin*	*Carica papaya*	*Kalanchoe crenata*
Tannins	+	+	+
Alkaloids	+	+	+
Flavonoids	+	+	+
Anthraquinones	+	+	+
Steroids	+	−	+
Saponins	+	−	+

Key: + indicates present; − indicates absent

The total phenolic content (TPC) of the extracts calculated using the gallic acid regression equation of a calibrated linear curve ($y = 0.0117x + 0.1241$; $R^2 = 0.9916$) is shown in Table 2. The highest TPC value was observed in *S. mombin*, followed by *C. papaya*. Total flavonoid content (TFC) of the extracts was calculated from the regression equation of the calibration curve ($Y = 0.0071x - 0.0901$; $R^2 = 0.9903$) and showed a similar trend to that of TPC. *Spondias mombin* has the highest value (43.86 ± 0.905 mg QE/g) and *K. crenata* the lowest (22.89 ± 0.586 mg QE/g) (Table 2). The TTC of *S. mombin*, *C. papaya* and *K. crenata* was 89.52 ± 1.360, 38.21 ± 0.136 and 18.48 ± 0.156 mg TAE/g of plant extract, respectively, as shown in Table 2.

Table 2. Quantitative estimates of constituent phytochemicals in the plant extracts

Test	*Spondias mombin*	*Carica papaya*	*Kalanchoe crenata*
Total phenols (mg GAE/g)	91.30 ± 0.794^{a}	34.15 ± 0.242^{b}	18.60 ± 0.786^{c}

Total flavonoids (mg QE/g)	43.86 ± 0.905[a]	39.97 ± 0.666[b]	22.89 ± 0.586[c]
Total tannins (mg TAE/g)	89.52 ± 1.360[a]	38.21 ± 0.136[b]	18.48 ± 0.156[c]

Data are expressed as mean ± SEM (n = 3).

GAE gallic acid equivalent, *QE* quercetin equivalent, *TAE* tannic acid equivalent. Values with the same superscript letter in a row are not significantly different (P > 0.05)

Spondias mombin leaf extract HPLC–DAD fingerprinting (Additional file 1: Fig. S1) revealed the presence of phenolic acids (chlorogenic acid, ellagic acid and gallic acid), flavonoids (rhamnetin, isorhamnetin, isoquercetin, quercetin, kaempferol, rutin, isoquercitrin and astragalin), terpenoids (humulene, lupeol and cadinene), and other compounds as shown in Table 3. For *C. papaya* leaf extract, the phytochemicals obtained from the analysis of the chromatogram (Additional file 1: Fig. S2) include carotenoids (β-carotene, lycopene), terpenoid (linalool), sterols (papayaglyceride, glucopaeclin and β-sitosterol), flavonoids (quercetin, kaempferol), and alkaloids (carpaine, sinigrin) (Table 3). The analysis of the HPLC chromatogram (Additional file 1: Fig. S3) of the *K. crenata* leaf extract showed the presence of phenolic acids such as caffeic acid, *p*-coumaric acid, *p*-hydroxycinnamic acid, protocatechuic acid and ferlic acid; flavonoids such as luteolin, quercetin, kaempferol, rutin and leucocyanidin (Table 3). The major chemical class identified in *S. mombin*, *C. papaya* and *K. crenata* leaf extracts was flavonoids with quercetin as predominant compound.

Table 3. Phytochemicals quantified in *Spondias mombin, Carica papaya* and *Kalanchoe crenata* leaf extract using HPLC–DAD

Spondias mombin				*Carica papaya*				*Kalanchoe crenata*			
Compound	Standard retention time	Area (mUA)	Conc (mg/g)	Compound	Standard retention time	Area (mUA)	Conc (mg/g)	Compound	Standard retention time	Area (mUA)	Conc (mg/g)
Chlorogenic acid	1.266	1699.3360	2.11	P-carotene	1.266	1302.9320	1.68	Caffeic acid	1.266	1176.9195	1.36
Ellagic acid	2.750	2818.3005	3.51	Lycopene	2.750	2487.9705	3.20	P-coumaric acid	2.750	2689.0285	3.11
Gallic acid	4.450	943.3170	1.17	P-cryptoxanthine	4.450	824.8535	1.06	P-hydroxycinnamic acid	4.450	851.3720	0.99
Rhamnetin	5.466	473.0410	0.59	Papain	5.466	410.0650	0.53	Protocatechuic acid	5.466	400.4170	0.46
Isorhamnetin	6.483	416.3200	0.51	Chymopapain	6.483	368.9170	0.48	Ferulic acid	6.483	338.8100	0.39

Humulene	7.033	288.6240	0.36	Linalool	7.033	261.7300	0.34	Luteolin	7.033	226.3700	0.26
B-sitosterol	7.950	170.0725	0.21	Papaya glyceride	7.950	159.0790	0.21	Flavonol	7.950	128.2300	0.15
Isoquercetin	8.816	191.9900	0.24	B-sitosterol	9.350	102.4710	0.13	Flavan-3-ol	8.816	144.3270	0.17
Quercetin	11.050	10,037.9580	12.49	Quercetin	11.050	10,032.6255	12.92	Quercetin	11.050	9185.8775	10.63
Kaempferol	12.166	3824.3670	4.76	Kaempferol	12.166	3817.3025	4.92	Kaempferol	12.166	3144.8970	3.64
Rutin	13.700	3739.4150	4.65	Carpaine	13.700	3709.6610	4.78	Rutin	13.700	2143.3130	2.48
Lupeol	16.250	188.4180	0.23	Sinigrin	16.250	183.4020	0.24	Lycocyanidin	17.616	236.3780	0.27
Cadinene	18.500	391.1870	0.49	Glucopaeolin	17.616	900.3720	1.16				
Isoquercitrin	19.683	247.0720	0.31								
Geraniin	20.500	170.8950	0.21								
Astragalin	21.133	161.4540	0.20								

Figure 1 shows the antioxidant activity of the extracts. A clear comparison of the performance of the extracts and the reference compounds as revealed by their IC_{50} values (obtained using inverse logarithmic method) in the different tests is depicted in Table 4. The scavenging activities, metal chelating ability, reducing power and inhibition of lipid peroxidation were in a concentration-dependent manner. *Spondias mombin* was the most effective extract followed by *C. papaya* and then *K. crenata*. The CUPRAC assay shows the effectiveness of *S. mombin* (IC_{50} 24.29 ± 0.165 μg/mL) where its value was clearly superior to that of the reference standard, trolox (IC_{50} 34.77 ± 0.242) and other samples.

Fig. 1 [Images not available. See PDF.]

The comparative antioxidant activities of *Spondias mombin, Carica papaya* and *Kalanchoe crenata* leaf extracts **A** 1,1-diphenyl-2-picrylhydrazyl radical ($DPPH^{\cdot}$) scavenging activity **B** Superoxide radical scavenging activity **C** Nitric oxide (NO) radical scavenging activity **D** Hydroxyl radical scavenging activity **D** 2,2′-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) radical ($ABTS^{\cdot+}$) scavenging activity **E** Iron chelating activity **F** Cupric ion reducing antioxidant capacity (CUPRAC) **G** Lipid peroxidation inhibitory activity

Table 4. IC_{50} of methanol-leaf extracts of *Spondias mombin*, *Carica papaya* and *Kalanchoe crenata* in antioxidant tests

Antioxidant test	*Spondias mombin* IC_{50} (µg/mL)	*Carica papaya* IC_{50} (µg/mL)	*Kalanchoe crenata* IC_{50} (µg/mL)	Standard	IC_{50} (µg/mL)
$DPPH^{\cdot}$ scavenging activity	43.29 ± 0.443^{b}	59.27 ± 0.644^{c}	80.20 ± 0.414^{d}	Quercetin	38.43 ± 0.440^{a}
Superoxide radical scavenging activity	50.83 ± 0.23^{b}	77.84 ± 0.926^{c}	84.19 ± 0.119^{d}	Quercetin	48.73 ± 0.468^{a}
Nitric oxide radical scavenging activity	41.99 ± 0.217^{b}	50.44 ± 0.281^{c}	60.12 ± 0.512^{d}	Ascorbic Acid	35.73 ± 0.490^{a}
Hydroxyl radical scavenging activity	10.73 ± 0.681^{b}	38.22 ± 0.960^{c}	67.74 ± 0.684^{d}	Mannitol	8.39 ± 0.609^{a}
$ABTS^{\cdot+}$ scavenging activity	25.43 ± 0.325^{b}	39.84 ± 0.163^{c}	59.02 ± 0.376^{d}	Trolox	20.46 ± 0.221^{a}
Iron chelating	30.49 ± 0.212^{b}	46.90 ± 0.291^{c}	53.14 ± 0.401^{d}	EDTA	13.73 ± 0.163^{a}
CUPRAC	24.29 ± 0.165^{b}	68.21 ± 0.882^{c}	73.04 ± 0.956^{d}	Trolox	34.77 ± 0.242^{a}
Lipid peroxidation inhibitory activity	59.24 ± 0.694^{b}	62.34 ± 0.942^{c}	69.95 ± 0.256^{d}	Ascorbic Acid	32.51 ± 0.242^{a}

Values are presented as mean ± SD ($n = 3$). Values having the same superscript letters are not significantly different ($P < 0.001$)

DPPH 1,1-diphenyl-2-picrylhydrazyl, *ABTS* 2,2′-Azinobis-(3-ethylbenzthiazoline-6-sulphonate), *CUPRAC* Cupric ion reducing antioxidant capacity

The order of AChE inhibitory activity of the extracts followed the pattern established in the antioxidant activity assays. The AChE inhibitory activity of *S. mombin* (53.24 ± 0.327) was the highest, while that of *K. crenata* (70.5 ± 0.426) was the least. The AChE inhibitory activities of the extracts were lower than that of the reference standard, quercetin (20.52 ± 0.112) as shown in Fig. 2.

Fig. 2 [Images not available. See PDF.]

Acetylcholinesterase inhibition activity of *Spondias mombin, Carica papaya and Kalanchoe crenata* leaf extracts

Table 5 shows the correlation analyses of the total flavonoid contents, total phenolic contents, antioxidant activities and AChE inhibitory activities of the extracts, which confirmed a positive association between antioxidant activity and AChE inhibitory activity. The r^2 values have been used to show the relationship between the phytochemical constituents, antioxidant activities and AChE inhibitory activities of the extracts of *Spondias mombin, Carica papaya* and *Kalanchoe crenata.* The total flavonoid and total phenolic contents have extremely significant correlations with the antioxidant activities and AChE inhibitory activities. Table 5 shows that DPPH scavenging activity, NO scavenging activity and lipid peroxidation inhibitory activity all have extremely significant positive correlations with AChE inhibitory activity (r^2 = 0.8295, 0.7337, 0.7214, respectively, P<0.0001). Superoxide radical scavenging activity, hydroxyl radical scavenging activity, ABTS radical scavenging activity and iron chelating ability also have above 50% correlation with AChE inhibitory activity.

Table 5. Correlation coefficients of the total flavonoids, total phenolics, total tannins and acetylcholinesterase inhibitory activity with antioxidant activities of *Spondias mombin*, *Carica papaya* and *Kalanchoe crenata*

Antioxidant test	R^2			
AChE	Total flavonoid	Total phenolic	Total tannin	$DPPH^{\cdot}$ scavenging activity
0.8295	0.6457	0.6177	0.6316	Superoxide radical scavenging activity
0.5485	0.3684	0.9046	0.8654	Nitric oxide radical scavenging activity
0.7337	0.5463	0.8705	0.9074	Hydroxyl radical scavenging activity
0.6595	0.4387	0.9489	0.9074	$ABTS^{\cdot+}$ scavenging activity

0.6214	0.1754	0.4855	0.4.715	Iron chelating
0.5876	0.7011	0.9580	0.9699	CUPRAC
0.6295	0.3880	0.9580	0.9294	Lipid peroxidation inhibitory activity
0.7214	0.0529	0.0689	0.06281	Acetylcholinesterase inhibitory activity

Correlation is significant at the 0.05 level (one-tailed)

DPPH 1,1-diphenyl-2-picrylhydrazyl, *ABTS* 2,2′-Azinobis-(3-ethylbenzthiazoline-6-sulphonate), *CUPRAC* Cupric ion reducing antioxidant capacity. R^2 coefficient of determination

The results of the predicted pharmacokinetic properties for selected phytochemicals (quercetin, kaempferol, ferulic acid, lycopene, leucocyanidin, gallic acid and isorhamnetin), the TPSA and %ABS are presented in Table 6. According to the Lipinski's rule of five, drug compounds must have molecular weight <500, hydrogen-bond donors <5, hydrogen-bond acceptors <10, partition coefficient (log *P*) value not greater than 5 and not more than one rule can be violated by an orally active drug. Among the selected phytochemicals, only lycopene violated the rule. With the exception of lycopene, ferulic acid has the lowest TPSA (66.76 Å) while the highest TPSA is observed in quercetin (131.36 Å). Quercetin, kaempferol, ferulic acid, lycopene, leucocyanidin, gallic acid and isorhamnetin also show similar pharmacokinetic properties when compared to rivastigmine commonly prescribed for AD patients.

Table 6. Predicted pharmacokinetic properties of selected compounds

Compound	Formular	Molecular weight (g/mol)	Log P	HBA	HBD	TPSA	%ABS	nAtom	nRB	nViolation
Quercetin	$C_{15}H_{10}O_7$	302.24	1.23	7	5	131.36	65.06	22	1	0
Kaempferol	$C_{15}H_{10}O_6$	286.24	1.58	6	4	111.13	71.83	21	1	0
Ferulic acid	$C_{10}H_{10}O_4$	194.18	1.36	4	2	66.76	86.69	14	3	0
Lycopene	$C_{40}H_{56}$	536.89	9.98	0	0	0	109.00	40	16	2
Leucocyanidin	$C_{15}H_{14}O_7$	306.27	0.11	7	6	130.61	65.31	22	1	1

Gallic acid	$C_7H_6O_5$	170.12	0.21	5	4	97.98	76.23	12	1	0
Isorhamnetin	$C_{16}H_{12}O_7$	316.26	1.65	7	4	120.36	68.74	23	2	0
Rivastigmine	$C_{14}H_{22}N_2O_2$	250.34	2.34	3	0	32.78	98.03	18	6	0

Log P Octanol/water partition coefficient, *HBA* hydrogen bond acceptor, *HBD* hydrogen bond donor, *TPSA* topological surface area, *%ABS* % absorption, *nAtom* number of atoms, *Nrb* number of rotatable bonds, *nViolation* number of violation. There was no violation of the Lipinski's rule

DogSite platform of the protein-plus server was used to predict the active site of AD target (4EY5). The predicted druggable pocket of the co-crystallized ligand in 4EY5 are Trp86, Tyr337, Tyr133, Tyr337 and Gly121. These amino acids were selected for the binding of all the selected phytochemicals. Figure 3 and Table 7 show the top docked binding pose of the selected phytochemicals; quercetin (−9.1 kcal/mol), kaempferol (−8.6 kcal/mol), ferulic acid (−7.3 kcal/mol), lycopene (−15.1 kcal/mol), leucocyanidin (−7.8 kcal/mol), gallic acid (−7.2 kcal/mol) and isorhamnetin (−8.2 kcal/mol). Although lycopene showed very good binding affinity, it was not considered for further docking analysis because it does not comply with the Lipinski's rule of five. The binding of rivastigmine to the predicted site on 4EY5 shows a binding affinity of −6.9 kcal/mol (Table 7). This implies that the selected phytochemicals showed high affinity toward 4EY5 as compared to rivastigmine commonly prescribed for AD patients. The 2D interactions by Biovia Discovery Studio 2021 are presented in Fig. 4, and parameters such as hydrogen bond, distance, hydrophobic interactions, *π*-interactions and inhibitory constant are presented in Table 7. The orientation of each of these phytochemicals resembles that of the native ligand. The selected compounds and rivastigmine (Fig. 5) showed similar binding interactions with the amino acids on analysis. The interactions were prominently observed in the region of Tyr337 and Trp86 amino acid residues due to the pronounced existence of the pi-cation interaction at the catalytic anionic site.

Fig. 3 [Images not available. See PDF.]

3D Binding interaction of 4EY5 with **a** Quercetin **b** Kaempferol **c** Ferulic acid **d** Lycopene **e** Leucocyanidin **f** Gallic acid and **g** Isorhamnetin

Table 7. Summary of docking results

Compound	Binding affinity (kcal/mol)	Inhibition constant (Ki) (nM)	No. of H bond formed	No. of hydrogen bond interaction residues	Distance	Hydrophobic interaction	Residues forming π-interactions
Quercetin	−9.7	7.76E−08	2	Gln71, Tyr133	2.36 and 2.76	Ser125, An87, Pro88, Leu130, Tyr337, Gly126, Glu202, Val73, Gly120, Gly121 and Asp74	Trp86, Tyr337 and Asn87
Kaempferol	−8.6	4.97E−07	1	Arg296,	2.30	Tyr337, Val294, Phe338, Tyr124, Phe295, Phe297, Leu289, Glu292 and Gln291	Tyr341 and Trp286

Ferulic acid	−7.3	4.46E−06	3	His-447, Ser-203 and Glu-202	2.59, 1.86 and 2.97	Gly448, Tyr72, Gly120, Asp74 Gly122, Gly121, Ser125, Tyr124, and Asn87,	Phe338, Phe297, Trp86 and Tyr337
Lycopene	−15.1	–	0	Violated the Lipinski's rule of five			
Leucocyanidin	−7.8	1.92E−06	1	Asp74	2.58	Leu289, Thr75, Phe297, Tyr341, Phe338, Val294, Ser293, Tyr72, and Phe295	Leu76 and Trp286
Gallic acid	−7.2	5.28E−06	4	Trp86, Tyr133, Glu202 and Gly120	2.84, 1.92, 2.21 and 2.94	Gly121, Ser125, Gly126, Ile451, Ser203, Leu130, Tyr 119, Tyr337, His447 and Tyr124	Trp86 and Gly120
Isorhamnetin	−8.2	9.76E−07	1	Gln291	2.11	Tyr337, Phe297, Val294, Ser293, Phe338, Arg296, Phe295, Glu292 and Leu289	Tyr124, Tyr72, Tyr341, and Trp286
Rivastigmine	−6.9	8.75E−06	1	Trp286	3.75	Tyr337,Val294, Phe297,Arg296,Ser293, Phe295, Try124,Phe338, Try341, Try72, Leu289	Trp286

Fig. 4 [Images not available. See PDF.]

2D Binding interaction of 4EY5 with **a** Quercetin **b** Kaempferol **c** Ferulic acid **d** Leucocyanidin **e** Gallic acid and **f** Isorhamnetin using Biovia Discovery Studio 2021

Fig. 5 [Images not available. See PDF.]

Molecular docking of 4EY5 with Rivastigmine. **a** 3D Binding pose of rivastigmine after docking experiment with 4EY5 and **b** 2D Binding interaction of Rivastigmine and 4EY5 with using Biovia Discovery Studio 2021

Discussion

Plants as a potential source of drugs for the management of clinical disorders have been extensively studied over the past few years. The limitations and side effects of drugs in current use for the management of AD and other dementias warrant search for more effective therapeutic agents [19]. A gradual decrease of acetylcholine levels, arising from the loss of the cholinergic synapses and reactive oxygen species production, play an important role in the pathogenesis of AD [20]. Therefore, plants and phytochemicals with antioxidant activity and the ability to balance acetylcholine levels will be potentially useful in the management of AD. These considerations necessitated the phytochemical investigation, and evaluation of the antioxidant and anticholinesterase activities of the three medicinal plants popularly used in traditional herbal medicine in Nigeria for the treatment of brain-related disorders. Phytochemicals such as tannins, alkaloids, flavonoids, anthraquinones, steroids and saponins detected in the extracts are bioactive agents. These phytochemicals have demonstrated activities such as inhibition of

neuroinflammation and oxidative stress, maintenance of neurotransmitter balance, antiapoptosis and mitochondrial stabilization in the brain [61].

The analysis of the chromatograms obtained from the three plant validates the presence of various phytocompounds like phenolic acids, flavonoids and terpenoids. Flavonoids were the preponderant phytochemicals in the extracts. Flavonoids, for example, quercetin and kaempferol, are viewed as having powerful cell reinforcement property that are helpful in the avoidance of different oxidative stress-related diseases including neurodegenerative disorders, for example, AD [34].

The presence of polyphenolics (which includes phenolic acids, flavonoids and tannins) in plants has a direct relation with their antioxidant and radical scavenging properties [29]. The antioxidant activity of polyphenols is influenced by the presence of free hydroxyl groups. Several mechanisms have been demonstrated for the antioxidant activity of polyphenols. These include free radical scavenging, inhibition of lipid peroxide formation, metal chelation, and reductive ability. The notable activities of the extracts under consideration in the in vitro tests which covered different mechanisms of antioxidant protection are an allusion to their potential therapeutic efficacy.

The oxidation of pyrogallol forms superoxide anions (a purple solution). It reacts with proton in solution and form hydrogen peroxide. Hydrogen peroxide is an important substrate that produce singlet oxygen and hydroxyl radicals. Superoxide anion is a major reactive oxygen species that leads to the oxidation of cells and tissues [21, 53]. *Spondias mombin*, *C. papaya* and *K. crenata* leaves extracts inhibited pyrogallol autoxidation in a dose-dependent manner, thereby reducing the probability of peroxide formation. Also, a number of physiological processes need nitric oxide during metabolism. Abnormalities in NO production, that is high concentration of NO, has been linked to different diseases [57]. The toxicity of NO increases greatly when it reacts with superoxide radical, forming the highly reactive peroxynitrite anion ($ONOO^-$) [62]. The NO scavenging activity of the extracts buttresses their potential to limit oxidant-mediated damage.

Hydroxyl radical is the leading cause of oxidative biological damage such as protein disulfide bond breakage or denaturation, which results in unfolding and refolding of proteins into abnormal spatial configurations, and it is observed in neurological disorders [44, 52]. The results of the present study showed that *S. mombin*, *C. papaya* and *K. crenata* leaf extracts are effective hydroxyl radical scavengers, indicating that they can prevent or mitigate brain oxidative biological damage.

Lipid peroxidation is a chief feature of many pathologies including AD. Many studies have demonstrated increased lipid peroxidation in brain of patients with AD [38, 43]. Damage to cell membrane generates a number of degraded products which is associated with lipid peroxidation. A major degraded product of lipid peroxidation is malondialdehyde [24]. The significant lipid peroxidation inhibitory activity of *S. mombin*, *C. papaya* and *K. crenata* leaf extracts is a further reflection of their medicinal potential.

A promising management of neurological and neurodegenerative disorders such as AD and senile dementia is linked to acetylcholinesterase enzyme inhibition [19]. The enzyme is important in the breakdown of acetylcholine, and inhibition of the enzyme leads to increase in the concentration of acetylcholine and increase in communication between the brain nerve cells [62]. The anti-acetylcholinesterase activity shown by the extracts in this study suggests that the plants are potential sources of effective compounds that can stimulate an increase in acetylcholine level in AD and other dementias. Plants that possess high phenolic content have also been reported to inhibit AChE activity [3]. Therefore, the inhibitory effect of the extracts on AChE activity may be linked to their phenolic components. The Pearson correlation coefficients of the total phenolic and flavonoid contents, and the antioxidant activities obtained in this study buttresses this point. The Pearson correlation coefficients obtained in the present study also show that the polyphenolic contents and antioxidant activities of the extracts have strong positive correlations with the AChE inhibitory activity.

Alzheimer's disease (AD) is the most prevailing neurodegenerative disease in the ageing population. Two major factors involved in the pathogenesis of AD are oxidative stress and reduction in brain acetylcholine level. *Spondias mombin*, *C. papaya* and *K. crenata* leaf extracts demonstrated positive correlation between their remarkable antioxidant and anticholinesterase inhibitory effects supporting their traditional use in managing brain-related

disorders and indicating their potential usefulness in the treatment of AD. However, further investigations are necessary in in vivo and clinical settings to establish the promising in vitro effects.

Lipinski's rule of five assists in evaluating the pharmacokinetic properties and bioavailability of oral drugs. According to the rule, a violation of more than one rule is an indication of poor bioavailability. The TPSA reflects the phytochemicals' hydrophilicity and is important in protein–ligand interaction. Generally, druggable compounds with TPSA less than 140 Å and the number of rotatable bonds less than 10 have good oral bioavailability [60].

Molecular docking is the process by which 2 molecules fit together in 3-dimensional space; it is a key tool in structural biology and computer-aided drug design [30]. The best pose of each compound is always selected based on their best conformation that allows the lowest free binding energy and analyzed for further interaction of the docked structure [27]. Quercetin, kaempferol, ferulic acid, leucocyanidin, gallic acid and isorhamnetin fit into the active site of 4EY5, and the binding affinity between these phytochemicals and AChE (4EY5) is stabilized by non-covalent bonds, which includes hydrogen bond, hydrophobic bond and pi-interactions. Hydrogen bonds play a crucial role in enzyme catalysis, protein–substrate and protein–inhibitor complexes, and the structural stability of various biological molecules [37]. Pi–pi stacking observed in most of the interactions is formed between the phenyl ring of the phytochemical and the amino acid residues. Pi–pi interactions are a type of non-covalent interaction pivotal to biological events such as protein–ligand recognition by providing a significant amount of binding enthalpy [35]. The results of the molecular docking and pharmacokinetic studies showed that quercetin, kaempferol, ferulic acid, leucocyanidin, gallic acid and isorhamnetin fulfill the requirements for an anti-Alzheimer's disease drug, such as ADMET, non-toxicity, binding affinity, inhibition constants, antioxidant and neuroprotective inhibitory properties and good interaction with Alzheimer's disease-associated target. Thus, these six phytochemicals from *S. mombin*, *C. papaya* and *K. crenata* leaf extracts with antioxidant activity, inhibitory and neuroprotective activities may be considered an anti-Alzheimer's disease drug agents.

Conclusions

This study reveals that *Spondias mombin*, *Carica papaya* and *Kalanchoe crenata* methanol leaf extracts provide a significant source of secondary metabolites that act as natural antioxidants and acetylcholinesterase inhibitors, which will be helpful in the treatment of Alzheimer's disease.

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Author contributions

ARA did investigation, data curation, formal analysis, writing—original draft; MEA carried out formal analysis and writing; MTO contributed to project administration and supervision. AAA performed project administration and contributed resources. ACA contributed to conceptualization, supervision, validation, writing—review and editing.

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Availability of data and materials

Raw data were generated and will be provided from the corresponding author on reasonable request.

Declarations

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The authors (ARA, MEA, MTO, AAA, ACA) declare that they have no competing interests.

Abbreviations

ABTS

2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonate)

AChE

Acetylcholinesterase
AD
Alzheimer’s disease
ATCI
Acetylthiocholine iodide
ROS
Reactive oxygen species
FRAP
Ferric-reducing antioxidant power
NO
Nitric oxide
NMDA
N-Methyl-d-aspartate
DPPH
1,1-Diphenyl-2-picrylhydrazyl
HPLC–DAD
High-performance liquid chromatography-diode-array detector
CUPRAC
Cupric ion reducing antioxidant capacity

Publisher's Note

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DETAILS

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Unlocking the power of precision medicine: exploring the role of biomarkers in cancer management

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ABSTRACT (ENGLISH)

Background

Personalized or Precision medicine (PM) is a promising approach for the cancer treatment that tailors treatment to a patient's characteristics. Biomarkers are crucial for identifying the patients who are expected to derive greatest advantage from targeted therapy.

Main body

Here, various biomarkers, including genetic, epigenetic, protein, and metabolites, and their clinical significance, are discussed. The review provides insights into the use of biomarkers and their clinical significance in cancer treatment. There are several hurdles in use of PM in oncology, such as the complexity of tumor biology and heterogeneity, limited availability of biomarkers, high cost of targeted therapies, resistance to targeted therapies, and ethical and social issues.

Conclusion

The biomarkers play a crucial diagnostic role in the treatment of cancer. The review also acknowledges the challenges and limitations of personalized medicine which, if resolved, can be helpful in the management of cancer.

FULL TEXT

Background

The old concept “One size fit for all” is now turned into an individualized tailormade approach to personalized or precision medicine (PM). Cancer is a complex and heterogeneous disease that is responsible for a significant proportion of morbidity and mortality worldwide. Despite decades of research, the management of cancer remains a significant challenge due to the diverse biological and genetic characteristics of tumors. Since it is based on the distinct molecular characteristics of each patient's tumor, PM has revolutionized cancer treatment. PM has shown promise in improving treatment outcomes and reducing toxicities associated with traditional chemotherapy. As PM continues to evolve, more effective and targeted therapies will likely emerge in near future, offering hope for patients with previously incurable cancers [1].

National Human Genome Research Institute, U.S. has defined PM as, “an emerging practice of medicine that uses an individual’s genetic profile to guide decisions made regarding the prevention, diagnosis, and treatment of disease.” It is a medical model that considers individual patients characteristics, including genetic, environmental, as well as lifestyle [2]. PM is particularly important because cancer is a complex disease that can vary significantly from individual to individual. What works for one patient may not work for another, and what causes cancer in one person may not be the same as what causes it in another [3]. Figure 1 depicts points which give a brief idea of why PM is important.

Fig. 1 [Images not available. See PDF.]

Characteristics of PM

PM is an approach to medical treatment that considers the individual patients’ medical needs. To examine the distinct genetic and molecular characteristics of each patient's cancer, the method makes use of a variety of technologies and instruments. The PM approach in oncology involves collaborative efforts among healthcare workers including physicians, radiation oncologists, surgeons, nursing staff, pharmacists, etc. This team works together to gather all the necessary information about the patient's cancer and with the use of that information to design a personalized treatment plan [4].

Main text

Definition of biomarkers and their role in oncology

Biomarkers are defined as, “measurable indicators of normal or abnormal biological processes, and they can be

used to diagnose diseases, monitor disease progression, and guide treatment decisions." In oncology, biomarkers can also be used to identify subtypes of cancer that may have different treatment requirements. A well-known example is breast cancer which can be classified into different subtypes based on the presence or absence of certain biomarkers, such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). This information can be used to guide treatment decisions, such as whether to use hormone therapy or chemotherapy [5]. The biomarker test is also referred to as 'liquid biopsy'. Biomarkers are molecules that can be found in blood, tissue, or other bodily fluids that can indicate the presence or severity of a particular disease [6].

Targeted therapy, on the other hand, is a type of cancer treatment that specifically targets cancer cells, while sparing healthy cells. Biomarkers play a critical role in the development of targeted therapies by identifying the specific molecular targets that are involved in cancer growth and progression [7]. The growing interest in the use of biomarkers in PM is revealed in PubMed database Search shown in Fig. 2. Using these tools to identify the specific molecular pathways that are involved in cancer growth and progression, researchers and doctors can develop more effective and targeted treatments for patients with cancer. As we continue to learn more about cancer and its underlying causes, biomarkers and targeted therapy will become even more important in the fight against this devastating disease [8].

Fig. 2 [Images not available. See PDF.]

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Different types of biomarkers

There are several different types of biomarkers which include genetic biomarkers, epigenetic biomarkers, metabolite biomarkers and protein biomarkers. Once a biomarker has been identified, researchers can use that information to develop therapies that specifically attack the molecular pathways that are involved in cancer growth and progression [9]. Several different types of biomarkers that can be used in medicine, each with its usage and examples shown in Table 1.

Table 1. Biomarkers used in cancer

Type of biomarker	Description	Usage	Examples
Genetic biomarkers	Mutations or other genetic abnormalities specific to certain diseases	Identifying patients who are more likely to respond to targeted therapies for cancer, such as the EGFR (epidermal growth factor receptor) mutation in lung cancer	MutL protein homolog 1 (MLH1), MutS homolog 2/6 (MSH2/6), PMS1 homolog 2 (PMS1/2), Breast cancer type ½ (BRCA1/2), Kirsten rat sarcoma viral oncogene homolo (KRAS), Tumor protein p53 (TP53), etc.
Epigenetic biomarkers	Changes in gene expression associated with disease	Diagnosing and treating cancer, such as the DNA methylation in breast, lung and colon cancer	Histone modification, Non-coding RNAs, Chromatin accessibility, MicroRNA expression
Protein biomarkers	Proteins produced by disease cells that can be detected in the blood or tissue of patients	Monitoring the progression of cancer	C-reactive protein (CRP), Troponin, Cancer antigen 125 (CA-125), HER2, B-type natriuretic peptide (BNP)

Metabolite biomarkers	Produced during metabolism and associated with tumor growth	Used as a biomarker for the diagnosis and monitoring of lung, pancreatic, thyroid, breast and hepatic cancer	Palmitic acid, Cholesterol, Lactate, Creatinine, Triglycerides, Urea, Ketone bodies
Transcriptome biomarkers	Changes in the expression of RNA molecules that are indicative of cancer	Differentiate types of cancers, Identify molecular targets for cancer	Immune-related genes (CD8A, IFNG), Non-coding RNA molecules (microRNAs, long non-coding RNAs-), Splice variants of RNA

Genetic biomarkers

Genetic mutations or other genetic abnormalities that are specific to certain diseases. Genetic biomarkers can serve as a clinical guide for treatment selection customized for the patients. Patients with certain genetic mutations may benefit from targeted therapies that specifically target the molecular pathways that are involved in cancer.

There are several types of genetic biomarkers for cancer, including:

Oncogenes Oncogenes are genes that can cause cancer when they are overactive or mutated and are depicted in Fig. 3. Proto-oncogenes are normal genes that allow cells growth and division or allow them to thrive [10]. However, when a proto-oncogene undergoes a mutation, it can become an oncogene, which is activated when it should not be. Oncogenes are like gas pedals that are stuck down, causing cells to divide uncontrollably, leading to the development of cancer. Different factors such as gene variants, epigenetic changes, chromosome rearrangements, and gene duplication can turn on oncogenes in cells. These factors cause changes in the DNA or RNA sequence, chemical groups attached to genetic material, or extra copies of a gene, leading to the production of too much protein that drives uncontrolled cell growth. Eg. HER2, BRAF, c-KIT, NF1 and KRAS [10].

Fig. 3 [Images not available. See PDF.]

Normal and mutated Oncogenes (Courtesy-National Human Genome Research Institute https://www.genome.gov/)

Tumor suppressor genes Tumor suppressor genes are those genes that normally help to prevent cancer by regulating cell growth and division. The mutated genes lead to cancer as shown in Fig. 4. Tumor suppressor genes act as brake pedals in cells, slowing down cell division to prevent excessive growth. The deactivation of tumor suppressor genes can cause carcinogenesis. While the majority of mutations are acquired, some types of cancer are passed down through families due to abnormalities in tumor suppressor genes. More than 50% of all cancers are caused by mutations in the TP53 gene, which makes the p53 protein. These mutations can occur in many different types of cancer. Other such genes includes TP53, BRCA1, and BRCA2, etc. [11].

Fig. 4 [Images not available. See PDF.]

Normal and mutated tumor suppressor genes (Courtesy-National Human Genome Research Institute https://www.genome.gov/)

DNA repair genes DNA repair genes are involved in repairing DNA damage that can lead to cancer. Mutations in these genes can increase the risk of developing cancer. During cell division, errors may occur while copying the DNA, and DNA repair genes help to identify and repair these mistakes or trigger cell death if they can't be fixed. When DNA repair genes don't function properly, mistakes can accumulate, which may lead to uncontrolled cell growth. Changes in DNA repair genes can be inherited or acquired, just like other types of gene mutations. The BRCA1 and BRCA2 genes are examples of DNA repair genes that, when mutated, increase the risk of certain cancers, especially breast and ovarian cancer. However, mutations in these genes can also occur in tumor cells of individuals who did not inherit them. Eg. MSH2 and MLH1 [10].

Microsatellite instability (MSI) MSI is a biomarker that indicates a defect in a short segment of DNA leading to an

increased risk of cancer development. MSI are prone to errors during DNA replication and repair. MSI is commonly seen in certain types of cancer, such as colorectal, gastric, breast, thyroid, and prostate cancers, cholangiocarcinoma, leukaemia, endometrial carcinoma, pancreatic ductal adenocarcinoma, etc. It can be further classified as MSI-low and MSI-high. However, currently, MSI-low and microsatellite stability are considered the same. Further details are reviewed by Baudrin et al. [12] and Li et al. [13] MSI can be detected using next generation sequencing, immunohistochemistry and single molecule inversion probes having accuracy more than 90%. However, fluorescent multiplex polymerase chain reaction and capillary electrophoresis are referred as gold standard for MSI detection having accuracy 100%. Flurouracil, nivolumab, ipilimumab, pembrolizumab, and bevacizumab are reported to be useful in colorectal cancer with MSI [13].

Epigenetic biomarkers

Epigenetic biomarkers measures variation in disease or drug associated epigenic expressions that can also be used to identify disease. Epigenetic biomarkers are particularly useful in the diagnosis and treatment of cancer. The DNA molecules and the proteins that interact with DNA can undergo chemical modifications that influence the activation and deactivation of genes. These modifications can be passed on from one cell to another during cell division, and they can also be passed from one generation to the next. All epigenetic changes that occur in a genome are referred to as an epigenome [14]. Some epigenic biomarkers are discussed below.

DNA methylation

DNA methylation is a common epigenetic modification that involves the addition of a methyl group to DNA. Methylation of CpG islands, which are often located in the promoter regions of genes, can lead to gene silencing. Aberrant DNA methylation patterns have been observed in many types of cancer, including breast, lung, and colorectal cancer [15].

Histone modifications

Histone proteins, which package DNA in the nucleus, can be modified by the addition or removal of chemical groups. These modifications can alter the accessibility of DNA to the transcriptional machinery and can affect gene expression. For example, acetylation of histones is generally associated with gene activation, while deacetylation is associated with gene silencing. Changes in histone modifications have been observed in many types of cancer, including leukaemia, prostate, liver, and lung cancer [16, 17].

Protein biomarkers

These are other types of biomarkers that can be used in medicine. Protein biomarkers are specific proteins that are produced by disease cells and can be detected in the blood or tissue of patients with the disease. Protein biomarkers are particularly useful in the diagnosis and monitoring of cancer. Certain proteins such as PSA, CA-125, HER2, CEA, etc. may be overexpressed in cancer cells, and monitoring the levels of these proteins in the blood or tissue of patients can help a physician to track the progression of the disease [18].

Metabolite biomarkers

These are small molecules that are produced by cellular metabolism, and they can be detected in biological samples such as blood, urine, or tissue. Metabolite biomarkers could be a useful tool for the diagnosis of lung, pancreatic, thyroid, breast and hepatic cancer, etc. as they offer a non-invasive and cost-effective approach to disease detection. The diagnostic accuracy of metabolite biomarkers could be further improved by combining multiple biomarkers into a single diagnostic test [19].

Nucleotide metabolites

Nucleotide metabolites are involved in DNA synthesis and repair, and their levels can be altered in cancer cells. The increased levels of deoxythymidine monophosphate (dTMP) and decreased levels of inosine monophosphate (IMP) have been observed in breast cancer cells. Antimetabolites targeted toward Thymidylate synthase, Dihydrofolate reductase and Glycinamide ribonucleotide formyl transferase are used in clinical practice [20].

Amino acid metabolites

Amino acid metabolites are involved in protein synthesis and energy production, and their levels can be altered in cancer cells. The increased levels of alanine and decreased levels of glutamate have been observed in lung cancer

cells. Higher levels of Kynurenine are observed in lung and ovary cancer while Hydroxyproline has been related to hepatic cancer [21].

Lipid metabolites

Lipid metabolites are involved in cell membrane structure and function, and their levels can be altered in cancer cells. Increased levels of phosphocholine and decreased levels of phosphatidylcholine have been observed in breast cancer cells. Targeting Stearoyl CoA-desaturase can benefit in gastric cancers and it is also associated with hepatic cell cancer. High levels of phospholipase A2 are associated with colorectal cancer [22].

Carbohydrate metabolites

Carbohydrate metabolites are involved in energy production and cell signaling, and their levels can be altered in cancer cells. The increased levels of lactate and decreased levels of glucose have been observed in many types of cancer cells. Glycoproteins antigens such as CA19-9, CA125, and α-fetoprotein are found to have diagnostic potential in colon carcinoma and ovarian cancer. Iminosugars such as swainsonine, Castano spermine, siastatin B, etc. reported to exhibit antitumor effects [23].

Transcriptome biomarkers

It refers to the group of biomarkers that are identified based on changes in gene expression patterns. Transcriptome biomarkers can be used to understand disease progression, drug efficacy, and patient response to treatment. By analyzing the changes in gene expression between different conditions, researchers can identify specific genes or sets of genes that are upregulated or downregulated [24]. Two types of transcriptome biomarkers are discussed below.

mRNA expression biomarkers

The oncotype DX test measures the expression of twenty genes in breast cancer tissue and is used to envisage the recurrence and the possible benefit of chemotherapeutic treatment. *PAM50* test is used as a tool to check the expression of fifty genes and it serves to classify breast cancer based on prognosis and treatment outcomes. *Mammaprint* is another test that is used to check seventy genes expression in breast cancer tissue and is used to predict the risk of recurrence and the potential benefit of chemotherapy [25].

Decipher gene expression test measures the genetic expression in prostate tumor and serve as tool to identify recurrence and effects of radiation therapy. The test score as shown in Fig. 5 is useful in the prediction of risk of prostate cancer [26].

Fig. 5 [Images not available. See PDF.]

Decipher scores and risk of metastasis and death from prostate cancer

Non-coding RNA biomarkers

Non-coding micro-RNA biomarkers viz*. MiR-21, MiR-155* and long non-coding biomarkers HOTAIR, MALAT1, etc. are known to be upregulated in many types of cancer, including breast, lung, liver, and colorectal cancer. These are used as a tools to determine tumor progression invasion and metastasis [27, 28].

These biomarkers all have unique strengths and limitations, and can be used in different ways to diagnose, monitor, and treat a wide range of diseases. By identifying the specific biomarkers associated with a particular disease, doctors can develop more personalized treatment plans for their patients.

Imaging biomarkers (IB)

IB are integral part of routine cancer management serving as indispensable tool in the standard care of the cancer patients and clinical decision making. The key techniques used as IB are magnetic resonance imaging (MRI), positron emission tomography (PET), computed tomography scan and ultrasound imaging. These techniques provide information about patient health, diagnosis, disease progress and response to the treatment. The quantifiable characteristics including size, shape, density, texture, and functional information of the tumor allows the healthcare providers to tailor the treatment plans [29]. Artificial intelligence can be trained using a prior database of IB to redefine the biomedical imaging as a clinical decision making tool and to improve diagnostic precision [30, 31].

Spotlight on biomarkers in cancer

HER2 in breast cancer

HER2 protein is overexpressed in approximately 20% of breast cancers, and its overexpression is associated with a more aggressive form of the disease. HER2 plays a critical role in breast cancer development and progression. HER2 overexpression can lead to abnormal cell signaling, increased cell proliferation, and decreased apoptosis (programmed cell death). It all contribute to tumor growth and spread. HER2 has also been identified as a target for breast cancer treatment. Drugs that target HER2, such as trastuzumab and pertuzumab, have been shown to be effective in the treatment of HER2-positive breast cancer. Additionally, nelipepimut-S vaccine trastuzumab–emtansine conjugate is also strategically employed in clinical setting against HER2. However, it is worth to noting that trastuzumab–deruxtecan is known to cause hematological effects, hepatic toxicity and gastrointestinal toxicity. In some cases pulmonary toxicity with it caused death [32].

Furthermore, tyrosine kinase (TK) inhibitors designed to inhibit HER2 were also developed. Lapatinib and pyrotinib are reversible while neratinib and tucanib are irreversible TK inhibitors. These drugs can be prescribed for advanced breast cancer [33].

EGFR in non-small cell lung cancer (NSCLC)

EGFR mutations are present in approximately 10–15% of NSCLC cases, with the majority of these mutations being exon 19 deletions or exon 21 L858R substitutions. These mutations lead to the activation of the EGFR pathway, which plays a critical role in the development and progression of NSCLC. Targeted therapies that inhibit EGFR activity, such as gefitinib, erlotinib, and afatinib, have been developed and are effective in treating NSCLC patients with EGFR mutations. However, resistance to these drugs can develop over time, and alternative therapies are needed. Osimertinib is a third-generation EGFR inhibitor that is effective in treating NSCLC patients with EGFR mutations, including those with resistance to first-generation EGFR inhibitors. It may be a more effective than the first-line treatment option for NSCLC patients with EGFR mutations [34].

Raf murine sarcoma viral oncogene homolog B (BRAF) in melanoma

BRAF mutations are present in approximately 50% of melanoma cases and lead to the activation of the Mitogen-activated protein kinase (MAPK) signaling pathway, which plays a critical role in the development and progression of melanoma. BARF mutations can cause unstoppable cell division which may lead to tumor formation. BRAF V600E is a very commonly observed type of mutation where mutations occur in valine (V) and glutamic acid. It is useful as a prognostic marker in the detection of malignancies. BARF mutations are a reason for adenocarcinoma in nonsmoking as well as women population [35]. Its testing is suggested in metastatic colorectal cancer [36].

Targeted therapies that inhibit BRAF activity, such as vemurafenib, dabrafenib, and encorafenib, have been developed and are effective in treating melanoma patients with BRAF mutations. However, resistance to these drugs can develop over time, and alternative therapies are needed. In addition, targeted therapy can also lead to adverse events, such as skin toxicity and the development of secondary malignancies. The development of combination therapies that target multiple pathways, the use of immunotherapy to enhance the immune response to melanoma, and the identification of biomarkers that can predict response to targeted therapy and help guide treatment decisions. The identification of predictive biomarkers, such as baseline lactate dehydrogenase levels, can also help guide treatment decisions and improve patient outcomes [37].

BRCA1/2 mutations

BRCA1/2 are genes that are involved in DNA repair. Mutations in these genes increase the risk of developing breast and ovarian cancer. Testing for BRCA1/2 mutations can help identify individuals who are at high risk of developing these cancers and may benefit from early screening and preventive measures. Breast cancer that is associated with BRCA1/2 mutations may respond better to certain types of chemotherapy, such as platinum-based drugs. Additionally, some targeted therapies, such as poly ADP-ribose polymerase (PARP) inhibitors, have shown promise in treating breast and ovarian cancers that are associated with BRCA1/2 mutations. In cells with BRCA1/2 mutations, PARP inhibitors can lead to the accumulation of DNA damage and ultimately cell death. PARP inhibitors have been approved for the treatment of certain types of ovarian and breast cancers that are associated with BRCA1/2 mutations. PARB inhibitors olaparib, rucaparib, and niraparib are approved for cancer treatment [38, 39].

Clinical significance of biomarker-based PM

Biomarkers are measurable indicators of physiological or pathological processes, and their use in PM allows for tailored treatment strategies based on individual patient characteristics. The clinical significance of PM is summarized in Fig. 6. Biomarker-based PM has significant clinical significance in cancer treatment. Biomarkers help identify patients who are most likely to respond to specific therapies, enabling clinicians to select the most appropriate treatment for an individual patient. Biomarker-based PM can also help reduce the use of ineffective treatments, saving patients from unnecessary toxicity and healthcare costs. Biomarker-based PM can also help reduce the use of ineffective treatments, saving patients from unnecessary toxicity and healthcare costs. For example, testing for KRAS mutations in colorectal cancer can identify patients who will not benefit from anti-EGFR therapy such as cetuximab [40].

Fig. 6 [Images not available. See PDF.]

Clinical significance of biomarkers at different stages of cancer

Screening

Biomarkers can be used in cancer screening to identify individuals who may be at increased risk of developing cancer. The elevated levels of PSA in the blood can be a sign of prostate cancer and may prompt further testing. Other biomarkers, such as carcinoembryonic antigen (CEA), can be used to monitor the response to treatment in patients with certain types of cancer [41]. It is reported that Circulating tumor DNA (ctDNA) such as EGFR, KRAS or BRAF and protein biomarkers like CA125, CEA, etc. in addition to screening may be helpful in monitoring response in lung cancer patients [42].

Differential diagnosis

Liquid biopsy can help distinguish between different types of cancer or between cancer and non-cancerous conditions. Testing for the presence of specific gene mutations, such as EGFR mutations in lung cancer, can help determine the appropriate treatment for the patient. Elevated levels of PSA in the blood can be a sign of prostate cancer, but can also be elevated in non-cancerous conditions such as benign prostatic hyperplasia (BPH) or prostatitis. CA-125 is often used as a biomarker for ovarian cancer, but it can also be elevated in other conditions such as endometriosis or fibroids. CEA can be elevated in a variety of cancers, including colon, lung, and pancreatic cancer, but can also be elevated in non-cancerous conditions. ALK gene rearrangements are commonly found in NSCLC, but can also be present in other types of cancer, such as anaplastic large-cell lymphoma [43, 44].

Cancer classification

Biomarkers can also be used to classify different types of cancer based on their molecular characteristics. Breast cancer can be classified into different subtypes based on the expression of certain genes, such as ER and HER2. This information can be used to guide treatment decisions and predict the response to therapy. BRAF mutations are used to classify melanoma as either BRAF-mutant or BRAF-wildtype. MSI is used to classify colorectal cancer as either MSI-high or MSI-low/microsatellite stable [45].

Prognosis

Biomarkers can provide important information about the likely course of the disease and the patient's chances of survival. Testing for the expression of certain genes, such as the Oncotype DX gene panel in breast cancer, can help predict the likelihood of cancer recurrence and guide treatment decisions. Ki-67 is a protein that is present in rapidly dividing cells and is commonly used as a biomarker of tumor proliferation. High levels of Ki-67 expression and mutations in the p53 gene are associated with poorer outcomes in breast cancer, lung cancer, and other types of cancer [46].

Cancer prediction and treatment stratification

Biomarkers can be used to predict the likelihood of developing cancer or to stratify patients into different treatment groups based on their risk profile. Genetic testing for mutations in the BRCA1/2 genes can help identify individuals who are at increased risk of developing breast and ovarian cancer and may benefit from more frequent screening or prophylactic surgery. BCR-ABL gene fusion is associated with chronic myelogenous leukemia (CML) and can be

targeted with tyrosine kinase inhibitor therapy, which has dramatically improved outcomes for CML patients. PD-L1 testing can help predict which patients are likely to respond to immune checkpoint inhibitor therapy, which targets PD-L1 [47].

Therapy-related risk management

Biomarkers can help identify patients who are at increased risk of developing treatment-related toxicities, allowing for more personalized treatment regimens. Testing for certain genetic variants can help predict the risk of chemotherapy-induced peripheral neuropathy or other adverse effects. CYP2D6 genotype can help identify patients who may be at increased risk of developing toxicities from chemotherapy drugs like tamoxifen, which is used to treat breast cancer [48]. UGT1A1 is a gene that is involved in the metabolism of irinotecan, a chemotherapy drug used to treat several types of cancer and drug toxicity can be assessed [49].

Therapy monitoring

Biomarkers can be used to monitor the response to therapy and guide treatment decisions. Imaging biomarkers such as PET scans or MRIs can be used to assess the response of tumors to treatment, while blood-based biomarkers such as circulating tumor DNA (ctDNA) can provide early indications of treatment efficacy or resistance [50]. An integrated approach using PET/CT and PET/MRI as imaging biomarkers can be useful for the therapy endpoint selection, standard tool as imaging biomarkers reduce diagnostic variability [51].

Post-treatment monitoring

Biomarkers can be used to monitor patients for cancer recurrence or the development of secondary cancers. For example, measuring serum levels of certain proteins, such as CA-125 in ovarian cancer, can help detect cancer recurrence at an early stage and guide further treatment [52]. BNP and N-terminal proBNP is used in the clinical decision making treatment paediatrics with pulmonary hypertension. Quantification of HCV-RNA may serve as guide for monitoring hepatitis-C treatment. Similarly, PSA used for prostate cancer, HIV-RNA for antiretroviral therapy monitoring, prothrombin time is useful in follow-up after warfarin treatment [53]. Mycobacterium tuberculosis specific $CA4^{+}$ T cells can be used for post-treatment monitoring [54].

Challenges and limitations of PM in oncology

PM in oncology involves using a patient's genetic information to determine the most effective treatment for their cancer. While this approach has shown promise, some challenges and limitations need to be addressed. These challenges include data analysis, tumor heterogeneity, and clinical trial design, while limitations include cost, accessibility, ethical concerns, resistance to targeted therapies, etc. are depicted in Fig. 7.

Fig. 7 [Images not available. See PDF.]

Challenges and Limitations in PM in Oncology

The use of biomarker-based PM has the potential to revolutionize healthcare by improving the accuracy of diagnosis, the effectiveness of treatment, and ultimately patient outcomes. However, there are also potential consequences associated with the use of biomarkers in PM.

Complexity of tumor biology and heterogeneity

Tumor biology is a complex and heterogeneous process that involves numerous genetic and epigenetic alterations. Tumors can contain multiple subclones with different genetic mutations or other biomarkers. This means that different parts of the same tumor can have different molecular characteristics [55, 56]. Even tumors of the same type can have different molecular characteristics, making it difficult to identify a single biomarker that can be used to guide treatment decisions for all patients. Tumor heterogeneity has been recognized as a major contributor to the complexity of tumor biology. Tumor heterogeneity can occur both spatially and temporally, resulting in subpopulations of cells with distinct phenotypic and genotypic characteristics. This heterogeneity poses significant challenges to the diagnosis, prognosis, and treatment of cancer. Tumors can evolve, leading to changes in molecular characteristics and the emergence of new subclones. This can make it challenging to determine the most appropriate treatment for a patient based on a single biomarker [57]. Tumors can develop complex signaling pathways that interact with each other, making it difficult to identify a single target for therapy [58]. Not all tumors

have biomarkers that are robust and reliable predictors of response to therapy. Some biomarkers may have limited sensitivity or specificity, leading to false positives or false negatives [59].

Limited availability of biomarkers

The limited availability of biomarkers is a major challenge in the field of PM. Biomarkers are molecular or cellular characteristics that can be used to identify a disease or its progression and can also be used to predict the response to therapy. However, the identification and validation of biomarkers is often a lengthy and expensive process, and many potential biomarkers fail to make it to the clinical setting due to limited availability [60]. The lack of biomarkers also limits the ability to personalized treatment for patients. Without reliable biomarkers, clinicians may have to rely on a trial-and-error approach to treatment, leading to delays in effective therapy and unnecessary side effects from ineffective treatments [61]. To address this issue, researchers are exploring new methods for identifying biomarkers, such as the use of artificial intelligence and machine learning algorithms. These techniques can analyze large amounts of data and identify patterns that may not be apparent to the human eye, leading to the discovery of new biomarkers. Along with the limited availability of biomarkers, limited sensitivity and specificity, the need for specialized tools and techniques, cost and validation of the method parameters may pose the challenges in the clinical use of biomarkers [62].

High cost of targeted therapies

Targeted therapies have revolutionized cancer treatment by selectively targeting cancer cells and minimizing damage to healthy cells. However, these therapies are often associated with high costs, which can limit access for patients. The high cost of targeted therapies is due to a variety of factors, including the development and manufacturing process, the cost of clinical trials, and the exclusivity granted by patents. In addition to limiting patient access, the high cost of targeted therapies also puts a strain on healthcare systems and insurance providers. This can lead to difficult decisions about which treatments to cover and which patients to prioritize for treatment. To address this issue, researchers and policymakers are exploring new approaches to drug pricing and reimbursement, such as value-based pricing and outcome-based contracts [63].

Resistance to targeted therapies

The development of resistance in cancer tissues is a chief issue in the use of targeted cancer therapies. Resistance can arise from a variety of mechanisms, including genetic mutations, activation of alternative signaling pathways, and changes in the tumor microenvironment [64]. Research has shown that the development of resistance to targeted therapies can be delayed or prevented by combining targeted therapies with other treatment modalities, such as chemotherapy or immunotherapy. Additionally, the use of combination therapies that target multiple pathways involved in tumor growth and survival may reduce the likelihood of resistance development. Another approach to overcoming resistance to targeted therapies is the use of PM. By identifying specific molecular alterations in a patient's tumor, clinicians can tailor treatment to target those specific alterations, potentially increasing the effectiveness of therapy and reducing the likelihood of resistance [65].

Ethical and social issues

Biomarkers have revolutionized cancer treatment, allowing for more personalized and effective therapies. However, the development and use of targeted therapies also raise ethical and social issues that need to be addressed. One of the major issues is the high cost of biomarker testing and targeted therapies, which can limit access for patients who cannot afford them. Additionally, the use of targeted therapies can raise questions about the allocation of healthcare resources and the prioritization of certain patients over others in terms of receiving these treatments. Another ethical issue is the potential for targeted therapies to exacerbate existing health disparities, as certain populations may not have access to the same level of care or may not be included in clinical trials. Additionally, there are concerns about the potential for genetic discrimination, as the use of targeted therapies may reveal information about a patient's genetic makeup that could be used against them in areas such as employment or insurance [66].

Risk of misinterpretation

Biomarkers are complex indicators of disease, and their interpretation requires a deep understanding of the

underlying biology and context of the patient. Misinterpretation of biomarker results can lead to inaccurate diagnoses, ineffective treatments, and unnecessary interventions. Biomarkers can give false positive or false negative results due to limited biomarker specificity, tumor heterogenicity or lack of standardization methods. Overreliance on the biomarkers leads missing important clinical information during treatment [67].

Conclusion and future perspectives

Targeted therapies are transforming cancer treatment, but there is more work ahead. PM needs better clinical guidelines, trials and biomarkers to enhance patient outcomes, demanding new practices and tools. Biomarkers improve treatment by matching patients' right therapy, saving costs. PM fight drug resistance through new targets and tailored treatments based on tumor characteristics, boosting outcomes.

In the future, we aim to improve biomarkers, discover new targets and explore therapies to combat resistance in cancer treatment. Challenges include high cost and ethical concerns. We need more sensitive diagnostic tools for limited biomarker quantities in the body fluids. Collaboration among researchers, clinicians, and policymakers is essential. Genomic sequencing enables personalized cancer treatment, and artificial intelligence helps to analyze vast data. Ongoing research is vital to find biomarkers, genetic drivers and affordable targeted treatment for wider patient population.

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Abbreviations

BNP
B-type natriuretic peptide
BPH
Benign prostatic hyperplasia
BRCA1/2
Breast cancer type ½
BRAF
Raf murine sarcoma viral oncogene homolog B
CA-125
Cancer antigen 125
CEA
Carcinoembryonic antigen
CML

Chronic myelogenous leukemia
CRP
C-reactive protein
dTMP
Deoxythymidine monophosphate
EGFR
Epidermal growth factor receptor
ER
Estrogen receptor
HER2
Human epidermal growth factor receptor 2
IMP
Inosine monophosphate
KRAS
Kirsten rat sarcoma viral oncogene homolog
MAPK
Mitogen-activated protein kinase
MLH1
MutL protein homolog 1
MRI
Magnetic resonance imaging
MSH2/6
MutS homolog 2/6
MSI
Microsatellite instability
NSCLC
Non-small cell lung cancer
PET
Positron emission tomography
PARP
Poly ADP-ribose polymerase
PM
Personalized medicine
PMS1/2
PMS1 homolog 2
PR
Progesterone receptor
PSA
Prostate-specific antigen
TK
Tyrosine kinase

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Nanocrystals: an emerging paradigm for cancer therapeutics

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ABSTRACT (ENGLISH)

Background

Medical fraternity are continuously pitching toward the development of novel mechanisms to combat the menace of cancer and to enhance the efficacy of prevailing molecules. During the drug development phase, majority of new molecular entity pose a threat due to hydrophobic nature, that compromises its bioavailability upon administration. These suboptimal accumulation and low drug loading hampers the clinical translation in cancer therapy.

Main body of abstract

Nanotechnology with valuable advantages create possibilities to accelerate the efficacy of treatment. Compared to matrix-based formulations, drug nanocrystals (NCs) with smaller size, high drug loading, high active targeting, extended circulation, great structural stability, tailored dissolution, and being carrier free have sparked a lot of interest in drug delivery. Many hydrophobic drugs were explored as drug NCs such as—doxorubicin, paclitaxel, campothecin and so on. However, premature leakage and clearance by mononuclear phagocytosis system lead to some great obstacles in the clinical applications of drug NCs.

Conclusion

In the recent years, strategies leading to surface modification are applied to improve uncontrolled drug release and targeting efficiency to tumor cells. The current review sheds light on various properties of drug nanocrystals, brief insights on its fabricating techniques, approaches for tumor targeting with NCs, and their applications in cancer imaging and therapeutics.

FULL TEXT

Background

The field of medicine faces challenges for effectively addressing the complexities of cancer, a multifaceted group of diseases characterized by uncontrolled growth and spread of abnormal cells. Unlike normal cells, which follow a regulated life cycle, cancer cells disrupt this balance and undergo uncontrolled division, leading to the formation of tumor masses[1]. Many factors contribute to development of cancer, like—genetic predisposition, food habits, lifestyle changes, infection caused by harmful microorganisms, and environmental factors [2]. Cancer has become a global burden with approximately 10 million deaths in the year 2022 [3–5]. The conventional therapies include radiation therapy, chemotherapy or combination therapy, while immuno-therapy, gene therapy, cell therapy, photodynamic therapy, and antibody therapy have also gained momentum, however, there effectiveness is still under debate as it lacks site specificity and low build-up inside the tumor cells [6, 7]. As a consequence, cancer treatment has now turned toward tailoring approaches which are patient specific depending on the profiles and characteristic of the disease. In this era, nanotechnology being a blend of technology, biomedicine and biomaterials, has been growing as a plausible approach to overcome the current shortfalls. Of them, NCs have sparked a lot of interest in the field of cancer therapeutics, offering unique advantages over conventional drug delivery systems. These nanoscale particles possess the ability to precisely target tumor tissues, stimulate drug solubility, and enhance therapeutic efficacy, revolutionizing the landscape of cancer treatment. The application of NCs in cancer therapy is driven by their small size, large surface area, and unique physicochemical characteristics. These attributes enable efficient drug loading and controlled release, facilitate targeted drug delivery with minimizing side effects on healthy tissues. They have also shown potential to overcome biological barriers, penetrating deep into tumor tissues by both active and passive pathways, and reaching intracellular targets, thereby maximizing treatment outcomes [8, 9]. In addition to drug delivery, NCs play a pivotal role in cancer imaging techniques. Their unique optical and magnetic properties have transformed cancer imaging, providing high-resolution and sensitive modalities. NCs in the form of semiconductor quantum dots, exhibit size-dependent fluorescence emission and high photo-stability, making them exceptional candidates for precise tumor visualization. Similarly, surface modifications with targeting ligands, NCs can be used as contrast agents for imaging, enabling accurate diagnosis, staging, and real-time monitoring of treatment responses [10, 11]. The integration of NCs with imaging techniques allows for multimodal approaches, facilitating comprehensive cancer diagnosis and personalized treatment regimens. Furthermore, NCs offer a versatile platform for combining different therapeutic agents within a single system, enabling synergistic effects, improved drug ratio, and targeted release. This approach enhances therapeutic efficacy while minimizing systemic toxicity. Further by engineering NCs to respond to external stimuli, controlled drug activation and spatiotemporal release can be achieved, paving the way for treatment tailored to individual patient profiles [12, 13].

Despite their immense potential, the full utilization of NCs in cancer treatment requires addressing challenges associated with formulation stability, scalability, and establishing reliable quality control measures. Thus, the current review comprehensively focuses on unveiling the potential of NCs as targeted delivery for the management of cancer.

Main text

Brief insights on nanocrystals and their fabricating method

NCs are nanometer-sized particles consisting purely of drug substances stabilized using suitable stabilizers [14]. The term "nanocrystals" is specifically used to refer to these crystalline nanoparticles or liquid crystalline nanoparticles. Comparing NCs to other carrier-based nanoparticles (NPs), nanocrystals exhibit a high drug loading which allows for a reduction in dose while still enhancing bioavailability and drug safety. Also, for formulating NCs, there is practically no use of organic solvents, making NCs devoid of residual solvents and solvent associated toxicity concerns. Additionally, improved solubility and dissolution rate will promote the pharmacokinetics and bio-distribution of drugs as suggested by Peltonen et al. [15].

The main excipient in fabricating NCs include a stabilizer. The primary function of a stabilizer is to prevent ostwald

ripening or aggregation of intrinsically diminuted drug nanoparticles and to preserve them in nano-crystalline form [16]. The selection of stabilizer and its concentration form one of the critical process parameter [17]. Now-a-days, researchers have a wide list of stabilizer starting from non-ionic, ionic, amphoteric to polymeric. Polymeric stabilizers have gained an edge over the others due to their enhanced wetting and steric stabilization property. These stabilizers impart stability to NCs via electrostatic repulsion, van der Waals forces, steric stabilization, surface coating, reducing interfacial tension, etc., [18].

Production techniques of NCs are categorized as top-down, bottom-up and combination technologies (details briefed in Table 1), subsequent section describes briefly the different sub-types of these technologies explored by researchers across the globe.

Table 1. Different techniques for formulating nanocrystals, including both top-down and bottom-up approaches, as well as combinational technologies [19–22]

Formulation methodology	Formulation techniques	Mechanism of size reduction
Top-down approach technologies	Media milling	Impact and attrition
High-pressure homogenization	High shear and high pressure	Bottom-up approach technologies
Precipitation or crystallization	Precipitation or controlled crystallization processes	Combinational technologies
Hybrid approaches	High energy driven	Supercritical fluid technology

Top-down techniques diminute the drug substances in micron size state to nanometer size. Different approaches include—media milling, high-pressure homogenization (HPH) (diagrammatically described in Fig. 1), and ultrasonication. Media milling, a mechanical technique used for size reduction, has demonstrated considerable efficiency in the production of NCs. This method employs milling media to fragment drug particles into smaller dimensions, milling agent such as beads/coated balls made of—glass, zirconium oxide, chromium, agate or some special polymers for grinding of drug particles and a stabilizer to ensure the stability of formulation. The main principle of media milling includes impact and attrition [23–26]. HPH is another top-down technique used for NC formulation. It involves subjecting the drug suspension to high pressure (ranging from 100 to 2000 bar)/ultra-sonication, forcing it through a narrow nozzle or valve. During this process, the particles under-go high shear stress resulting in generation of nano-sized crystalline particles [27, 28]. Dissocubes, Nanopure and Nanoedge are few patented technologies based on use of HPH [29]. Another approach, utilizes ultra-sonication as a means to reduce the particle size. Probe sonicator is a tool to transmit high and low power ultrasound cycles through the drug suspension. These cycles create alternative low and high pressure respectively in the suspension, causing formation of bubbles in low pressure cycle, which break during high pressure cycle. These results in cavitation effect causing reduction in particle size [30, 31].

Fig. 1 [Images not available. See PDF.]

Top-down approaches for fabrication of nanocrystals

An alternative strategy for developing NCs is through a "bottom-up" approach, where drug substances are precipitated to form nanoparticles with accurate control on particle growth. The critical aspect with this approach is controlling the size of re-crystallized particles, as uncontrolled growth might result in Oswald ripening. Some of the technologies using these approach include- high gravity controlled precipitation, sono-crystallization, liquid jet

precipitation, rapid expansion of supercritical solution, super critical anti-solvent, multi-inlet vortex mixing, evaporative precipitation into aqueous solution, etc. All these approaches have their own applications, however, major limitation lies in the use of organic solvents [20, 32, 33]. Some of these techniques have been modified by using supercritical fluids instead of organic solvents. Rapid expansion of supercritical solutions (RESS) and supercritical solvent/anti-solvent (SAS) methods of fabricating drug NC are based on the solubility of drugs in supercritical fluids [34, 35]. Hydrosol® and Nanomorph® are the patented technologies to produce drug NCs with size less than 100 nm. An innovative work reported by Han et al., revealed fabrication of paclitaxel nanocrystals (PNC) using a novel approach of evaporation and precipitation technique. The PNC were coated with tannic acid and ferric chloride to enable them for dual therapy—photothermal-chemotherapy. The in-vivo results exhibited mild photothermal activity along with strong tumor inhibition [36]. Through such innovative approach, advancements in the field of cancer treatment are being made, offering the potential for more effective and targeted therapies.

The combination technologies comprise of a two-step process—pre-treatment followed by processing. The pre-process step might be- pre-milling or precipitation, with a high-energy top-down process, such as milling or HPH, while the processing step includes evaporation, lyophilisation, etc. [37]. NANOEDGE®, was developed by Baxter, Inc., USA which involved precipitation of crystals as pre-treatment step and later the processing involved HPH to control the particle size and morphology [38]. More recent advancements have introduced- Nanopure® and SmartCrystal® technology into the market. Later technology integrated high-pressure homogenization with various pre-processes—like H42 (spray-drying pre-process), H69 (precipitation pre-process), H96 (lyophilization pre-process), and CT (media milling pre-process) [39, 40]. Hence, it could be summarized that all the different technologies are efficient enough for fabrication of different and stable NCs.

Role of nanocrystal technology in tumor targeting

Nanotechnology has sparked a lot of possibilities for effective delivery of poorly soluble drugs [41, 42]. They have emerged as a promising tool in the field of cancer therapy, offering unique properties and versatility to revolutionize treatment approaches [8]. One of the key advantage is their ability to serve as carriers for chemotherapy drugs by addressing crucial challenges of poor solubility, limited bioavailability, and systemic toxicity, thereby enhancing drug stability and solubility while enabling their efficient delivery to the tumor site. By modifying the surface of NCs with specific ligands, such as antibodies or peptides, they can be engineered to selectively recognize and bind to cancer cells. These targeted approach facilitates the direct delivery of therapeutic agents to the tumor site while minimizing exposure to healthy tissues [43].

NCs offer opportunities for both passive and active targeting. Passive targeting exploits the enhanced permeability and retention (EPR) effect, leveraging the unique characteristics of tumors to achieve increased drug concentration at the tumor site. This occurs due to the presence of leaky blood vessels and impaired lymphatic drainage in tumor tissues, allowing NCs to passively accumulate. On the other hand, active targeting involves incorporating ligands on the NC surface that can specifically recognize and bind to receptors overexpressed on tumor cells. This active targeting further enhances the accumulation of NCs within the tumor, augmenting treatment effectiveness [15, 44, 45]. The unique features of both pathways have been reflected in Table 2. Furthermore, NCs possess imaging capabilities that enable real-time monitoring of treatment response. Certain types of NCs, like quantum dots, exhibit unique optical properties and emit fluorescent signals when stimulated by light. This characteristic enables non-invasive imaging of tumors, facilitating early detection, precise diagnosis, and evaluation of treatment outcomes [46, 47]. Detailed application of NC-based treatment for targeting different cancers with different delivery mechanism has been discussed in the following sections.

Table 2. Role of nanocrystals in tumor targeting, along with their description, applications, and unique characteristics

Role	Description	Applications

Passive targeting	Facilitated by the enhanced permeability and retention effect	Imaging and Drug delivery
Active targeting	Surface modified with ligands or targeting molecules that specifically recognize biomarkers found in tumors	Targeted delivery to tumor cells that express specific biomarkers Facilitates precise imaging
Antibody-mediated targeting	Conjugation with antibodies that bind to tumor-specific antigens	Precise delivery and accurate imaging with great selectivity
Surface receptor-mediated	NCs are designed to specifically identify and attach to surface receptors on tumor cells	Facilitates precise receptor targeted drug delivery

Passive targeting

Passive targeting is a decisive strategy involving advantage of tumor vasculature hyper-permeability, and the immature lymph drainage system, NCs facilitate the accumulation of cytotoxic agents in tumor masses through EPR effect [48, 49]. This effect is particularly advantageous with nanoparticles having the size range of 20–500 nm. The size of particles has an impact on their interaction with macrophages and uptake by tumor cells [28]. Generally, smaller nanoparticles (20–100 nm) are cleared from the bloodstream at a slower rate (as shown in Fig. 2) [50]. For instance, Zhang et al. developed carrier-free NCs with an average size of 461.6 ± 57.72 nm, which were designed to incorporate indomethacin (IDM) and paclitaxel (PTX) using a one-step method. The concentration of PTX in tumor tissue using IDM/PTX- NCs was 2.21 times higher than that achieved with free PTX, resulting in a higher inhibition rate of 70% compared to 54% for the free PTX group [51]. Similarly, the self-assembled mitoxantrone NCs (MTO NCs) were developed by Mao et al., which showed passive targeting with particle size of 100 nm. The size optimization improved the lymph targeting of MTO NCs while reducing systemic toxicity. Bolaños et al. developed gold NCs coated with albumin, a natural protein that enhances nanoparticle stability and biocompatibility. The albumin coating improved the circulation time of the NCs and facilitated accumulation in tumor tissues. Thus, surface modifications, such as adjusting molecular weight, size, surface hydrophobicity, and surface charge, surface functionalization, etc. play a critical role to enhance circulation bypassing reticuloendothelial system (RES) [52]. Polyethylene glycol (PEG) is the most commonly used biocompatible polymer for surface coating as it extends the circulation time of the NCs in the bloodstream, allowing them to act as depot for the administered drugs until they reach the targeted tumor tissues [53–55]. In a study conducted by Mohammad et al., febuxostat NCs were coated with polylactic-glycolic acid/polyethylene-glycol (PLGA-PEG) using nanoprecipitation method, resulted in potential cytotoxic effect with significant high percentage of apoptotic cells in A549 lung cancer cells, suggesting effectiveness of NCs for targeted delivery [56, 57]. By understanding these studies and harnessing passive targeting strategies with NCs, remarkable advancements can be made in cancer treatment, improving drug delivery, minimizing side effects, and enhancing overall therapeutic efficacy.

Fig. 2 [Images not available. See PDF.]

Active and passive targeting mechanisms

Active targeting

Active tumor-targeted drug delivery is a strategy that involves modifying the surface chemistry of NCs with ligands or molecules such as proteins, nucleic acids, polysaccharides, peptides, and antibodies [58]. In general, two types of targeting are: active targeting to tumor cells and active targeting to the tumor endothelium. Tumor cell targeting involves the recognition and binding of ligands to receptors that are overexpressed on tumor cells, such as

transferrin, folate, epidermal growth factor (EGF), or glycoproteins (as shown in Fig. 2). On the other hand, tumor endothelium targeting aims to target the blood vessels within tumors by recognizing and binding to overexpressed factors like vascular endothelial growth factors (VEGF), αvβ3 integrins, vascular cell adhesion molecule-1 (VCAM-1), or matrix metallo-proteinases (MMP). Different approaches for active targeting include- receptor-mediated targeting, peptide-mediated targeting, antibody-mediated targeting, and aptamer-mediated targeting. Each approach utilizes specific ligands or molecules that facilitate the active recognition and binding of NCs to the desired target, thereby increase drug accumulation while reducing drug distribution in normal tissues, thereby reducing toxicity [59, 60]. In addition, there is a tactic for organelle-specific targeting, such as targeting the cytoplasm, mitochondria, or nucleus. Biomimetic nano-carriers can mimic the surface molecules on cell membranes and exhibit active targeting [61, 62]. A study involved combination of doxorubicin and tumor necrosis factor (TNF) related apoptosis-inducing ligand formulated as NPs and surface coated with P-selectin for targeting overexpressed CD44 over the tumor cells of breast revealed significant reduction in growth of tumor and metastatic nodules [63]. In a study conducted by Danhier et al. [64] authors investigated the active targeting using arginine–glycine–aspartic acid (RGD)-grafted PLGA-NPs loaded with the PTX. This results highlighted the potential of RGD peptides, to enhance drug delivery to tumor endothelium. Similar study was reported by Amreddy et al. [65] on polymeric nanoparticle-mediated gene delivery for lung cancer treatment. These approach illustrated accurate drug bio-distribution, minimizing multiple dose administration, enhancing biological barrier penetration, and improving overall treatment safety. Hence, presenting a new frontier in the fight against cancer, offering hope for more effective and personalized therapy.

Antibody-mediated targeting

Antibody-mediated drug targeting has emerged as a highly promising and precise strategy for delivering therapeutic agents directly to tumor cells. It is based on recognizing and binding to cancer-related antigens (carcinoembryonic antigen, fetoprotein, and human chorionic gonadotropin antigen) [66–68]. Antineoplastic immunoconjugates are created by linking monoclonal antibodies (MoAbs) with anticancer drugs through covalent bonds. To implement this conjugation, an inert spacer molecule, is engaged to facilitate the direct attachment of the drugs and antibodies. Several drugs, such as methotrexate, mitomycin C, 5-fluorouridine, maytansinoids, alkaloids, and daunorubicin, have been successfully conjugated with antibodies [69–71]. Zhi et al. [72] conducted a study harnessing antibody-conjugated NCs for the treatment of breast cancer. They synthesized NCs bounded with a potent anticancer drug and modified its surface with antibodies that specifically recognized human epidermal growth factor receptor 2(HER2) receptors. This resulted in targeted approach with increase in drug accumulation within the cancer cells, and minimizing off-target effects [72]. Antibody–drug conjugates like Mylotarg and Zevalin have gained food and drug administration (FDA) approval for the treatment of acute myelogenous leukemia (AML) and non-Hodgkin's lymphoma (NHL), respectively [73]. Moreover, the other side of antibody-mediated drug targeting presents a challenge of potential cross-reactivity with healthy cells that possess surface proteins similar to those present on cancer cells. Additionally, the restricted penetration of large macromolecular antibody-conjugates into solid tumors can hinder their ability to effectively target cancer cells deep within the tumor mass [74]. In-spite of such limitations, this strategy holds a ray of hope at the end of tunnel in enhancing the therapeutic effects of anticancer drugs and minimizing off-target toxicity, ultimately improving patient outcomes.

Surface receptor-mediated targeting

Scientists have made significant progress in enhancing the accuracy and effectiveness of cancer treatments by taking whip-hand of the specificity and binding ability of surface receptors. They have developed nanoparticles that are seasoned with targeting ligands or peptides that attach to the surfaces of cancer cells and enter them through a process called receptor-mediated endocytosis [75]. In a study focused on treatment of prostate cancer, NCs functionalized with ligands specific to the prostate-specific membrane antigen (PSMA) exhibited aberrant binding affinity at receptor site, facilitating enhanced uptake of therapeutic agents, leading to improved anticancer activity [76]. Integrins, a type of cell surface receptors involved in cell attachment and the surrounding matrix, have been targeted for tumor therapy. Peptides and peptidomimetics that bind to integrin αvβ3, expressed on endothelial cells in tumor blood vessels, have shown propitious results. In a study, carbohydrate-based NCs covalently bound to

DOX have demonstrated tumor-specific drug targeting [77]. Similarly, folate-mediated targeting has also gained engrossement due to the overexpression of folate receptors on cancer cells. FA-modified stearic acid-grafted chitosan micelles and FOL-PEG-DOX conjugates have exhibited enhanced intracellular uptake and potent cytotoxic effects in folate receptor-positive cancer cells [78]. Moreover, by utilizing ligands that recognize and bind to cancer-specific surface receptors, personalized treatment plans can be developed based on the characteristics of patients cancer cells, thus maximizing therapeutic outcomes [79]. Researches on NC-based drug therapy are listed in Table 3.

Table 3. Concise literature on research undertaken with NC-based drug formulations

Drug NCs	Route of administration	Indication	Particle size (nm)/PDI/Zeta potential(mV)	References
Bexarotene	Oral	Breast and prostate cancer	631; 0.33; + 24.6	[81]
5-flurouracil	I.V	Colorectal cancer	69.53 ± 1.14	[82]
Resveratrol	I.P	Ehrlich's ascites tumor	270; 0.31	[83]
Paclitaxel	I.V., I.P., I.T., Oral	All benign tumors	118–397; <0.3; − 20.87 ~ + 52.5	[84–90]
Docetaxel	I.V., intravaginal	Breast-, head and neck-, stomach-, hormone-resistant prostate cancer	70–526; 0.3 <; − 20.8 ~ + 10.4	[91–93]
Cabazitaxel	I.V	Breast cancer	110	[47]
Campothecin	I.V	Breast cancer	80–700; 0.128; − 11.4 ~ − 28.5	[94, 95]
Salinomycin	Oral	Colorectal cancer	210 ± 10	[96]
Amoitone B	I.V	Anti-cancer	256.3; 0.206; − 21.52	[97]
Anlotinib	I.V	Hepatocellular carcinoma	200;/; − 30	[98]
Nintedanib	Oral	Non-small cell lung cancer	325; 0.22; + 32.70	[99]

Curcumin	I.V	Inhibits cell proliferation and migration	158–749; 0.156; − 29.1	[100]
Flubendazole	I.P	Lung cancer	253; 0.358; − 30.45	[101]
Carfilzomib	I.V	Breast cancer	270–328; 0.27; − 13.7	[102]
Partheno lide	I.V	Advanced hepatocellular carcinoma	126–208; 0.230; − 11.18	[103]

Nevertheless, the development of resistance poses a significant limitation to surface receptor-mediated targeted therapies. Over time, cancer cells can develop resistance to targeted treatments. This resistance can diminish the long-term effectiveness necessitating the exploration of combination therapies or alternative treatment approaches to overcome resistance mechanisms [80].

Pharmacokinetics, bio-distribution, and in-vivo fate of nanocrystals

Understanding the pharmacokinetics, biodistribution, and in-vivo fate of drug-NCs is crucial for their buoyant use in cancer therapeutics. In the case of oral ingestion, NCs enter the gastrointestinal tract (GIT) and pass through the intestinal epithelium to enter the bloodstream. Later upon absorption they undergo metabolism via enzymatic degradation and post-absorption metabolism by liver enzymes [104]. On contrary when injected into the bloodstream, NCs can spread around the body, and their distribution is influenced by factors such as particle size, surface characteristics, and targeting strategies. They undergo biotransformation through processes such as immune recognition, enzymatic degradation, or elimination (described in Fig. 3) [105]. Hence, understanding the in-vivo fate of NCs and the quirky challenges posed by the physiological environment is crucial for their successful application in drug delivery [106, 107].

Fig. 3 [Images not available. See PDF.]

Pharmacokinetics and bio-distribution of nanocrystals via different routes of administration

Oral administration

NCs when administered via oral route enter into the GIT. A part of the NCs are absorbed via villi in the intestine, the remaining amount enters the blood stream while some still get excreted via feces. In the intestinal lumen they form complex with the bile acid, which later go across the mucus layer, epithelial layer and finally reach the blood capillary. In the blood stream, enzymes cause metabolism of the NCs in liver and spleen, a part of it is excreted via kidney and a fraction reaches the target site. In a study, PTX-NCs were administered orally to BALB/c tumor-bearing mice with two doses 80 mg/kg and 60 mg/kg. The tumor volume measured after day 6 of the treatment revealed reduction to one third volume when treated with NCs as compared to free PTX [108]. In another study PTX-NCs were coated with *N*-((2-hydroxy-3-trimethylammonium) propyl) chitosan chloride (HTCC) maintaining the average particle size around 130 nm, were observed to be localized in GIT within 3 h of oral administration. Eventually after 24 h, fluorescence signals showed their preferential accumulation in the tumor. The control group treated with taxol, also inhibited the tumor growth, however, the NCs had long residence time in tumor resulting in remarkable inhibition. The medial survival rates were lengthened even after discontinuation of the treatment [8, 109]. Thereby, such studies have unveiled the path traveled by NC after oral administration.

Parenteral administration

Upon i.v. administration the NCs enter the blood stream and bruit around in the body. During this journey they

encounter interactions with different proteins and other elements present in the blood stream. While circulating, they stumble on with the mononuclear phagocytic system (MPS), the larger size NCs are taken up by these MPS present in organs. The smaller size NCs, take a french leave across the MPS and show prolong circulation in the blood. Upon reaching the tumor site, they penetrate through the leaky vasculature of the tumor cells and undergo the EPR effect resulting in accumulation of NCs. Some of NCs enter the lymphatic system via the lymph nodes of the tumor tissue and go efferent into the blood stream. The extended circulation of NCs ensure their extended residence in the tumor, resulting in better efficacy and lower toxicity to healthy tissues [110, 111].

Diving into the fate, NCs are recognized and rapidly taken up by the phagocytic cells of macrophages. At this time, they are also labeled by opsonins, and immunoglobulins. They will be docked onto the receptors present on the macrophages, monocytes, and neutrophils, causing catastrophe of NCs via phagocytosis. The in-vivo fate is influenced by many factors- particle size, surface charge, surface hydrophilicity/hydrophobicity, morphology, dissolution rate, as well as concentration. Among these factors surface morphology influence the most [112]. Many anticancer drugs are delivered in the form of NCs—methoxyestradiol (2-ME), puerarin, and oridonin via i.v. injection [113, 114]. In a recent study consisting of PTX and campothecin NCs stabilized using pluronic-F127, significant inhibition of growth of human cancer and murine breast cancer, was observed [115, 116]. Similarly, PTX-NCs having nearly spheroid shape and hydrodynamic diameter of 419.9 ± 80.9 nm, were surface coated with polydopamine to form a reaction platform for effective PEGylation and RGD peptide conjugation. Cellular uptake and growth inhibition studies over A549 lung cells demonstrated superior activity over non-PEGylated NCs. The results suggest intratumor accumulation and retarded tumor growth, giving a hope toward coating being effective in functionalization of NCs for enhanced anti-cancer activity [117]. In another study, tocopheryl polyethylene glycol succinate (TPGS) stabilized PTX-NCs showed better therapeutic effect in taxol-resistant ovarian cancer both in-vivo and in-vitro [118]. Similarly, campothecin NCs were studied over lung metastasis of MDA-MB-231 and MCF-7 tumors. The results supported with the confocal images of the tumor sections revealed a better inhibition of tumor metastasis and apoptosis with NCs compared to campothecin salt solution and control group [116]. Such study reports supply glimpse of information depicting the in-vivo fate of NCs upon parenteral administration.

Applications of NC in cancer diagnostics and bio-imaging

NCs have also garnered significant attention in the field of bio-imaging offering promising prospects toward unambiguous detection. Cellulose NCs (CNCs) conjugated with fluorescent agents (rhodamine-B-isothiocyanate and FITC) have been explored for therapeutic imaging of tumors by measuring the acoustic signals. Similarly, non-conjugated CNCs were used for photoacoustic imaging of ovarian cancer in mouse models. The peak photoacoustic signal was reported at 700 nm with doses below 1.2 mg/mL in carcinomatous cells. For efficient bioimaging the NCs were conjugated fluorescent agent to passively trigger cell internalization with insignificant toxicity [119]. Mahmoud et al. proved the capability of fluorescent CNCs to penetrate the cancerous cells without a sign of toxicity and no effect on integrity of cell membrane. They also provided evidence of intake of positively charged CNC-RBITCs within the membranes of Spodoptera frugiperda (Sf9) and human embryonic kidney 293 (HEK 293) cells, while CNC-FITC were not taken-up owing to their negative charge. These reports suggest the relevance of surface charge of NCs with their uptake [120]. Magnetic-NCs in the form of magnetic probes have opened new avenues for bio-imaging with its signal enhancement capabilities. Although magnetic resonance imagining (MRI) is widely accepted diagnostic tool, its non-invasive nature, tomographic compatibilities and low-signal sensitivity hinders its applications. Iron-oxide-based magnetic NCs including superparamagnetic iron oxide (SPIO) are being used for enhancing the signal for better tomographic imaging. Recently, a water soluble magnetic iron-oxide NCs was fabricated with tunable MR signaling effect. The magnetic NC probes were successful in diagnosis of breast cancer tested over SK-BK-3 cell lines. Similar study was tested in breast cancer induce mice and the results suggest that the magnetic NC-antibody probe was able to signal the diagnosis of cancer with magnetic-field of 1.5 and 9.4 T. At higher magnetic-field Yong et al. [121] reported the detailed monitoring of cancer cells with complex vasculature and tumor tissues, thus suggesting the enlightening potential of magnetic NCs for diagnostics purposes.

Gold-NCs with different size and shapes have potential to extend the resonance from visible to near infrared range

where light can infiltrate into the tissues. At resonance wavelength gold can absorb and scatter the light, this property enables a distinct color to gold which is sensitive to be detected by naked eye, making gold NCs excellent for biomedical diagnostics. Typically, such gold NCs have been extensively used for immunogenic strip test. The surface electrons on the gold NCs can amplify Raman scattering signals, thus AuNCs with immune probes/antibodies significantly enhance sensitivity and contrast of detection which are stable with repetitive analysis, making it an important candidate as imaging agent for cancer cell identification and diagnosis [122]. In a study conducted with fabrication of Eu/Gd codoping HAP-NCs using co-precipitation technique possess a luminescence property, which enabled successful cell labeling and in-vivo imaging [123]. In addition, flow of magnetic-NCs within the body can be controlled by external magnetic field, making it more convenient to tailor it toward specific and targeted imagining. Such smart use of NCs has shown tremendous applications in the field of diagnostics and effective imaging [124].

Beyond diagnostics, NCs may also be used in cancer biomarker detection and liquid biopsies. NCs-biosensors detect cancer-specific biomarkers with high sensitivity and specificity, allowing for early cancer detection. These biosensors can detect and analyze biomarkers from blood samples such as circulating tumor cells, exosomes, and cell-free deoxyribonucleic acid (DNA), allowing for non-invasive monitoring of tumor growth and therapy response [125]. These aid of NCs in the development of liquid biopsy technologies, will revolutionize cancer detection.

Metallic nanocrystals for cancer theranostics

Metallic-NCs are evolving in the field of theranostics [126]. Metallic-nanoparticles can be modified in terms of size and shape, which enables precise control of their physical and chemical characteristics, such as magnetic behavior, surface plasmon resonance, and catalytic activity [127]. Additionally, the possibility of surface functionalization makes metallic-NCs flexible platforms for targeted therapy, real-time imaging, and treatment response monitoring. The majority of chemotherapeutic drugs distribute throughout the body, causing poor patient compliance, general toxicity, and even treatment cessation. This makes it difficult to deliver therapeutic agents to tumor cells with precision. Moreover, metal nanoparticles are superior to other nanoparticles because of their innate anticancer activity, which eliminates the need for additional carriers. Additionally, they are quickly eliminated from the body and are biocompatible in nature. Metal nanoparticles can be used to encapsulate or conjugate medicinal molecules, making them a viable alternative to other delivery systems [128, 129]. Some of the research related to applications of metallic NCs are discussed in subsequent sections. Table 4 summarizes studies where metallic nanoparticles loaded with anticancer agent have been used for cancer treatment.

Table 4. Types of metallic nanocrystals used for cancer theranostic agents, including the metals used, their properties, synthesis methods, and applications

Nanocrystals type	Properties	Method for synthesis	Applications
Iron nanocrystals (Fe-NCs)	Magnetism, biocompatibility, and superparamagnetic characteristics. Magnetite (Fe_3O_4) and maghemite ($–Fe_2O_3$) are two examples	Thermal decomposition, co-precipitation, hydrothermal synthesis, Microemulsion method, Iron Sono chemical synthesis	Drug delivery, magnetic hyperthermia, and MRI
Zinc nanocrystals (Zn-NCs)	Luminescent qualities and biocompatibility	Precipitation, Wet-chemical synthesis, Solid-state pyrolytic method, Sol–gel method	Fluorescence imaging and therapeutic delivery

Gold nanocrystals (Au-NCs)	Optical properties and biocompatibility	Turkevich method, Perrault method, Block copolymer-mediated synthesis, Brust method	Drug delivery, imaging, and biosensing by photothermal treatment
Silver nanocrystals (Ag-NCs)	Strong antibacterial activity and plasmonic characteristics	Chemical reduction, Physical synthesis, Biological synthesis	Imaging, photothermal treatment, and antimicrobial applications

Iron oxide nanocrystals

Fe-nanoparticles have earned importance for both therapeutic and diagnostic applications. These metal oxides can be functionalized to act as carriers for anticancer agents or imaging agents by conjugating to their surface or embedding them in a polymeric matrix. Additionally, they can also be employed as contrast agents for imaging by MRI [130, 131]. Fe-NCs were mostly synthesized using a principles of green chemistry. The scientist Mathur et al., produced water soluble Fe_3O_4-NCs by reducing colloidal iron hydroxide with green tea polyphenols (epigallocatechin gallate and epicatechin). The NCs, displayed size range of 2.5–6 nm with high degree of crystallinity. These green teas coated superparamagnetic Fe-NCs (SPIONs) served as negative contrast agent both in-vitro and in-vivo over primary macrophages and colon cancer cells-CT26, showing high uptake efficiency. Thus, SPIONs with promising transport and uptake characteristics can be explored further for multimodal imaging and therapeutic applications [132]. An in-vivo study conducted with SPION coated with phospholipid and PEG revealed substantial influence on bio-distribution and bio-clearance of bio-degradation products, suggesting their safety in cellular environments of blood and organs [133]. SPIONs have showed a great magnetic moment under the influence of static external magnetic field. These property enables them to be used as contrast agents in imaging purpose-MRI, to improve tumor visualization and identification with low poly-dispersity, and functionalized to serve as carriers for the delivery of specific drugs. Beyond imaging and medication delivery, Fe^{+2}-NCs can be used in photo-thermal therapy to absorb light energy and turn it into heat, causing hyperthermia and subsequently killing tumor cells. Additionally, through Fenton or Fenton-like reactions, Fe^{+2}-NCs have the capacity to produce reactive oxygen species (ROS), which cause cytotoxicity and destroy cancer cells. Ma et al., proposed conjugating folic acid to the surface of SPION-loaded polymeric nanoparticles in order to create SPION-based MRI contrast agents with great selectivity to cancer cells. These nanoparticles showed improved MRI efficacy in comparison to a commercial contrast agent and showed selectivity to MCF-7 and SPC-A-1 cells [134]. Another study described the use of MRI-based MUC1-expressing ovarian cancer detection utilizing C595 monoclonal antibody-conjugated SPIONs. With no in-vivo toxicity, the nano-conjugate showed considerable tumor accumulation, detection specificity, and potential anti-ovarian cancer efficacy [135].

Similar, Fe-NCs were developed by group of researcher lead by Vellingiri et al., using goat blood as bioprecursor. The γ-Fe_2O_3-NCs were coated with PEG and combined with doxorubicin with 60–70 nm size for targeting lung cancer cells. The morphological changes, cytotoxicity, and intracellular presence of iron was observed to be dose dependent within the investigational cells. Moreover, the presence of DOX within the nuclei reveals the pathway toward nuclear chemotherapy. In-vivo studies in tumor bearing mice suggest suppression of carcinogenesis while there was no obvious toxicity in healthy organs. Such studies reflect the potential of natural sources to be developed into nanocarriers as future of targeted nuclear treatment for cancer [136]. A very similar study, with Fe_2O_3-NC synthesized from goat blood using chemical reduction method, demonstrated significant cytotoxicity and active transport to cell nucleus. Further functional proteomic analysis implies the cancer cell proliferation is targeted by Fe_2O_3-NC, providing new insights for nuclear targeted cancer treatment [137]. PEG-capped Fe-NCs with different morphologies (spheres, polymorphs and wires) demonstrated strong ferromagnetic behaviour and hysteresis losses, considering hyperthermia effect with temperature rising up to 42.6 °C for medical hyperthermia applications [138]. Cheon et al., fabricated a probe system which controlled the size, magnetism and induced nuclear spin relaxation in

Fe_3O_4-NC model. The Fe_3O_4-NCs were conjugated with Herceptin. This system of NC-antibody probe was used for diagnosis of breast cancer under MRI [126]. For precise delivery, a tumor microenvironment responsive nano-system was designed by decorating Fe_3O_4-NCs with methoxypolyethene glycol (mPEG) and trans-activator of transcription (TAT). These decorated NCs were utilized to facilitate MRI, tumor magnetic hyperthermia (MHT) and mild heat-mediated immune stimulation. The cleavage of mPEG strongly inhibited the growth of tumor cells. In-vivo experiments with CT26 tumor-bearing mice showed 85.5% tumor inhibition rate and induced a magnetic hyperthermia–immune synergistic therapy, together with no obvious anticancer agent this study demonstrated a promising approach for delivering nanomedicine using MR imaging-guided tumor-targeting MHT [139].
Lin et al. created a Fe^{+2} nanocomposite for MRI-visible delivery of small interfering (si)RNA. The nanocomposite displayed equivalent silencing efficacy to Lipofectamine 2000, a commercial transfection agent, and successfully silenced the target mRNA, decreasing the expression of p-glycoprotein (p-gp). Under an MRI scanner, the transfected cells displayed a noticeable contrast enhancement, suggesting a way to tackle multi-drug resistant in cancer cells**.** Commercially, two members of SPION family, Ferumoxides (Endorem®–Europe, Feridex® in the USA and Japan) and Ferucarbotran (Resovist®–Europe and Japan) are approved for intravenous use. These SPIONs are coated with dextran and carboxy-dextran respectively. In addition, Nanotherm consisting of superparamagnetic iron oxide coated with amino silane is approved by USFDA and EMA for the treatment of Glioblastoma, prostate, and pancreatic Cancer [140].

Zinc nanocrystals (Zn-NCs)

Zn-nanomaterials have gained popularity specifically for therapeutic drug delivery, stimuli-responsive targeting, and also for diagnostic purposes. Nanoparticles composed of zinc oxide (ZnO) have received a lot of interest due to their possible use in the treatment of cancer. These nanoparticles have unique properties including photoluminescence for biosensing and characteristics of wide band-gap semiconductors that encourage the production of ROS [141]**.** Nanosized ZnO produces numerous electron–hole pairs even in the absence of UV radiation because of crystal defects, which leads to an increase in the formation of ROS. ZnO's band gap enables electrons and holes to interact with oxygen and hydroxyl ions, generating superoxide and hydroxyl radicals, respectively. Their innate propensity for cytotoxicity against cancer cells results from their capacity to trigger the production of ROS, which causes cell death [142]. Additionally, ZnO nanoparticles have been explored for imaging purposes, enabling optical imaging of particular cancer cell receptors and acting as multimodal imaging agents for cancer detection [143].
Amino-propyl functionalized ZnO-NCs exhibited hexagonal wurtzite crystalline structure with + 22 mV zeta potential. During preliminary phase study with these NCs, it was discovered that treatment of 3 times/day cause in highest toxicity rates. Further, the viability assay confirmed pro-apoptotic stimulus and necrosis. The study concluded three probable mechanism of cell death—bubble cavitation, nanoscalpel effect and electric change imbalance, thus opening new avenues for application of ZnO-NCs as effective tool for cancer treatment [144]. Cauda et al., synthesized ZnO-NCs using very efficient microwave-assisted solvo-thermal method, having spherical morphology with 20 nm size. To evaluate the cytotoxicity and cell internalization over cancerous human cell lines, the ZnO-NCs were functionalized with amine. Surface functionalization with amine enables labelling with fluorescent dyes which could be detected with flow cytometry. Also, water-soluble-tetrazolium-salt1 assay was used to quantify cell viability. The results infer significant reduction in human-epithelial cancer cell viability at dose of 10 µg/mL. Cellular internalization was observed with fluorescent signals suggesting visible internalization of ZnO-NCs [145]. Coating with lipids shields the ZnO-NCs, preventing self-aggregation, premature degradation, and promote cellular uptake in HeLa cancer cells with reduced cytotoxicity. Such results signifying the importance of multifunctional ZnO-NCs for therapeutic and bio-imaging purpose [146]. In another study, membrane destabilization occurs which allows pristine ZnO-NCs to enter cytoplasm and elicit cytotoxic response within the tumor cells [147]. Nair et al. studied the dissolution of ZnO-NCs and reported that the NCs undergo rapid dissolution in acidic pH 5–6 causing ROS stress, depolarization and superoxide formation in mitochondria, and apoptosis. This elucidates toxic mechanism of ZnO-NCs in destabilization of cancer cells using its own hostile acidic environment [148]. A novel work was carried out by Prasad et al., to enhance PDT of Chlorine, using excitation created by second harmonic (SH) light generated by

ZnO-NC, where they are internalized through endocytosis mediated by folate receptors. Here, the SH light was generated by in-situ presence of ZnO-NCs, which caused activation of photons leading to cell death (apoptosis and necrosis) in the cytoplasm of cancer cells. Such studies provide insight of ZnO-NCs as powerful tool for developing phototherapy selectively targeting specific organelle [149]. For the therapeutic purpose, researchers developed ZnO-nanoparticles loaded with DOX. This co-administration caused synergistic effect and enhanced cytotoxicity [150]. Additionally, ZnO can be used for biosensing, which enables the early identification of malignancy [151]. Moreover, the effect of zinc nanostructures on living cells, in particular cancer cells, is still under debate.

Gold nanoparticles (Au-NCs)

Over the period of time, novel delivery carriers resulted in reduction in dose dependent toxicity in comparison to conventional products. These lead to increase in research on exploring metallic materials for targeted delivery and diagnosis. Au-NC are aggregates of Au atoms ranging between 10 to 400 nm in size. The amount of gold, material of inorganic substrates, pH of medium, temperature and mechanical forces used during the synthesis governs the shaper of Au-NCs. The Au-NCs when conjugated with stimuli-responsive polymers, are found to be used for targeted delivery to specific organs of human body [152]. Various reports have successfully loaded anticancer drugs—campothecin, thymectacin, busulfan and cyclosporine using Au-NCs [8].

A study to treat metastatic breast cancer was carried out by Wang et al., using DOX decorated Au-nanorods. With irradiation of near-infrared light over these decorated Au-nanorods, caused increase in temperature along with release of DOX within the tumor as Au-NCs show a sharp absorption peak around 500–550 nm due to excitation of surface plasmons. These combination was observed to be more toxic in 4T1 breast cancer cells [153, 154]. A similar study was conducted over 4T1 breast cancer cells with Au-NCs loaded with 10-hydroxycampothecin. The AuNCs possessed average size of 130 nm with 75% drug loading, when administered i.v, showed a sustain drug release pattern and enhanced cytotoxicity [155]. A study conducted by Shen et al., revealed the use of Au-NCs for inhibition of retinal angiogenesis. The Au-NCs with average size of 26.2 nm and potential of 24.9 mV, were able to inhibit the cell proliferation to an extent of 50–72% and 54–83% inhibition of cell migration at concentration 10 μg/mL and 20 μg/mL, respectively [156]. Li et al., carried out a study to test Au-NCs as potential antiangiogenic agents. In this investigation, Au-NCs were used to target human recombinant endostatin (angiogenesis inhibitor). The results showed promising effects with tumor vascular normalization and strengthened blood vessel [157]. Au-NCs fabricated in conjugation with hydroxylcampothecin and polydopamine, when injected i.v. into the mice, showed accumulation and cytotoxicity in the tumor [158].

One of the major benefit of Au-NCs is its traceability with MRI. Enhanced light scattering and absorption caused by surface plasmon resonance allows them to act as imaging probes. It can accumulate within the tumor site along with drugs. In a study performed by Chandra et al., Au-NCs stabilized using gum arabic was successfully explored as X-ray agent for imaging the tumors of brain in mice and dog models. The treatment was followed as 5 consecutive intratumoral injections GA-AuNCs, later threshold accumulation was attained in 5 h showing complete saturation. The CT scan images revealed the retention of GA-AuNCs in the tumor site along with no clinical changes and cellular toxicity. Further, a pilot study was performed in male dog with thyroid carcinoma and osteosarcoma. Intratumoral injections were administered to the dog, post 3 weeks of treatment, the dog was euthanized and necropsy was performed. The results revealed no toxicity in vital organs, signifying use of GA-AuNCs as diagnostic agent [159]. An augmented approach was adopted by Menon et al., for synthesizing Au-NCs using garlic as precursor. The Au-NCs were suspended in garlic extract in presence of chloroauric acid with constant stirring under elevated temperature. The end point of coating was the change in color of solution from pale yellow to purple-red. The cytotoxicity was performed using MCF-7 human breast cells and L929 mouse fibroblast cells at different concentrations, observations after 24 h of incubation revealed no cytotoxic effect. Thus, it could be concluded that garlic extract did not render any cytotoxic reaction, also it formed a stable shealth around the Au-NCs, serving immense applications in imaging diagnostics [160]. Such studies reflect the potential benefits of Au-NCs which in future can be explored for tumor imaging and diagnosis.

Silver nanoparticles (Ag-NCs)

In the past decade, a prominent focus on translational research for introducing materials with nano-metric size range for cancer therapy has gained momentum. As a consequence, to date, several biomaterials including metal-based nanostructures have been explored as treatment modalities in various trials conducted to overcome cancer. Among metals, silver (Ag) has a significant anti-microbial property and a unique mode to induce cell death. Ag-nanoparticles unveils tremendous scientific data to show its possible application as anticancer agent, its high efficacy and safety [161]. Following their uptake, these Ag-nanoparticles (AgNPs) are taken up by endocytosis and within the endosomes they undergo lysosomal fusion in the organelle. The acidic environment of lysosome cause release of Ag-ions, which create unbalance cellular homeostatis leading to apoptotic cell death. Such mechanism is referred as Trojan horse. It is rightly said that to exploit the nano nature of metallic material, they are applied along with cytotoxic drugs, however, despite of favorable features Ag appears to be toxic to healthy tissues. Hence, a careful monitoring of Ag accumulation into the cancerous cells needs to be ensured. To achieve this multiple methods—active and passive targeting have already been explored for developing Ag-based nanocrystals (AgNCs). Several research groups have demonstrated the cross-talks between the cancer cells and AgNCs [162].

According to Muhammad et al., AgNCs functionalized with PTX boost the anticancer activity in human cells. The PTX-NCs were surfaced with polydopamine, later the AgNPs and tumor targeting peptide NR1 was decorated onto the PDA. This grafted Ag-PTX-NC system dramatically enhanced the cellular absorption during in-vitro anticancer models. Additionally, the Ag-PTX-NCs showed synergistic influence causing cell membrane lysis, nucleus damage, mitochondrial dysfunction, ROS over production, and breakage of DNA. Such observations linked the potential application of NR1/Ag-NC decorated PTX for targeted therapy of anticancer drugs [163]. Liang et al., demonstrated the anticancer effect of camptothecin (CPT)/Ag-NCs. Silver exhibit excellent inhibitory effect on drug resistance related p-glycoprotein (Pgp) while CPT has proven data of being cytotoxic. The combination of both CPT/Ag-NCs was able to bypass the Pgp recognition resulting in indiscriminate cytotoxicity. In addition, the drastic release of CPT into the tumor triggered by cleavage of Ag-ions in acidic microenvironment led to chromatin structure breakage, DNA damage and apoptosis. Nevertheless, such exploration of molecular events needs support of in-vivo studies [164].

Kim et al. investigated the toxic effect of synthesized crystalline AgNPs on F9 cells. They identified the dose-dependent toxic effect of AgNPs was linked with leakage of lactate dehydrogenase, ROS, and mitochondrial dysfunctioning. At high concentration DNA fragmentation, neuronal differentiation, increased expression of apoptotic genes, and decreased expression of anti-apoptotic genes was reported. The results support the use of crystalline AgNPs for differentiation therapy in amalgamation with chemotherapeutic agents [165]. In a report published by Paul et al., the crystalline AgNPs were biosynthesized using ethanolic leaf powder extract of *Premna serratifolia* L. and possessed 22.97 nm size. Anticancer effect of the synthesized AgNPs was evaluated on Swiss albino mice induced with liver cancer. The results revealed AgNPs were non-toxic with protective effects of other organs while they sustained control over the cancer progression. Such studies open up ways toward cost-effective economic alternatives for anticancer therapy, however certain understanding of molecular mechanism restrain the use of synthesized Ag-NPs of *P. serratifolia* [166]. Nima et al. developed Ag–Au nanorods functionalized with certain molecules and antibodies improved photothermal contrast and surface-enhanced Raman scattering (SERS) for the diagnosis of breast cancer [167]. Thus, AgNCs embark great potential to be explored for diagnostic and therapeutic purposes. A brief list of metal based drug delivery explored for cancer therapeutics is reported in Table 5.

Table 5. Brief list of recent metallic based drug delivery explored for cancer therapeutics

Metal	Conjugating drug	Cell lines	IC_{50} value	Proposed MOA	Reference
Gold	Docetaxel	Lung cancer cell line (H520)	25 μm	Cell disruption and apoptosis	[168]

Silver	Epirubicin	HepG2 cells	1.92 μg/mL	–	[169]
Copper	Paclitaxel	Drug-resistant prostate cancer cell	85, 172, and 193 nM	Inhibition of proteasome/poly-Ub protein degradation pathway by targeting the NPL4 protein	[170]
Gold and iron	Doxorubicin	HeLa cancer cells	2.3 μg/mL	Cytoplasmic internalization and cell death	[171]
Silver	Methotrexate	Breast cancer cells MCF-7	258.6 μg/mL	Blocks the synthesis to tetrahydrofolate	[172]
Silver	Acety-lshikonin and beta-dimethyl-acrylshikonin	Human chronic myeloid leukaemia	100 nM	ROS induced toxicity	[173]
Gold	Doxorubicin	Glioma carcinoma cell line (LN 229)	4 μg/mL	DNA intercalation and cell death	[174]

Regulatory challenges toward formulation of drug NCs

In addition to opportunities and challenges, hurdles pertaining to approval of NCs by the regulatory bodies are one of the major obstacle in its commercialization. A bridge needs to be build-up with validation, toxicity, reproducibility and stability of NCs produced in academic setting to enable collaboration with industries. A helping hand with adopting minimal standards for conducting pre-clinical studies was offered by US-FDA and European medical agency (EMA), to promote clinical translation of academic-industry collaboration. On the other hand, following good manufacturing practice (GMP) becomes crucial while demonstrating the promise of technology for effective drug delivery and diagnostics. However, following GLP might increase the overall cost, but with previously demonstrated proof of concept, the collaborators may seek funding from different organization for the said purpose. Pre-clinical studies might also need placebo controlled treatment regimens for appropriate evaluation of safety and efficacy. In these context, FDA has initiated the Nanotechnology Regulatory Science Research Plan. The plan aims to address the gap in scientific knowledge required to make regulatory assessment of NCs hassle free. The plan includes major criteria- physio-chemical characterization, preclinical models, risk characterization, risk assessment, and risk communication open for discussion with collaborators [177]. A fruitful outcome of the plan is the establishment of Nanotechnology Characterization Lab, that performs characterization of the different nanoparticles received from government funded research labs, academia, and industry. These platform integrates academic-industry collaborations to gather relevant data for filing Investigational New Drug application. Thus, such resource utilization enables academic-industry collaborations for effective translation of the NC-based therapeutics established by academic to reach market [178].

A part from these, a significant consideration must also be given to selection of demographics for performing clinical trials. Recently, a phase 2 clinical study failed to achieve its primary end point for treating lung cancer, and prostate cancer using BIND-014 (PSMA-targeted docetaxel nanocrystals). This results reflect the need for designing clinical

trials, considering selection of patients based on EPR, tumor heterogeneity, presence of target receptors, ability of NCs to bind to receptors, and the simultaneous need for diagnostics during the study are some of the crucial parameters for achieving better outcomes [175]. Another NC-based product called as Panzem Nanocrystal Colloidal Dispersion is currently under phase 2 clinical trials, being explored for its potential effect on prostrate and ovarian cancer. Some of the NC-based formulation approved for commercialization by FDA are listed in Table 6. A pioneering effort in this direction was adopted by Merrimack Pharmaceuticals, to determine the accumulation of ferumoxytol iron nanoparticles (FMX) using quantitative MRI and may predict the response to nanoliposomal-irinotecan. The study concluded that the tumors with high accumulation of FMX were more responsive to nano-liposomes [176]. This study was based on simultaneous quantitative estimation using MRI, and we believe that such initiatives along with pre-selection of patients will turn out to be a bridging bench-bed gap. Thereby promoting the commercialization of highly potent NCs with detailed safety and efficacy profiles along with backbone of clinical data.

Table 6. Drug NCs with approval of commercialization

Drug	Product	Method of manufacturing	Route	Indication
Cabotegravir/rilpivirine	Cabenuva	Media milling	I.M	AIDS
Meloxicam	Anjeso	Media milling	I.V	Analgesics
Aripiprazole Auroxil	Aristada	High pressure homogenization	I.M	Schizophrenia
Paliperidone Palmitate	Invega Trinza	HPH	I.M	Schizophrenia
Dantrolene sodium	Ryanodex	Media milling	I.V	Malignant hyperthermia
Nepafenac	Ilevro	Media milling	Drops	Analgesic and anti-inflammatory
Theophylline	Theodur	Media milling	Oral	Bronchiectasis
Naproxen sodium	Naprelan	Media milling	Oral	Anti-inflammatory
Fenofibrate	Triglide	HPH	Oral	Reduce Cholesterol/Triglycerides
Megestrol acetate	Megace ES	We media milling	oral	Appetite stimulation
Cannabinoid	Cesamet	Co-precipitation	Oral	Nausea and vomiting
Aprepitant	Emend	Wet media milling	Oral	Antiemetic
Tizanidine	Zanaflex	Wet media milling	Oral	Muscle relaxant
Diltiazem	Herbesser	Media milling	Oral	Angina

Morphine sulfate	Avinza	Media milling	Oral	analgesia
Methyl phenidate	Ritalin Focalin	Media milling	Oral	Attention Deficit Hyperactivity Disorder
Sirolimus	Rapamune	We media milling	Oral	Immune suppression

Conclusion and future prospects

Delivery of BCS class II and IV is challenging yet a promising pitch for research. NC-based drug delivery has emerged with incomparable drug loading over other carrier-based drug delivery systems for poorly soluble drug substances. Over the last two decades, NC technology have shown useful and prevailing role in the treatment of cancer providing improved drug targeting and delivery. Current research on these drug substances deals with strategies to improve their absorption and selective delivery to tumor cells, leading to emergence of surface functionalization and usage of novel excipients. To add cherry on the top, their functionalization with targeting ligands offers excellent dynamism to control the growth of tumors. With the on-going research, different new techniques have also evolved for the production of NCs. These techniques involve combination of top-down and bottom-up approaches to effectively rationalize their advantages. Nevertheless, the clinical efficacy of drug NCs depends on number of factors. The present review highlighted multifaceted applications of different types of NCs in the management of cancer. Metal based NCs have offered simultaneous application in diagnostic and therapy with targeted drug delivery, thereby revolutionizing cancer management and therapy. The drug-metal NC complex enable tracking within the body owing to their interaction with light, thus offer an efficient therapy in addition to chemotherapy. Despite these facts, their clinical translation and market authorization is challenging due to certain bottlenecks. Toxic effects are possible due to their tiny size and large surface area which boosts reactivity with the biological targets. Numerous problems with respect to long-term sustainability and safety of nanoparticles has always remained unresolved. Inflammation, genotoxicity, and organelle failure in cells are few dose dependent toxic effects of nanocrystalline particles. Also, the activation of oxidative enzymes results in increase in concentration of free radicals and in-turn increase oxidative stress and cell damage. Moreover, they have a great potential if the risks and toxicity are controlled from production to treatment. Certain ways including coating with polymers and with the concept of green synthesis, a relatively safe way for the use of metallic nanoparticles can be en-routed.

As far as biological challenges are concerned, the bridge between the disease pathology and human heterogeneity is required. Also, the physicochemical properties of drug NCs should primarily focus on overcoming biological barriers to achieve target and reduce build-up in nonspecific organs. Unfortunately, lack of attention toward these aspects, cause failure observed during the translation of promising NCs in clinical trials. These factors could be deterrent for pharmaceutical industries for investing on commercialization of NC-based products. To cope with this challenge, there is a dire need for inclusive evaluation of preclinical data with special emphasis on efficacy, safety, pharmacokinetics (ADME profile), targeting efficiency and stability with appropriate tumor induced animal models. Also, reproducibility of the results must be validated using different animal models, such practise will boost the reliability toward the anticancer therapy. For most of commercialized products, EPR-mediated accumulation has been reported in most of the animal models. Moreover, the tumors are heterogeneous and may possess inter and intra patient variability. With NC-based products, the amount of cellular uptake and drug release kinetics inside the target sites can be tailored, moving away from the traditional concept of one-size-fits all.

Although most of the research about drug NCs on cancer therapy are still in preclinical development, the motivation arises from those products which have reached the market, with enhanced circulation time, site specific cellular uptake, long retention in tumor environment and reduction in dose-dependent toxicity. Also, decorated NCs with targeting ligands have generated a ray of hope for cancer therapy. Even, NCs can be labeled with dyes/contrast agents to further augment visualization and theranostic applications. In nutshell, NC technology has profound

opportunities to mitigate cancer therapy and with gradual maturation of drug NCs, commercialization of NC-based products will emerge like a boon for cancer therapeutics.

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Author contributions

VP conceptualized the work; AP, KP and VP wrote the manuscript; MSR and RP critiqued the manuscript; AR formatted the manuscript.

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Abbreviations

NCs
Nanocrystals
HPH
High-pressure homogenization
RESS
Rapid expansion of supercritical solutions
PNC
Paclitaxel nanocrystals
EPR
Enhanced permeability and retention
PTX
Paclitaxel
MTO NCs
Mitoxantrone nanocrystals
RES
Reticuloendothelial system
PEG
Polyethylene glycol
PLGA-PEG
Polylactide-co-glycolide-Polyethylene glycol
MMP
Matrix metallo-proteinases
TNF
Tumor necrosis factor
PSMA
Prostate-specific membrane antigen
FA

Folic acid
I.V.
Intravenous
GIT
Gastrointestinal tract
MPS
Mononuclear phagocytic system
TPGS
D-a-tocopheryl polyethylene glycol 1000 succinate
CNCs
Cellulose nanocrystals
FITC
Fluorescein isothiocyanate
RBITC
Rhodamine-b-isothiocyanate
MRI
Magnetic resonance imaging
AuNCs
Gold nanocrystals
DNA
Deoxy ribonucleic acid
Fe-NCs
Iron nanocrystals
Zn-NCs
Zinc nanocrystals
Ag-NCs
Silver nanocrystals
SPIONs
Superparamagnetic iron nanocrystals
ROS
Reactive oxygen species
DOX
Doxorubicin
mPEG
Methoxypolyethene glycol
TAT
Trans-activator of transcription
UV
Ultra violet
ZnO
Zinc oxide
Pgp
P-glycoprotein
CPT
Camptothecin
US-FDA
United States Food and Drug Administration

EMA
European medical agency
GMP
Good manufacturing practice
BCS
Biopharmaceutical classification system
MOA
Mechanism of action

Publisher's Note

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Integrating in silico molecular docking, ADMET analysis of C.verticillata with diabetic markers and in vitro anti-inflammatory activity

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ABSTRACT (ENGLISH)

Background

Over the past decade, various research studies have proved the interconnection between the inflammatory pathways and diabetes complication in clinical condition. The present study evaluated the anti-inflammatory and antioxidant activity. Further, the sample was tested for its pharmacokinetics properties and the best compounds were docked with the diabetic markers (DPP IV (PDB-ID: IJ2E) and SGLT2 (PDB-ID: 7VSI)).

Results

C.verticillata showed a good hydrogen peroxide (78.3 ± 0.34%, IC_{50} = 287.81 µg/ml) and superoxide scavenging activity (52.7 ± 1.26%, IC_{50} = 796.15 µg/ml). In addition, the sample was checked for its anti-inflammatory activity with protein denaturation (57.4 ± 0.19%, IC_{50} = 471.5 µg/ml) and proteinase inhibition assay (68.3 ± 0.48%, IC_{50} = 213.42 µg/ml). Further, the bioactive compounds detected from HPLC-ESI-MS/MS analyzed sample were checked for its drug likeliness by checking its ADME properties and toxicological parameters. It has been observed that except Loliolide, all the other compounds have followed the physicochemical parameters and proved to exhibit drug likeliness characteristics. The bioactive compounds that follow the Lipinski's rule were taken further for in silico molecular docking analysis with the diabetic protein markers (DPP IV and SGLT2). Docking results revealed that

Pyro pheophorbide a with DPP IV and Dihydromonacolin L acid with SGLT2 have recorded a maximum docking score of (−9.4 kcal/mol) and (−9.2 kcal/mol), respectively.

Conclusion

The observed results suggest that the identified and selected bioactive compounds from *C.verticillata* can be considered as a potential target molecule for the management of oxidative stress induced diabetic condition. Furthermore, the study also provides an insight on the effectiveness of the compounds on reducing the inflammation as well.

FULL TEXT

Background

Inflammation is characterized as a host defense mechanism which involves the activation of immune system to remove the foreign antigen from the host system. The visual interpretations of inflammation include swelling, increased temperature at the site of injury, pain and redness [1]. It is achieved by the enhanced production of reactive oxygen species by the action of inflammatory cells [2], where it aids in the removal of invasive microorganisms. Nevertheless, prolonged production of ROS (reactive oxygen species) leads to oxidative stress and illness leading to chronic inflammation. Moreover, chronic inflammation leads to various kinds of illness like cancer, neurological diseases, diabetes, cardiovascular diseases, and so on.

According to [3], oxidative stress may be a major factor in the pathophysiology of diabetes-related macrovascular and microvascular complications which end up inflammation. The emergence of endothelial dysfunction is a warning sign of such damage. Numerous evidences are there to support the relationship between the immune and metabolic systems. It has been proved that the inflammatory pathways are the primary mediators of diabetes [3]. Research findings had already revealed the interconnection between diabetes and inflammation, where the mediators of inflammation like IL-6 and CRP (C-reactive protein and plasminogen activator inhibitor-1) have shown to be elevated in different diabetic conditions [4].

Increasing evidences propose that prolonged activation of pro-inflammatory pathways in the action of insulin might contribute to metabolic disorders like T2D (type 2 diabetes) [5]. Currently existing treatment strategies for type 2 diabetes mellitus, such as insulin delivery and oral anti-diabetic medications, are either ineffective or have negative side effects. Hence, it is critical to continue looking for an effective treatment that can assist people with type 2 diabetes and eventually cure them. Therefore, there is an increased demand in search for the new marine algae-based bioactive compounds with minimum or no side effects to the patients. Hence, the present study focuses on the search of novel bioactive compounds for the effective management of type 2 diabetes that has an effect on reducing oxidative stress induced inflammation. In addition, our work also focuses on the usage of computational tools to verify the ADMET (absorption, distribution, metabolism, excretion and toxicity) properties of the bioactive compounds as well as the in silico molecular docking analysis with the diabetic protein markers (DPP IV (Dipeptidyl peptidase) and SGLT2 (sodium-glucose cotransporter-2). This work will open the way for the use of marine bioactive compounds as an alternative in the curative treatment of diabetes.

Methods

The collection of four green marine algae (*Chaetomorpha crassa, Caulerpa racemosa, Caulerpa verticillata and Caulerpa scalpelliformis*) from Gulf of Mannar (9°28′N, 79°18′E) and extraction of the marine algal extract were followed as described by [6]. Briefly, the algal samples were collected and extracted for its phytochemicals and the antioxidant potential of the algal extracts was assessed based on three different antioxidant assay (ABTS, metal chelation and phosphomolybdenum assay). Moreover, the samples were also subjected to antidiabetic assays (α-amylase and α-glucosidase enzyme inhibition). Among the four algal extracts, *C.verticillata* showed a good antioxidant and anti-inflammatory activities. Hence, further analysis was carried out only with *C.verticillata*.

In vitro antioxidant assays

Hydrogen peroxide scavenging assay

The ability of the ethanolic extract of *C.verticillata* to scavenge hydrogen peroxide was measured by following the

protocol using ascorbic acid as a standard [7]. To 1 ml of extract of varying concentration (100–800 µg/ml), 600 µl of 2 mM H_2O_2 prepared in phosphate buffer (pH 7.4) was added. The reaction mixture was completely mixed and incubated for 10 min at room temperature, and the absorbance was measured at 230 nm in UV–Vis spectrophotometer (1650 Shimadzu, Japan). The same protocol was followed for the standard ascorbic acid, the scavenging potential was calculated, and the percentage of inhibition was calculated using the following equation:% of inhibition=Abs C-Abs TAbs C×100

Abs T = Absorbance of test sample; Abs C = Absorbance of control.

Superoxide radial scavenging assay

The superoxide radical scavenging activity was measured based on the protocol followed by [8]. The superoxide radicals were generated by adding 500 µl of NBT (nitro blue tetrazolium) (0.3 mM), 500 µl of NADH (*nicotinamide adenine dinucleotide)* (0.936 mM), 500 µl of Tris HCL buffer (16 mM, pH 8.0) and 1 ml of algal extract with increasing concentration (100-800 µg/ml). The reaction was initiated by the addition of PMS (phenylmethanesulfonic acid) (0.12 mM), and the tubes were incubated at 25 °C for 5 min. The absorbance was measured at 560 nm. A similar treatment was given to the standard ascorbic acid, and the inhibition percentage was expressed using the following equation:%of inhibition=Abs C-Abs TAbs C×100

Abs T = Absorbance of test sample; Abs C = Absorbance of control.

In vitro anti-inflammatory assays

Protein denaturation assay

Denaturation of protein was assessed by the protocol followed by [9]. To 1 ml of 1% BSA (bovine serum albumin), 1 ml of marine algal extracts (100–800 µg/ml) and 1 ml of phosphate buffer saline (pH 6.4) were added. Once the reaction mixture was mixed, the tubes were incubated at 70 °C in water bath for 10–15 min. The tubes were allowed to cool, and the turbidity was measured in UV–Vis spectrophotometer at 660 nm. The protein denaturation inhibition percentage was calculated according to the formula as follows:%inhibition of protein denaturation=Abs C-Abs TAbs C×100

Abs T = Absorbance of test sample; Abs C = Absorbance of control.

Proteinase inhibitory assay

The proteinase inhibition was measured as described by [10]. To 1 ml of 20 mM Tris–Hcl buffer (pH 7.4), 0.06 mg of trypsin and 1 ml of algal extract (100–800 µg/ml) were added. The reaction mixture was vortexed completely and incubated for 30 min at 37 °C. Furthermore, 1 ml of 0.8% casein (w/v) was added to stop the reaction, and the tubes were centrifuged for 5 min at 2500 rpm. The absorbance of the supernatant was measured at 210 nm. The inhibition percentage was calculated by the following equation:Proteinase inhibition%=Abs C-Abs TAbs C×100

Abs T = Absorbance of test sample; Abs C = Absorbance of control.

HPLC–UV-ESI MS/MS analysis of *C.verticillata*

The HPLC-MS (high-pressure liquid chromatography–mass spectrometry) system consisted of a MS pump, autosampler, UV detector, and a triple-quadrupole, ESI QTOF high-resolution mass spectrometer (Bruker, USA) with Met Frag software for data acquisition and analysis. The sample separation was carried out using Agilent poroshell C_{18} reverse phase column (150 mm×4.6 mm), 2.7 µm particle size (part number: 683975-902), protected with a security guard cartridge (Zorbax eclipse, C_8, 4.6×12.5 mm, 5µ). The sample was analyzed according to the standard protocol with some minor modifications [11]. Two different mobile phases were used, like Solvent A: water (with 0.1% formic acid), and Solvent B: acetonitrile (with 0.1% formic acid), with a column temperature of 25˚C and UV detector at 280nm. The flow rate was 300µl/min, and 10µl of sample was injected. The samples eluting out from UV detector were then directed to triple-quadrupole tandem mass spectrometer with an electrospray interface (ESI), operating in complete scan mode from m/z (50–2200). Mass spectra were acquired in positive modes with capillary voltage at 4500 V, end plate offset 500 V, nebulizer gas at 60 psi, dry gas 12.0L/min, and capillary dry temperature at 220 °C. The identification was achieved and processed using Met Frag workstation software.

ADME analysis and pharmacokinetics toxicity prediction analysis

The canonical structures of the four aforementioned compounds from HPLC-ESI-MS/MS analysis were retrieved

from Pubchem database (www.pubchem.ncbi.nlm.nih.gov). The selected compounds were subjected to ADME analysis. For this, the compounds were analyzed for its physicochemical limitations, pharmacokinetics investigation and drug-likeness of the compounds with use of SWISSADME (http://www.swissadme.ch/index.php). Furthermore, the toxicity prediction for the oral consumption was also calculated using Protox ii (tox.charite.de). This toxicity prediction helps in evaluating the carcinogenicity, hepatotoxicity, immunotoxicity and also the toxicological pathways [12].

In silico molecular docking analysis

Preparation of ligands and protein molecule

The crystal structures of the selected bioactive compounds (ligands) of *C.verticillata* were downloaded from Pubchem database. The 3D SDF structure of DPP IV (PDB-ID: IJ2E) and SGTL2 (PDB-ID: 7VSI) protein was retrieved from the protein data bank (www.rcsb.org). The protein was carefully validated using Ramachandran plot before the docking process using MolProbity server [13]. Further, the protein molecules were prepared by removing the water molecules, adding hydrogen atoms, removing the heteroatoms and bounded other polypeptide inhibitors. The modified protein molecule was used for the docking study [12].

In the current study, PyRx 0.8 version (https://pyrx.sourceforge.io/) software was used for docking purpose, which included Open babel, Auto and AutoDock Vina. Furthermore, the protein and the ligand molecules were transformed to pdbqt format using Autodock tools. Open babel was used to minimize the energy, and blind docking was performed. For a promising protein–ligand binding, the active binding regions were recognized using Biovia discovery studio tool. Analysis of the docked proteins is performed once docking is complete and visualized by BioVia, Discovery studio, 2020. Further, the inhibition constant was calculated to find out the binding efficacy of the ligand and protein molecule using the following equation [14]

1

$Ki=e[-\Delta G/RT]$ where $R = 1.985 \times 10^{-3}$ kcal/mol (universal gas constant; $T = 298.15$ K (temperature);

ΔG = Binding energy.

Statistical analysis

All the tests were carried out in triplicate, and the data were represented as mean ± SE. The experimental results were statistically analyzed using GraphPad prism 9 and Microsoft Excel 2010.

Results

The ability of the ethanolic extract of *C.verticillata* has shown that the extract has a strong ability to scavenge hydrogen peroxide radicals at a dose-dependent manner. At a highest concentration of 800 µg/ml, *C.verticillata* extract has shown a maximum scavenging activity of 78.3 ± 0.34% with an IC_{50} of 287.8 µg/ml compared to the standard ascorbic acid (71.8 ± 1.15% at 200 µg/ml) (Table 1). The superoxide radical scavenging activity was determined using PMS-NADH method. The reduction in absorbance at 560 nm showed that the algal extract has the ability to quench the superoxide radicals, where the ethanolic extract of *C.verticillata* at a maximum concentration of 800 µg/ml has observed to show 52.7 ± 1.26% (IC_{50} = 796.15 µg/ml) when compared with the standard ascorbic acid (69.7 ± 1.02%, IC_{50} = 115.5 µg/ml) at 200 µg/ml.

Table 1. Percentage of inhibition of scavenging activity of *C.verticillata* and IC_{50} values

Algae name / Standard	In vitro antioxidant assays

Hydrogen peroxide scavenging assay (%) and IC_{50} (µg/ml)		Superoxide scavenging assay (%)and IC_{50} (µg/ml)		*Caulerpa verticillata* (800 µg/ml)
78.3±0.34	287.81	52.7±1.26	796.15	Ascorbic acid (200 µg/ml)

Result are expressed as Mean±SE of three replicates (n=3); the data were statistically analyzed by two-way ANOVA using GraphPad Prism. The difference was considered significant when p<0.05

Protein denaturation assay was carried out to measure the efficiency of the algal extracts to inhibit the denaturation of the proteins. *C.verticillata* has recorded a maximum inhibition of 57.4±0.19% (IC_{50}=471.5 µg/ml) with respect to the standard drug diclofenac of 96.4±0.25% (IC_{50} of 9.4 µg/ml). In Table 2, the effect of *C.verticillata* extract on anti-proteinase activity is presented. The extract has recorded a good level of proteinase inhibition of about 68.3±0.48% (IC_{50}=213.42 µg/ml) with a significance of p<0.05. The results have shown a good anti-proteinase activity in comparison to the standard aspirin (94.4±0.22%, IC_{50}=19.7 µg/ml). The results clearly indicate that the extract has the potential to bind with the cell surface by stabilizing the membrane with minor alterations on the charges of the cells.

Table 2. Summary of anti-inflammatory activity of *C.verticillata*

Algae name / Standard	In vitro anti-inflammatory assays			
Protein denaturation assay (%)and IC_{50} (µg/ml)		Proteinase inhibition assay (%)and IC_{50} (µg/ml)		*Caulerpa verticillata* (800 µg/ml)
57.4±0.19	471.5	68.3±0.48	213.42	Diclofenac (100 µg/ml)

96.4 ± 0.25	9.4	–	Aspirin (100 µg/ml)

Results are expressed as Mean ± SE of three replicates (n = 3); the data were statistically analyzed by two-way ANOVA using GraphPad Prism. The difference was considered significant when p<0.05

C.verticillata was subjected to HPLC-ESI-MS/MS analysis for the identification of the bioactive compounds. It was identified as 2-Palmitoylglycerol, Pyro pheophorbide a, Dihydromonacolin and Loliolide are reported for its antioxidant and antidiabetic activities. The fragmentation of mass spectrum was carefully analyzed for its *m/z* values (Fig. 1). The chemical structure of the ESI-MS identified compounds is listed in Table 3. The compound peaked at 33.8 min retention time (R_t) was identified as Loliolide ($C_{11}H_{16}O_3$) with a molecular ion peak at *m/z* 197.11 $[M+H]^+$. Further, the compound at R_t = 56.6 min was identified as Dihydromonacolin L acid ($C_{19}H_{32}O_4$) consisting of molecular ion peak at *m/z* 325.23 $[M+H]^+$. The peaks at 59.0 min and 78.6 min were identified as 2-Palmitoylglycerol ($C_{19}H_{38}O_4$) and Pyro pheophorbide a ($C_{33}H_{34}N_4O_3$) containing a molecular ion peak at *m/z* 331.28 $[M+H]^+$ and *m/z* 535.26 $[M+H]^+$, respectively. Additionally, the fragmentation pattern of mass spectra was already reported in our previous published data [15]. The bioactive compounds after HPLC-ESI-MS/MS analysis were checked for its physicochemical properties like lipophilicity, solubility, bioavailability, number of hydrogen atoms, TPSA, BBB, and Lipinski rule; rotatable bonds were evaluated carefully using SWISSADME software tool. Firstly, Fig. 1 represents the bioavailability radar charts, which gives a quick glimpse of six important physicochemical properties like lipophilicity, size, polarity, flexibility and saturation. The compounds which fall under the pink area in radar charts refer that the compound comes under the property of drug likeliness (Fig. 2).

Fig. 1 [Images not available. See PDF.]

HPLC–ESI–MS chromatogram of *C. verticillata* at positive mode

Table 3. ADME analyses of bioactive compounds of *C.verticillata.*

Name of the bioactive compound	Molecular formula	Molecular Weight	H donor	R bonds	H acceptor	Solubility	Logp 0/w	BA score	GI	BBB	Lipinski
2-Palmitoylglycerol	$C_{19}H_{38}O_4$	330.5	2	18	4	MS	4.7	0.55	High	Yes	Yes
Loliolide	$C_{11}H_{16}O_3$	196.2	1	0	3	VS	1.5	0.55	High	Yes	Yes
Dihydromonacolin L acid	$C_{19}H_{32}O_4$	324.4	3	7	4	S	2.9	0.56	High	Yes	Yes
Pyro pheophorbide a	$C_{33}H_{34}N_4O_3$	534.6	3	5	6	MS	3.9	0.56	High	No	Yes

H donor Hydrogen donor, *R bonds* Rotatable bonds, *B.A score* Bioavailability score, *H acceptor* Hydrogen acceptor, *GI* Gastrointestinal absorption, *BBB* Blood brain barrier

Fig. 2 [Images not available. See PDF.]

Radar charts of the bioactive compounds for drug likeliness calculated by Swiss ADME software
All the leading ligand molecules (2-Palmitoylglycerol, Loliolide, Pyro pheophorbide a and Dihydromonacolin L acid) were observed to follow maximum drug-likeness limitations. Moreover, all the compounds were observed to possess a good gastrointestinal absorption and solubility. Further, the compounds were subjected to toxicity prediction, where LD_{50} plays an important role in determining their level of toxicity, where it includes immunotoxicity, carcinogenicity, TPSA value and octanol/water partition (LogP) value (Table 4). All the four compounds were thoroughly evaluated for its pharmacochemical and physicochemical properties for in silico molecular docking analysis. The Ramachandran plot analysis of the protein (DPPIV) clearly revealed that most of the residues lie within the favored regions that indicate the protein quality (Fig. 3). Except Loliolide, docking was performed for all the other three compounds with the diabetic protein markers like DPP IV (PDB: IJ2E) and SGLT2 (PDB: 7VSI). Docking scores were assessed between the ligands and the proteins. The results revealed that Pyro pheophorbide a has a strong binding affinity with DPP IV (−9.4 kcal/mol), followed by Dihydromonacolin L acid (−7.7 kcal/mol) and 2-Palmitoylglycerol (−5.3 kcal/mol) (Fig. 4a–f).

Table 4. Toxicity prediction of *C.verticillata.* LD_{50}- lethal dose, TPSA- topological polar surface area

Name of the compound	Predicted LD_{50} (mg/kg)	Predicted toxicity class	Carcinogenicity	Immuno toxicity	TPSA	Octanol/water partition (log P)
2-Palmitoylglycerol	5000	5	Inactive	Inactive	66.76	4.36
Loliolide	34	2	Active	Inactive	46.53	1.41
Dihydromonacolin L acid	11,800	2	Inactive	Inactive	77.76	3.23
Pyro pheophorbide a	1300	4	Inactive	Inactive	106.64	5.34

Fig. 3 [Images not available. See PDF.]

The Ramachandran plot of DPPIV (PDB ID: 1JJ2E) retrieved from MolProbity web
Fig. 4 [Images not available. See PDF.]

a–f 3D interactions of the ligands (2-Palmitoylglycerol, Pyro pheophorbide a and Dihydromonacolin L acid) and proteins (DPP IV and SGTL2). **g**, **h** Acarbose with DPP IV and SGTL2)

Discussion

The physiological and biological condition of the marine seaweeds that persist in varying conditions like temperature, pH, salinity and various other factors leads to the production of various phytoconstituents and potential bioactive compounds as a measure of defense mechanism. These seaweeds contain a variety if bioactive compounds that has the potential to quench the free radicals. It is well known that hydrogen peroxide is present everywhere in the environment at a low concentration. Earlier studies have reported that it is a weak oxidizing agent with the capability of crossing the cell membranes quickly and acts in response to Cu^{2+} and Fe^{2+} ions leading to the formation hydroxy radicals [16]. These hydroxyl radicals have the potential to initiate lipid peroxidation that leads to the damage of

DNA. Hence, from the results it is very clear that the algal extract has the capability of scavenging hydrogen peroxide where the occurrence of potential phytoconstituents would have involved in electron donation to neutralize the radicals [17].

It is to be noted that enzymatic antioxidants like superoxide dismutase and catalase are involved in the decomposition of hydrogen peroxide to oxygen and water. Hence, changes in the enzymatic antioxidants due to hyperglycemic condition could lead to inactivation of superoxide dismutase where its glycosylation leads to decrease in activity [18]. Therefore, diabetes-induced oxidative stress increases the concentration of pro-inflammatory cytokines that results in the inflammation [19]. Many scientific reports had already proved that increased production of TNF-α has a strong link with obesity induced insulin resistance that leads to type 2 diabetes [20]. The present findings indicate that the extract has the potential to inhibit the denaturation of the protein by stabilizing the membrane of the protein. Various factors like heat, hypotonic environment, etc., can lead to membrane damage of the protein molecule that further results in the leakage of the inner lysosomal contents. The extracellular release of the membrane further leads to inflammation in the tissue. Hence, it is proved that the anti-inflammatory activity of *C.verticillata* extract provides an evidence for the stabilization of the membrane by inhibiting the discharge of lysosomal constituents [21].

Previous reports have evidenced that prolonged discharge of inflammatory intermediates would upregulate the expression of genes involved in acute and chronic inflammatory diseases by inactivating the anti-proteinases. Earlier reports of [22] have stated that uninhibited levels of proteolysis by the proteinase enzyme can result in various patho-physiological symptoms. The equilibrium of anti-proteinases and proteinases is to be maintained and regulated. Under stress conditions, the ROS will inactivate the anti-proteinases by which the balance gets disturbed resulting in uncontrolled damage of proteins causing increased tissue injury. Therefore, a natural remedy is required to have both anti-inflammatory and antioxidant property that can lessen the ROS-induced stress and regulate the immune function. Hence, *C.verticillata* could be the right choice to prevent the rupture of the HRBC membrane, by which the erythrocyte membrane gets stabilized. By stabilizing the lysosomal membrane, the inflammatory process gets regulated and prevents the release of inflammatory mediators. Our current results of *C.verticillata* have provided the evidence for the aforementioned activities.

Currently, many effective medications made from natural sources are discovered utilizing computer-aided drug design approaches because of the ongoing advancements in computer science. Nowadays, computer-aided drug design is employed to anticipate ADMET features of bioactive compounds, resulting in early stage drug development [23]. The motivation for this in silico techniques is due to the reduced cost and time factor required when compared to traditional ADMET profiling. With respect to drug development, it is important to consider the concept of inhibition constant (*Ki*) that helps to prioritize the bioactive compounds as efficient inhibitors for therapeutic purpose. The inhibition constant of the compounds is listed in Table 5 which is expected to lie between the ranges of 0.1–1.0 μM [13]. The molecular docking results clearly implies that there is a strong relationship with respect to the docking score (ΔG) and the inhibition constant (*Ki*). The inhibition constant was calculated according to the formula as mentioned in the methodology section (Eq. (1). The results revealed that the compounds that showed maximum binding energy (ΔG) (DPP IV: Pyro pheophorbide a (−9.4 kcal/mol) possess a lower inhibition constant (*Ki*= 1.31 μM). From this, it is evident that higher binding energy and lower inhibition constant indicate a stronger and a stable ligand–protein interaction [24] since it provides a deeper insight about the compound to regulate the particular biological pathways. Also, these bioactive compounds were subjected to SWISS ADME software to evaluate the drug likeliness and the physicochemical properties of the compounds, toxicity prediction and in silico molecular docking. These preclinical compounds have the ability to get absorbed to the surface of the receptor that is again circulated into the target site. Further, these compounds will be metabolized by the liver and excreted out of the system to avoid toxicity [25]. In the present study, almost all the bioactive compounds have followed the pharmacochemical and physicochemical parameters (molecular weight: less than 500D), Log S which refers the solubility. The result showed that all the compounds except Loliolide were inactive for immunotoxicity and carcinogenicity, whereas Loliolide showed positive to carcinogenicity. Additionally, the LD_{50} value of Loliolide was

34 mg/kg; hence, it comes under class 2 of toxicity, which is fatal if swallowed.

Table 5. In silico molecular docking score of the bioactive compounds of *C.verticillata*

Name of the bioactive compound	Diabetic Protein targets	Docking score (kcal/mol)	Inhibition Constant K_i (μM)
2-Palmitoylglycerol	1J2E	−5.3	0.000134
Dihydromonacolin L acid	−7.7	0.0000021	Pyro pheophorbide a
−9.4	1.318256	2-Palmitoylglycerol	7VSI
−6.6	0.0000147	Dihydromonacolin L acid	−9.2
1.862087	Pyro pheophorbide a	−8.7	4.365158

Considering the Ramachandran plot analysis, the widespread of ψ (psi) and φ (phi) angles clearly specifies the flexibility and potential ability of the protein to undergo conformational changes on binding of the ligand molecule [14]. These observations helped in accounting for the protein dynamics in molecular docking studies (Fig. 3). Except Loliolide, docking was performed for all the other three compounds with the diabetic protein markers like DPP IV (PDB: IJ2E) and SGLT2 (PDB: 7VSI). Docking scores were assessed between the ligands and the proteins. The results revealed that Pyro pheophorbide a has a strong binding affinity with DPP IV (−9.4 kcal/mol), followed by Dihydromonacolin L acid (−7.7 kcal/mol) and 2-Palmitoylglycerol (−5.3 kcal/mol). Earlier reports suggest that DPP IV was considered as a target to treat type 2 diabetes by inactivating glucagon inhibitory peptide and GLP-1 (glucagon-like peptide) [26]. Additionally, DPP IV inhibition was suggested to show a beneficial effect on postprandial glucose levels [27]. Moreover, it is proved that Pyro pheophorbide has the ability to inhibit alpha amylase and lipase by which it decreases the absorption of triglycerides present in the intestine that prevents from hyperlipidemia and obesity [28]. It has been evidenced that administration of Pyro pheophorbide a has significantly reduced the blood glucose level in normal and diabetic mice by inhibiting alpha amylase and alpha glucosidase enzymes [29]. Similarly, 2-Palmitoylglycerol has the ability to activate GPR119 which is predominantly expressed in pancreatic-β cells, where its activation leads to induce the secretion of insulin and GLP-1 [30].

Since studies had shown that the incretin hormones, glucose-dependent insulin tropic polypeptide (GIP) and glucagon-like peptide-1 are important regulators of postprandial insulin production; inhibition of DPP4 might be a great choice of interest to treat type 2 diabetes. It is also proved that 2-Palmitoylglycerol has the potential of reducing the inflammation and additionally minimizes the level of fatty acid oxidation [31]. Hence, strong interaction of the ligands (2-Palmitoylglycerol, Pyro pheophorbide a and Dihydromonacolin L acid) with the active site of DPP IV protein proves that these compounds could be a great choice for the management of type 2 diabetes (Fig. 2a, c and e). $SGTL_2$ (sodium–glucose co-transporters) plays a very important role in the reabsorption of glucose in the kidney. Inhibition of $SGTL_2$ increases the excretion of glucose in the urine by decreasing the glucose level in the plasma [32]. Existing literature suggests that the primary method by which SGLT2 reduces inflammation and oxidative stress was its capacity to minimize the negative impact of an overabundance of nutrients on cells and tissues, especially on the adipose tissues. When adipose tissue is exposed to an overabundance of nutrients, it produces more pro-inflammatory hormones and cytokines) TNF, IL-1, IL-6 and leptins) [33].

In our present study, Dihydromonacolin L acid was shown to dock a maximum score of -9.2 kcal/mol with SGTL2 followed by Pyro pheophorbide a (−8.7 kcal/mol) and 2-Palmitoylglycerol (−6.6 kcal/mol), which directly implies that the compound has the ability to reduce the glucose level and lessen the effects of inflammation induced oxidative

stress as well (Fig. 2b, d and f). Previously, it was reported that the Dihydromonacolin L acid was proved to be a potential inhibitor in the biosynthesis pathway of cholesterol [34]. Hence, it protects from LDL oxidation by inhibiting HMG-CoA reductase [35]. Pheophorbide is a by-product of chlorophyll which is known for its anti-inflammatory and anticancer property, where it inhibits the high concentration of nitric oxide (NO) to suppress the induction of iNOS (nitric oxide synthase) [36]. Hence, these bioactive compounds were expected to be a possible choice for the treatment and management of type 2 diabetes.

Conclusion

The ultimate aim of the drug discovery research is to find effective medications through clinical and preclinical studies. In this context, the integration of docking and ADMET studies has provided a deeper insight into the understanding about the ligand–protein interaction and pharmacokinetics of the tested compounds. The results of the present study conclude that *C.verticillata* has the potential to inhibit the upregulation of blood glucose level. Moreover, the extract has the ability to reduce oxidative stress induced inflammation as well. The bioactive compound extracted (2-Palmitoylglycerol, Dihydromonacolin L acid, Loliolide and Pyro pheophorbide a) from *C.verticillata* could be considered as one of the promising agents for treating T2D. Even though a number of bioassay experiments were performed for compound's to evaluate its ADMET profile, they are quite time-consuming and costly. This emphasizes on the importance of in silico predictions for its ADMET properties. Ideally, ADME, toxicity prediction and in silico analysis have revealed that the aforementioned bioactive compounds have a greater affinity to DPP4 and SGTL2. The outcome of the analysis suggests that the docked bioactive compounds were found to be non-hazardous and the computed drug analysis needs further validation to find its effectiveness in treating oxidative stress and inflammation in diabetic condition. To ensure the inhibitors of DPP4 and SGTL2 from marine algal source, the analyses necessitate further validation through in vivo experiments. We believe that expanding this research by integrating proper in vivo experiments along with ADMET and in silico analysis gave a deeper insight about the bioactive compounds from marine seaweeds as a potential therapeutic candidate in the treatment of oxidative stress induced diabetes. Hence, this holistic approach will eventually pave the way for the advancement in drug discovery to produce more safer and effective therapeutic agents.

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Author contributions

SDE contributed to conceptualization, investigation and formal analysis; MA contributed to conceptualization, methodology, and original draft writing—review and editing.

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Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations

DPP IV

Dipeptidyl peptidase 4

SGTL2
Sodium-glucose cotransporter-2
ROS
Reactive oxygen species
CRP
C- reactive protein
IL-6
Interleukin 6
T2D
Type 2 diabetes
ADMET
Absorption, distribution, metabolism, excretion and toxicity
ABTS
2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
NBT
Nitro blue tetrazolium
NADH
Nicotinamide adenine dinucleotide
PMS
Phenylmethanesulfonic acid
BSA
Bovine serum albumin
HPLC-MS
High-pressure liquid chromatography–mass spectrometry
ESI-MS
Electrons spray ionization–mass spectrometry
BBB
Blood–brain barrier
TPSA
Topological polar surface area
R_t
Retention time

Publisher's Note

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Multifaceted applications of micro/nanorobots in pharmaceutical drug delivery systems: a comprehensive review

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ABSTRACT (ENGLISH)

Background

Drug delivery systems (DDSs) encompass a wide range of methods, including oral, injectable, and topical routes of administration, all tailored to meet specific patient needs. Micro and nanorobots, equipped with pioneering propulsion mechanisms that convert external energy sources into precise movements, have revolutionized drug delivery. This cutting-edge technology ensures highly efficient drug delivery, particularly when targeting specific targets within intricate physiological environments. In contrast to traditional drug delivery approaches that rely on bloodstream circulation, engineered micro/nanorobots have autonomous mobility, enabling drug delivery to previously unreachable areas.

Main body of the abstract

Integrating micro/nanorobots into drug delivery raises vital safety and biocompatibility issues. These encompass material selection, degradation in-vivo, overcoming biological barriers, controlled movement, external interference, immune response, chemical reactions, systemic effects, long-term impact, and real-time monitoring. While micro/nanorobots hold immense transformative potential, they confront significant hurdles in their journey toward practical applications. Chief among these challenges are concerns regarding biocompatibility, ensuring that these tiny devices do not trigger adverse reactions. Long-term safety remains a critical issue, as understanding the effects of prolonged exposure and potential accumulations within the body and navigating complex biological environments with precision is another obstacle.

Short conclusion

The paper summarizes how to explore the various ways in which micro/nanorobots can be employed to enhance drug delivery, including their precision, targeting capabilities, and adaptability to different physiological conditions. Additionally, the review seeks to highlight the transformative potential of these technologies and their impact on the pharmaceutical industry.

FULL TEXT

Background

Drug delivery systems (DDSs) encompass diverse approaches for administering therapeutic agents. These methods involve various routes of drug administration, encompassing oral, injectable, and topical routes, tailored to specific patient needs [1]. DDSs incorporate a range of drug formulations and delivery devices, addressing the complexities of biotechnology-based therapeutics like proteins and peptides. Advanced techniques, such as cell and gene therapies, offer precision in treatment delivery. Nanoparticles play a pivotal role, serving as carriers for drugs and even as pharmaceuticals and diagnostics themselves [2]. Targeted drug delivery, particularly in cancer therapy, has seen significant progress. Overcoming blood–brain barrier challenges is vital, and DDS refinements pave the way for personalized medicine. The ideal DDS aims to optimize therapeutic efficacy while minimizing side effects [3].

Micro and nanorobots have emerged as revolutionary drug delivery systems, leveraging innovative propulsion mechanisms that convert external energy sources into precise movements. This advanced technology offers exceptional efficiency in drug delivery, especially when targeting specific sites within complex physiological environments [4]. These micro and nanobots have demonstrated remarkable capabilities, including the encapsulation, transport, and direct delivery of therapeutic substances to disease sites. This targeted approach not only enhances therapeutic efficacy but also significantly reduces systemic side effects associated with potent drugs. In essence, micro and nanobots represent a transformative leap in drug delivery, enabling precise, site-specific treatment with minimal collateral impact [5]. Unlike conventional drug delivery methods relying on bloodstream circulation for drug transport, engineered micro/nanorobots possess autonomous mobility, facilitating drug delivery to otherwise inaccessible regions [6]. These micro/nanorobots are powered either externally, through magnetic fields, light, acoustics, or electric fields, or internally, via chemical reactions. While micro/nanorobots hold promising potential, the majority of current research remains confined to in-vitro experiments, with in-vivo investigations still in

their nascent stages. Further biological studies are imperative to substantiate the in-vivo drug delivery efficacy of micro/nanorobots [7].

However, PLA (Polylactic Acid) and TPU (Thermoplastic Polyurethane) are two different types of biocompatible polymers that play important roles in the development of nanorobots/microrobots for drug delivery systems [8]. PLA is a biodegradable and biocompatible polymer derived from renewable resources, such as corn starch or sugarcane which can be used to construct the structural components of nanorobots/microrobots, providing a stable and non-toxic framework for drug delivery systems. Therefore, PLA-based nanorobots can be designed to release drugs at a controlled rate as the polymer gradually degrades, making it suitable for sustained drug delivery [9]. Its versatility and compatibility with various drug formulations make it a popular choice for encapsulating and delivering therapeutic agents. On the other hand, TPU is another biocompatible polymer known for its flexibility and durability which can be used to create flexible components or coatings for nanorobots/microbots, allowing for improved mobility and adaptability within the body's complex environment [10]. TPU-coated nanorobots can navigate tight spaces, overcome obstacles, and potentially target specific tissues or cells more effectively. Its ability to withstand mechanical stress and maintain stability under different physiological conditions makes TPU valuable in enhancing the functionality and maneuverability of drug-delivery microrobots [11]. In drug delivery systems, both PLA and TPU can be utilized to design and manufacture nanobots/microbots tailored for specific applications.

The review aims to provide a thorough examination of the diverse roles that micro/nanorobots play in drug delivery, encompassing their design, functionalities, and mechanisms of action. Furthermore, it seeks to elucidate how these innovative systems enable targeted drug delivery, controlled release, and navigation within physiological environments. Additionally, the review assesses the challenges and advancements in this field, including biocompatibility, safety concerns, and recent technological developments. Ultimately, it aspires to offer insights into the transformative potential of micro/nanorobots in revolutionizing drug delivery and advancing healthcare.

Main text

Types of micro/nanorobots in pharmaceutical drug delivery systems

The types of micro/nanorobots that are prevalent in current pharmaceutical drug delivery technologies can be classified into two broad types as shown in Tables 1 and 2.

Table 1. Types of micro/nanorobots based on size

Types	Classification based on size
1. Microbots	– Typically, 1 μm to several hundred μm in size
– Larger than nanoparticles but smaller than macroscopic robots	– Suited for navigating through blood vessels and tissues
2. Nanorobots	– Typically, in the nanometer (nm) to micrometer range
– Substantially smaller than microbots	– Ideal for precise targeting at the cellular or molecular level

Table 2. Types of micro/nanorobots based on functionality

Types	Functionalities

1. Active Propulsion Robots	– These micro/nanorobots possess self-propulsion mechanisms to navigate within the body
– Examples: Microswimmers, Microrobotic capsules, Light-powered microbots, Magnetic field-driven microbots, Acoustically-driven microbots, and Chemically-powered microbots	2. Passive Transport Robots
– These micro/nanorobots rely on external factors or carriers for transport within the body	– Examples: DNA nanorobots, Nanoparticle-based carriers, Molecular shuttles, and Biomolecule carriers

Magnetic small-scale robots hold significant promise in the biomedical field due to their advantages in actuation. Recent research has witnessed notable advancements in the design, fabrication, and application of these devices, aiming to enhance their performance for potential clinical use [12]. An overview of recent progress in small-scale biomedical robots has been reviewed and studied emphasizing their development, capabilities, and existing challenges. It also suggests alternative biomedical applications for some technologies. The study of Koleoso et al., 2020 underscores the need for continued efforts to enhance the functionality and reliability of these robots, particularly in clinical contexts, and offers recommendations for advancing their commercialization [13]. According to Azar et al., 2020, the essential components of nanorobots, including sensors, actuators, and nano controllers, draw upon previous research to showcase diverse designs. It advances beyond theoretical discussions to delve into the practical aspects of manufacturing and implementation. Significantly, it emphasizes recent innovations in drug delivery, detection, and manipulation, providing insights into their motion mechanisms and the current manufacturing methods in use [14]. Control and navigation strategies for nanorobots are explored in-depth, emphasizing the importance of mathematical positioning concepts in modeling these intricate devices [15]. Several studies presented various algorithms that enable precise control, underscoring their significance in the field. Furthermore, studies presented numerous applications that underscore the effectiveness of nanorobots across various domains. These applications encompass a wide range of functions, including diagnostics, treatments, target detection, and even complex surgical missions. These devices serve as a crucial link between theoretical analyses and practical manufacturing approaches [7, 14]. Importantly, it sheds light on future implementation concerns and emerging research gaps, emphasizing the need for continued investigation and innovation [14]. In a nutshell, the practical integration of nanorobots can be implemented in critical areas such as optical surgeries, cell manipulation, and cancer treatment. It contributes significantly to the advancement of nanorobotics and its potential impact on healthcare and other fields.

Targeted drug delivery mechanisms using micro/nanorobots

The use of micro/nanorobots capable of efficiently harnessing a variety of energy sources to facilitate motion has the potential to bring about a significant transformation in the pharmaceutical field, particularly in the realm of targeted drug delivery. Through the precise delivery to specific tissues or anatomical sites and controlled release mechanisms, drugs can be directed toward their intended destinations [16]. Targeted delivery encompasses a range of actuation energy sources, including self-propulsion via substances like hydrogen peroxide and enzymes, external propulsion driven by factors such as light, electricity, acoustics, and magnetic fields, as well as propulsion driven by motile microorganisms like bacteria, sperm cells, contractile cells, and immune cells as shown in Fig. 1.

Fig. 1 [Images not available. See PDF.]

Energy sources to design and develop micro/nanorobots for precise drug delivery

Targeted and/or precise drug delivery is generally processed by using two major technologies—exogenous power-driven method and endogenous power-driven methods.

Exogenous power-driven technology

In exogenous power-driven Micro/Nanorobots, due to their micro/nano-size, drug-delivery robot systems face the challenge of countering Brownian motion to achieve autonomous movement within complex bodily fluids. According to Hu et al., 2020, an external power source is typically employed to enable the controlled and coordinated locomotion of these micro/nanorobots. Commonly utilized sources of external power include magnetic fields, electric fields, light energy, acoustic waves, and heat energy [17–21]. A combination of these driving modes in practical design is often employed to create micro/nanorobots with diverse functionalities [7]. Magnetic propulsion is one of the exogenous power-driven methods to prepare Micro/Nanorobots in drug delivery which often involves the design of helical swimmers, which are inspired by the flagella of micro-organisms [17]. These helically shaped micro/nanorobots mimic the rotary corkscrew motion of bacterial flagella, enabling them to move through bodily fluids through interaction with external magnetic fields [22]. Researchers frequently combine these artificial bacterial flagella (ABF) with drug-loaded liposomes for drug delivery applications. For example, in a study conducted by Qiu et al. in 2014, they developed a microrobot consisting of two components. The first component is a titanium-coated ABF that enables precise 3D navigation within fluids when subjected to rotating magnetic fields. The second component is an outer temperature-sensitive liposome that controls the release of the drug based on temperature regulation. This innovative approach shows potential for enhancing targeted drug delivery in pharmaceutical applications [7, 23]. Electric field propulsion is also a widely used method in micro/nanorobotics, offering precise control and versatile applications in drug delivery and other fields [7]. Another significant illustration of exogenous power-driven micro/nanorobots is found in the Janus colloidal system. This system utilized a combination of electric and magnetic energy to facilitate independent movement and cargo retrieval [24]. It consists of metal-dielectric Janus colloids that respond to a high-frequency electric field (0.5–2.5 MHz). These colloids are characterized by a hemisphere coated with nickel, allowing them the ability to be guided in a specific direction by a magnetic field. This capability facilitates precise cargo delivery by predefining the path that the micro/nanorobot will follow. Rahman et al., 2017 introduced a rotational nanomotor structure using carbon nanotubes, which exhibited rapid responsiveness and ultra-high-speed movement when exposed to an electric field [25]. This motion was driven by the alignment of water dipoles induced by the electric field, demonstrating exceptional performance in water. However, its behavior in simulating more complex human systems or body fluids remained unexplored. Incorporating multiple energy sources, nanoparticles can achieve directional movement. Guo et al., 2018 also presented an approach for regulating the movement of catalytic nanomotors through the application of electric fields in conjunction with light energy [18]. In brief, electric field propulsion in micro/nanorobotics offers exciting possibilities for precise and programmable movement. The Janus colloidal system, with its dual responsiveness to electric and magnetic fields, exemplifies the potential for autonomous cargo delivery [26]. Carbon nanotube-based nanomotors exhibit rapid movement under electric fields, although their performance in complex biological environments requires further investigation. Finally, the combination of electric fields and light energy provides a controllable means to steer catalytic nanomotors [7, 26]. These advancements hold great promise for applications in drug delivery and other fields where precise, directed movement at the micro/nanoscale is essential. Light energy serves as another frequently employed method in micro/nanorobotics, offering high controllability and programmability, typically used in a supplementary role. Wang et al., 2018 studied that it enables directional movement of nanorobots through the modulation of light parameters such as frequency, polarization, intensity, and propagation direction [19]. A notable example is the work of Zhan et al., 2019 who harnessed the linear dichroism property of Sb_2Se_3 nanowires to create an artificial swimmer. This swimmer incorporated two cross-aligned dichroic nanomotors, and its movement was guided by adjusting the polarization direction of incident light [27]. This approach showcases the potential for precise control of nanorobot movement using light energy, making it a valuable tool in micro/nanorobotic applications. Light energy not only serves as a direct driving force for micro/nanorobots but can also catalyze redox reactions within them, leading to propulsion through the generation of chemical gradients or bubbles [28, 29]. For instance, Wang et al., 2019 developed a Cu_2O@N-doped carbon nanotube (Cu_2O@N-CNT) micromotor powered by glucose and activated by visible-light photocatalysis [30]. This micromotor exhibited several advantages, including non-toxicity, high biocompatibility, and environmental friendliness. It showcased impressive movement and 3D motion control

within a biological environment. However, challenges persist when transitioning to in-vivo applications, mainly due to the limited ability of visible light to penetrate tissues [7, 30]. Utilizing an external power source, ultrasound power-driven micro/nanorobots show great potential in the field of advanced targeted drug delivery. Their outstanding biocompatibility and dependability make them a promising option, and they rely on external power-driven technology for their functionality. Commonly, nanowires, typically composed of gold, serve as the primary carriers for these ultrasonically driven nanorobots [20]. The template electrodeposition method plays a pivotal role in the design of ultrasound-propelled micro/nanomotors. This method involves creating a concave cavity at one end of the nanomotor through the deposition of a sacrificial copper layer. When subjected to ultrasound waves directed at the concave end, the nanomotor is propelled forward by the resulting pressure gradient [31]. Furthermore, ultrasound is frequently integrated with magnetic fields to enable precise control. For example, Victor and his team devised a magnetically guided three-segment nanowire motor with Au–Ni–Au segments, harnessing ultrasound for propulsion [32]. Changing the magnetic field's orientation enables ultrasound-propelled particles to move in all directions. The feasibility of precise drug delivery has been confirmed by introducing a polymeric section containing pH-sensitive drugs into the nanomotor. In acidic conditions, these drugs can be released, improving the selectivity of drug delivery. Additionally, Garcia-Gradilla et al., 2014 have developed an ultrasound-propelled nanorobot featuring four segments, including Au-Ni-Au and Au wire components [33]. This innovative approach also showcases the versatility and potential of ultrasound-powered nanorobots for advanced drug delivery applications.

Endogenous power-driven technology

In endogenous power-driven approaches, nanorobots rely on endogenous power sources for self-propulsion, primarily driven by chemical or biological reactions [34]. These micro/nanorobots are typically asymmetric and often coated with catalysts to harness continuous chemical energy from their surroundings. A prevalent approach involves converting chemical energy into a driving force using redox reactions, with the decomposition of hydrogen peroxide being a widely employed method. Hydrogen peroxide possesses an unstable chemical bond that readily breaks down into water and oxygen when catalyzed by various agents like metals, enzymes, or an alkaline environment. This concept has been extensively employed in nanorobot designs, including bimetallic nanorods, hollow Janus particles, vesicular polymers, and more [35–37]. These nanorobots hold great promise, particularly in the field of targeted drug delivery [38, 39]. Janus particles, a class of particles with distinct compositions and structures on their two hemispheres, have also contributed to this field. Wu and colleagues, for instance, engineered polymer multilayer Janus capsules capable of self-propulsion using 0.1% hydrogen peroxide as fuel at physiological temperatures [36]. This approach underscores the potential of nanorobots powered by chemical reactions, opening exciting avenues for various applications, including targeted drug delivery. While the concept of using chemical energy conversion has been extensively explored, its practical application within living organisms has been constrained due to the inherent toxicity of the commonly employed "fuel," hydrogen peroxide [40]. Researchers have sought alternative, safer materials to power nanorobots. One such substitute is magnesium, renowned for its high biocompatibility. Magnesium can react with water to produce bubbles, serving as a propellant, making it a promising replacement for the hazardous hydrogen peroxide [40, 41]. Another approach involves harnessing biocompatible enzyme-catalyzed reactions for self-propulsion. This method utilizes non-toxic fuels like glucose and urea to drive nanorobots. For instance, researchers developed a core–shell nanorobot based on mesoporous silica, demonstrating self-propulsion capabilities in ionic media [43]. They functionalized the nanorobot with urease, an enzyme that catalyzes the breakdown of urea into carbon dioxide and ammonia. This enzymatic reaction allowed the nanorobot to move autonomously and release drugs. Importantly, when loaded with Doxorubicin (Dox), these nanorobots exhibited remarkable efficacy against HeLa cells. This success can be attributed to the synergistic effects of enhanced drug release and the presence of ammonia generated through the catalytic reaction [42, 43]. These inventive strategies showcase the potential of utilizing biocompatible materials and enzyme-catalyzed reactions to power nanorobots, thereby mitigating safety concerns and advancing their application in drug delivery within biological systems. Endogenous chemical energy propulsion offers an advantage by minimizing the need for constant micro/nanorobot control, focusing instead on guidance toward the target, often via magnetic attraction. This approach uses gas

generated from chemical reactions to reverse robot movement, well-suited for the gastrointestinal tract. However, chemical energy-driven nanorobots have drawbacks. Controlling their direction proves challenging, and they are sensitive to ionic environments, potentially disrupting motion. A significant limitation is their power continuity; they may exhaust energy as reactions proceed. Moreover, safety concerns surround the "fuel" and reaction products when applied within organisms, posing a substantial obstacle to their practical use [7]. These issues warrant further investigation and research in the future to address these challenges and unlock the full potential of chemical energy-driven self-propelled nanorobots in various applications, including targeted drug delivery within living organisms. The comparison between exogenous and endogenous power-driven Micro/Nanorobots in Pharmaceutical drug delivery systems has been detailed in brief in below Table 3.

Table 3. Simplified comparison between exogenous and endogenous power-driven micro/nanorobots in pharmaceutical DDS

Aspects	Exogenous power-driven Micro/Nanorobots	Endogenous power-driven Micro/Nanorobots
Power source	External energy sources (e.g., magnetic fields, ultrasound)	Internal energy sources (e.g., chemical reactions, enzymes)
Control	Requires external control and guidance mechanisms (e.g., magnetic field manipulation)	Self-propelled and guided by internal processes or cues
Navigation	Controlled externally, often using precise guidance systems	May exhibit less precise navigation, influenced by internal conditions
Energy supply	Constant energy supply required from external sources	Utilizes internal energy generation, potentially limited by available substrates
Targeted drug delivery	Achievable with precise external control	Potential for targeted delivery, guided by internal conditions
Safety concerns	Limited safety concerns related to the energy source	Safety concerns related to the choice of reactants and products
Potential for in vivo use	Commonly used in controlled laboratory settings	Holds promise for in vivo applications, subject to further research
Examples	Magnetic or ultrasound-guided nanorobots	Enzyme-catalyzed nanorobots using the body's chemicals

Apart from these types of micro/nanorobots, 4D printed micro/nanorobots are also some of the promising approaches in pharmaceutical DDS. 4D printed nano/microrobots are innovative vehicles for drug delivery in the pharmaceutical field [7, 44]. These tiny robots are constructed using advanced 4D printing technology, which allows them to change shape or behavior in response to external stimuli.

Safety and biocompatibility considerations for micro/nanorobots in drug delivery systems

Incorporating micro/nanorobots into DDS brings forth critical safety and biocompatibility concerns that must be addressed for successful application within the human body. These concerns encompass various aspects of nanorobot design, function, and interaction with biological systems. Key considerations include—biocompatible materials, in-vivo degradation, biological barriers, controlled mobility, external interference, immune response,

chemical reactions, systemic effects, effects on long-term use, and real-time monitoring and feedback mechanisms. The biocompatible materials used in nanorobot construction or designs must be ensured that the materials are non-toxic and biocompatible is paramount. Materials should not trigger adverse immune responses or tissue damage [45]. Considering in-vivo degradation, the nanorobots should be designed to degrade naturally or be eliminated from the body without leaving harmful residues or by-products inside the physiological systems [5]. Taking biological barriers into account, several research suggested that overcoming barriers such as the blood–brain barrier (BBB), and ensuring that micro/nanorobots can safely traverse them without causing damage is crucial for targeted drug delivery [46, 47]. According to Arvidsson and Hansen, 2020, it must be noted that nanorobots must be controlled to prevent unintended movement and potential harm to healthy tissues. Precise navigation and guidance mechanisms are essential for its development [48]. Protection against external interference, such as magnetic fields or other external stimuli, is vital to maintain micro/nanorobot stability and prevent unintended actions [15]. Avoiding excessive immune responses that could neutralize nanorobots or trigger inflammation is critical for long-term functionality. The chemical reactions within nanorobots, especially endogenous energy sources, must be carefully selected to avoid toxicity and ensure safe reaction products [7, 15]. The assessment of potential systemic effects of nanorobot deployment, including impacts on organ functions or overall homeostasis, is essential [48]. Understanding the long-term effects of nanorobot presence in the body is necessary to evaluate safety over extended periods [7, 48]. Implementing real-time monitoring and feedback mechanisms to track nanorobot behavior and address any safety issues promptly [7, 11].

Addressing these safety and biocompatibility concerns is critical to harnessing the full potential of micro/nanorobots in drug delivery systems while minimizing risks to patient's health and well-being.

Overcoming the limitations of endogenous and exogenous power-driven micro/nanorobots

However, endogenous/exogenous systems may have limitations in terms of biocompatibility. 4D-printed microrobots can be designed to carry larger drug payloads due to their controlled and customizable structural properties [7]. This can improve the drug delivery efficiency compared to endogenous/exogenous systems.

4D printing enables the precise fabrication of micro robots, tailoring their size, shape, and material properties to specific drug delivery tasks. These robots can be programmed to navigate through complex biological environments with controlled motion. They can swim, crawl, or even fold and unfold to reach target locations [44]. 4D-printed microrobots can be designed to deliver drugs to precise locations within the body, improving drug efficacy while minimizing side effects. They can respond to various stimuli, such as changes in temperature, pH, or magnetic fields, allowing for on-demand drug release. These microrobots can be multifunctional, combining drug release mechanisms with sensors or imaging capabilities, enhancing their utility in diagnostics and therapy [49]. Using biocompatible materials, 4D-printed nano/microrobots reduce the risk of adverse reactions in the body, enhancing patient safety.

For example, biomaterials such as Polylactic Acid (PLA) and Thermoplastic Polyurethane (TPU) can play important roles in 4D printed nanorobots for pharmaceutical drug delivery systems. PLA can be used as a structural material for 4D-printed nanorobots, providing a stable and biocompatible framework. Its rigidity and strength are useful for maintaining the structural integrity of the nanorobots. It is biodegradable, which can be advantageous in drug delivery systems [8, 9]. The nanorobots can be designed to degrade gradually, releasing the drug payload over time, and making it suitable for sustained drug delivery. Its 4D printing properties allow for the precise fabrication of intricate and customizable shapes, enabling the design of nanorobots with specific geometries for optimal drug delivery performance [9, 11, 44].

While, TPU is known for its flexibility and elasticity, which can be valuable for 4D-printed nanorobots in navigating through the body's complex and dynamic environments [11, 44]. It allows the nanorobots to bend and adapt to obstacles, enhancing their mobility. It can be used as a coating or an outer layer for the nanorobots. This coating can protect the nanorobots from degradation, provide better manoeuvrability, and reduce friction when in contact with biological tissues. TPU coatings can reduce the risk of aggregation of nanorobots in bodily fluids, ensuring a smoother and more efficient drug delivery process [10, 11, 44]. It can be incorporated into the 4D printing process to

create components with shape-changing capabilities in response to external stimuli, improving the nanorobots' ability to reach target locations and release drugs as needed. By leveraging PLA and TPU in 4D-printed nanorobots, pharmaceutical drug delivery systems can benefit from the combination of structural stability, biodegradability, customizability, flexibility, and responsive behavior [9, 10]. These properties make PLA and TPU valuable materials for designing advanced nanorobots capable of precise drug delivery in complex biological environments [8].

The design of 4D-printed nanorobots can minimize immune responses, reducing the risk of rejection or other immune-related issues. They can carry a higher drug payload compared to some traditional drug delivery systems, improving efficiency [50]. The customizability of 4D-printed microrobots offers the potential for personalized drug delivery, addressing individual patient needs. These robots can be engineered to reduce the risk of aggregation in bodily fluids, ensuring smooth drug transport.

4D-printed microrobots are engineered to move with more precision and control in response to external cues, improving their ability to reach target sites effectively [44, 49]. Endogenous systems may not offer the same level of control. 4D printing technology allows for the integration of multiple functionalities into a single microrobot, such as drug release mechanisms, sensors, and imaging capabilities, making them versatile tools for drug delivery. Endogenous/exogenous micro/nanorobots can sometimes aggregate in the bloodstream or other bodily fluids, leading to potential blockages. 4D-printed microrobots can be designed to minimize aggregation risks. 4D printing technology allows for efficient and scalable production of micro robots, potentially reducing costs and improving accessibility compared to endogenous/exogenous systems, which may be more challenging to manufacture.

Recent insights on in-vivo *applications of micro/nanorobots drug delivery*

Several kinds of research are still being undertaken on animal models to demonstrate the use of micro/nanorobots in targeted drug delivery systems, however, the studies on human volunteers are yet to be done to draw clear justification for the use of these technologies in clinical phase trials. A few recent reports on the use of drug-loaded micro/nanorobots have been presented in Table 4.

Table 4. Recent in-vivo studies of nanorobots in pharmaceutical drug delivery

Animal Models	Micro/Nano-robots used	Loaded Drug	Targeted Region	Key Findings	Refs.
Mice	Gold Nanorobots	Doxorubicin (Dox)	Tumor	– Enhanced drug delivery Tumor shrinkage	[51]
Rats	Magnetic Nanorobots	Paclitaxel (PTX)	Bloodstream	– Improved drug circulation Improved efficacy	[52]
Rabbits	Polymer Nanorobots	Methotrexate (MTX)	Joints	– Reduced inflammation Targeted therapy	[53]
Guinea Pigs	Lipid Nanorobots	Insulin	Gastrointestinal	– Controlled insulin release Controlled efficacy	[54]
Monkeys	DNA Nanorobots	Antiviral Drug	Brain	– Enhanced brain drug delivery	[7, 55]

From Table 4, it is evident that micro/nanorobots have emerged as promising tools in pharmaceutical drug delivery, offering precise and targeted delivery of therapeutic substances within the body. These tiny robotic systems, typically at the nanoscale, provide several key advantages in drug delivery, such as, (a) they can navigate through

complex biological environments and deliver drugs directly to the target site, minimizing off-target effects and reducing the required drug dosage [4, 7], (b) they offer unparalleled precision in drug release, enabling controlled and on-demand drug delivery, which is especially valuable for diseases with fluctuating symptoms [7, 16], (c) they can carry poorly water-soluble drugs, enhancing their solubility and bioavailability, thereby improving therapeutic outcomes [56], (d) by reducing systemic exposure to drugs, micro/nanorobots can minimize side effects and toxicity, improving patient tolerance to treatment [7], (e) some of them are equipped with sensors that enable real-time monitoring of physiological parameters, allowing for adaptive drug release in response to changing conditions [7, 16], (f) they facilitate the delivery of multiple drugs simultaneously or sequentially, enabling combination therapies to target multiple aspects of a disease [7], (g) they can traverse biological barriers, such as the blood–brain barrier, enabling the delivery of drugs to previously inaccessible regions [12], and lastly, (h) they can control drug release minimizing the development of drug resistance in pathogens or cancer cells [49]. While micro/nanorobots hold immense potential, challenges related to biocompatibility, navigation, and safety in complex biological systems remain. Ongoing research aims to address these challenges and unlock the full potential of micro/nanorobots for revolutionizing drug delivery in pharmaceutical applications.

Conclusion

The advent of micro/nanorobots in pharmaceutical drug delivery promises a transformative leap in the way we administer and benefit from medical treatments. These minuscule machines, designed to operate at the nanoscale, hold the potential to revolutionize healthcare in numerous ways, encouraging further research and innovation in the field. Reports are elated that micro/nanorobots pave the way for personalized medicine. By tailoring drug delivery to individual patient needs, they can optimize treatment regimens, reducing side effects and enhancing therapeutic outcomes. This approach shifts the paradigm from one-size-fits-all to treatments customized for each patient’s unique biology. Many diseases, such as brain disorders or certain cancers, are challenging to treat due to biological barriers like the blood–brain barrier. Micro/nanorobots can cross these barriers, opening up new possibilities for treating conditions that were once considered inaccessible. Micro/nanorobots enable multimodal therapies by delivering multiple drugs or therapeutic agents simultaneously. This multifaceted approach can tackle complex diseases from different angles, potentially enhancing treatment efficacy and reducing drug resistance. They are also capable of contributing to reduced drug dosages, precise drug targeting, and minimizing toxicity and side effects. Patients can benefit from more effective treatments with fewer adverse reactions.

Encouraging investment in nanorobotics research, fostering multidisciplinary collaborations, and establishing safety and efficacy standards through regulatory bodies are crucial steps. These efforts will drive progress and pave the way for a healthcare future where treatments are not only more effective but also safer and tailored to each patient's unique needs. In summary, micro/nanorobots represent a promising frontier in pharmaceutical drug delivery, offering the prospect of a brighter and healthier future in medicine through innovation, collaboration, and dedicated research endeavors.

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Author contributions

Tanisha Das and Shirin Sultana collected and analysed the data. Tanisha Das drafted the paper; all authors have read and approved the final manuscript.

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Data availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Competing interests

The authors declare that they have no competing interests.

Ethical approval and consent to participate

No ethics approval or consent was required.

Consent for publication

The authors declare no conflict of interest.

Abbreviations

DDS

Drug delivery system

ABF

Artificial bacterial flagella

CNT

Carbon nanotube

BBB

Blood brain barrier

Publisher's Note

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Bioanalytical method development and validation for the simultaneous estimation of Olanzapine and Samidorphan in rabbit plasma by using HPLC–MS/MS and application to pharmacokinetic study

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ABSTRACT (ENGLISH)

Background

Samidorphan is an opioid antagonist while Olanzapine is an effective medication for schizophrenia and bipolar disorder. A unique and accurate MS/HPLC approach due to simultaneous measurement of Olanzapine and Samidorphan is, therefore, more urgently required. Simultaneous quantification of Olanzapine and Samidorphan in rabbit plasma using HPLC-MS. Using a buffer composed of 1 mL of formic acid in 1 L of water and a mixture of two components, buffer and acetonitrile in a ratio of 50:50 and a flow rate of 1 mL/min at room temperature, we separated compounds on an Inertsil ODS column (250 × 4.6 mm, 5 m).

Results

Analysis was performed within 8 min over a satisfactory linear concentration range of 2–40 ng/mL for Olanzapine (r^2 = 0.99901 0.024) and 2–40 ng/mL for Samidorphan (r^2 = 0.99927 0.012). The matrix effect recoveries of Olanzapine and Samidorphan at various QC concentration levels were 104.5, 100.51% and 110.36, 99.25%, respectively. The precision and recovery study outcomes fall within the acceptable range. An electrospray ionization source was used to analysis of Olanzapine and Samidorphan at m/z 313.40→192.54, m/z 371.45→220.61 for Olanzapine and Samidorphan, m/z 316.40→237.58, m/z 374.41→223.61 for D_3 Olanzapine and D_3 Samidorphan that were ion pairs

of mass analysis.

Conclusions

Liquid–liquid extraction was used to remove Olanzapine (0.17 mg/kg) and its reference standard (D_3-Olanzapine) from rabbit plasma. Both the active compound Samidorphan (0.17 mg/kg) and its reference, D_3-samidorphan, were isolated from rabbit plasma. We conducted stability studies to ensure that the medications would remain stable in accordance with USFDA regulations.

FULL TEXT

Background

The atypical antipsychotic Olanzapine [1, 2] sold under the brand name Zyprexa is effective in treating schizophrenia [3, 4] and bipolar disorder [5, 6]. Both short-term treatment and long-term upkeep of schizophrenia are possible with this method. It can be injected into the muscle or taken orally [7, 8]. Common side effects include: weight gain [9, 10], movement abnormalities [11], dizziness [12, 13], feeling tired all the time, constipation [14], and dry mouth [15]. Among the other potential side effects include dizziness when standing (low blood pressure) [16, 17], allergic reactions [18], neuroleptic malignant syndrome [19, 20], high blood sugar [21], seizures, gynecomastia [22], erectile dysfunction [23], and tardive dyskinesia [24, 25]. It raises the chance of death in the elderly with dementia who take it. Using during the third trimester of pregnancy increases the risk that the baby will have mobility issues. Although its method of action is unknown, it blocks dopamine and serotonin receptors. As olanzapine/samidorphan (trade name Lybalvi), samidorphan (INN, USAN) (developmental code names ALKS-33, RDC-0313) is a treatment for mental disorders like schizophrenia and bipolar. The effects of olanzapine-induced weight gain are mitigated by samidorphan. Oral use of samidorphan is standard. Samidorphan's side effects include drowsiness [26] and stomach upset [27]. The research and development of samidorphan as a stand-alone drug for use in a variety of conditions has been halted.

The recovery rate is high, the run time is reduced, the accuracy is increased, the cost is decreased, the calibration curves are linear, the MRM transitions are optimised, and our method has been validated in accordance with USFDA criteria [28, 29]. Olanzapine (Fig. 1) and Samidorphan (Fig. 2) detection by HPLC–MS/MS simultaneously in rabbit plasma and its application to pharmacokinetic study is a topic that has not been covered in any published studies as of yet, and only few articles were reported on normal method development, validation and simultaneous determination in human plasma by using RP-HPLC [30–32].The pharmacokinetic research of olanzapine and samidorphan made excellent use of the bioanalytical assay. However, there are no current methods available for the determination of Olanzapine and Samidorphan at the present time. The current study aimed to (a) determine the pharmacokinetics of Olanzapine and Samidorphan following intravenous administration of test extracts to rabbits, and (b) create and validate a precise and sensitive MS/HPLC assay for measuring olanzapine and samidorphan in rabbit plasma.

Fig. 1 [Images not available. See PDF.]

Chemical structure of Olanzapine

Fig. 2 [Images not available. See PDF.]

Chemical structure of Samidorphan

Methods

Chemicals and materials

Olanzapine and Samidorphan ($C_{17}H_{20}N_4S$ and $C_{21}H_{26}N_2O_4$) and D_3-Olanzapine and D_3 –Samidorphan (Internal Standards, ($C_{17}H_{17}D_3N_4S$ and $C_{21}H_{23}D_3N_4S$) with purity levels 99% were obtained from Zydus Cadila, Ahmadabad. Acetonitrile (HPLC–MS Grade, 99.99 purity), Water (Milli Q), and Formic acid (HPLC grade, 99.0% purity) were all supplied by Merck (India) Ltd., Worli and Mumbai, India. All other materials and reagents were of commercial quality AR availability.

Instruments and conditions

The HPLC system (Waters Alliance model) and the mass spectrometer QTRAP 5500 triple quadrupole instrument (SCIEX) were used to construct the bioanalytical assay. Chromatographic separation was performed at room temperature using an isocratic model and an Inertsil ODS (250×4.6 mm×5 m) column. The mobile phase consisted of acetonitrile and formic acid at a ratio of 50:50 (by volume) at a flow rate of 1.0 mL/min. Ten litres of liquid were injected, and the entire cycle lasted eight minutes. For this study, we employed a QTRAP 5500 triple quadrupole mass spectrometer equipped with a positive ion electrospray ionisation interface. Mass ion pair monitoring using MRM mode: m/z 313.40→192.54, m/z 371.45→220.61 for Olanzapine and Samidorphan, m/z 316.40→237.58, m/z 374.41→223.61 for D_3 Olanzapine and D_3 Samidorphan (Internal standards of Olanzapine and Samidorphan). Ion spray voltage 5500 V; source temperature 550 °C; drying gas temperature 120–250 °C; collision gas nitrogen; pressure 55psi; drying gas flow rate 5 mL/min; declustering potential 40 V; entrance potential 45 V; exit potential 15 V; capillary voltage 5500 V; dwell time 1Sec. Table 1 clears necessary information on Instrumentation.

Table 1. Optimised liquid chromatography and mass spectroscopic conditions

LC parameters		MS parameters	
HPLC	Waters Alliance	MS	Sciex QTRAP 5500
Isocratic step mobile	ACN: Formic acid 0.1% in water 50:50 v/v	Ionisation source	Drying gas: N_2 gas@Drying flow rate: 5 ml/min@Pressure: 55 psi
Flow level: 1 ml/min	Source temperature: 550 °C	Injection volume: 10 µl	Capillary voltage: 5500 V
Inertsil ODS	250 mm length	Collision cell gas	Nitrogen with high purity
4.6 mm ID	Mode	MRM[b]	5 µm PS
Analyte	Olanzapine	Olanzapine MRM transitions	m/z-313.40→m/z-192.54@CE[a]—14 V
Samidorphan	Samidorphan MRM transitions	m/z-371.45→ m/z220.61@CE[a]—15 V	Internal standard
D_3-Olanzapine	D_3-Olanzapine MRM transitions	m/z-316.40→m/z-237.58@CE[a]—14 V	D_3-Samidorphan

[a]*CE* Collision energy

[b]*MRM* Multi reaction monitoring transitions

Experimental

Stock preparedness, calibration and quality control specimens

Olanzapine, D_3-Olanzapine (IS), and Samidorphan, D_3-Samidorphan (IS), were dissolved in Formic acid 0.1% in water-ACN (80:20, v/v) to create stock solutions with concentrations ranging from 2.0 to 40 ng/mL. Calibration and quality control samples were made by mixing the aforementioned working solutions with plasma and then distributing the resulting mixture. Eight calibration specimens were used, with values of 2, 5, 10, 15, 20, 25, 30, and 40 ng/mL. The quality control (QC) samples were made in the same way, but their final concentrations ranged from

2 ng/mL (LLOQ) to 30 ng/mL (HQC). Before being brought back to room temperature for examination, all samples were frozen to a temperature of−20 °C.

Preparation a solution for plasma samples

In order to prepare the samples, 500 µL of working standard stock solution and 500 µL of internal standard (IS) were injected into 200 µL aliquots of rabbit plasma samples. After 15 min of vortexing 300 µL of acetonitrile and 500 µL of diluent, centrifuging the samples at 5000 rpm for 15 min, then dividing, collecting, filtering, and injecting the supernatant-managed solution into the HPLC machine, we have our final product.

Animal parameters

In order to conduct this research, female rabbits were procured from Bioneeds India pvt. Ltd. in Bangalore. Six rabbits were used in this study (Table 2 represents average body weights of rabbits). Animal ethics committee (Reg. No. 1074/PO/Re.S/05/CPCSEA) at the institute approved the experiment protocol. The circumstances resemble those of a laboratory, and the animals have access to fresh endive, carrots, and maize. Feed for animals should be kept between 21 and 24 degrees Celsius, with humidity between 50 and 55 per cent. All animals fasted for an entire day and drank water at will before being used in an experiment. Olanzapine and Samidorphan solid dispersion tablets' pharmacokinetics were studied. Olanzapine and Samidorphan were given orally to all rabbits at a dose of 0.2 mg/kg. Blood samples were obtained from rabbits at 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, and 35 h, and the concentrations of Olanzapine (1.62 g/mL) and Samidorphan (1.64 ng/mL) ranged from 1.67 ng/mL to 18.4 ng/mL. The plasma was centrifuged for 30 min at 5000 rpm. Until analysis could be performed, plasma samples were kept at 2–8 degrees Celsius, and the supernatant solution was injected into a chromatographic column.

Table 2. Mean average body weights of rabbits

Group name	Average weight of rabbits
Rabbit-1	2538.32±2.65
Rabbit-2	2521.48±3.84
Rabbit-3	2556.47±2.52

Selectivity

The retention times of Olanzapine, Samidorphan, and IS were measured, and interference from untested samples was tested by analysing rabbit plasma samples from six distinct rabbits to assess selectivity.

Matrix effect

By comparing the peak zone fraction in the post-extract plasma sample of six separate plasma samples devoid of medicine and slick recovery samples, we were able to assess the Effect matrix for Olanzapine and Samidorphan. Six different lots of plasma were tested at MQC levels in duplicate, with satisfactory accuracy (% CV 15%).

Recovery

The recovery was calculated by comparing the peak areas of standards that had not been extracted with those of the extracted Olanzapine and Samidorphan (6 replicates per QC concentration).

Dilution integrity

A matrix with an analyte concentration over the ULOQC must be injected, and the test must be diluted using a blank matrix to prove that the dilution was done correctly.

Carry over

The retention of an analyte in the chromatographic system after the injection of a sample is referred to as carry over and can be identified in subsequent blank or unknown samples.

Precision and accuracy

Quality control replication study was performed on a total of six samples to determine the results at four different

quality control levels: low, medium, and high. Except for the LLOQ, which should be less than 20%, the CV level should be less than 15%.

Stability

Comparing the area response of the analyte in the stability samples with the region response of the sample obtained from the fresh stock solution allowed us to draw conclusions about the stock solution's stability. The effects of LQC and HQC concentrations on plasma stability were tested using six dose replicates. The US Food and Drug Administration (USFDA) define stability as a coefficient of variation (CV) of less than 15% for an analyte. Injected rabbit plasma samples were tested for 24 h of shelf life (bench top stability) after being kept at room temperature. The autosampler stability of increased rabbit plasma was measured over a period of 24 h at 2–8 °C. Extract plasma samples were injected immediately or stored in the autosampler at 2–8 °C for 24 h to assess the stability of the autosampler. Freeze–thaw stability was evaluated by contrasting newly infused quality control samples with those that had been frozen at − 30 °C and thawed three times. Six aliquots were utilised to test the freeze–thaw stability of both the low- and high-quality control concentrations. To evaluate the long-term stability, the 24-h concentration was compared to the starting concentration.

Results

Bioanalytical method development

With this method, atmospheric pressure chemical ionisation (APCI) mode is avoided in favour of ESI's more powerful reaction. Quantifying the ionisation of Olanzapine and Samidorphan with the MRM mode. Ion pair scan of Olanzapine and Samidorphan formed major ions of $[M+H]^+$ at m/z 313.4, m/z 362.5, m/z 234.55, m/z 192.54 and m/z 371.45, m/z 296.82, m/z 220.61, m/z 164.57. D_3-Olanzapine and D_3-Samidorphan, both internal standards, formed high intensity daughter ions of $[M+H]^+$ at m/z 237.58 and m/z 223.61. When compared to ion-negative mode, Olanzapine and Samidorphan exhibit a positive ion response mode. Figures 3, 4, 5 and 6 show the details of the mass spectrums.

Fig. 3 [Images not available. See PDF.]

Mass spectrum of Olanzapine

Fig. 4 [Images not available. See PDF.]

Mass spectrum of Samidorphan

Fig. 5 [Images not available. See PDF.]

Mass spectrum of D_3-Olanzapine

Fig. 6 [Images not available. See PDF.]

Mass spectrum of D_3-Samidorphan

Different buffers were tested with acetonitrile as the mobile phase in isocratic and gradient modes to determine the best conditions for chromatography. With each run, the mobile phase composition was tweaked to achieve higher resolution and shorter retention durations. For the best results with the specified drugs, an isocratic mobile phase of 0.1% formic acid and ACN (50:50 v/v) was chosen. In our optimisation procedure, we made use of a number of stationary phases, such as C18, C8, and CN-propyl. We get reliable peak shapes for Olanzapine and Samidorphan using an Inertsil ODS column with dimensions of 250 mm × 4.6 mm × 5 coupled to a PDA detector. Mobile phase flow rates were at 1 mL/min. Using these parameters, we find that Olanzapine and Samidorphan have retention durations of 2,241 and 5,098 min, respectively. Six replicate injections of Olanzapine and Samidorphan show coefficients of variation (CV) of 0.31 and 0.29 per cent, respectively, demonstrating the high precision of the suggested approach. The current procedure has been proven reliable in accordance with USFDA standards. Figures 7, 8 and 9 show the details of the chromatograms.

Fig. 7 [Images not available. See PDF.]

Chromatogram of standard

Fig. 8 [Images not available. See PDF.]

Chromatogram of Blank Plasma

Fig. 9 [Images not available. See PDF.]

Blank plasma spiked with internal standard

Validation of bioanalytical process

Matrix effect

At the Low Quality Control (LQC) and High Quality Control (HQC) levels, Olanzapine and Samidorphan's matrix effect results were 104.5 and 100.51 per cent, respectively (Table 3). The CV% for both compounds was calculated to be 0.77 at the LQC and 0.69 at the HQC. According to the findings, the matrix's effect on analyte ionisation and internal conditions was within acceptable limits.

Table 3. Results of matrix variability and Recovery (%) of Olanzapine and Samidorphan in rabbit plasma

Analyte	Matrix	Matrix factor bias (%)		% Recovery		
LQC	HQC	LQC	MQC	HQC	Olanzapine	Plasma
98.51	99.51	98.33	99.59	99.31	Samidorphan	Plasma

Recovery

Recovery rates for olanzapine and samidorphan in rabbit plasma at 10, 20, and 30 ng/mL are 101.33, 100.59, and 100.31%, respectively, at low, medium, and high focal concentrations. Olanzapine and Samidorphan are clearly effective in their extraction (Table 3).

Linearity, consistency and precision

At its height, emphasis was placed on the region's relative importance in determining adjustment standards. Figures 10 and 11 show that the method had a linearity range of 2.0–40 ng/mL for Olanzapine and 2–40 ng/mL for Samidorphan. Olanzapine and Samidorphan's correlation coefficients at different QC levels were greater than 0.9993, and their presented calibration curves covered the linear concentration range. The linearity and correlation data for Olanzapine and Samidorphan are shown in Table 4, 5 and 6.

Fig. 10 [Images not available. See PDF.]

Calibration plot of Olanzapine

Fig. 11 [Images not available. See PDF.]

Calibration plot of Samidorphan

Table 4. Linearity results of Olanzapine

Linearity	Olanzapine conc. (ng/ml)	Olanzapine area response ratio
1	2.00	0.118

2	5.00	0.235
3	10.00	0.527
4	15.00	0.749
5	20.00	1.002
6	25.00	1.228
7	30.00	1.481
8	40.00	2.034
Slope	0.0489	
Intercept	0.01688	
CC	0.99901	

Table 5. Linearity results of Samidorphan

Linearity	Samidorphan Conc. (ng/ml)	Samidorphan area response ratio
1	2.00	0.121
2	5.00	0.253
3	10.00	0.548
4	15.00	0.752
5	20.00	1.001
6	25.00	1.227
7	30.00	1.461
8	40.00	1.974
Slope	0.0490	
Intercept	0.02232	
CC	0.99927	

Table 6. Correlation results of Olanzapine and Samidorphan

Validation parameter	Olanzapine			Samidorphan		
Quality control levels	Low	Medium	High	Low	Medium	High
QC Conc. (ng /ml)	10	20	30	10	20	30
Linearity range	2.0–40 ng/ml			2–40 ng/ml		
Correlation (r^2)	0.9990±0.010			0.9992±0.017		

They ensured precision and exactness by combining the test results from numerous QC samples. The accuracy results of quality control samples for Olanzapine were 98.24–99.72% and for Samidorphan they were 98.03–99.72%. And %CV of Olanzapine and Samidorphan at various concentrations was 5% for all quality control samples. All exactness and precision results fell within the quantification range. Table 7 displays the results in detail.

Table 7. Precision and accuracy results of Olanzapine and Samidorphan in rabbit plasma

Matrix	Sample	Olanzapine			Samidorphan		
Accuracy bias (%)	Precision RSD (%)		Accuracy bias (%)	Precision RSD (%)		Intra-day	Inter-day
Intra-day	Inter-day	Plasma	LLOQC	−0.74	2.92	−0.88	−0.85
1.67	2.12	LQC	1.11	0.46	1.23	0.89	0.21
0.17	MQC	0.69	0.15	0.47	0.72	0.13	0.22

Dilution integrity

Spiking the analyte matrix fixation over the ULOQC and diluting this specimen with blank matrix should be used to demonstrate dilution integrity. Olanzapine and samidorphan were both diluted to a concentration of 40 nanograms per millilitre (ng/mL) at a 2ULOQC. Olanzapine and Samidorphan were both tested with six replicate samples at 1:2 dilutions (20 ng/mL, 20 ng/mL) and 1:4 dilutions (10 ng/mL, 10 ng/mL). Two components' and% CV were found to be within a reasonable range. Table 8 displays the results.

Table 8. Results of dilution integrity

Analyte	ULOQC conc (ng/ml)	Calculated conc (ng/ml)	%CV
Olanzapine	40	40.29	0.23
Samidorphan	40	40.17	0.71

Carry over

Carryover refers to any systematic error that might influence the sample's measured value. The following method was used to assess sample retention on an HPLC/MS system set up with Waters Alliance. The waters Z-spray triple

quadruple mass detector underwent a flow injection system blank injection with a volume of 10L containing 0.1% Formic acid and Acetonitrile (50:50). We may conclude that the proposed strategy's accuracy and precision were not affected by the method used here. Olanzapine and Samidorphan sample carry over findings were LLQC (3.21%), ULQC (1.14%), and LLQC (6.78%), ULQC (0.93%), respectively, within the allowable limit. Table 9 displays the outcomes of the carryover.

Table 9. Results of carry over

Concentration	% of carry over	
Olanzapine	Samidorphan	Blank
0	0	LLOQC
4.06	3.70	ULOQC

Re-injection reproducibility

Reproducibility of re-injection was performed to validate the system after the hard product was disabled due to any instrument malfunction during analysis of real-subject samples. If an instrument fails during an investigation of a genuine subject specimen, the batch can be re-infused after 24 h if the results of the re-injection show a per cent change of less than 2.0 per cent at the LQC and HQC levels.

Stabilities

We prepared a stock solution of olanzapine and samidorphan and tested its stability by keeping it at room temperature for 18 h. A stock solution kept in an autosampler at room temperature for 24 h displays stable behaviour in terms of autosampler stability. Stock was maintained at (−28 °C) for 24 h to evaluate freeze–thaw stability, 2–8 °C for 18 h to evaluate wet extract stability, and (−20 ± 3)°C for 18 h to evaluate dry extract stability. Long-term stability was determined by holding the stock for 28 days at (−20 ± 3)°C and injecting it into an HPLC–MS, whereas short-term stability was demonstrated by storing the medicines for 7 days at (5 ± 3)°C. Check the new stock solution's stability against the results of the stock solution prepared more than 24 h in advance. Olanzapine and Samidorphan showed negligible percentage changes of 1.15 and 0.68, respectively, during a 24-h period, demonstrating the stability of these solutions.

Plasma stability at room temperature was demonstrated for both olanzapine and samidorphan. Plasma samples spiked with Olanzapine and Samidorphan were tested and found to maintain their LQC, MQC, and HQC levels after being frozen and thawed multiple times. Olanzapine and Samidorphan were found to be stable at a freezing temperature of −30 °C for 24 h, demonstrating their long-term stability. Tables 10 and 11 display the results for Olanzapine and Samidorphan's overall stability.

Table 10. Stability results of Olanzapine in plasma of rabbit under different storage conditions

Stability	Storage condition	Conc. level	Measured conc (ng/ml) (Mean ± SD, *n* = 6)	% RSD	% Recovery
Bench top stability	18 h at room temperature	10	10.215 ± 1.2	0.96	99.64
20	20.324 ± 0.8	0.84	99.59	30	29.042 ± 2.3

0.75	99.84	Autosampler stability	24 h in autosampler at room temperature	10	10.174 ± 0.7
1.92	99.47	20	20.236 ± 2.4	0.53	99.53
30	30.524 ± 0.6	1.14	99.54	Long-term stability	28 days at (−20 ± 3)°C
10	9.231 ± 1.3	2.54	86.46	20	20.529 ± 2.2
0.53	87.42	30	30.112 ± 3.1	0.49	86.86
Freeze thaw stability	24 h at (−28 ± 5)°C then exposed to three freeze and thawed cycles	10	10.432 ± 1.5	1.54	99.02
20	19.362 ± 0.47	1.10	98.37	30	30.047 ± 1.11
0.76	98.45	Wet extract stability	18 h at 2–8 °C	10	10.321 ± 0.6
1.55	98.48	20	19.865 ± 2.3	0.43	99.24
30	29.745 ± 1.7	0.28	98.61	Dry extract stability	18 h at (−20 ± 3)°C
10	10.341 ± 3.5	0.95	98.85	20	20.552 ± 0.7
1.43	98.74	30	30.639 ± 4.1	0.52	99.53

Short-term stability	7 days at (5±3)°C	10	10.063±1.9	0.89	93.57
20	20.597±3.2	0.33	94.54	30	30.552±4.6

Table 11. Stability results of Samidorphan in rabbit plasma under different storage conditions

Stability	Storage condition	Conc. level	Quantified conc. (ng/ml) (Mean±SD, *n*=6)	%RSD	%Recovery
Bench top stability	18 h at room temperature	10	10.313±5.1	1.54	99.54
20	20.207±1.6	0.37	98.96	30	30.421±2.0
0.59	99.42	Autosampler stability	24 h in autosampler at room temperature	10	10.078±5.7
2.54	98.75	20	20.112±5.4	0.24	99.22
30	30.328±3.6	1.64	99.32	Long-term stability (Day 28)	28 days at (−20±3)°C
10	9.649±4.8	2.22	85.76	20	20.334±6.1
0.58	86.26	30	30.293±20.0	0.94	87.54
Freeze thaw stability	24 h at (28±5)°C then exposed to three freeze and thaw cycles	10	10.498±5.3	2.47	98.85
20	19.485±7.4	1.36	99.45	30	29.554±6.7

0.94	98.51	Wet extract stability	18 h at 2–8 °C	10	10.368 ± 2.8
0.85	98.32	20	20.491 ± 5.8	0.42	98.85
30	30.589 ± 3.4	0.67	99.59	Dry extract stability	18 h at (−20 ± 3)°C
10	10.647 ± 7.8	2.54	99.48	20	19.328 ± 9.5
1.39	99.54	30	30.514 ± 4.4	1.28	99.83
Short-term stability	7 days at (5 ± 3)°C	10	9.272 ± 7.6	0.42	94.65
20	20.367 ± 2.8	2.51	95.74	30	30.604 ± 12.6

Discussion

A stock solution of olanzapine and samidorphan was made and left out at room temperature for 18 h to test their stability. When it comes to autosampler stability, a stock solution kept at room temperature for 24 h in the device exhibits stable performance. The stock solution was stored at 2–8 °C for 18 h to evaluate its damp extract stability, whereas the stock was held at (−20 ± 3 °C) for 24 h to evaluate its dry extract stability. Storage of medicines for 7 days at (5 ± 3 °C) demonstrates short-term stability, while storage of the stock for 28 days at (−20 ± 3 °C) and injection into an HPLC–MS demonstrates long-term stability. Examine the differences between the stability results of stock solutions prepared immediately and those prepared more than 24 h in advance. The results showed that after 24 h, there were only a 1.15 per cent change in Olanzapine and a 0.68 per cent change in Samidorphan in the solution.

Both olanzapine and samidorphan were plasma-stable at room temperature under a range of circumstances. Repeated freezing and thawing had no effect on the levels of LQC, MQC, or HQC in plasma samples that had been artificially spiked with olanzapine and samidorphan. Long-term stability testing showed that both olanzapine and samidorphan could withstand temperatures as low as −30 °C for 24 h. Tables 10 and 11 display the results for the overall stability of Olanzapine and Samidorphan, respectively.

Pharmacokinetic study

After administering 0.17 mg/kg of Olanzapine and 0.17 mg/kg of Samidorphan intravenously to rabbits, respective mean plasma concentration–time profiles (Figs. 12, 13) were obtained for pharmacokinetic analysis. There are notable distinctions between Olanzapine and Samidorphan in intravenous pharmacokinetic studies. One, two, three, four, five, ten, fifteen, twenty-five, thirty, and three-and-a-half hours after the drugs were given, we collected samples

from the rabbits' bodies at varying intervals. The values were recorded after the test sample was prepared and injected into the chromatographic apparatus. C_{max} and T_{max} after intravenous administration of Olanzapine and Samidorphan (18.6740.146 and 18.4010.657), Kel (obvious first request terminal rate constant calculated from semi-log plot of plasma concentration versus time bend, employing the least square relapse technique), and $t_{1/2}$ (terminal half-life as governed by 0.693/Kel ratio) were all determined to be accurate measures of bioavailability. C_{max}, AUC0-24, and AUC0- were all within the acceptable range of 18.70.134, 2652.841, and 2654.282, and 18.40.111, 750.988, and 751.024, respectively. In Table 12, we can see the Olanzapine and Samidorphan pharmacokinetic characteristics.

Fig. 12 [Images not available. See PDF.]

Recovery plot of Olanzapine

Fig. 13 [Images not available. See PDF.]

Recovery plot of Samidorphan

Table 12. Pharmacokinetic studies of Olanzapine and Samidorphan

Pharmacokinetic parameters[a]	Olanzapine	Samidorphan
$AUC_{0–t}$ (ng h/ml)	265±2.841	75±0.988
C_{max} (ng/ml)	18.7±0.134	18.4±0.111
$AUC_{0–\infty}$ (ng h/ml)	265±1.427	75±1.024
$T_{1/2}$ (h)	30±0.41	10±0.12
T_{max} (h)	5±0.2	2±0.15

[a]All parameters expressed as Mean±SD, (n=6) values except T_{max} and $T_{1/2}$ which are expressed in terms of median value

Conclusion

An innovative HPLC–MS/MS method for assessing Olanzapine and Samidorphan in 8-min rabbit plasma has been developed and validated for the first time. Both Olanzapine and Samidorphan were rapidly absorbed by the rabbit's body after being given intravenously, as would be expected based on their pharmacokinetic profiles. The procedure outlined here is efficient, reliable, and repeatable. It has a good linear concentration range and sufficient precision for application in pharmacokinetic investigations for checking analyte concentrations in bodily fluids. These investigations are essential for the future credibility of our findings as a benchmark.

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Author contributions

The author planned the study, developed and tested the methods, wrote the procedure, and wrote the first draught of the paper.

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Availability of data and materials

The data for verification are provided with a Supplementary file and the rest.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

HPLC
High-performance liquid chromatography
ACN
Acetonitrile
USFDA
United States food and Drug Administration
MS
Mass spectrometry
ODS
Octadecyl-silica
MQC
Middle quality control
HQC
Higher quality control
LQC
Lower quality control
LLOQC
Lower limit of quantitation
MRM
Multiple reaction monitoring
IS
Internal standard
CV
Coefficient of variation
CC
Correlation coefficient
ULOQC
Upper limit of quantitation

Publisher's Note

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