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Original Research

OPEN ACCESS

Decreasing α-synuclein Aggregation by Ethanol Extract of Keluwih (Artocarpus camansi) Leaves on Rotenone-Induced Adult Zebrafish as Parkinson's Diseases Model

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Abstract: The prevalence of Parkinson's disease is increasing every year. This progressive disease is characterized by the loss of neurons in the substantia nigra due to the presence of α-synuclein aggregates. Keluwih leaves (Artocarpus camansi) are known to have activity in inhibiting acetylcholinesterase, as well as being an antioxidant and anti-inflammatory. The aim of this study was to evaluate the effect of ethanol extract of A. camansi leaves on the levels of α-synuclein in male and female adult zebrafish induced with rotenone. The zebrafish were induced with rotenone at a concentration of 5 µg/L for 28 days, along with the administration of 96% ethanol extract of A. camansi leaves at doses of 2.5, 5, 7.5, or 10 mg/L. The media was changed every 48 hours to maintain the concentration of rotenone and extract. After 28 days, α-synuclein levels were examined using immunohistochemistry. The administration of ethanol extract of A. camansi leaves can reduce the average levels of α-synuclein in male and female adult zebrafish, with the optimum dose being 2.5 mg/L. Therefore, it can be concluded that the administration of ethanol extract of A. camansi leaves can be used as an alternative treatment for Parkinson's disease.

Keywords: *α-synuclein, Artocarpus camansi, Parkinson disease, Zebrafis.*

INTRODUCTION

The prevalence of Parkinson's disease (PD) has doubled over the last 25 years. Disability and mortality rates caused by Parkinson's disease are higher and grow faster than other neurodegenerative diseases. In 2019, Parkinson's disease morbidity rose to 5.8 million cases of disability and 329,000 cases of death.[1] This data places PD second-ranked as the most common disease, especially in people over 60 years old, then the cases are estimated to double in 2030 while increasing frequently each year.[2]

Parkinson's disease is characterized by the progressive loss of neurons in the substantia nigra, which is affected by decreasing dopamine production and Lewy bodies (LB) accumulation. Those conditions affect the presence of α-synuclein aggregates. The formation of LB impairs the ubiquitin-proteasome degradation process, causing mitochondrial dysfunction and then failure of adenosine triphosphate (ATP) production.[3] Mitochondrial dysfunction results in abnormal regulation of calcium ions involved with the increasing calcium ion level, thus it is toxic for neurons and accumulates α-synuclein aggregates.[4] Accordingly, mitochondrial dysfunction leads to dopaminergic damage and peripheral motor nerve degeneration in experimental animals.[5,6]

The common animal used for the PD model is the zebrafish (*Danio rerio*) due to the uniqueness of its ventral telencephalon, which is similar to the human striatum.[4,7] Parkinson's disease in zebrafish is induced by rotenone.[8] Rotenone,

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one of the commonly used pesticides, is a neurotoxin that penetrates the cell membrane and causes complex I mitochondrial dysfunction and oxidative stress production,[8] consequently dopaminergic damage and peripheral motor nerve degeneration.[4]
Traditionally, the plant *keluwih* (*Artocarpus camansi*) use in seizure treatment.[9] *Artocarpus camansi* that belongs to the Moraceae family spreads in various countries such as Indonesia, India, Malaysia, Africa, Australia, Brazil and others. *Keluwih* leaves have known potential as antioxidants, anti-inflammatory agents, antibacterial agents, and antivirals. The phytochemicals in ethanol extract of keluwih leaves show several active compounds, such as flavonoids, alkaloids, tannins, triterpenoids, and phenolics (Jangtap, 2010; Solichah et al., 2021).[10,11] Other studies also proved that several genera of Artocarpus including *A. camansi* contain artomunoisoxanthone, artocommunol CC, artochamin D, artochamin B, and dihydroartomunoxanthone from leaves extract, which have potential as antioxidants to fight oxidative damage.[10] *Keluwih* contains compounds such as stilbenoids, arylbenzofurans, and abundant flavonoids.[10] The flavonoid compounds from *keluwih* leaf extract confirmed inhibiting acetylcholinesterase (AChE) enzyme activity with anticholinergic and antioxidant effects.[12] Those properties are effective in neurodegenerative disease treatment. This study aims to evaluate the effectiveness of ethanol extract of *keluwih* leaves on α-synuclein levels in rotenone-induced adult zebrafish, both male and female, as PD patient models.

MATERIAL AND METHOD

Zebrafish

A wild type strain of black zebrafish, both male and female, were acquired from Tulungagung cultivators in East Java, Indonesia, and were identified by Airlangga University, Faculty of Fisheries and Maritime Affairs in Surabaya, East Java using identification letter number 074/ULMKILP/UA.FPK/12/2022. Late adulthood zebrafish, 9-12 months of age with 0.08 g body mass and 30.6 ± 0.95 mm length were chosen for the study to represent human brain of elderly [13], which is the age range commonly Parkinson's disease sufferers. The zebrafish were acclimatized for seven days and were maintained in accordance to the standard procedures set by the research ethics committee of Airlangga University (No: 3.KEH.159.11.2022).

A. camansi Leaf Extraction

Keluwih leaves (*A. camansi*) were obtained from the UPT Herbal Laboratory Materia Medika Batu in Malang, East Java. The study was conducted under the determination letter number 074/124/102.20-A/2022. To extract the active compounds from the *A. camansi* leaves, a dry powder weighing 200 grams was mixed with 96% ethanol (Merch) in a ratio of 1:10 and macerated for three cycles of 24 hours each. The resulting liquid extract was then concentrated into a thick extract using a Rotavapor® apparatus. Different concentrations of concentrated extract were prepared by weighing 2.5 grams of extract then dissolved in 500 ml, homogenized to obtain a stock solution of 5000 mg/L, then diluted to obtain Theconcentration of extract were 2.5 mg/L, 5 mg/L, 7.5 mg/L, and 10 mg/L.

Rotenone and *A. camansi* Treatment

To induce a Parkinson's disease model in zebrafish, a concentration of 5 µg/L rotenone in DMSO (Sigma R8875) was added to a 2L water volume in a 25 x 16.5 x 12.5 cm aquarium, simultaneously the extract of *A. camansi* was administered in different concentrations (2.5, 5, 7.5, and 10 mg/L). The zebrafish male and female were placed in difference aquarium to compare the effects. Then, the aquarium water was refreshed every 2 days to maintain the rotenone concentration. The water temperature in the tank was tightly controlled between 24-25.5℃, and a light-dark cycle of 14:10 was established.[14] Feeding the zebrafish was three times daily

with Tetra Bit and Color Tropical Flakes from Tetra Sales, Blackburg, Germany. This treatment conducted along 28 days.

Analysis of α-synuclein concentration with Immunohistochemistry (IHC) Technique

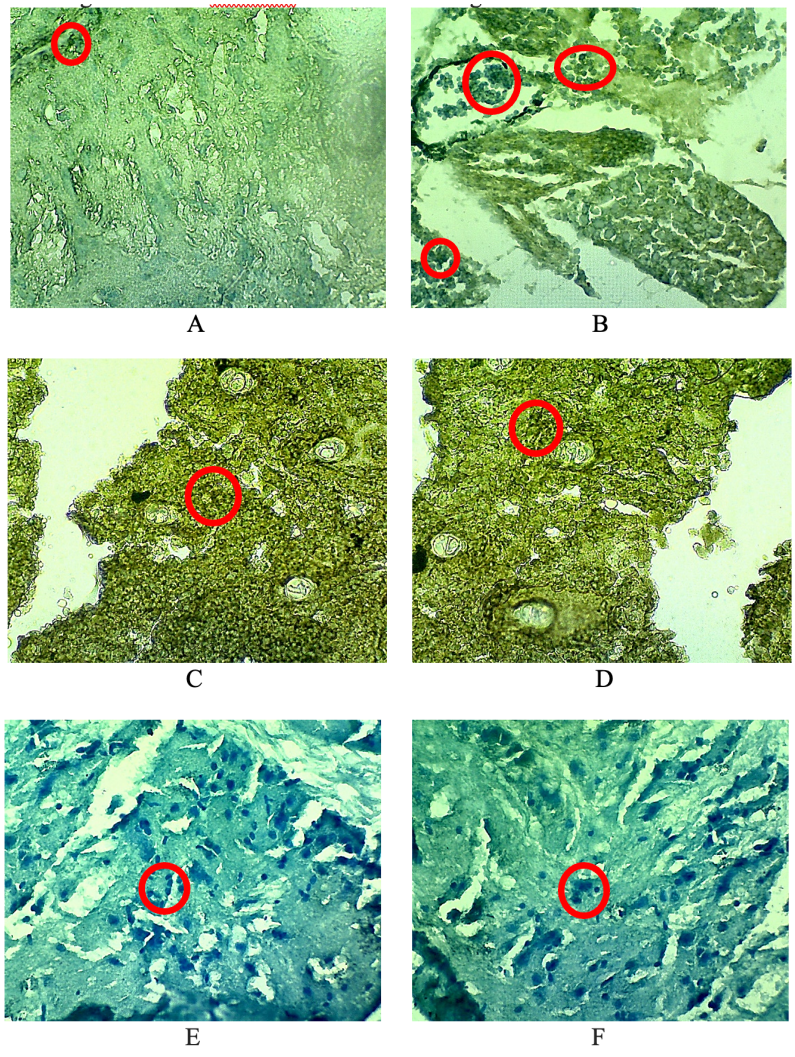
After 28 days, the animal was decapitated, the brain was isolated carefully and immediately immersed in a formalin buffer and prepared in paraffin blocks. The paraffin blocked samples were sectioned at a thickness of 0.4 mm for immunohistochemical staining. For staining, the slides were deparaffinized and subjected to the Millipore IHC procedure (Cat No. DAB 500). In this study, α-synuclein (Sigma) was used as the primary antibody, and the qualitative or quantitative expression of α-synuclein (brown color staining) was observed. Twenty fields of view in different areas were observed using a microscope on each slide at a magnification of 1000x, then quantified to obtain the average values of each treatment group were obtained from three replications (3 slides).

Data analysis

The data collected from each treatment group were subjected to statistical analysis using SPSS version 29. A one-way ANOVA test ($p < 0.05$) was employed for the analysis. The results are presented as the mean ± standard deviation (SD) for each respective treatment group.

RESULTS AND DISCUSSION

The image below shows where the positive cells are brownish (Qualitative), then quantified to obtain the average value of α-sinuclein levels shown in Figure 2.



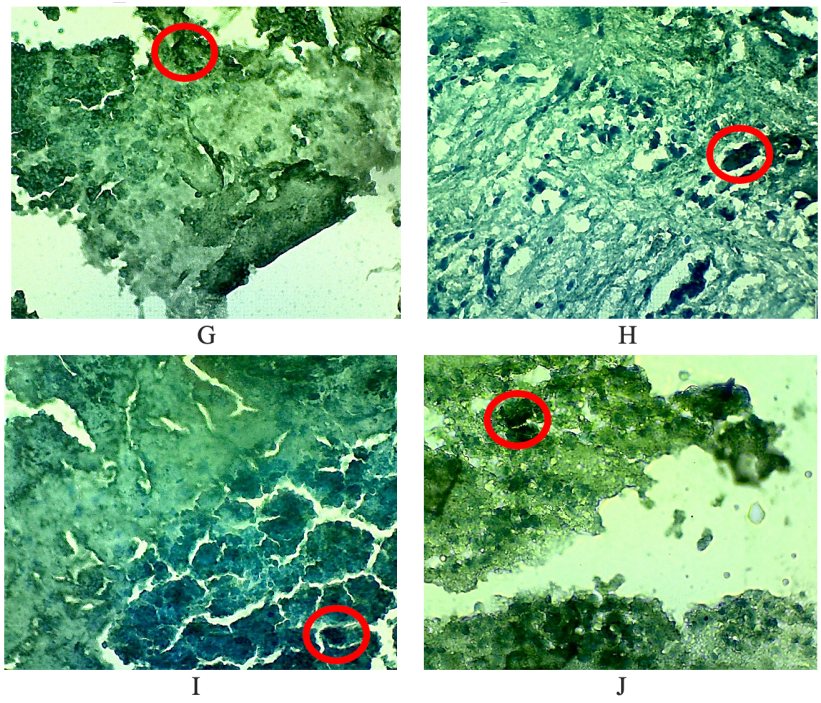


Figure 1. Staining Result of Brain Zebrafish to Evaluate Alpha-Synuclein Aggregation (brownish, in red circle). A: Control; B: Control Negative / Rotenone treatment; C: 2.5 mg/L leave extraction from adult male; D: 2.5 mg/L leave extraction from adult female; E: 5 mg/L leave extraction from adult male; F: 5 mg/L leave extraction from adult female; G. 7.5 mg/L leave extraction from adult male; H. 5 mg/L leave extraction from adult female; I. 10 mg/L leave extraction from adult male; J. 10 mg/L leave extraction from adult female

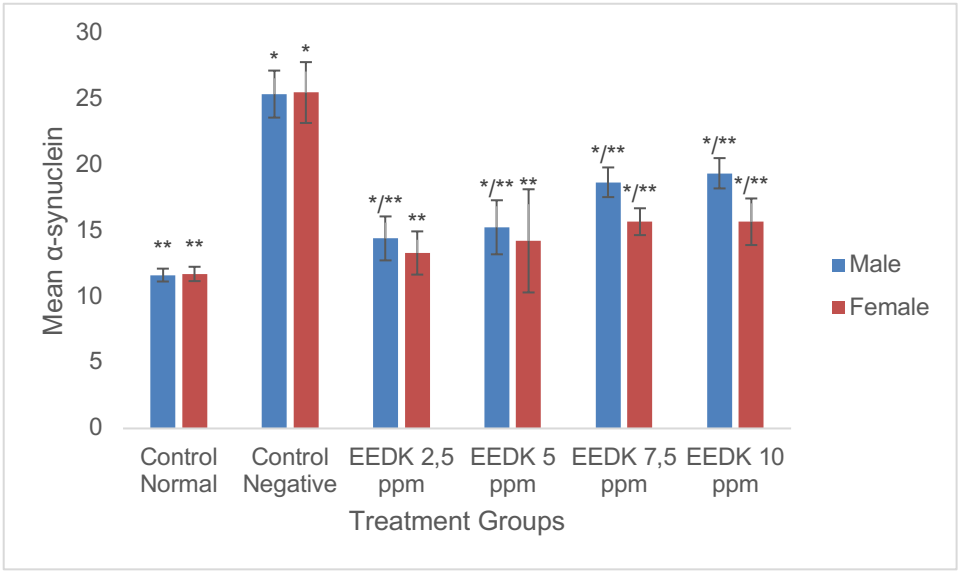


Figure 2. The Mean of α-synuclein Aggregation in Adult Zebrafish as Parkinson's Diseases Model with Ethanol Extract of *Keluwih* Leaves. **Significantly different with control normal, **Significantly different with control negative*

Upon administration, rotenone notably escalated the levels of α-synuclein in both male and female adult zebrafish, presenting a significant deviation from the control group ($p < 0.05$). Contrastingly, the introduction of *A. camansi* extract resulted in a marked decline in α-synuclein levels when juxtaposed with the control group. A comparison of α-synuclein aggregation between untreated and *A. camansi*-treated samples, as visually demonstrated in Figure 1, revealed a more subdued brownish color, indicative of α-synuclein aggregation, in the latter. Statistical evaluation substantiated this visual observation, revealing a significant improvement in α-synuclein levels following *A. camansi* treatment ($p<0.05$). These findings imply that *A. camansi* extract may have potential as a therapeutic agent in mitigating α-synuclein-related pathologies in rotenone-induced Parkinson's models in zebrafish.

Rotenone, owing to its potent lipophilic attributes, permeates cellular membranes with ease and speed, and infiltrates the brain with striking rapidity.[15] The inherent toxicity of rotenone incites a domino effect of damaging consequences, foremost among them being the impairment of mitochondrial electron transport. This, in turn, results in respiratory failure and the ultimate demise of the cell, or apoptosis.[16]

Rotenone's initial onslaught triggers oxidative stress, culminating in alterations to DNA, lipids, and protein folding, and subsequently sparking neurodegenerative changes.[17] These changes precipitate lipid modifications, thereby prompting mitochondrial dysfunction. The intricate interplay between mitochondrial damage and respiratory failure constitutes a hazardous cycle, spurred on by the dysfunction of electron transport.

Interestingly, respiration is intrinsically linked to ATP production, a cornerstone in facilitating axonal transport and cell metabolism.[18] This entire gamut of deleterious conditions inexorably leads to neuronal apoptosis, especially impacting the dopaminergic neurons of the substantia nigra. This, in turn, engenders a considerable reduction in dopamine production, a process vital to the neuron's survival.[19]

Moreover, the synergistic action of rotenone toxicity and oxidative stress induced by mitochondrial dysfunction has the potential to modify α-synuclein formation. α-synuclein, a presynaptic protein ubiquitous in various brain regions, under physiological conditions assumes a random formation, naturally unfolding rather than aggregating.[20] However, upon exposure to unfavorable conditions such as low pH, organic solvents, high temperatures, metal ions, oxidative stress, and pesticides like rotenone, α-synuclein tends to aggregate in cells. This is believed to play a crucial role in the pathogenesis of Parkinson's disease (PD), as such aggregation is predominantly found in neuronal cells.[21]

Investigations into the aggregation of α-synuclein in zebrafish models have revealed that it primarily takes place in axons and is highly toxic, often preceding neuronal death.[22,23] Such aggregation is associated with a reduced lifespan in fish models.[24] Further demonstrated that α-synuclein aggregation disrupts cellular microtubules and impairs mitochondrial axonal transport, owing to its high affinity binding to lipid structures such as cell membranes and organelles.[25] Thus, mitochondrial dysfunction in PD patients is influenced not only by rotenone exposure but also by α-synuclein aggregation.

Interestingly, the present study exhibits that the administration of an ethanol extract of *A. camansi* leaves can lower α-synuclein levels in rotenone-induced Parkinson's models in zebrafish. The improvement observed across all doses was significantly different from the control group, with the most optimal dose, based on α-synuclein measurements, being 2.5 mg/L. The constituents of the ethanol extract of *A. camansi* leaves flavonoids, alkaloids, tannins, triterpenoids, and phenolics known to be involved in the cellular pathology of Parkinson's, appear to have a synergistic effect on α-synuclein levels.

A. camansi leaves have been shown to inhibit acetylcholinesterase, the enzyme responsible for neuromelanin formation in the human brain. Neuromelanin, when increased significantly, can contribute to dopamine neurotoxicity and precipitate severe neurodegeneration. Therefore, the inhibition of neuromelanin by *A. camansi* might help stave off dopamine neurotoxicity and neurodegeneration.[26]

Additionally, the antioxidant and anti-inflammatory properties of the *A. camansi* leaf extract could potentially stabilize the synthesis, availability, and kinetics of dopamine.[18] This is further buttressed by the extract's capacity to prevent α-synuclein from aggregating, which would otherwise be neurotoxic, and inhibit the development of dopaminergic neuron degeneration.

CONCLUSION

Treatment of *keluwih* (*A. camansi*) leaves with 96% ethanol extract can be used as an alternative Parkinson's Disease ailment due to the ability of decreasing α-synuclein aggregation measurement, both male and female adult zebrafish. The lowest dose in this research of 2.5 mg/L is the optimum dose of 96% ethanol extract of *keluwih* leaves in adult male and female zebrafish. It is necessary to carry out further tests on other markers to ensure the pharmacological effects that contribute to PD pathology, and in silico tests can be carried out to determine compounds that have an effect.

AUTHORS' CONTRIBUTIONS

Marisca Evalina Gondokesumo: prepared the samples, designed the protocols, executed the protocols, wrote the manuscript, submit and revision the manuscript. Krisyanti Budipramana: reviewed and supervised the manuscript. Putu Dea Angelita Putri and Ni Putu Diah Nopitasari: data collection. Martanty Aditya and Liza Yudistira Yusan: data analytic and visualization statistically. All authors have read and approved the final manuscript.

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DATA AVAILABILITY STATEMENT

The utilized data to contribute in this research are available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals.

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Review Article

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Potential dyes from edible mushrooms for human health

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Abstract: Colors (dyes or pigments) have been applied in many sectors of human life, such as textile industries, food, and medicine, thus becoming a crucial factor that cannot be neglected. The origin of color can be obtained from nature or synthetic sources. Nowadays, synthetic colors are used more often than natural ones. However, the use of synthetic colors needs to be considered as they have the potential to cause health problems and contribute to waste issues. On the other hand, natural color sources are dominated by the plant kingdom, such as mushrooms, which are advantageous for health, more economical, and environmentally friendly. The method used in this review was to explore the literatures that discus dyes or pigments from macrofungi or mushrooms. Furthermore, the dyes or pigments were classified from edible or medicinal mushroom, then dyes or pigments were categorized based on their chemical structure. Mushrooms of various genera and species produce different colors that belong to constituent melanin, terpenoids, carotenoids, quinone, styrylpyrone, azulene, and pteridine. Therefore, natural colors are very promising for application in human health, due to their active compounds potency as anticancer, anti-HIV, antioxidant, and antimicrobial. In addition, pigments containing azulene structures from mushrooms are developed as solar cells and UV protection.

Keywords: Color, Dyes, Health, Mushroom, Pigments.

INTRODUCTION

Coloring agents have been involved in many areas of human life, and we can distinguish things through shapes and colors. The application of color is inseparable from life since it is involved in the textile industries, painting, photography, food, and beverages such as yeast for making beer, bread, meat, and medicine.[1] Plants are the main natural source of color and the most widely used coloring agent, but plants have many limitations including the different weather and geography. Due to the intensity and stability of synthetic colors after washing as well as the fact that they were unaffected by changing weather and geography, synthetic colors began to replace many natural colors by the end of the 1900s. Nevertheless, synthetic colors have some problems, especially regarding environmental waste that induces cancer; therefore motto “back to nature” is back to life.[2,3,4]

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There are two types of colors: dyes and pigments with the main difference in their solubility. Dyes are soluble in water, while pigments are inorganic and organic compounds which is insoluble in water. The diameter of pigments around 1-2 microns suggested a microscope to see it.[5,6] According to the origin, the color can be derived from natural or synthetic sources. The natural color can be produced from plants, animals, and mushrooms. The term mushroom is used to refer to only the fruiting bodies of fungi that are usually used for food. In comparison fungi mention the overall bodies, including the fruiting bodies and the underground.[7] Both micro- and macrofungi can produce the natural color. In the Greek language, macros mean large; therefore macrofungi can be seen by the eyes. On the other hand microfungi need a magnifying glass to be observed.[8] The synonym of macrofungi is mushroom.[9]

One of the famous microfungi is *Monascus* sp., which gives the red color to red rice (angkak). Microfungi like *Aspergillus ochraceous, A. sulphureus*, and *A. glaucus* make viopurpurin, which gives things their purple color. *Monascus* sp. gives things their red color from canthaxanthin and ankaflavin compounds. *Aspergillus* sp. also produces viomellein, which gives a reddish brown color, catenarine (red color), and variecolorquinone (yellow color).[4]

REVIEW METHOD

This review started with collecting some literatures from Pubmed (107), Frontiers (1133), and Springer (1755). The keywords were (“Pigments” OR “dyes”) AND (from); AND (“Edible”); AND (“Mushroom” OR “Macrofungi”). The articles have been collected then selected using inclusion criteria including full-text accessability and the articles containing the chemical structure. The exclusion criteria were articles not relevant to the topic and articles containing molecular biology and genetics.

RESULTS AND DISCUSSION

MELANIN

The first step of selecting suitable solvents for extraction depends on their polarity and chemical structures. For aglycones structure, the medium polarity until less polarity solvents are preferable but if the glycosylated structure requires more polar solvents. Many extraction techniques are performed at room temperature or at a higher temperature. The popular one is maceration because it can be done at room temperature with an simple procedure. The other methods of extraction are percolation, soxhlet, reflux, ultrasound, supercritical fluid, and microwave.[10] Melanin is a polymer with insoluable characteristics in water, organic solvents, and cold or hot acids. Moreover, any modification to improve the dissolution potentially disarrangess the structure. Nevertheless, melanin is soluble in alkali and can be bleached by oxidizing agents. Thus, with its special characteristics, melanin is challenging to be isolated.[11,12]

There are three pathways producing melanin in fungi (Figure 1). The first pathways to produce 1,8-dihydroxy naphthalene (DHN) start with *malonyl CoA* or *acetyl* CoA precursor. DHN-melanin is melanin without nitrogen in its structure. The second-way melanin is by *glutaminyl-hydroxybenzene* (GHB), which is made when the tyrosinase enzyme changes. The third-way melanin is made from the amino acids eumelanin and pheomelanin.[13] *Agrocybe cylindracea* produces the melanin coloring agent, which gives a dark color. According to data from *Fourier transform infrared spectroscopy* (FTIR) and UV-Vis, the 3,4-dihydroxyphenylalanine melanin groups that *A. cylindracea* produces are structurally similar to synthetic tyrosine melanin.[14] There are 4 forms of melanin, including allomelanin (blue), GHB-melanin (green), eumelanin (pink), and pyomelanin (red).[4] *According to Kim et al. (1997), A. cylindracea also makes the indole colors 6-hydroxy-1H-indole-3-acetamide and 6-hydroxy-1H-indole-3-*

carboxaldehyde. These can stop lipid peroxidation in rats with IC_{50} of 3.9 and 4.1 µg/ml.

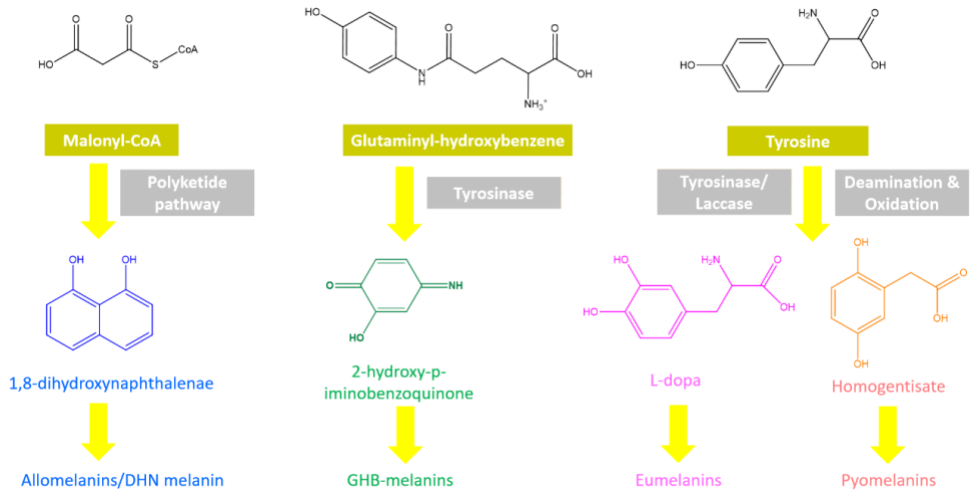

Figure 1. Pathways of melanin production

TERPENE

Terpene possesses varied structures from linear to cyclic, with a low molecular weight to a high molecular weight, volatile to non-volatile, simple terpene into modified terpene. Different terpene structures can induce different polarities, thus requiring particular extraction techniques. The structure of linear terpene or cyclic terpene commonly comprises hydrocarbons. The longer the hydrocarbon chain, the more solubility to non-polar solvents. Monoterpene or sesquiterpene with carbons less than 15 tends to be volatile and has low polarity and low molecular weight. Mostly, the volatile terpene can be extracted using distillation or organic solvent or even modern ones such as microwave-assisted extraction (MAE). The longer hydrocarbon chain will affect their elution. For example, a terpene with longer carbon chains will elute faster than with shorter ones. Still, the unique one is that cyclic terpene will eluate earlier than non-cyclic terpene, even though they have the same number of carbons. This happened because cyclic terpene gives a more compact structure to be eluated.[16]

Terpene is one of the metabolite substances that macrofungi produce with the basic structure of isoprene. Terpenes are classified into:[17]

a. Monoterpene : 2 units isoprene or 10 atoms of carbon (C_{10})
b. Sesquiterpene : 3 units isoprene or 15 atoms of carbon (C_{15})
c. Diterpene : 4 units isoprene or 20 atoms of carbon (C_{20})
d. Triterpene : 6 units isoprene or 30 atoms of carbon (C_{30})
e. Tetraterpene : 8 units isoprene or 40 atoms of carbon(C_{40})

Terpene constituents from fungi have been used for a long time to increase human health. Ganoderma (Reishi) has been recorded since ancient times as a medicinal mushroom in China as well as in Japan to increase the immune system. In the book of *Shennong Bencaojing* and *Chinese Pharmacopeia* (2010), Ganoderma is documented as a medicinal mushroom.[18] Colossolactone V-VIII compounds contain the skeleton structure of steroid terpenoid from *G. colossum* that inhibit HIV-1 protease with IC_{50} of 5-13 ppm while a yellow oil, ganomycin I, showed IC_{50} of 7.5 ppm.[19,20] In vitro, ergosterol, ergosterol peroxide, and 5,6-dehydroergosterol isolated from *G. lucidum* could inhibit breast cancer MDA-MB-231 (*triple negative breast cancer*) by decreasing the expression of cyclin D1,

AKT1, AKT2, dan BCL-XL.[21] A yellow powder, leucocontextin D and L, have been successfully isolated from *G. leucocontextum* and leucocontextin E, a yellow oil, showed its activity against endocervical cancer SMM-7721, leukemia cancer K562, and breast cancer MCF-7.[22]

Besides terpenes, fungi also produce meroterpenoids with different kinds of structure and color thus having different pharmacological activities. Meroterpenoids have at least two main structures. Terpenoids structure come from the mevalonate pathway, and the non-terpenoids structure comes from pathways like shikimate, amino acid, polyketide, and more. There are also four parts to the non-terpenoid structure: the shikimate-terpenoid moiety, the indole-terpenoid moiety, the polyketide-terpenoid moiety, and others.[23] One of the edible mushrooms that synthesize meroterpene is *Albatrellus fletti* which also produces grifolin, neogrifolin, and confluentin. The IC_{50} of grifolin, neogrifolin, and confluentin were around 24 up to 35.4 µM for colon cancer HT-29 and SW48 and also cervix cancer HeLa cells. The purple oil, albatrellin, from *A. confluens* was able to kill lung cancer Hep52 cells with an IC_{50} 1.55 µM.[24,25] Another purple oil called grifolinone B was found in *A. caeruleoporus* and it was able to stop the production of nitric oxide (NO) triggered by lipopolysaccharide with an IC_{50} value of 22.9 µM.[26] In Finland, *A. ovinus* is categorized as an edible mushroom and is usually used as a culinary ingredient because it contains a lot of minerals.[27]

CAROTENOIDS

Carotenoid pigments are part of terpene, especially tetraterpene (C_{40}), found in many plants, photosynthetic bacteria, algae, animals, and mushrooms. Isolating carotenoids is exciting due to the complexity of the pigments and the matrix. Carotenoids are very sensitive to heat, light, acids, oxygen, and long-time exposure extraction. The advisable solvent to extract is a non-polar solvent for non-polar carotenoids, whereas polar solvents are more suitable to extract the more polar carotenoids. The presence of water in the matrix can disrupt the extraction steps. The water can be removed by boiling or heating, but it will degrade and isomerize the structure of carotenoids. In order to minimize the water content and to protect the carotenoids, the matrix can be dehydrated by freeze drying method. However, if the matrix also presents a mixture of water-soluble compounds and sugar, it must be separated first before the freeze-drying step.[28]

The characteristic feature of carotenoid is the presence of chromophore functional groups that reflect red, yellow, and orange color in fruits, vegetables and more than 600 isomers have been found. *Chantarellus cibarius* is commonly called “Golden chanterelle” because the color of the cap is golden yellow until orange due to the presence of canthaxanthin. The compound canthaxanthin belongs to the carotenoids that are easy to degrade by heating or drying. *C. enelensis* is “Albino chanterelle” which lack canthaxanthin thus gives white fruiting bodies but still has an apricot scent like “Golden chanterelle” and is edible.[29] The ability of carotenoids to bind radical scavengers allows for their identification.[30] In addition to being an antioxidant, the methanol extract from *C. cibarius* was also able to fight cervical adenocarcinoma HeLa, breast cancer MDA-MB-453, and leukemia K562. The methanol extract can also inhibit *angiotensin-converting enzyme* I (ACE) to decrease blood pressure by 0.063 ppm. The methanol extract is also potent against gram-positive bacteria especially *E. faecalis.*[31]

Carotenoids in *Cordyceps militaris* turned the fruiting bodies of these fungi from yellow to orange. However, some albino *Cordyceps* have also evolved due to mutations.[32] *Cordyceps* sp. is a rare fungus that lives in high mountains, primarily in Bhutan and Nepal at elevations ranging from 3400 to 4100 feet and temperatures ranging from -10°C to -20°C. *Cordyceps* comprises two Greek words: “cord” or “club” means lower part, and “ceps” or “head” means upper part since these fungi live in the head of a worm (*Hepialus armoricanus*). *Cordyceps* sp. is an entomopathogenic fungus with many popular names such as “Chinese

Caterpillar Fungus" and "Worm in Winter and Grass in Summer".[33] This rare mushroom has been shown to maintain the immune system and cure chronic diseases. The existence of these fungi has become very scarce due to the complex life cycle of the worm and the environment. The hunting of *Cordyceps* sp. was forbidden until 2003, was just legalized in 2004, and became the main livelihood for the society living in the mountains.[34,35]

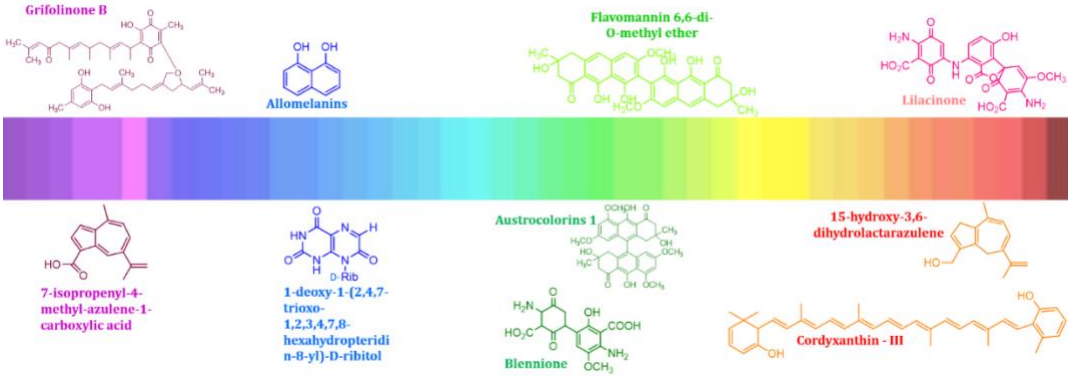

Figure 2. The variety of chemical structures yielded from mushrooms

UV radiation from sunlight and blue light from electronic gadgets could destroy the retina. Supplementation of extract water of *C. militaris* twice a day at 10 mg/kg could protect the retina from those lights by decreasing the hypertrophy of Muller cells and increasing the GSH level to accelerate the functional recovery of visual acuity and sensitivity. The major compound from the water extract was cordyxanthin, which has a similar structure to that of carotenoids (Figure 2). The structure of cordyxanthin is unique compared to other carotenoids since it contains more hydroxyl groups than carotenoids, making it more soluble in water.[36] Moreover, *Cordyceps* sp. is also rich in ergothioneine, cordycepin, γ-aminobutyric acid (GABA), polysaccharides, and other important compounds for therapy or as *functional foods* to stay healthy. Cordycepin became the most wanted antivirus during COVID-19.[37]

QUINONE

There are two forms of anthraquinones: aglycones and glycosylated anthraquinones. For aglycones, they can be extracted using less polar solvents, whereas the glycosylated anthraquinones will be suitable to be extracted using more polar solvents. Interestingly, anthraquinone can be soluble in alkaline; thus, the color can be used as a guideline to distinguish anthraquinones or, anthrones or dianthrones. Hydroxyls that attach to position numbers 1 and 2 of anthraquinone in an alkaline solution will give a blue-violet color, while substitution in position numbers 1 and 8 has a red color. The yellow color of anthrones and dianthrones in an alkaline solution will quickly turn to red color. Acid can be used to hydrolyze the glycosylated anthraquinones into free anthraquinones, but some C-glycosides are resistant to being hydrolyzed.[10,38]

Quinones are two keto groups at positions 9 and 10 that define the skeleton structure of anthraquinones, which derive from anthracene. More than 700 anthraquinones and their derivatives have been found with color gradations ranging from green yellowish to green blueish.[39,40] Anthraquinone structure dominated the variety of color in mushrooms.[41] *Cortinarius* sp. is also one of the mushrooms producing anthraquinone. Citreorosein 6,8-dimethyl ether gives an orange color; 1-hydroxy-3-methyl-2-isopropanyl-6,8-dimethoxyanthraquinone gives a reddish-orange color; rufoolivacin A, C, and D are red color while leuocorufoolivacin; Verbindung Cr11; and Verbindung Cr60 have the same yellow

color isolated from *C. purpurascens*. All of these compounds were tested for their antioxidants, and leuocorufoolivacin showed the best antioxidant with an IC_{50} of 3.88 ppm.[42] *C. purpurascens* and *C. violaceus* are not only edible but also interesting in the color of their purple fruiting bodies thus increasing appetite. The fruiting bodies of *C. violaceus* also give a purple color due to the presence of an amino acid called (R)-β-dopa.[43]

In 1987, Gill and Steglich successfully isolated the yellow color of emodin, physcion, and physicion 1-O-methyl ether from the *Dermocybe* subgenus *Cortinarius.* Some rare anthraquinones were also successfully isolated, including orange needles of fallacinol (6-O-methoxycitreorosein); the bright yellow-green of flavomannin (6,6-di-O-methyl ether), the green color of atropisomer austrocolorins A1, and powder of yellow-green B1. Thirteen species of *Cortinarius* contained emodin, physion, austrocortirubin, austrocortilutein, and torosachrysone. These chemicals were able to stop *S. aureus* with IC_{50} values ranging from 0.7 to 12 ppm. However, only emodin (IC_{50} 2.0 ppm) and physicion (IC_{50} 1.5 ppm) were able to stop *P. aeruginosa.*[44] Anthraquinone compounds also have the potential to be developed as anticancers since their structure is similar to that of anthracycline commonly used in chemotherapy extracted from bacteria.[45,46,47,48]

Pycnoporus cinnabarinus is a white root fungus, also commonly named *Cinnabar polypore,* since the color of the fruiting bodies is reddish orange. Three compounds, cinnabarin, cinnabarinic acid, and tramesanguin, were successfully isolated from *P. cinnabarinus.* In Africa, this fungus can be prepared for culinary and cosmetics purposes; it was indeed listed in the traditional pharmacopeia of Africa, but in Europe, this fungus is forbidden. Native Africans and South Americans used *Pycnoporus* to cure some skin lesions and illnesses.[49,50] Smania et al. (2003) looked into how cinnabarin killed cells and viruses. They found that up to 1000 mg/kg of cinnabarin did not hurt mice and could lower rabies virus titers by up to four times.[51]

STRYLPYRONE

Styrylpyrone compounds are commonly found in fungi, especially Basidiomycetes, although they are also found in Angiosperm and Pteridophytes. Unfortunately, the abundance of mushrooms that contain styrylpyrones is rare. Thus, the utility remains still limited. *Inonotus hispidus* (Shaggy Bracket) has been used as folk medicine in China and Europe. The Compendium of Materia Medica and Shennong's Classic of Materia Medica mentioned *I. hispidus* as Sanghuang. Local people in Northeast China used it to release dyspepsia, and in Xinjiang, it was formulated to reduce indigestion, ulcers, and cancer.[52] From *I. hispidus* two compounds have been successfully isolated, a yellow color, called hispolon and hispidin. Hispolon with the skeleton structure styrylpyrone showed anti-virus and immunomodulator activities. Styrylpyrone can be extracted using polar or semi-polar solvents such as ethanol and ethyl acetate.[53,54]

There are abundant pigment derivates of styrylpyrone in fungi from Hymenochaetaceae mostly from the genera *Phellinus* and *Inonotus.* Interestingly, compounds with a styrylpyrone structure are also present in primitive plants such as Zingiberaceae, Ranulaceae, Annonaceae, Lauraceae, and Piperaceae to build an immune system against bacteria and wounds. In fungi, styrylpyrone is derived from phenylalanine amino acids, which have many functions against predators, molecular signaling, and pigmentation. Pigment derivative styrylpyrone in fungi might have a similar function as flavonoids in plants.[55]

AZULENES

Azulenes mean blue since the name azul originally comes from the Arabic “azur” and Spanish “azul.” Both Arabic and Spanish mean blue color. On the other hand, naphthalene, which is the isomer of azulene, has no color or is colorless.[56] Azulene contains isoprene moiety with its derivatives, including guaiazulene and chamazulene. Both of them belong to sesquiterpene. Therefore, Tala et al. (2017) and Patino et al. (2017) used polar solvents to extract azulene, such as ethanol or methanol, followed by a purification step based on molecular weight separation like Sephadex LH-20. The blue color comes from azulene compounds isolated from *L. indigo*, which are usually used for culinary purposes, while *L. delicious* contains orange isomer gum. The compounds of 15-Hydroxy-3,6-dihydrolactarazulene and 15-hydroxy-6,7-dihydrolactarazulene also contain purple solid 7-isopropenyl-4-methyl-azulene-1-carboxylic acid.[57] Azulene and its derivatives are also developed for many applications especially solar cells, components of optoelectronics, and sensors.[58]

Lactarius indigo commonly forms symbioses with plants or trees thus being classified as ectomycorrhiza mostly in pine trees. Ectomycorrhiza is a mutual relationship between fungi and trees. The mycelium of the fungi interacts with the root of the tree then both become partners. There are so many benefits for plants or trees in the presence of ectomycorrhizal fungi. Ectomycorrhizal fungi can revive plants and trees that have suffered damage from fire, corrosion, or heavy metals. Ectomycorrhizal fungi can also repair the fertility of the soil due to flooding, soil erosion, and clearcutting. Ectomycorrhiza could prevent corrosion because it can add roots to the tree thus making the tree stronger. All fungi can absorb and digest heavy metals thus ectomycorrhizal fungi could hinder heavy metals from sticking to the root. The ectomycorrhizal fungi themselves can live from the plants or trees because of the carbon they provide to their lives.[59]

Peet et al. (2016) made 18 different kinds of azulene structural derivatives and tested them against HIV-1 on virus-like particles, TZM-BL cell lines, and infectious HIV-1 in U2OS. The findings showed that 2-hydroxyazulenes stopped the replication of the HIV-1 virus (IC_{50} 2–10 μM) and stopped HIV-1 from infecting other people (IC_{50} 8–20 μM). These results suggested that the derivatives of azulene could be developed as HIV antiretroviral candidates.[61] Another species of *Lactarius*, which is also colorful and interesting to explore is *L. lilacinus*. There are lilacinone derivatives of aminobenzoquinone in the fruiting bodies of *L. lilacinus* that give the plant its red color and blennione, a green color, that comes from *L. blennius.*[62]

PTERIDINES

The word of pteridines in Greek means wing (pteron) since it was first discovered in the wings of butterfly. Pteridines are heterocycles consisting of fused pyrazine and pyrimidine rings. Based on its structure, pteridines are classified into lumazines and pterins.[63,64] Probably the most popular compounds with pterin structures are riboflavin (vitamin B_2) and folic acid (vitamin B_9). Riboflavin, a yellow color, was successfully isolated from *R. xerampelina* but it will degrade into lumichrom if exposed to light. An analog of lumichrom that is also photoactive is 3N-methyl riboflavin isolated from *Panellus serotinus* and lampteroflavin from *Omphalotus japonicus.*[66,67] In the 1950s, methotrexate, a pteridines derivatives that is also an antifolate medicine, was usually used to treat tumors. The characteristic of pterin compounds is heterocyclic with low molecular weight. Folates are also called conjugated pterins because the structure contains a para-aminobenzoilglutamine moiety.[65]

The first lumazine was isolated from Basidiomycetes, 1-(5-amino-2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl) amino-1-deoxy-D-ribitol was added. A blue-violet fluorescence of 1-deoxy-1-(6-methyl-2,4,7-trioxo-1,2,3,4,7,8-

hexahyropteridine-8-yl)-D-ribitol and 1-deoxy-1-(2,4,7-trioxo-1,2,3,4,7,8-hexahydropteridin-8-yl)-D-ribitol were first isolated from higher fungi. *Russula* sp. also has russupteridine-yellow I, II, IV, and V.[68] In 1969, Gluchoff successfully isolated three russularhodines with a red color, three russula cyanines with a blue color, and seven russula xanthines with a yellow color. The abundance of lumazines in nature is less than that of pterins thus their pharmacological activities are still undiscovered.[69,70]

Lactarius and *Russula* genera are members of the same order and family, which are Russulales and Russulaceae. The microscope examination of their spores is similar but can still be distinguished easily by a morphology test. A specific and unique feature of *Lactarius* is the presence of latex usually white milk this is called a milky cap if their fruiting bodies are squeezed while *Russula* produces no latex. A yellow-orange gum, ochroleucins A_1, isolated from *Russula ochroleuca* and *R. viscida,* will turn red if reacted with base. Sontag et al. (2006) and Clericuzio et al. (2008) used EtOAc or dichloromethane or hexane to extract pigments from Russula.[71,72]

CONCLUSION

Based on the review results obtained, natural colors are very promising for application in human health, due to their active compounds potency as anticancer, anti-HIV, antioxidant, and antimicrobial. In addition, pigments containing azulene structures from mushrooms are developed as solar cells and UV protection. However, further research needs to be done in this regard.

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All authors contributed equally to this work.

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DATA AVAILABILITY STATEMENT

The utilized data to contribute to this investigation are available from the corresponding author on reasonable request.

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There is no conflict of interest.

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Abstract: Coffee is a drink that is commonly consumed by Indonesian people. Coffee contains caffeine, cafestol, and kahweol which can increase lipid levels, including increasing triglyceride levels. Triglycerides are substances consisting of glycerol linked to fatty acid groups. The purpose of this study was to describe blood triglyceride levels in coffee addict students at Anwar Medika University using the GPO-PAP method. This study uses a quantitative descriptive design with a cross-sectional survey approach. This research was conducted in May 2023. The research sample consisted of 48 samples which were determined using a purposive sampling technique which was taken at random. The univariate analysis used is the distribution of frequencies and percentages which describe the presentation of data for one variable. This research test obtained an average triglyceride level of 158.7 mg/dl. Based on age category, maximum triglyceride levels were 204.73 mg/dl with an average of 152.26 mg/dl. Based on lifestyle, the maximum triglyceride level is 189.93 mg/dl with an average of 166.43 mg/dl. Based on the frequency of coffee consumption, the maximum triglyceride level is 160.64 mg/dl with an average of 158.51 mg/dl. Based on physical activity, the maximum triglyceride level is 173.88 mg/dl with an average of 160.60 mg/dl. Based on the type of coffee consumed, the maximum triglyceride level was 166.76 mg/dl with an average of 164.57 mg/dl. So, it can be concluded that excessive coffee consumption can increase triglyceride levels in the body

Keywords: Coffee, Triglycerides, College Students.

INTRODUCTION

Coffee is a drink commonly consumed by Indonesian people. The distinctive aroma of coffee has its attraction for consumption. Indonesia is the third largest coffee-producing country in the world after Brazil and Vietnam with a total production of 748 thousand tons or 6.6% of world coffee production in 2016. From 2016 to 2019 the level of coffee consumption in Indonesia increased every year. It is predicted that this increase will increase by an average of 8.22% every year.[1] The increase in coffee consumption in Indonesia is due, in part, to people's lifestyles which have made processed coffee drinks a daily activity for their daily needs. However, the amount of coffee consumption in Indonesia still reaches 300 thousand tons and is still far below other countries.[2]

The habit of drinking coffee has spread and become a culture in various regions, including among students, from urban to rural areas on the island of Java. Students also have a large level of coffee consumption due to internal and external

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factors. One of them is students who work while studying with heavy physical activity, then need coffee to prevent drowsiness and refresh the body. This causes the level of coffee consumption among young people to remain very high.[3]

The ingredients contained in coffee consist of more than a thousand molecules of different substances, including phenolic compounds, vitamins, minerals, and alkaloids which can increase lipid levels. The caffeine, cafestol, and kahweol compounds contained in coffee can also increase triglyceride levels.[4] Kalel et al.'s 2020 research on the effect of coffee consumption on serum lipid profiles in adults concluded that individuals who drink coffee show a higher risk of developing dyslipidemia.[5]

Triglycerides are a type of fat that is transported in the blood and stored in the body's fat tissue. Triglycerides are the main constituent of lipids in the body. Triglycerides are used by the body to provide energy in metabolic processes, small amounts of triglycerides are also used throughout the body to form cell membranes. Triglyceride levels are an indicator of body health, excessive triglycerides in the body can narrow blood vessels and increase the risk of heart attack.[6] Several factors that can influence triglyceride levels include age, gender, lifestyle, frequency of coffee consumption, hormone levels, and obesity. Triglyceride levels in the body can be controlled with a healthy lifestyle and avoiding foods or drinks that can increase lipid levels.[7] This research aims to determine the description of triglyceride levels in the blood of students who are coffee addicts at Anwar Medika University using the GPO-PAP method.

MATERIAL AND METHOD

This research uses a quantitative descriptive method with a cross-sectional survey approach. The sampling technique in this research was purposive sampling which was taken randomly. Triglyceride examination uses the GPO-PAP (Glycerol Peroxidase Phosphate Acid) enzymatic colorimetric method. The principle of the GPO-PAP method is that triglycerides will be enzymatically hydrolyzed as glycerol and free acids with specific lipase to form a colored complex whose levels can be measured using a photometer. The intensity of the color formed can be determined by measuring the absorbance in the wavelength range 480-550 nm.[8,9] This research was carried out in May 2023, at the Medical Biology Laboratory on the 4th floor of Anwar Medika University.

The tools used in this research were a tourniquet, red vacuum tube, 3cc syringe, micro lab 300 photometer, yellow and blue tip, 500-1000 ul and 50-5 ul micropipette, serology tube, and serology tube rack. The materials used in this research were 70% alcohol, blood serum/plasma samples, GPO-PAP triglyceride reagent, tissue, and labels/labels.

The procedure of this research was:

1. Pre-analytical, including sample preparation, labeling, venous blood sampling,
2. Analytical, including making serum/plasma and checking triglyceride levels as follows:

	Blanko	Test
Reagen	1000ul	1000µl
Sample	-	10µl

- Pipette 1000µl of triglyceride reagent with a micropipette into a serology tube then pipette 10µl of serum sample.
- Homogenize, then incubate for 5-10 minutes.
- Read on the Microlab 300 instrument and record the results.

3. Post-analytic, including interpreting the results according to the reference value for triglyceride examination and recording the results then continuing with data analysis

The data analysis used in this research is manually using a Microsoft Excel computer with univariate analysis to analyze each variable from a study. The univariate analysis used is frequency and percentage distribution which describes the presentation of data for one variable.[10]

RESULTS AND DISCUSSION

Based on the research that has been carried out, it is known that several characteristics of research subjects are presented in Table 1.

Table 1. Research Subject Characteristic

Characteristic	Total	
	N	%
Age (tahun)		
18-19	9	17
20-21	13	23
22-23	11	26
24-25	15	34
Drinking Coffee Frequency		
< 3 glasses/day	23	49
> 3 glasses/day	25	51
Physics Activities		
Usually, physics activities	30	47
Seldomphysics activities	18	53
Lifestyle		
Smoker	16	57
Non-Smoker	32	43
The amount of triglycerides		
Normal <150 mg/dl	28	58
High >150 mg/dl	20	42

Based on Table 1, the characteristics of the 48 research subjects based on triglyceride levels were divided into the normal category (<150 mg/dl) for 48 (58%) respondents and the high category (≥150 mg/dl) for 28 (42%) respondents. Age characteristics are categorized as ages ranging from 18-25 years with a frequency of coffee consumption of <3 cups/day and >3 cups/day as well as physical activity and lifestyle which are categorized into groups of regular exercise, rarely exercise, smoking, and non-smoking.

Tabel 2. Statistic Distribution of the amount of Triglycerides

Variable	N	Mean	SD	Min	Max
The amount of triglycerides (mg/dl)	48	158.7	67.089	62	304

Based on Table 2, the results of the statistical analysis of triglyceride levels in this study showed that the average triglyceride level was 158.7 mg/dl with the lowest triglyceride level being 62 mg/dl and the highest triglyceride level being 304 mg/dl. This can be caused by factors such as age, physical activity, frequency of daily coffee consumption, lifestyle, and the type of coffee consumed. Increasing age affects the decline in the function of the hormones estrogen and testosterone in distributing fat, thus allowing fat to accumulate in the body.[7] Consuming coffee over a long period can also cause an increase in lipids in the body, the compounds

in coffee can speed up the process of narrowing and blockage of blood vessels which function to carry oxygen to the heart. Excessive levels of caffeine, cafestol, and kahweol compounds in the body will disrupt and inhibit triglyceride metabolism in the blood, causing lipid build up in blood vessels. Free fatty acids released due to excessive lipid accumulation can inhibit lipogenesis and will inhibit serum triacylglycerol clearance, resulting in increased blood triglyceride levels or hypertriglyceridemia.[11,12]

Examination of triglyceride levels is an important parameter in the lipid profile which can help diagnose a disease or determine risk factors for coronary heart disease and detect metabolic syndrome, as well as monitor the effectiveness of lipid-lowering therapy. Based on analysis of the results of research conducted by researchers, an overview of triglyceride levels in coffee addicted students at Anwar Medika University was obtained with an average triglyceride level of 158.7 mg/dl. This can be caused by factors such as age, physical activity, frequency of daily coffee consumption, lifestyle and the type of coffee consumed. Consuming coffee over a long period of time can cause an increase in lipids in the body, the compounds in coffee can speed up the process of narrowing and blockage of blood vessels which function to carry oxygen to the heart. The habit of consuming coffee is closely related to the emergence of lipid disorders, including increased triglyceride levels.[13]

Table 3. The Distribution of the amount of triglyceride based on the coffee type

Coffee Type	The amount of trigliseride		Mean of trigliseride (mg/dl)
	<150 mg/dl	≥150 mg/dl	
Black coffee	10	9	166.76
Sachet coffee	13	16	162.38
Total	48		164.57

Based on Table 3, the results of the analysis of triglyceride levels in students who are coffee addicts based on the type of coffee consumed, namely black coffee and sachet coffee, showed that the average triglyceride level was 164.57 mg/dl. black coffee and sachet coffee. both contain caffeine, cafestol, and kahweol, but in different amounts or levels.[14,15] Consuming black coffee tends to have higher triglyceride levels compared to those consuming sachet coffee.[16,17] This is because black coffee has a caffeine content of 85-185 mg/cup, kahweol 6-12 mg/cup, and cafestol 4-6 mg/cup. Sachet or instant coffee has a caffeine content of 30-90 mg/coffee sachet in 150 ml of water, kahweol 0.2-0.6 mg/cup, and cafestol 0.1 mg/cup.[4,7,18,19]

Triglycerides in the body will be converted into fatty acids and glycerol which are stored in adipose tissue and then absorbed through the intestines and distributed widely throughout the body. Excessive free fatty acids in the blood, some will be used as an energy source and some will be taken to the liver as raw material for the formation of triglycerides. Free fatty acids will become triglycerides again and become part of VLDL (Very Low-Density Lipoprotein) because there has been a process of inhibiting the beta oxidation mechanism in the liver so that the lipids produced will be very rich in triglycerides. Excessive levels of caffeine, cafestol and kahweol compounds in the body will disrupt and inhibit triglyceride metabolism in the blood, causing lipid buildup in blood vessels. Free fatty acids released due to excessive lipid accumulation can inhibit lipogenesis and will inhibit serum triacylglycerol clearance, resulting in an increase in blood triglyceride levels or hypertriglyceridemia. In addition, the accumulation of lipids will cause adipose cells to be unable to store triglycerides adequately, which will trigger an increase

in LDL (Low Density Lipoprotein) and ultimately an increase in triglyceride levels in the body. Caffeine is a central nervous system stimulant which can increase heart rate and contribute to the occurrence of supraventricular tachycardia (heart rhythm disturbance). The caffeine compound content in coffee has the function of stimulating nervous system activity and increasing heart function, but if caffeine is consumed in excess, caffeine will be toxic by inhibiting nervous system mechanisms and can increase triglyceride levels. Kahweol is a compound usually found in coffee that can cause the degradation of toxic substances and is protective against aflatoxin B1 if consumed in excess. The cafestol compound contained in coffee can also increase triglyceride levels by inhibiting the beta oxidation mechanism, preventing the breakdown of triglycerides into energy so that triglyceride levels in the blood will increase.[20-22]

The picture of triglyceride levels increasing in the 24-25year age category is triglyceride levels. This is because with increasing age the function of the body's organs will decrease due to aging which is a risk factor for functional disorders. The age factor will make a person less physically active and increase the risk of developing more functional disorders compared to a younger age. Increasing age significantly increases the risk of degenerative diseases in both men and women. As we age, there will be a decline in the function of various body organs, making it difficult to achieve balance in triglyceride levels. An increase in lipids in the blood is associated with a decrease in the elimination of lipids as bile salts and a decrease in receptors that mediate the clearance process of LDL. This can result in triglyceride levels tending to increase more easily. Age factors influence the deterioration of body functions, including stiffness of blood vessels (shrinking and aging). In men, serum triglyceride concentrations increase to a peak in middle age, while in women they continue to rise until the age of 70 years. Increasing age also affects the decline in the function of the hormones estrogen and testosterone in distributing fat, thus allowing fat to accumulate in the body. The danger is that if this fat buildup sticks to the walls of blood vessels, it will narrow blood flow, especially if the blood vessels are old. This condition will result in blocked blood vessels and increased triglyceride levels which can cause a decrease in HDL.[23,24]

The limitation of this research is that this research was carried out semi-quantitatively using a Microlab 300 type photometer so that the measurement results were less accurate because they were influenced by several factors, including the pipetting angle being less precise so that the ratio of reagent and blood volumes was not appropriate because pipetting was still done manually outside of the tool for reacting the sample. with reagents so that it will affect the results of measuring falsely low or high triglyceride levels. The cleanliness factor of the tube can also influence the results of measuring triglyceride levels because the researchers did not use disposable tubes so it is possible that when washing the tube it was not clean enough so that soap residue was still attached to the walls of the tube which will affect the results of measuring triglyceride levels because triglyceride examination tends to be sensitive compared to lipid profile examination. other. Contamination during the reaction or incubation process can also be a factor in the high or false results of measuring triglyceride levels because this research still uses semi-quantitative methods where the process is still carried out outside the equipment which allows contamination to occur. Future researchers are expected to be able to develop this research by using more sophisticated tools to obtain more accurate research results and avoid several limitations.[25]

Control efforts that can be made to monitor triglyceride levels are by paying more attention to health, for example having regular check-ups at least once every 3 months to determine body condition including triglyceride levels, in addition to maintaining diet, lifestyle, and increasing physical activity and reducing the habit of consuming coffee every day. so that health problems do not occur, for example increasing triglyceride levels in the body.[12,13,26-28]

CONCLUSION

Based on the research results obtained, it can be concluded as follows, examination of high triglyceride levels ≥150 mg/dl with a percentage of 58% and triglyceride levels which are classified as normal, namely <150 mg/dl with a percentage of 42%. The average triglyceride level was 158.7 mg/dl with the lowest triglyceride level being 62 mg/dl and the highest triglyceride level being 304 mg/dl. High triglyceride levels in many respondents were caused by several factors including age, physical activity, frequency of daily coffee consumption, lifestyle, and type of coffee consumed. Excessive coffee consumption can increase triglyceride levels in the body.

AUTHORS' CONTRIBUTIONS

Amellya Octifani: designed the protocols, and executed the protocols. Tarisa Suci Novianti: prepared the samples, and data collection. Farida Anwari: data analytics and visualization statistically. Arif Rahman Nurdianto: wrote the draft manuscript. Fery Setiawan, Arif Rahman Nurdianto, and Rizal Fauzi Nurdianto: reviewed and supervised the manuscript, and wrote the final manuscript. All authors have read and approved the final manuscript.

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DATA AVAILABILITY STATEMENT

The utilized data to contribute in this research are available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals.

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Bioactivity of Nelumbo nucifera extract on sperm recovery due to 2-methoxyethanol exposure: In vivo and in silico study

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Abstract: One of the causes of male infertility is influenced by the compound 2-Methoxyethanol (2-ME) that can increase Reactive Oxygen Species (ROS), which affect sperm quality. This study aims to analyze the effects of various doses of *Nelumbo nucifera* extract on sperm quality in mice. Methods: Male ddy mice were divided into six equal groups. The negative control group was given aquades for 28 days, the positive control group was injected with 0.05 ml of 2-ME for 7 days, compare control group was injected with 0,05 ml 2-ME and *clomiphene citrate* 50 mg for 21 days. The treatment groups were injected with 0.05 ml of 2-ME for 7 days followed by injection different doses of *N. nucifera* extract, namely 50 mg/kg body weight (BW), 150 mg/kg BW, and 450 mg/kg BW for 21 days. At the end of the experiment, the mice were sacrificed, and sperm suspensions were collected from the epididymis to measure morphology, concentration, and motility. In silico testing was performed by preparing ligands and the GSK3b protein receptor using PyMOL, and then tested to determine the binding energy using PyRx. Results: The administration of *N. Nucifera* extract can significantly improve sperm morphology, concentration, and motility ($p<0.05$). The dose of 450 mg/kg BW has a pronounced protective effect. Quercetin is the compound of *N. nucifera* extract with the highest inhibition of non-receptor protein kinase and the most significant antioxidant effect. *N. nucifera* extract can improve the decline in sperm quality caused by exposure to 2-Methoxyethanol.

Keywords: *Nelumbo nucifera*, sperm recovery, 2-methoxyethanol, in-vivo, in-silico.

INTRODUCTION

Infertility is a health issue that is increasingly prevalent in Indonesia. One of the causes of male infertility is exposure to chemicals that pollute the environment, with detrimental effects on sperm quality.[1,2] 2-Methoxyethanol (2-ME), used in plastic production and as a solvent for water-based organic materials,[3,4] is implicated in this context. 2-ME can enter the human body through various routes, including skin absorption, inhalation, and the consumption of contaminated food containing this compound. Once inside the body, 2-ME undergoes metabolism to a more toxic compound, namely methoxyacetic acid (MAA).[5,6,7] MAA then spreads throughout the body and accumulates in the testes, an organ highly sensitive to this substance. MAA is known to cause an increase in Reactive Oxygen Species (ROS) and lipid peroxidation, leading to decreased sperm motility and morphology.[8,9]

The toxic effects of 2-ME also result in increased expression of the cortactin gene, particularly evident in spermatocytes. Cortactin is a protein involved in the

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regulation of cell cytoskeleton and cell movement. Elevated cortactin levels cause damage to cell function, contributing to the degeneration and necrosis of spermatocytes. Cortactin activity is induced by a non-receptor kinase enzyme, which is linked to the regulation of endocytosis processes and sperm formation.[10,7] Antioxidants may serve as inhibitors of non-receptor kinase. To counteract the negative effects of oxidation induced by 2-ME, substances with strong antioxidant properties are required.

Nelumbo nucifera, an abundant aquatic plant, has not been optimally utilized as an antioxidant source.[11] Recent research shows that ethanol extracts of *N. nucifera* reveals that the plant contains essential phytochemical components such as phenols, flavonoids, tannins, alkaloids, saponins, steroids, terpenoids, glycosides, coumarins, and quinones[12]. This study demonstrates that lotus extract can enhance sperm viability and reduce lipid peroxidation levels by up to 80%. The petals of *N. nucifera* extract have activity as antioxidant against mancozeb, it's an toxic agent on male reproductive expecially on spermatogenesis, the mayor chemical compound in *N. nucifera* petals is tochopherol and flavonoid.[13]

On this research aims to explore the potential of *N. nucifera* petal extract in protecting and repairing damaged sperm due to 2-ME exposure, as well as how ligand activities of lotus phytochemical compounds interact with the glycogen synthase kinase-3. Glycogen synthase kinase-3 (GSK-3β) is a serine/threonine kinase that regulating various signaling pathways, GSK-3β GSK-3β plays a role in depleting beta-catenin, which subsequently enters the cell nucleus to regulate the expression of specific genes. GSK3b increase in the expression of the cortactin gene, which is particularly prominent in spermatocytes as receptor or GSK-3β as a kinase inhibitor playing a key role in the regulation of various signaling pathways.[14,15]

This study is intended to identify the optimal dosage of *N. nucifera* petal extract with potential compounds and to identify potential compounds that may serve as candidates for anti-infertility drugs. In this context, the research holds significant relevance in the field of phytopharmaceuticals and can contribute to our understanding of the potential use of lotus petals as a natural alternative medicine to address male infertility issues.

MATERIAL AND METHOD

In Silico Testing

Phytochemical compounds present in lotus petal extract were subjected to Druglikeness evaluation using Lipinski's rule through the Way-2-drug web server. Sample preparation involved collecting target protein through the Protein Data Bank and ligand samples through the Pubchem website. The next step included the sterilization of unnecessary molecules, including water molecules on the target protein. For ligands, minimization and docking were performed using PyRyx software. The docking results were visualized using PyMoL software to understand the interactions between ligands and proteins.

Ethical Approval

All procedures in this research, including the use of mice as animal models, were approved by the Ethics Committee, Department of Research and Community Service, Brawijaya University, East Java, Indonesia, with the number No: 370-KEP-UB-2023.

Plant Material

Nelumbo nucifera was collected from Jotosanur Reservoir, Lamongan, East Java in June 2023. The material was identified and authenticated in the Biology Laboratory, Department of Biology, Universitas Muhammadiyah Lamongan.

Preparation of N. Nucifera Ethanol Extract and Suspension

Lotus flower petals were cut into small pieces and dried in an oven at 40°C for 60 minutes. The dried petals (500 grams) were ground electrically and macerated with 96% ethanol for 3 days at room temperature. The extract was then filtered and

concentrated with a rotary evaporator and heated in a water bath at 70°C. To make the suspension, the extract was homogeneously mixed with distilled water. Concentrations used were 0.5%, 1%, and 2%.[16]

Preparation of 2-Methoxyethanol Suspension

For a five-day stock, seven milligrams of lead acetate were weighed and dissolved in warm distilled water. After dissolution, all remaining distilled water was added to obtain a volume of 15 ml in the Na-CMC solution.

Materials and Equipment

The tools used included an analytical balance, beakers, stirring rods, pipettes, petri dishes, 1cc syringes, Eppendorf tubes, glass slides, cover glasses, slide boxes, digital scales, probes, light microscopes, micropipettes, mouse cages with food and water, Pasteur pipettes, Eppendorf tubes, a Neubauer counting chamber, a rotary vacuum evaporator, and a water bath. The materials used in this study included Lotus Flower Petal Extract, ddy strain mice, filter paper, ethanol 96%, ethanol 70%, distilled water, Nigrosin, NaCl 0.9%, 2-Methoxyethanol, Neutral Buffered Formalin (NBF 10%), Dpph, Chloroform, Methanol, hematoxylin-eosin tissue stain.

Animals

Thirty adult male ddy mice aged 6-7 weeks, 25-30 grams in weight, were obtained from the Center for Veterinary and Pharmacy, Surabaya, East Java. They were kept under standard laboratory conditions (temperature 28-30°C, light/dark cycle 12 hours/12 hours) and provided with food and water ad libitum.

Experimental Design

After one week of acclimatization, the animals were randomly divided into six equally sized groups (n=10) as follows: negative control group received distilled water for 28 days (KN), positive control group received subcutaneous injection of 0.05 ml 2-ME at a dose of 200 mg/kg BW for 7 days (KP), comparative control group received subcutaneous injection of 0,05 ml 2-ME at a dose of 200 mg/kg Bw for 7 days and continously injection with commercial medicine (*Clomiphene citrate*) 50 mg for 21 days (K0). Treatment groups received subcutaneous injection of 0.05 ml 2-ME at a dose of 200 mg/kg BW for 7 days. Subsequently, subcutaneous injection of 0.2 ml with different doses of *N. nucifera* petal extract was given to each treatment group. The first treatment group received 50 mg/kg BW (P1), the second group received 150 mg/kg BW (P2), and the third group received 450 mg/kg BW (P3) for 21 days. After the procedure, all mice were sacrificed using chloroform. The cauda epididymis was then collected, and sperm suspension was made for sperm analysis.

Sperm Analysis

In this study, spermatozoa suspension was used to measure motility, morphology, and sperm count. For sperm motility analysis, 10µL was placed in a hemocytometer chamber and analyzed under a light microscope. Ten sperm were observed for the duration of their movement and measured using a stopwatch. For sperm count evaluation, in a sperm solution, 10µL of sperm suspension was transferred to each hemocytometer counting chamber and left for 5 minutes. Then, sperm heads were counted with a light microscope at 40x magnification and expressed as million/ml of suspension.[17] Sperm morphology was also determined using the eosin-nigrosin staining method. For this purpose, spermatozoa suspension was dropped on a glass slide to make a smear preparation and air-dried. The smear preparation was fixed with methanol, stained with 1% eosin and nigrosin solution, and allowed to dry. The preparation was rinsed with distilled water and dried.[18] The preparation was observed under a light microscope at 400x magnification to determine the morphology of 100 mouse sperm. The final step was to calculate the percentage of normal and abnormal sperm.

RESULTS AND DISCUSSION

The Lipinski test is conducted in the early stages of in silico testing in the field of vaccine and drug design.[19] The Lipinski test comprises a set of empirical rules used in drug research and pharmacology to identify the potential of new drug molecules, aiming to predict whether a molecule is likely to be a successful drug. Molecules with a molecular weight of less than 500 Daltons are more likely to penetrate cell membranes and reach biological targets within the body. The Log P (octanol-water partition coefficient) measures how well a molecule dissolves in fat compared to water. Molecules with a Log P of less than 5 tend to have better bioavailability. The number of hydrogen bond donors (H-Donor) should be less than 5, as molecules with too many hydrogen atoms functioning as donors tend to have less chemically stable properties. Similarly, the number of hydrogen bond acceptors (H-Acceptor) should be less than 10, as molecules with too many atoms that can function as hydrogen acceptors tend to have less chemically stable properties.[20]

Table 1. Lippinski Result Test

Ligands	Molecular Weight (<500)	Log P (<5)	Hydrogens Binding Donor (<5)	Acceptors (<10)	Explanation
Astragalin	448.0	-0,43	7	11	Not Eligible
Diosmetin	**300.0**	**2,42**	**3**	**6**	**Eligible**
Delphinidin	303.0	2,42	6	7	Not Eligible
quercetin	**302.0**	**2.01**	**5**	**7**	**Eligible**
Trifolin	448.0	-0.43	7	11	Not Eligible
Quercitrin	448.0	0.29	7	11	Not Eligible
Isoquercitrin	464.0	-0.73	8	12	Not Eligible
Myricetin	318.0	1.71	6	8	Not Eligible
Syringetin	**346.0**	**2.32**	**4**	**8**	**Eligible**
Cyanidin	**287.0**	**2,71**	**5**	**6**	**Eligible**
Peonidin	**301.0**	**3.02**	**4**	**6**	**Eligible**
petunidin	**317.0**	**2.72**	**5**	**7**	**Eligible**
asimilobine	**267.0**	**2,81**	**2**	**3**	**Eligible**
N-Nornuciferine	**281.0**	**3,11**	**1**	**3**	**Eligible**
Nuciferine N-Oxide	**311.0**	**3,09**	**0**	**3**	**Eligible**
O-Nornuciferine	**281.0**	**3,15**	**1**	**3**	**Eligible**
Norjuziphine	**285.0**	**2,53**	**3**	**4**	**Eligible**
Florinbundine	**281.0**	**3,15**	**1**	**3**	**Eligible**
Pronuciferine	**311.0**	**2,51**	**0**	**4**	**Eligible**
Clomiphene	405.5	5,95	0	2	Not Eligible

Lipinski rule of five is important for determining compounds such as drug candidate molecules. In this study, it was found that there are 13 bioactive compounds that match Lipinski rules of five, namely: chlorogenic Diosmetin, Quercetin, Syringetin, Cyanidin, Peonidin, Petunidin, asimilobine, N-Nornuciferine, Nuciferine N-Oxide, O-Nornuciferine, Norjuziphine, Florinbundine, and Pronuciferine. The PASS Online analysis results show that all drug candidate

compounds have good activity (Table 1). In Table 1, during the Lippinski test, clomiphene does not meet the criteria. However, clomiphene serves as the reference drug in this case. In pharmaceuticals, a compound developed into a drug undergoes a rigorous evaluation process to maintain a balance between effectiveness and potential risks before production. However, it can be concluded that some phytochemical compounds of the flavonoid group in lotus petal do not meet the criteria as potential drug candidates.

Table 2. Glycogen synthase kinase-3β (GSK-3β) Inhibitor Docking Result

Ligands	Type	Protein Target	Pa	Pi	Binding Energy	Hydrogen Bindings
Astragalin	Flavonoid	GSK-3β	0,837	0,004	-7,6	6
Diosmetin	Flavonoid	GSK-3β	0,922	0,002	-7,5	3
delphinidin	Flavonoid	GSK-3β	-	-	-7,3	2
quercetin	Flavonoid	GSK-3β	0,809	0,005	-9,1	5
Trifolin	Flavonoid	GSK-3β	0,837	0,004	-7,7	2
Quercitrin	Flavonoid	GSK-3β	0,863	0,004	-8,3	6
Isoquercitrin	Flavonoid	GSK-3β	0,843	0,004	-7,6	6
Myricetin	Flavonoid	GSK-3β	0,958	0,001	-7,6	2
Syringetin	Flavonoid	GSK-3β	0,945	0,002	-7,2	3
Cyanidin	Flavonoid	GSK-3β	0,863	0,004	-7,3	8
peonidin	Flavonoid	GSK-3β	-	-	-7,3	2
petunidin	Flavonoid	GSK-3β	-	-	-7,1	4
asimilobine	Alkaloid	GSK-3β	0,418	0,058	-8,2	0
N-Nornuciferine	Alkaloid	GSK-3β	0,318	0,125	-8,0	1
Nuciferine N-Oxide	Alkaloid	GSK-3β	-	-	-7,7	1
O-Nornuciferine	Alkaloid	GSK-3β	0,352	0,095	-8,3	0
Norjuziphine	Alkaloid	GSK-3β	0,347	0,099	-7,0	2
Florinbundine	Alkaloid	GSK-3β	0,352	0,095	-8,3	1
Pronuciferine	Alkaloid	GSK-3β	0,077	0,043	-7,5	0
Clomiphene	kontrol	GSK-3β	0,643	0,029	-7,2	0

The molecular docking results aim to determine the stability of the interaction between the ligand and the target protein. The lowest binding affinity indicates the maximum level of stable interaction indicating that the ligand inhibitory activity against the target protein is larger, the negative values indicate an increasing energy.[21] All bioactive compounds have activity against target proteins (Table 2). However, the compounds to be analyzed next are those with the lowest binding affinity and that follow Lipinski rules of five. Quercetin from flavonoids group and Floribundine from alkaloids group are examples of such compounds. Based on the data in Table 2 on the bioactivity of ligands as kinase inhibitors, the flavonoid group shows a greater potential for activity compared to inactivity. The compound quercetin, with the highest binding energy of 9.1 kcal/mol and 5 hydrogen bonding sites, demonstrates the most significant inhibitory potential against non-receptor protein kinase. and in this context, Quercetin emerges as the compound with the highest potency, followed by other compounds from alkaloid group, Floribundine with the highest binding energy of -8,3 kcal/mol and 1 hydrogen bonding sites, demonstrates the most significant inhibitory potential against non-receptor protein kinase.

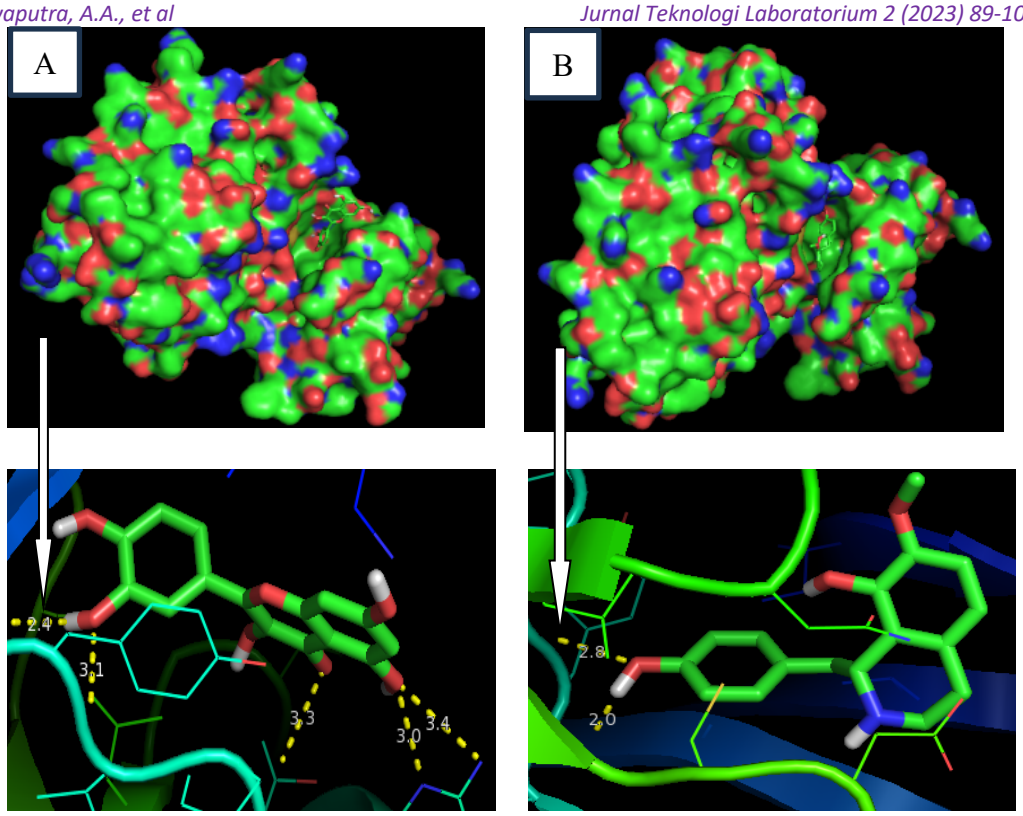

Figure 1. Interaction of ligands with the catalytic site of GSK-3β kinase. The structure of GSK-3β is depicted in granular form, with the catalytic site marked by hydrogen bonds represented by dashed yellow lines. (A. Quercetin, B.Floribundine)

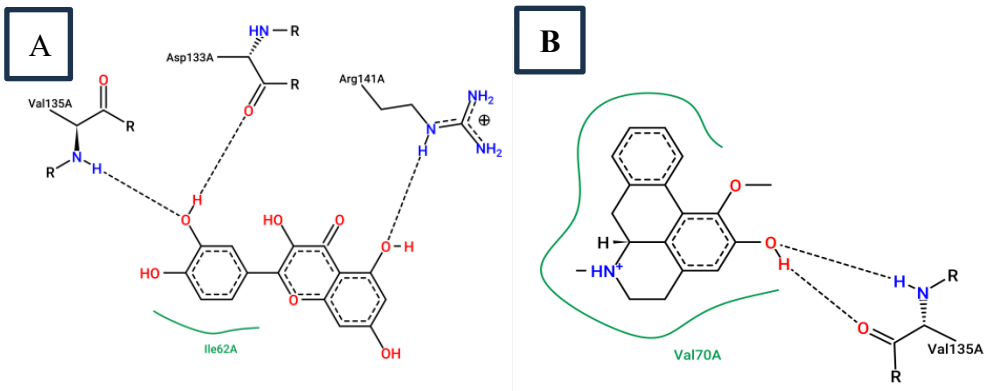

Figure 2. The binding visualization 2D of A) Quercetin and B) Floribundine targeting GSK-3β

Glycogen synthase kinase-3 (GSK-3β) is a serine/threonine kinase that regulating various signaling pathways, GSK-3β plays a role in depleting beta-catenin, which subsequently enters the cell nucleus to regulate the expression of specific genes. GSK-3β increase in the expression of the cortactin gene, which is particularly prominent in spermatocytes. The increased activity of cortactin is induced by non-receptor kinase enzymes (GSK-3β) Cortactin activated become a protein involved in the regulation of cell cytoskeleton and cell movement. The elevation of cortactin levels leads to cellular damage and dysfunction, contributing to the development of degeneration and necrosis in spermatocytes with the regulation of endocytosis processes and sperm formation.[14,10,15]

Some of these conserved sites are catalytic residues consisting of Aspartic acid (Asp), Arginina (Arg), and Valina (Val) this position can be used for molecular

docking in the development of multivariate anti-infertility. Based on the docking results (Figure 2), quercetin forms five hydrogen and hydrophobic bonds but only three bond connect with conservative residues (Asp 133A, Val 135A, Arg 141A). Meanwhile, floribundine has only one bond with catalytic residues Val135A. There are other conservative catalytic residues in this protein have the potential to bind to docked bioactive compound ligands, the presence of hydrogen bonds in catalytic residues aids in the stabilization of the ligands-target protein complexes, resulting in the desired interaction conformation play a role in the stability of drug molecules to trigger the inhibitory activity of the target protein.[22,23]

Table 3. In Vivo Test Result

Parameters	KN	KP	K0	P1	P2	P3
Motility (minutes/cell)	2,06±0,11	1,13±0,37	1,80±0,12*	1,03±0,11	3,10±0,15*	3,51±0,14*
Quantity (Million/ml)	22,1±2,38	10,8±0,97	12,8±3,39	12±1,44	20±1,09*	23±1,08*
Morphology normal (%)	99,1±1,95	77,5±2,29	72,6±3,31	71,4±3,20	81±2,42	83,2±2,37

Note: (*) indicates a significant difference compared to the control group ($p < 0.05$)

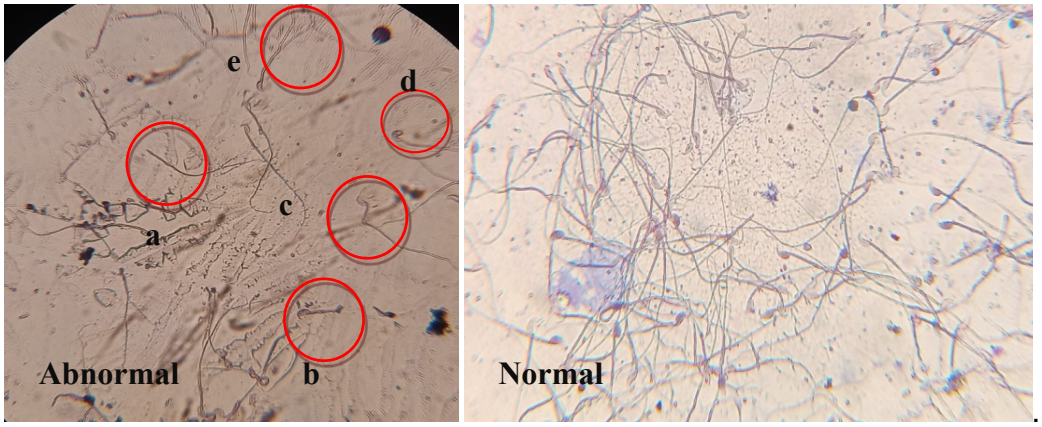

Figure 3. Normal and Abnormal Mature Mouse Spermatozoa (a. Headless, b. Tailless, c. Broken Neck, d. Folded Head Shape, e. Two-Tailed)

Spermatozoa Motility

The average mortality rate of sperm cells indicates that in the Positive Control group (KP), which was only injected with 2-ME, there is no significant difference compared to the Comparative Control group (K0). However, in groups P2 and P3, which received moderate and high doses of lotus petal extract, the sperm motility duration is above that of the out-of-room control group (KN). Meanwhile, in the P1 group, there has not been a significant increase in speed compared to the KN group.

Spermatozoa Quantity

Data from Table 3, obtained from the average of individuals in the treatment group, show that the cumulative sperm count in each chamber on the hemocytometer, when multiplied by 1,000,000, leads to the conclusion that, compared to P1 and P2 doses, the P3 group indicates a significant improvement in quantity, well above the KN group.

Spermatozoa Quality

Percentage calculations of sperm quality reveal that in groups P2 and P3, which were treated with lotus petal extract injections, the number of normal cells is higher compared to the Comparative Control group (K0), reaching only 72.6%. The percentage of normal cells increases with the rising extract dose. Along with the administration of ethanol

extract of Nelumbo nucifera, the percentage of observed normal morphology increasingly dominated. Table 3 shows that the percentage of sperm with normal morphology increased from 77.5% to 83.2%, approaching the percentage of the negative control. The decrease in abnormal cells indicates that lotus petal extract possesses antioxidant activity in influencing the development of degeneration and necrosis of spermatocytes caused by the effects of 2-Methoxyethanol (Figure 3).

2-ME causes damage to the spermatogenic process, as evidenced by the occurrence of azoospermia in the observation of sperm quality, the positive control group (KP) differs from the negative control group (KN), which was not given 2-ME. The effect of 2-ME on spermatogenesis can also be seen in the quantity and motility of spermatozoa in the positive control group (PC) and Negative (KN) in Table 3. The quantity, quality, and motility of spermatozoa in PC are always lower than NC, and this decrease indicates spermatogenesis disruption by 2-ME. Exposure to 2-ME in experimental animals causes a decrease in testis weight, a decrease in the quantity and motility of sperm, and an increase in the proportion of epididymal sperm with abnormal morphology. This indicates that these cells degenerate due to exposure to 2-ME.

Metabolite MAA in the body has toxic and teratogenic properties, thus inhibiting the formation of DNA and RNA in primary spermatocytes, especially in pachytene spermatocytes. Pachytene spermatocytes are the most active cells in synthesizing RNA. Therefore, these cells are the most sensitive to MAA, resulting in them becoming the most degenerated cells. MAA can also increase membrane permeability, causing excessive Ca^{2+} influx (overload).[6,7,4] This increase allows calcium ions to bind to calmodulin protein (Chin, 2000), calmodulin will activate the signaling pathway by binding to the active side of protein kinase, too much protein kinase will increase protein phosphorylation which further impacts cell apoptosis. In addition, protein tyrosine kinase can also cause an increase in the expression of cortactin genes. Cortactin is a protein involved in the regulation of cell cytoskeleton and cell movement. Cortactin connects "ectoplasmic specialization" (ES) with the actin cytoskeleton and is considered important in regulating the release of sperm from the seminiferous epithelium and facilitating the movement of spermatids. In short, this actin-binding protein controls the dynamics of the actin cytoskeleton through nucleation, elongation, closure, binding, assembly, cleavage, and depolymerization, thus facilitating changes in cell shape and the location of spermatids in the epithelium during spermiogenesis.[24] However, an excess of cortactin can affect the ability of sperm to pass through the seminiferous epithelium properly and uncontrolled changes in the actin cytoskeleton that can interfere with the structural integrity of cells and their normal function and cause cell damage and dysfunction, contributing to the degeneration and necrosis of spermatocytes[25,10]. Overload Ca^{2+} can inhibit oxidative phosphorylation, so energy supply becomes reduced as it is used to pump out Ca^{2+} ions, while energy is crucial for sperm motility.[24,26]

The lotus petal (*N. nucifera*) extract contains bioactive substances from secondary metabolite compounds such as quercetin, quercitrin, isoquercetin, myricetin which have the potential as antioxidant preparations.[27,28] Antioxidants are effective in counteracting the effects of oxidative reactions and the toxicity of 2-methoxyethanol. Not only can they protect DNA from oxidative damage by inhibiting lipid peroxidation, but they can also capture free radicals by providing additional electrons to unstable molecules. Moreover, they play a crucial role in maintaining cell signaling regulation by modulating signaling pathways involved in inflammatory responses, and help regulate the redox balance in cells, thus not interfering with the regulation in spermatogenesis.[13,29] Based In silico analysis, lotus flavonoid compounds have also been proven to have the potential as ligands capable of inhibiting non-receptor protein kinase.[30] Non-receptor protein kinase (GSK-3β) is active due to the redox imbalance of Ca^{2+} ions in cells caused by the toxicity of 2-Methoxyethanol.[31,32] By inhibiting the activity of this protein, the

compound content of lotus can bind to the active side of non-receptor kinase and prevent over-expression of cortactin genes that can be the cause of degenerative spermatogenic cells and lead to infertility.[26,33] Therefore, compounds in lotus petals show potential as candidates for anti-infertility drugs. In general, it can be concluded from this study that the optimal dose for preventing spermatocyte degeneration is the administration of lotus petal (*N. nucifera*) extract at a dose of 450 mg/kg BW (Group P3). However, further research in the field of phytopharmaceuticals is needed to consider lotus petals as a potential drug.

CONCLUSION

This study reveals that lotus petal extract, particularly the flavonoid quercetin, shows potential as a non-receptor kinase inhibitor targeting GSK-3β, a key protein in spermatocyte signaling pathways. The administration of 450 mg/kg BW of lotus petal (*N. nucifera*) extract significantly improves sperm quality and mitigates the negative effects of 2-ME. This suggests the potential of lotus petal (*N. nucifera*) extract as a natural remedy for male infertility issues.

AUTHORS' CONTRIBUTIONS

Angella Ananda Syaputra: Project administration, Conceptualization, In-silico analyze. Badriatul Musyarofah: Data curation, Writing- Original draft preparation. Amelia Kartika Reza; Yunita Ainul Kasanah: Methodology, Visualization, Investigation; Helga Syasya Qatrunnada: Analyze data; Putri Ayu Ika Setiyowati: Resources, Supervision, Validation, and Reviewing.

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DATA AVAILABILITY STATEMENT

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

DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals.

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Original Research

In vitro antibacterial activity of patchouli batik (Pogostemon Cablin) plant extract varieties from Southeast Sulawesi and South Sulawesi

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Abstract:
Indonesia has biodiversity traditional medicinal plants. One of the plants used is patchouli Batik (Pogostemon cablin) from South Konawe, Southeast Sulawesi and patchouli from Bulukumba, South Sulawesi. This experimental research aimed to determine the effect of stem and leaves extracts of patchouli Batik from South Konawe, Southeast Sulawesi and patchouli from Bulukumba, South Sulawesi, on Escherichia coli, Staphylococcus aureus and Streptococcus mutans using the diffusion method (Kirby-bauer). The diffusion test was expressed as the width of inhibition zone in millimetres The results showed that the total flavonoid content of the leaves and stems of patchouli Batik from South Konawe, Southeast Sulawesi was 301.823 mg QE/gr and 119.905 mg QE/gr, and the total flavonoid content of the leaves and stems of patchouli from Bulukumba, South Sulawesi was 474.120 mg QE/gr and 148.856 mg QE/gr. The phenolic content of the leaves and stems of patchouli batik from South Konawe, Southeast Sulawesi was 420.22 mg GAE/gr and 411.55 mg GAE/gr, while the phenolic content of the leaves and stems of patchouli from Bulukumba, South Sulawesi was 818.13 mg GAE/gr and 227.77 mg GAE/gr. Stem and leaves extracts have antibacterial effects on Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 43300, Streptococcus mutans ATCC 35668 with the largest diameter found in samples of Patchouli batik leaves extract with concentration of 100% in Streptococcus mutans bacteria of 16 mm, Escherichia coli bacteria of 15 mm and in patchouli leaves extract. from Bulukumba with concentration of 100% in Staphylococcus aureus bacteria was 15 mm.

Keywords: Patchouli Batik, Pogostemon cablin, Escherichia coli, Staphylococcus aureus, Streptococcus mutans.

INTRODUCTION

Chemical medications are commonly used to treat infectious wounds, however incorrect dosages potentially lead to negative effects such as bacterial

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resistance[5]. Consuming medications derived from plants, such as the binahong plant, becomes an option to prevent the negative effects of chemical drugs. The Binahong leaf extract is known to stimulate fibroblast cells and collagen formation which can accelerate the wound healing process[6,7]. Alkaloids, saponins, and flavonoids are contained in binahong leaves. Flavonoid compounds have anti-inflammatory activity and potentially prevent oxidation. Himawan shows that the combination of basil leaf and binahong leaf (ratio of 2:1) on ethanol extract has forceful antioxidant activity[8]. Saponin compounds are used as cleaning agents and antiseptics in wounds to kill or prevent the growth of bacteria[9,10]. Based on the research of Amerta, et.al. (2012) binahong was able to inhibit the growth of *S. aureus*[11]. Saponins also have the benefit of increasing fibroblast cells and stimulating the formation of collagen[12]. According to Miladiyah and Prabowo's (2012) research, Binahong leaf extract has the potential for wound healing in guinea pigs[13].

Wound healing is a transition process that involves a series of responses and complex interactions between cells and mediators. It is one of the most complex processes in physiology. The wound healing process is limited to local regeneration processes and is also strongly influenced by endogenous factors such as age, nutrition, immunology, use of drugs, metabolic conditions, bacterial infection, and location of the wounds[13,14]. The wound healing process is classified into three phases: inflammatory, proliferative, and remodeling[15]. The proliferative phase lasts from days 3 to 14 and is designed to strike a balance between scar tissue production and tissue regeneration[14]. According to Landénet et al. (2016), macrophages are granulocytes, endothelial cells, and collagen that form the extracellular and neovascular matrix[16]. The construction of new blood vessels from pre-existing blood vessels is known as angiogenesis[17].

Collagen is needed in the process of wound healing and scar tissue formation. Collagen begins to form at the proliferative stage of the injury which occurs on the 3rd day following the physical injury and keeps increasing until the 3rd week. The proliferative phase occurs at 3-14 days, characterized by the formation of granulation in the wound. In the form of fibronectin and cytokines, the extracellular matrix, wil lead fibroblast cells to proliferate. Proliferating fibroblast cells then will migrate to the wound surface, where fibrin threads had previously clotted the wound[18,19].

Fibroblast cells slowly develop on the wound surface and produce new collagen fibers during the proliferative phase[9,20]. Collagen fibers that have an irregular shape due to injury will be destroyed and replaced with new collagen. However, the amount of collagen produced is determined by the amount of collagen needed for the wound area. Collagen fibers that are formed will cover the wound surface and be strengthened by fibronectin's presence[21]. Collagen formation can be observed microscopically with Masson's Trichrome staining, which will be shown in blue on the preparation. This study is the first to examine the thickness of collagen tissue with Masson's Trichrome staining on day 8 of wound healing infected with S. aureus and treatment with binahong leaf extract.

MATERIAL AND METHOD

This research has been approved by the Research Ethics Commission of the Faculty of Public Health, University Muhammadiyah Semarang number 553/KEKP-FKM/UNIMUS/2021. This type of experimental research used a completely randomized design (CRD) with 4 research groups (2 control groups and 2 treatment groups). Table 1 shows the study group structure. The study population used white rats (Rattus norvegicus) aged ± 2.5 months with a weight of ± 200-250 gram. Research samples were obtained from rat skin tissue from each research group, with 3 replicate, and each replication was made into a tissue block and then each block made 5 slides.

Table 1. Research Group Design

Group	Descriptions
Normal Control Group (KN)	normal rats without any treatment
Negative Control Group (K-)	Rats treated incision and suspension of *S. aureus*
Treatment group 1 (P1)	Rats treated incision and suspension of *S. aureus*, and 25% binahong leaf extract.
Treatment group 2 (P2)	Rats treated incision and suspension of *S. aureus*, and 50% binahong leaf extract.

The experiment was started by culturing and preparing a suspension of *S. aureus* from the pure culture which was inoculated in a liquid BHI medium and then incubated at 37°C for 3-6 hours. Then *S. aureus* was inoculated on MC media (Mac Conkey) and incubated for ± 24 hours at 37°C. *S. aureus* colonies were grown, then injected into BHIA media, cultured for 24 hours at 37°C, then suspended in NaCl 0.9% using the standard Mc Farland 0.5. The next step is to make binahong leaf extract in a 96 percent alcohol solution using the maceration process. The resulting macerate was then evaporated using a rotary evaporator at 37-39oC to obtain a thick extract. The produced Simplicia was diluted according to the treatment group (25%, and 50%).

Experimental animal acclimation was carried out for 7 days at the Unimus Experimental Animal Laboratory. The wound region was administered a 20 L suspension of *S. aureus* after making an incision on the back skin of rats (2 cm length and 3 mm depth). Binahong leaf extract (up to 50 L) was applied to the wound area every morning and evening for 8 days, then flattened with a cotton bud. The paraffin method was used to prepare skin tissue slides, starting with the excision of the biopsy in the wound area (2x1cm and 3mm depth), followed by a fixation on 10% NBF. Dehydration using graded alcohol, clearing using xylol, and embedding using paraffin. The skin tissue was cut with a thickness of 5 micrometers, then the slides were stained with Masson's Trichrome (SkyTec Laboratories). Identification and measurement of collagen tissue thickness with a magnification of 400x as much as 5 fields of view on each preparation and then given a score according to Table 2. Collagen thickness data were analyzed using the difference test between groups with Maan Whitney.

Table 2. Criteria for Collagen Thickness Measurement Score[22,23,24]

Score	Description
0	Very low collagen thickness, 0% of collagen thickness in the wound area
1	Low collagen thickness, ≤25% of collagen thickness (marked in blue color) in the wound area
2	Medium collagen thickness, 25% of collagen thickness (marked in blue color) in the wound area
3	Thick collagen thickness, 50% of collagen thickness (marked in blue color) in the wound area
4	The thickness of collagen is very thick, 75% of collagen thickness (marked in blue color) in the wound area

.

RESULTS AND DISCUSSION

Identification of collagen tissue in skin tissue preparations by microscopic observation at 400x magnification indicated by the blue-colored section on Masson's Trichrome staining is presented in Figure 1. The results of the measurement of collagen thickness in each group are presented in Table 3. The P2 group had the same mean collagen thickness as the normal control group (KN), which had a score of 3 with 50% collagen thickness in the wound area. These results demonstrate that the administration of 50% concentration of

binahong extract for 8 days can increase the growth of collagen in the skin tissue S. aureus-infected wounds so that it has a thickness of collagen as in normal skin.

The collagen thickness score of the P1 group, which received a 25% concentration of binahong extract, was 2. These results showed that administration of 25% binahong extract for 8 days could increase collagen growth by 25% in the skin tissue S. aureus-infected wounds. While the negative control group, which treated incision and suspension of *S. aureus* on the 8th day, had not formed collagen. The results of the Maan Whitney test showed that there was no significant difference in collagen thickness in the normal control group and the P2 group administration 50% binahong leaf extract. Meanwhile, between KN groups with K- and P1, K- with P1 and P2 as well as between P1 and P2, there are differences. The results of these statistical tests are by the results of collagen thickness measurements in Table 3.

The average thickness of collagen in the negative control group (K-) was measured at 0%, which can be caused by *Staphylococcus aureus* infection. Bacterial infection can prolong the inflammatory period, impairing wound healing and reducing collagen activation in the wound area[5]. Meanwhile, the P1 and P2 groups showed a collagen thickness of 25% and 50% respectively in the wound area. The administration of binahong leaf extract at 25% and 50% concentrations led to an increase in collagen production. This is due to the presence of secondary metabolites in binahong leaf extract that can be utilized as medicine. Secondary metabolites found in binahong plants include flavonoids, saponins, terpenoids, alkaloids as well as tannins, and ascorbic acid[25].

The flavonoid content of the binahong extract, namely flavor, has been shown to enhance vascularization and decrease edema. Flavonoids also have anti-inflammatory and antioxidant activities, which can help to eliminate or alter free radicals. Free radicals can inhibit inflammatory processes as well as the contraction of the formed collagen tissue, interfering with the wound healing process[5]. The flavonoid content is also believed to help in wound repair[26].

Saponins have antibacterial, analgesic, and anti-inflammatory activities, as well as the ability to stimulate collagen formation[27]. Saponins can enhance wounds heal faster by stimulating fibroblast proliferation and myofibroblast differentiation. Saponins play a role in wound healing by stimulating the production of type I collagen, which is necessary during wound closure[28]. Saponins are also known to enhance the membrane's ability to activate cell hemolysis. Bacteria lyse when saponins interacted with them. Saponins, which increase the number of macrophages and release growth factors in the production of fibroblasts, and the synthesis of collagen for the wound area, can increase monocyte proliferation. Saponins can also help accelerate the migration of keratinocytes, which play an important role in the wound resurfacing process[6].

Ascorbic acid (vitamin C) is needed to stimulate prolyl-hydroxylase and lysyl hydroxylase enzymes in the process of forming hydrogen bonds as a molecular framework and stabilizing polypeptide interactions to produce procollagen. Furthermore, procollagen will be converted into collagen molecules by the enzyme procollagen peptidase[29,30]. In wound healing, ascorbic acid has an important role as an antioxidant, as demonstrated by cell proliferation, inflammatory suppression, and collagen tissue contraction[13].

CONCLUSION

The administration of 25% and 50% binahong leaf extract stimulated the formation of collagen in wound healing in rats infected with S. aureus on the 8th day; the thickness of collagen in the group with 50% binahong leaf extract was the same as in the normal rat group.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to this work.

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DATA AVAILABILITY STATEMENT

The utilized data to contribute to this investigation are available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals.

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Original Research

In vitro antibacterial activity of patchouli batik (Pogostemon Cablin) plant extract varieties from Southeast Sulawesi and South Sulawesi

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Abstract:
Aging is a physiological process that causes the decline of tissues and organs in an individual. The cause of aging is believed to be due to an increase in reactive oxygen species (ROS) which are oxidative, resulting in cell membrane damage and DNA mutations. One of the organs most frequently damaged is the pancreas which functions in maintaining the function of sugar in the individual's body. Damage to the pancreas causes degenerative diseases, one of which is Diabetes Mellitus, which often occurs in old age. The efforts to reduce the occurrence of ROS due to aging is to consume antioxidants. Andaliman fruit is a native plant of Sumatra which is often used as a cooking spice. This plant contains a lot of flavonoid substances that function as antioxidative, anti-inflammatory, antimutagenic and anticarcinogenic. The purpose of this study was to examine the effect of andaliman fruit extract on body weight, blood sugar levels, MDA levels, and histopathological features of the pancreas of the mice. The Research methods design is post-test only control group design .The samples used were 32 white rats which were divided into 4 groups, namely the control group and the andaliman fruit extract dose group of 100, 150, 200 milligrams per kilogram of body weight. Data analysis using One-way Analysis of Variance. The results showed that there was an effect of fruit extract on body weight, blood sugar levels, MDA levels and histopathological features of the pancreas

Keywords: Patchouli Batik, Pogostemon cablin, Escherichia coli

INTRODUCTION

Tartrazine, as a synthetic food coloring consumed for a long time, has a toxic effect by free radicals in large quantities, creating an imbalance in the body and triggering disturbances, namely liver function. Free radicals can be minimized with antioxidants to reduce free radicals in the body. Antioxidants can be found in fruits, including Andaliman fruit. This study aimed to determine the effect of ethanol extract from Andaliman fruit on SGOT-SGPT levels and histology of tartrazine-induced white rats. The research design used 25 white rats with 5 treatments and 5 replications for 30 days. The negative control group was induced by 1% CMC, positive control induced by tartrazine 15 mg/kg BW.

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Consuming medications derived from plants, such as the binahong plant, becomes an option to prevent the negative effects of chemical drugs. The Binahong leaf extract is known to stimulate fibroblast cells and collagen formation which can accelerate the wound healing process[6,7]. Alkaloids, saponins, and flavonoids are contained in binahong leaves. Flavonoid compounds have anti-inflammatory activity and potentially prevent oxidation. Himawan shows that the combination of basil leaf and binahong leaf (ratio of 2:1) on ethanol extract has forceful antioxidant activity[8]. Saponin compounds are used as cleaning agents and antiseptics in wounds to kill or prevent the growth of bacteria[9,10]. Based on the research of Amerta, et.al. (2012) binahong was able to inhibit the growth of *S. aureus*[11]. Saponins also have the benefit of increasing fibroblast cells and stimulating the formation of collagen[12]. According to Miladiyah and Prabowo's (2012) research, Binahong leaf extract has the potential for wound healing in guinea pigs[13].

Wound healing is a transition process that involves a series of responses and complex interactions between cells and mediators. It is one of the most complex processes in physiology. The wound healing process is limited to local regeneration processes and is also strongly influenced by endogenous factors such as age, nutrition, immunology, use of drugs, metabolic conditions, bacterial infection, and location of the wounds[13,14]. The wound healing process is classified into three phases: inflammatory, proliferative, and remodeling[15]. The proliferative phase lasts from days 3 to 14 and is designed to strike a balance between scar tissue production and tissue regeneration[14]. According to Landénet et al. (2016), macrophages are granulocytes, endothelial cells, and collagen that form the extracellular and neovascular matrix[16]. The construction of new blood vessels from pre-existing blood vessels is known as angiogenesis[17].

Collagen is needed in the process of wound healing and scar tissue formation. Collagen begins to form at the proliferative stage of the injury which occurs on the 3rd day following the physical injury and keeps increasing until the 3rd week. The proliferative phase occurs at 3-14 days, characterized by the formation of granulation in the wound. In the form of fibronectin and cytokines, the extracellular matrix, wil lead fibroblast cells to proliferate. Proliferating fibroblast cells then will migrate to the wound surface, where fibrin threads had previously clotted the wound[18,19].

Fibroblast cells slowly develop on the wound surface and produce new collagen fibers during the proliferative phase[9,20]. Collagen fibers that have an irregular shape due to injury will be destroyed and replaced with new collagen. However, the amount of collagen produced is determined by the amount of collagen needed for the wound area. Collagen fibers that are formed will cover the wound surface and be strengthened by fibronectin's presence[21]. Collagen formation can be observed microscopically with Masson's Trichrome staining, which will be shown in blue on the preparation. This study is the first to examine the thickness of collagen tissue with Masson's Trichrome staining on day 8 of wound healing infected with S. aureus and treatment with binahong leaf extract.

MATERIAL AND METHOD

This research has been approved by the Research Ethics Commission of the Faculty of Public Health, University Muhammadiyah Semarang number 553/KEKP-FKM/UNIMUS/2021. This type of experimental research used a completely randomized design (CRD) with 4 research groups (2 control groups and 2 treatment groups). Table 1 shows the study group structure. The study population used white rats (Rattus norvegicus) aged ± 2.5 months with a weight of ± 200-250 gram. Research samples were obtained from rat skin tissue from each research group, with 3 replicate, and each replication was made into a tissue block and then each block made 5 slides.

Table 1. Research Group Design

Group	Descriptions
Normal Control Group (KN)	normal rats without any treatment
Negative Control Group (K-)	Rats treated incision and suspension of *S. aureus*
Treatment group 1 (P1)	Rats treated incision and suspension of *S. aureus*, and 25% binahong leaf extract.
Treatment group 2 (P2)	Rats treated incision and suspension of *S. aureus*, and 50% binahong leaf extract.

The experiment was started by culturing and preparing a suspension of *S. aureus* from the pure culture which was inoculated in a liquid BHI medium and then incubated at 37°C for 3-6 hours. Then *S. aureus* was inoculated on MC media (Mac Conkey) and incubated for ± 24 hours at 37°C. *S. aureus* colonies were grown, then injected into BHIA media, cultured for 24 hours at 37°C, then suspended in NaCl 0.9% using the standard Mc Farland 0.5. The next step is to make binahong leaf extract in a 96 percent alcohol solution using the maceration process. The resulting macerate was then evaporated using a rotary evaporator at 37-39oC to obtain a thick extract. The produced Simplicia was diluted according to the treatment group (25%, and 50%).

Experimental animal acclimation was carried out for 7 days at the Unimus Experimental Animal Laboratory. The wound region was administered a 20 L suspension of *S. aureus* after making an incision on the back skin of rats (2 cm length and 3 mm depth). Binahong leaf extract (up to 50 L) was applied to the wound area every morning and evening for 8 days, then flattened with a cotton bud. The paraffin method was used to prepare skin tissue slides, starting with the excision of the biopsy in the wound area (2x1cm and 3mm depth), followed by a fixation on 10% NBF. Dehydration using graded alcohol, clearing using xylol, and embedding using paraffin. The skin tissue was cut with a thickness of 5 micrometers, then the slides were stained with Masson's Trichrome (SkyTec Laboratories). Identification and measurement of collagen tissue thickness with a magnification of 400x as much as 5 fields of view on each preparation and then given a score according to Table 2. Collagen thickness data were analyzed using the difference test between groups with Maan Whitney.

Table 2. Criteria for Collagen Thickness Measurement Score[22,23,24]

Score	Description
0	Very low collagen thickness, 0% of collagen thickness in the wound area
1	Low collagen thickness, ≤25% of collagen thickness (marked in blue color) in the wound area
2	Medium collagen thickness, 25% of collagen thickness (marked in blue color) in the wound area
3	Thick collagen thickness, 50% of collagen thickness (marked in blue color) in the wound area
4	The thickness of collagen is very thick, 75% of collagen thickness (marked in blue color) in the wound area

.

RESULTS AND DISCUSSION

Identification of collagen tissue in skin tissue preparations by microscopic observation at 400x magnification indicated by the blue-colored section on Masson's Trichrome staining is presented in Figure 1. The results of the measurement of collagen thickness in each group are presented in Table 3. The P2 group had the same mean collagen thickness as the normal control group (KN), which had a score of 3 with 50% collagen thickness in the wound area. These results demonstrate that the administration of 50% concentration of

binahong extract for 8 days can increase the growth of collagen in the skin tissue S. aureus-infected wounds so that it has a thickness of collagen as in normal skin.

The collagen thickness score of the P1 group, which received a 25% concentration of binahong extract, was 2. These results showed that administration of 25% binahong extract for 8 days could increase collagen growth by 25% in the skin tissue S. aureus-infected wounds. While the negative control group, which treated incision and suspension of *S. aureus* on the 8th day, had not formed collagen. The results of the Maan Whitney test showed that there was no significant difference in collagen thickness in the normal control group and the P2 group administration 50% binahong leaf extract. Meanwhile, between KN groups with K- and P1, K- with P1 and P2 as well as between P1 and P2, there are differences. The results of these statistical tests are by the results of collagen thickness measurements in Table 3.

The average thickness of collagen in the negative control group (K-) was measured at 0%, which can be caused by *Staphylococcus aureus* infection. Bacterial infection can prolong the inflammatory period, impairing wound healing and reducing collagen activation in the wound area[5]. Meanwhile, the P1 and P2 groups showed a collagen thickness of 25% and 50% respectively in the wound area. The administration of binahong leaf extract at 25% and 50% concentrations led to an increase in collagen production. This is due to the presence of secondary metabolites in binahong leaf extract that can be utilized as medicine. Secondary metabolites found in binahong plants include flavonoids, saponins, terpenoids, alkaloids as well as tannins, and ascorbic acid[25].

The flavonoid content of the binahong extract, namely flavor, has been shown to enhance vascularization and decrease edema. Flavonoids also have anti-inflammatory and antioxidant activities, which can help to eliminate or alter free radicals. Free radicals can inhibit inflammatory processes as well as the contraction of the formed collagen tissue, interfering with the wound healing process[5]. The flavonoid content is also believed to help in wound repair[26].

Saponins have antibacterial, analgesic, and anti-inflammatory activities, as well as the ability to stimulate collagen formation[27]. Saponins can enhance wounds heal faster by stimulating fibroblast proliferation and myofibroblast differentiation. Saponins play a role in wound healing by stimulating the production of type I collagen, which is necessary during wound closure[28]. Saponins are also known to enhance the membrane's ability to activate cell hemolysis. Bacteria lyse when saponins interacted with them. Saponins, which increase the number of macrophages and release growth factors in the production of fibroblasts, and the synthesis of collagen for the wound area, can increase monocyte proliferation. Saponins can also help accelerate the migration of keratinocytes, which play an important role in the wound resurfacing process[6].

Ascorbic acid (vitamin C) is needed to stimulate prolyl-hydroxylase and lysyl hydroxylase enzymes in the process of forming hydrogen bonds as a molecular framework and stabilizing polypeptide interactions to produce procollagen. Furthermore, procollagen will be converted into collagen molecules by the enzyme procollagen peptidase[29,30]. In wound healing, ascorbic acid has an important role as an antioxidant, as demonstrated by cell proliferation, inflammatory suppression, and collagen tissue contraction[13].

CONCLUSION

The administration of 25% and 50% binahong leaf extract stimulated the formation of collagen in wound healing in rats infected with S. aureus on the 8th day; the thickness of collagen in the group with 50% binahong leaf extract was the same as in the normal rat group.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to this work.

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DATA AVAILABILITY STATEMENT

The utilized data to contribute to this investigation are available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals.

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Case Report

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Clinical condition of patients with Obesity Hypoventilation Syndrome (OHS): case report

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Abstract: Obesity hypoventilation syndrome (OHS) or Pickwikian's syndrome consists of obesity, breathing-related sleep disturbances, and chronic daytime hypoventilation. Identifying OHS is essential due to the risk of clinical exacerbation leading to respiratory failure and the high mortality rate among untreated patients. A 55-year-old woman was admitted to the emergency room with two days of weakness and fatigue. She is unable to perform daily tasks due to shortness of breath. She was recently diagnosed with diabetes. The patient weighs 133 kilograms and has a height of 155 centimeters (Body Mass Index of 55,4 kg/m2). The patient's blood glucose was 351 mg/dl, with an abnormal result of HbA1C of 11.5%. The blood pressure tends to stage 1 hypertension, and arterial blood gas examination showed respiratory acidosis. We diagnosed her with obesity hypoventilation syndrome, type 2 diabetes, and stage 1 hypertension. During her five-day hospitalization, her condition improved. Correct diagnosis and management can improve the patient's quality of life and decrease the risk of comorbidities

Keywords: Cardiometabolic, Diabetes Mellitus, Hypertension, Obesity, Obesity Hypoventilation Syndrome

INTRODUCTION

Overweight and obesity represent an abnormal or excessive accumulation of fat that poses a health risk. Body mass index (BMI) is used to measure personal nutritional status. In the asian population, overweight is defined as BMI between 23-24,9 kg/m2, and obesity is a BMI of more than 25 kg/m2.[1] Southeast asian population, the prevalence of overweight and obesity tends to increase; previously, from 8% to 30% in the male population and from 8% to 52% in the female population.[1,2] The proportion of people with BMI >27 kg/m2 in the Indonesian population increased from 10.5% in 2007 to 21.8% in 2018.[1,3]

People with obesity correlate with cardiometabolic and respiratory disease. Obesity can trigger medical conditions known as obesity hypoventilation syndrome (OHS).[1,4,5] The prevalence of OHS is unknown in Indonesia or other countries due to a lack of population-based studies. However, the prevalence of OHS can be estimated at 0.15% to 0.3% in the United States.[6,7] Obesity hypoventilation syndrome (OHS) or Pickwikian's syndrome is a group of symptoms consisting of obesity, breathing-related sleep disturbances, and chronic daytime hypoventilation; after ruling out

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other causes of hypoventilation, such as pulmonary disease, chest wall deformity, hypothyroidism, or neuromuscular disease.[6,8]

Identifying obesity hypoventilation syndrome is vital because of the possibility of clinical exacerbation leading to respiratory failure and the high mortality rate in untreated patients. Symptoms of arterial hypertension and insulin resistance are more common in patients with OHS.[7,9] Comorbidities such as heart failure, coronary artery disease, and cor pulmonale are more common in patients with OHS, and the likelihood that such patients will require invasive mechanical ventilation or ICU admission is also increased. Patients with OHS use more healthcare resources.[6,7]

CASE REPORT

A 55-year-old woman was admitted to the emergency room with two days of weakness and fatigue. She also experiences nausea and dizziness. She is unable to perform daily tasks due to shortness of breath. Before she went to the hospital, she had experienced this symptom for an extended period of time. There is no illness comparable to cough, fever, and influenza. She was recently diagnosed with diabetes and began taking metformin 500 mg three times a day and glimepiride once daily. The patient has no asthmatic medical history and has never smoked.

On the physical examination, the patient looked at compos mentis respiratory rate of 24 times per minute and peripheral oxygen saturation of 96% in room air, which escalated to 99% after using a nasal cannula with 3 liters of oxygen per minute. Patient's blood pressure of 116/66 mmHg, and heart rate of 97 bpm. A pulmonary examination revealed normal condition and typical heart sound. Both legs were edema. The patient's weight is 133 kg, and a height of 155 cm (Body Mass Index 55.4 kg/m2). She had fat accumulation on the neck, chest, and abdomen. However, the patient was not measured for neck, chest, and abdominal circumstances.

According to anteroposterior chest radiography, had been an increase in broncho vascular pattern and cardiomegaly. The patient's ECG result was normal sinus at 97 bpm (Table 1). To rule out a COVID-19 diagnosis, our physician did a COVID-19 PCR which showed a negative result. The patient's blood glucose was 351 mg/dl, with an abnormal result of HbA1C of 11.5%. Slightly increase in ALT liver function of 43U/L (normal: <31 U/L), normal kidney function, and creatinine of 0.58 mg/dL. Troponin results were negative. Normal Hemoglobin (15.6 g/dl), erythrocyte of 5.36 /µL, leucocyte of 7.630/µL, thrombocyte of 226.000/µL Arterial blood gas analysis (ABGA) was conducted after using a nasal cannula with pH 7.356, PaCO2 of 30.7 mmHg, PaO2 194.5 mmHg, Bicarbonate (HCO3) of 17.4 mmHg, base excess of -6.2 mmol/L (Table 2).

Table 1. Diagnostic Test Results

Diagnostic Test	Results
Chest X-ray Antero Posterior	Increased broncho vascular pattern and cardiomegaly
Electrocardiography	Early on the Emergency ward : • Normal sinus rhythm 97 bpm After hospitalized : • Normal sinus rhythm 86 beats bpm, left ventricle hypertrophy with occasional premature ventricular complex and prolonged QT interval
Echocardiography	Impaired systolic LV function 48%, hypokinetic cardiac muscle in segment anteroseptal and anterior, and abnormal diastolic LV function

We consult patients with internists, cardiologists, and pulmonologists. She was diagnosed with obesity hypoventilation syndrome with type 2 diabetes and stage 1 hypertension. She was treated in the High Care Unit (HCU). Treatment at HCU lasted for three days. The patient improved her shortness of breath, although she still had to use nasal oxygenation at 3 liters per minute. The patient's respiratory rate each day is between 18-22 times per minute. The patient's blood pressure fluctuated during treatment, systolic was recorded between 115-149 mmHg, and diastolic was recorded at 80-95 mmHg. Electrocardiography examination on day 2 of HCU treatment showed a normal sinus rhythm of 86 beats per minute with occasional premature ventricular complex and prolonged QT interval. The echocardiogram revealed abnormal Left ventricular systolic function of 48%, hypokinetic cardiac muscle in the anterior and anteroseptal segments, and abnormal Left ventricular diastolic function.

The patient used to take two types of oral antidiabetic drugs, namely metformin, and glimepiride, since being diagnosed with type 2 diabetes mellitus. However, the drug was discontinued and replaced with insulin administration by our internist. This replacement therapy has obtained the patient's consent. At the beginning, the patient received eight units of rapid-acting insulin therapy three times a day. The dose of rapid-acting insulin is tapered up to 22 units. The examination results (Table 4) showed increasing doses of rapid-acting insulin followed by decreased blood glucose levels. Our cardiologist also gave her furosemide IV and carvedilol 6.25 mg tablet once daily for her hypertension. The patient received supervision from a pulmonologist and got IV ceftriaxone therapy twice daily for five days of treatment in the hospital. In addition to receiving drug therapy to improve the patient's clinical condition, our internist also regulates the patient's diet by limiting the intake of calories to 1900 kilocalories per day with foods that are low in salt.

After five days of hospitalization, the patient went home in good condition by continuing therapy, such as a rapid-acting insulin dose of 10 units in the morning. The patient was administered 22 units of Rapid action insulin daily, 10 mg of amlodipine orally once daily, and 6.25 mg of carvedilol orally once daily. She was asked to change her lifestyle to be healthier by losing weight according to the ideal body mass index, taking medication regularly, and consuming foods low in fat and salt.

Table 2. Inpatient Laboratory Test Results

Laboratory Test	Results	Normal Value
Blood Glucose	351	<140 mg/dl
HbA1C	11.5	<5.7 %
Troponin I	0.004	<0.002
SGPT	34	<31 U/L
BUN	4.3	9.81-20.1
Creatinin	0.58	0.50-0.90
Natrium	136	136-146 mmol/L
Kalium	3.6	3.5-5.1 mmol/L
Chloride	103	98-106 mmol/L
Albumin	3.77	3.5-5.0 g/dL
Haemoglobin	15.6	12-16 g/dL
Erythrocyte	5.360.000	4.0-5.0 /uL
Leukocyte	7.630	5.0-10 /uL
Thrombocyte	226.000	150.000-450.000 /uL

DISCUSSION

The pathophysiology of OHS is related to three primary mechanisms: 1) obesity-related changes in the respiratory system, 2) alterations in respiratory drive, and 3) breathing abnormalities during sleep. Identifying one predominant or a combination of these critical mechanisms in a patient is crucial to characterize

the OHS phenotype.[10] Obesity's nutritional status is a prominent clinical feature of this patient. The patient weighs 133 kg with a height of 155 cm (body mass index 55.4 kg/m2). According to the WHO western pacific region, the patient's BMI is classified as Obesity II (BMI> 30 kg/m2).[11] The incidence of OHS increases significantly, with a reported prevalence of nearly 50% of hospitalized patients with a BMI greater than 50 kg/m2. Obesity, especially severe, can be associated with significant changes in lung mechanics and respiratory muscle performance, causing significant reductions in total lung capacity, vital capacity, functional residual capacity, and increased residual volume.[5] It can lead to severe hypoxemia, hypercapnia, and other complications.[12] Clinically, patients with OHS may present with unexplained hypoxemia or symptoms such as excessive daytime sleepiness, fatigue, loud habitual snoring, nocturnal choking episodes, morning headaches, lower extremity edema, and low oxygen saturation.[6,12]

On physical examination, fat accumulated in the neck, chest, and abdomen. However, the clinician did not measure the neck, chest, and abdominal circumference. We recognize this as deprivation in our report. Patients with OHS are prone to have greater neck circumstances. The greater the neck circumstance is, the easier the upper airway may collapse. Obesity predisposes the upper airway to closure by reducing the pharyngeal size and enhancing collapsibility.[10,12,13]

The patient's respiratory rate reaches 24 breaths per minute, and we provide oxygen therapy with a nasal cannula of 3 liters per minute. Increased oxygen demand, followed by increased carbon dioxide production, even at rest, is common in patients with obesity. It creates an imbalance between the work demands of the respiratory muscles and the capacity to generate tension, resulting in the perception of increased breathing effort.[5] The oxygen saturation was 96% room air when she first came to the emergency ward. Hypoxia, especially chronic hypoxia, can be a predictor of glucose intolerance. Chronic repetitive hypoxic episodes increase the formation of reactive oxygen species (ROS) and cytokines, suppressing insulin secretion and worsening insulin sensitivity. Reactive oxygen species can contribute to the dysregulation of adipocytokines, thereby increasing insulin resistance. Intermittent hypoxia leads to sympathetic activation, chronic inflammation, and oxidative stress, reducing insulin sensitivity, augmentation of gluconeogenesis, and beta cell dysfunction (decrement of insulin secretion).[14,15]

Arterial blood gases were conducted after she was given oxygen. The results are obtained as follows; pH 7.356, PaCO2 of 30.7 mmHg, PaO2 194.5 mmHg, bicarbonate (HCO3) of 17.4 mmHg, base excess of -6.2 mmol/L (Table 3). Based on OHS criteria, there is daytime hypercapnia (arterial carbon dioxide tension (PaCO2) ⩾45mmHg at sea level) [6]. Arterial blood gas examination showed respiratory acidosis. However, the PaCO2 value does not fulfill the criteria for hypercapnia in OHS. We suspect that taking arterial blood gases after oxygen supplementation alters the patient's PaCO2 value. The bicarbonate value was 17.4 mmHg; this value did not match the OHS predictor (bicarbonate ≥27 mEq/L). Multivariate analysis showed that hypercapnia was associated independently with bicarbonate and oxygen saturation. In addition, HCO3 ≥27 mEq/L had high sensitivity and specificity for identifying OHS patients. A sensitive screening tool for daytime hypercapnia is an elevated bicarbonate level due to metabolic compensation of respiratory acidosis.[16,17] There are weaknesses in blood gas analysis. Blood gas analysis performed after receiving oxygen supplementation may change the compensatory mechanisms of the patient's body.

Cabrera Lacalzada and Díaz-Lobato [18] suggested categorizing OHS into mild, moderate, or severe based on daytime PaCO2, daytime arterial oxygen tension (PaO2), body mass index, and the respiratory disturbance index or apnea/hypopnoea index based on polysomnographic findings (Table 4). The presence of complications can also be taken into account. Complications such as pulmonary hypertension, cor pulmonale, left ventricular failure, polycythemia, or history of intensive care in the hospital occur in severe OHS. We categorized the

patient as mild OHS based on the results of arterial blood gas analysis (PaO2 value 194.5 mmHg), but the body mass index in this woman was in the severe OHS category (BMI 55.4 kg/m2). The polysomnographic examination was not performed because it was unavailable in our hospital. The patient has left ventricular failure as comorbidity, so we categorized her as having severe OHS for comorbidity criteria.

The criteria that are often used to assess patients with metabolic syndrome, that is, if three of the five criteria are present, namely central obesity (abdominal circumference ≥ 90 centimeters for Asian men and ≥ 80 centimeters for Asian women), triglycerides ≥ 150 mg/dL, or are on medication for hypertriglyceridemia, high-density lipoprotein cholesterol (HDL) < 40 mg/dL in men and < 50 mg/dL in women or are on medication to increase HDL cholesterol levels, systolic blood pressure ≥ 130 mmHg or diastolic ≥ 85 mmHg or are on medication for hypertension, and fasting blood sugar ≥100 mg/dl or type 2 diabetes mellitus.[19,20] Lipid profile examinations were not conducted on patients, but other criteria, such as body mass index 55.4 kg/m2, being treated for diabetes mellitus and hypertension, met the criteria for metabolic syndrome. The lipid profile examination was not carried out due to limited funds.

Table 3. Arterial Blood Gas Analysis Results

Arterial Blood Gas Analysis	Results	Normal Value
pH	7.356	7.35-7.45
PaCO2	30.7	35-45 mmHg
PaO2	194.5	83-108 mmHg
HCO3	17.4	21-28 mmol/L
BE	-6.2	-2.0 - +3.0 mmol/L
AaDO2	31.5	
SO2	99.6%	94 - 99%
Lactate	1.7	0.7 – 2.5 mmol/L

Extensive cohort studies in the general population have demonstrated an increased mortality risk in individuals with overweight and obesity. Patients with obesity, depending on the degree, distribution, and duration of obesity, are at increased risk of developing cardiovascular disease.[21] Patients with severe obesity hypoventilation syndrome are at increased risk of systemic hypertension, diabetes, metabolic syndrome, left ventricular hypertrophy with diastolic dysfunction, pulmonary hypertension, and hepatic dysfunction.[22,23] The results of blood pressure checks during hospitalization showed that the patient met the criteria for grade 1 hypertension (Figure 1). American Heart Association and American College of Cardiology published new guidelines for hypertension management and defined high hypertension as blood pressure at or above 130/80 mmHg. Stage 2 hypertension is blood pressure at or above 140/90 mmHg.[22]

Table 4. Factors Influencing Severity of Obesity Hypoventilation Syndrome: A Proposal For Classification Based on Functional Parameters

	Mild	Moderate	Severe
$PaCO_2$ (mmHg)	46-60	60-80	≥80
PaO_2 (mmHg)	≥70	60-70	≤60
BMI (kg/m^2)	30-40	40-50	≥50
Apnoea/hypopnoea index ($event.h^{-1}$)	<5	5-15	>15
Complications or comorbidities	No	No	Yes

The prevalence of hypertension in patients with OHS is very high, ranging between 55% and 88% (Masa et al., 2018). A study by Alawami et al. found that poor echocardiography views were reported in 33 patients (84.6%) out of 39 patients who had an echocardiogram. LV systolic dysfunction was found in eight patients (24%), and diastolic dysfunction was reported in 18 (60%) out of 30

patients in whom it could be assessed. There was right ventricular (RV) dysfunction in 13 patients out of 25 with good RV views on echocardiogram. Also, from Alawami et al. 16 patients (34%) documented evidence of recurrent atrial or ventricular arrhythmias either during admission to the hospital or after being diagnosed with OHS.[24] The patient's electrocardiogram showed abnormalities with left ventricular hypertrophy, occasional premature ventricular complex, and prolonged QT interval. The echocardiography showed LV systolic dysfunction. The results of a chest x-ray photo with cardiomegaly plus an increase in broncho vascular pattern (Figure 2).

The patient received carvedilol 6.25 mg once daily as an antihypertensive drug. Beta blocker therapy is appropriate in patients with NYHA class II or class III symptoms resulting from left ventricular systolic dysfunction. Carvedilol is the only agent labeled by the FDA for use in patients with heart failure. Patients in the carvedilol group felt better and were less likely to have a severe adverse event related to HF. Carvedilol was the choice of beta blocker for patients with hypertension, hypercholesterolemia, diabetes melitus, and peripheral arterial disease.[24,25]

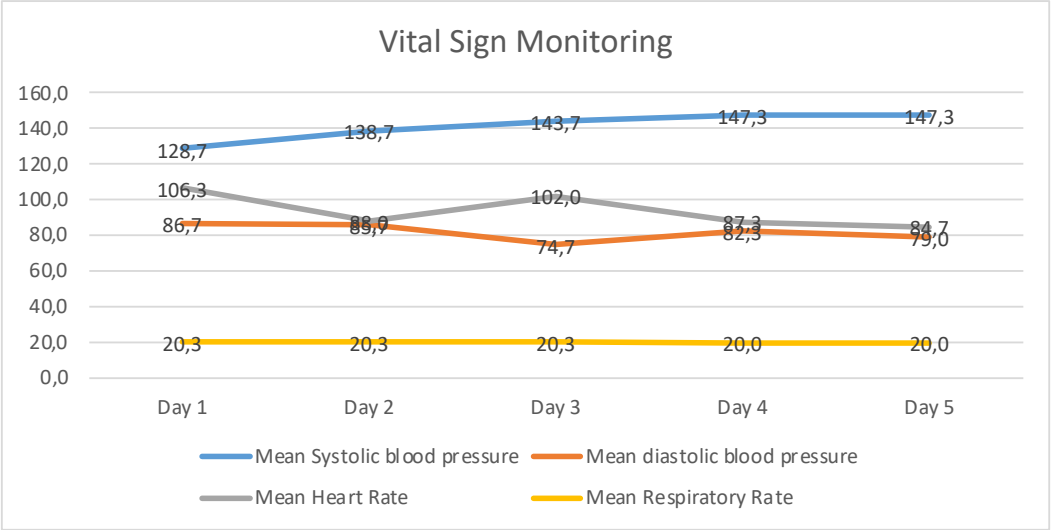

Figure 1. Vital Sign Monitoring

Diabetes is an independent predictor of mortality in OHS. Obesity hypoventilation syndrome should be treated as a systemic disease with respiratory, metabolic, and cardiovascular components that require a multi-model therapeutic approach.[23] In Macavei et al. study, diabetes prevalence was 17.7% (93/525) in the study population, and 60.3% (317/525) had a family history of snoring or sleep apnea. According to Cignarelli et al., sleep-breathing disorders may influence glucose and HbA1c levels independent of central Obesity.[26] In another study, the odds of OHS were 50% higher in those with diabetes mellitus. As measured by elevated HbA1c levels, extended hyperglycemia is associated with an increased basal metabolic rate in OHS via mechanisms of increased gluconeogenesis and lipid oxidation. A high resting metabolic rate had a significant relation with abnormal levels of HbA1c but not with high fasting glucose levels, indicating more of a long-term effect of poor glucose control than short-term effects.[24,26,27]

Based on the results of blood glucose and HbA1C tests, the patient has been diagnosed with type 2 diabetes mellitus. We monitored daily blood glucose and obtained the results in Table 5. Our doctor increased the insulin dose gradually to achieve optimal blood sugar control. Giving oral antidiabetic drugs is not chosen for patients. Internists choose blood sugar control using insulin management. Other co-morbidities suffered are reasons for using insulin. Besides that, the patient has also received education on insulin as a proper control measure to

improve blood sugar levels, and the patient has approved it. In the circumstances requiring relatively fast and precise blood sugar regulation, insulin is the best choice because it acts quickly, and the dose can be adjusted according to the results of blood glucose levels. The principle of insulin therapy is to start from a small dose which is increased gradually to prevent hypoglycemia.[28,29] The step of administering insulin therapy to this patient was started with a dose of four units of insulin, followed by eight units of insulin on the second day, sixteen units of insulin on the third day, twenty units of insulin on the fourth day, and twenty-two units of insulin on the fifth day. Insulin therapy was continued after the patient was discharged from the hospital.

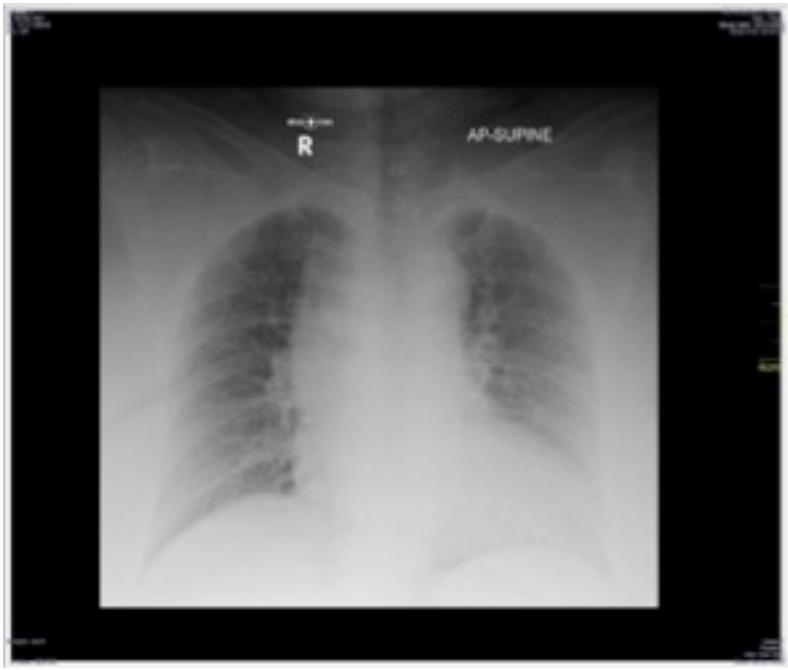

Figure 2. Chest X-Ray AP Position with Increased Broncho Vascular Pattern and Cardiomegaly

Table 5. Result of Daily Monitoring Blood Glucose

Treatment Day	Post Prandial Blood Glucose	Dose of Rapid Action Insulin
Day 1	351	4 units
Day 2	307	8 units
Day 3	236	16 units
Day 4	279	20 units
Day 5	226	22 units

Progress during treatment showed positive results, hypoxia improved, and the patient no longer used oxygen supplementation. The patient's blood sugar and blood pressure began to be controlled after taking medicine during hospitalized. She was discharged and continued antihypertensive medication and insulin therapy with an outpatient dose adjustment. The patient is advised to change her lifestyle, lose weight and increase her consumption of vegetables and fruit. The patient was asked to return for control one week later.

CONCLUSION

We present a case of obesity hypoventilation syndrome. Obesity and hypoxia are risk factors for worsening complications in patients with OHS. Handling obesity-related health problems such as hypertension and diabetes mellitus can improve the patient's condition more quickly with better outcomes. Patient

education is also essential to prevent worsening of the patient's condition. Correct diagnosis and good management can improve the patient's quality of life and reduce the risk of comorbidities such as heart failure, coronary artery disease, and cor pulmonale. In addition, it can reduce the cost and time of hospital care.

AUTHORS' CONTRIBUTIONS

Made Oka Heryana and Heru Widjono collected research data and wrote this journal. All authors have read and approved the final version of the journal.

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The utilized data to contribute in this journal are available from the author on reasonable request.

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Case Report

OPEN ACCESS

Therapy of resistant hypertension in patients with chronic kidney disease complications of anemia in hemodialysis: A case report

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Abstract: Chronic Kidney Disease (CKD) is closely related to hypertension. Increasing the severity of CKD is associated with more difficult blood pressure control. Appropriate therapeutic management is needed to prevent complications due to uncontrolled hypertension. We report the case of a 78-year-old female patient with a diagnosis of hypertension and end-stage CKD with a history of undergoing hemodialysis for 4 years. The patient has been taking antihypertensive drugs such as Angiotensin Receptor Blockers, Calcium Channel Blockers and Diuretics. However, the administration of three antihypertensive drugs still could not help achieve the expected blood pressure target where the systolic blood pressure was still above 160 mmHg. The patient also has anemia as a common complication of chronic kidney disease. Appropriate management of therapy with fourth-line therapy and hemoglobin repair is necessary to achieve improved clinical outcomes and reduce renal worsening.

Keywords: Hypertension, Chronic Kidney Disease, Anemia, Hemodialysis.

INTRODUCTION

Chronic Kidney Disease (CKD) is characterized by renal structural abnormalities or a progressive and irreversible decline in renal function.[1] CKD is defined as kidney damage lasting for ≥ 3 months due to structural or functional anomalies, with or without a reduction in Glomerulus Filtration Rate (GFR) to <60 mL/min/1.73 m².[2–4] CKD significantly contributes to hypertension development.[5,6] Chronic kidney dysfunction escalates blood pressure through mechanisms encompassing impaired sodium excretion, decreased baroreceptor sensitivity, heightened sympathetic nerve activity, and activation of the renin-angiotensin-aldosterone system.[7] The prevalence of hypertension increases proportionally with the severity of CKD stages.[2,8]

Hypertension emerges as a pivotal cardiovascular comorbidity among end-stage CKD patients undergoing hemodialysis.[9] Underlying pathophysiology

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revolves around excessive extracellular volume and kidney function alterations, influencing heightened vascular resistance and cardiac output.[10] Compared to healthy individuals, hemodialysis patients exhibit reduced urine volume, predisposing them to excess extracellular volume.[11,12] This fluid surplus drives changes in cardiac output and vascular resistance, key regulators of blood pressure.[13,14] Even with reduced GFR, renal renin secretion persists, triggering angiotensin II production and sodium retention, exacerbating extracellular volume expansion. As glomerular mass declines, sympathetic nerve overactivity ensues, contributing to increased cardiac output and vascular resistance.[15,16]

Hypertension management principles in hemodialysis patients involve preventing excess extracellular volume through antihypertensive diuretic therapy.[17,18] Inappropriate diuretic selection can lead to resistant hypertension. Regimen modification, including diuretic addition, dose escalation, or utilization of diuretics with distinct mechanisms, is viable.[2,19] Loop diuretics are preferred for GFR < 30 ml/min/1.73 m^2.[20] Kidney Disease: Improving Global Outcomes (KDIGO) recommends loop diuretics from CKD stage 4.[21] If tolerated, first-line Angiotensin-Converting Enzyme Inhibitors (ACE Inhibitors) or Angiotensin Receptor Blockers (ARBs) are also suggested for CKD patients with or without proteinuria due to their renoprotective benefits and ability to mitigate cardiovascular and renal event risks.[22–25]

CASE REPORT

A 78-year-old female elderly patient was admitted to the hospital with a diagnosis of grade II hypertension, stage 5 chronic kidney disease (CKD), anemia, constipation, and meteorism. The patient had been undergoing hemodialysis (HD) for the past 4 years. She presented with moderate nausea, abdominal pain, and black stools three days prior to admission. Vital signs revealed a heart rate of 84 beats per minute, systolic blood pressure above 160 mmHg, and SpO2 at 96%. Laboratory results indicated a hemoglobin level of 7.6 g/dL (L), PCV (*packed cell volume*) 23,5%(L), RBC 2,74 x 10^6 sel/µL (L), MCV 85,5 fl (N), MCH 27,7 pg (N), MCHC 32,5% (N), serum creatinine of 1.82 mg/dL, BUN of 10 mg/dL, urea of 22 mg/dL, and albumin of 5.03 g/dL. Stool examination revealed the presence of bacteria, leukocytes, and erythrocytes. On the second day of hospitalization, a repeat kidney function test showed an increase in serum creatinine to 3.28 mg/dL. Radiological findings indicated cardiomegaly with HHD configuration and aortosclerosis.

During her 9-day hospital stay, the patient received two units of blood transfusion, 14 TPM Kidmin, 1x8 mg ODR, 1x40 mg omeprazole, 3x500 mg Kalnex for 2 days, 3x1 15 mL Nucral syrup, 3x1 Dulcolax tablets, and Lactulax syrup. Initial antihypertensive therapy included furosemide 1x40 mg, which was discontinued after two days of use. The medication was switched to candesartan 1x16 mg and bisoprolol 1x5 mg. On the third day of medication use, these were halted, and amlodipine 1x5 mg was administered for a day. With the initiation of candesartan, blood pressure decreased from 180/83 mmHg to 155/88 mmHg. However, on the subsequent day, blood pressure rose again above 160 mmHg. The patient was discharged with amlodipine 1x5 mg, bisoprolol 1x5 mg, ranitidine 2x150 mg, and 3x1 15 mL Lacons syrup for home use.

RESULTS AND DISCUSSION

Uncontrolled hypertension is associated with increased risks of cardiovascular events, hospitalization, and mortality.[5] Thus, appropriate blood pressure control is essential for patients with chronic kidney disease (CKD) and hypertension to prevent adverse clinical outcomes. In this case report, we present a patient with end-stage CKD who experienced uncontrolled blood pressure despite treatment with three different classes of antihypertensive medications, a

condition known as resistant hypertension. According to the European Society of Cardiology (ESC) 2018 guidelines, resistant hypertension is defined as failure to lower systolic or diastolic blood pressure to values below 140 and 90 mmHg, respectively, despite combination therapy with an ACE inhibitor or ARB, calcium channel blocker (CCB), and thiazide or thiazide-type diuretic.[26,27]

In the double-blind crossover PATHWAY-2 study, spironolactone was compared to placebo, bisoprolol, and doxazosin. The results demonstrated that low-dose spironolactone (12.5-50 mg) led to greater reductions in both systolic and diastolic blood pressure compared to other therapies and placebo.[28] Based on these findings, ESC recommends managing uncontrolled hypertension in CKD with a combination of ARB, CCB, diuretic, and the addition of an aldosterone antagonist.[27] The American Heart Association (AHA) also shares a similar recommendation, suggesting spironolactone as the fourth-line treatment for resistant hypertension, followed by beta-blockers, alpha and beta-blockers, clonidine, or diltiazem).[29] If contraindicated, spironolactone may be substituted with bisoprolol and doxazosin.[26,27]

Anemia, a complication of CKD due to erythropoietin deficiency, can also occur. Research reports that CKD patients with a history of hypertension may experience anemia. Anemia is associated with hypertension, affecting the increase of endothelin-1 as a vasoconstrictor or increasing the sensitivity of angiotensin II. Hence, antihypertensive therapy is often accompanied by erythropoietin-stimulating agents (ESA) administration.[23] Subcutaneous erythropoietin administration can increase blood pressure by up to 10 mmHg in patients with chronic kidney disorders.[30]

The Kidney Disease Outcomes Quality Initiative (KDOQI) recommends a systolic blood pressure target of 140 mmHg before dialysis and ≤130 mmHg after dialysis. The JNC 8 guidelines suggest a more lenient target, recommending systolic and diastolic blood pressure below 150 mmHg and 90 mmHg, respectively).[24] Monitoring serum creatinine and BUN for adverse effects is essential when using ARB therapy.[25] Several studies have shown that the use of valsartan, captopril, and lisinopril can increase serum creatinine by 20–30%. In cases of persistent hyperkalemia, discontinuing ARB may be considered. Patient education regarding dietary and lifestyle modifications, as well as medication adherence, is crucial upon hospital discharge.

CONCLUSION

Based on the above case reports, the management of patients with chronic kidney disease complicated by hypertension is carried out in achieving appropriate blood pressure control targets and reducing the risk of kidney deterioration as well as cardiovascular events. Combination antihypertensive therapy consisting of ACE inhibitors or ARBs, calcium channel blockers, diuretics and spironolactone can be a therapeutic approach in patients with resistant hypertension.

AUTHORS' CONTRIBUTIONS

Yenry Sumarlim and Emilia Gan took research data and wrote this journal. Desantika Wuryana, M. Hari Pristantiningtyas, Herya Putra Dharma and Muhammad Muchlis chose cases in the hospital that could be used as case reports, as well as guiding the writing of this journal. Jainuri Erik Pratama, Adji Prayitno Setiadi and Marisca Evalina Gondokesumo reviewed and supervised the journal. All authors have read and approved the final journal.

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Case Report

OPEN ACCESS

Safety of metamizole analgesic therapy in patients with dyspepsia syndrome: A case report

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Abstract: The selection of drugs in patients who have experienced dyspepsia syndrome needs to be studied for its use. Non-steroidal anti-inflammatory drugs (NSAIDs) are a class of drugs that are effective in controlling various inflammatory conditions and treating inflammatory symptoms such as pain but are limited in their use in patients with dyspepsia. We report case of patients with metamizole NSAIDs during three days of hospitalization with a diagnosis of dyspepsia. Other side effects such as cardiovascular, neurological disorders and agranulocytosis can also occur with the use of metamizole. There were no side effects that exacerbated dyspepsia symptoms in patients or other side effects during the three days of using metamizol. It can be concluded that short-term metamizole therapy is safe for use in patients with dyspeptic syndrome. Concomitant use with gastric acid blocking agents such as H2 receptor antagonists may also reduce the effects of gastrointestinal disturbances.

Keywords: Metamizole, Gastrointestinal, Adverse effect, Dyspepsia

INTRODUCTION

Dyspepsia is a clinical syndrome consisting of symptoms of pain or burning that primarily occur in the epigastric or upper abdominal area, accompanied by symptoms of early satiety, bloating, nausea, and vomiting.[1–3] About 40% of individuals with dyspepsia complaints seek medical consultation, while 15% of dyspepsia patients are referred for further examination and management.[4] Dyspepsia is categorized into organic/structural dyspepsia and non-organic/functional dyspepsia. Organic/structural dyspepsia is caused by abnormalities such as peptic ulcers, gastritis, stomach cancer, and gastroesophageal reflux disease (GERD), whereas functional dyspepsia shows no abnormalities on physical examination and endoscopy.[5] Functional dyspepsia is associated with abnormal gastrointestinal motility, visceral hypersensitivity, genetic

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factors, H. pylori infection, post-infection factors, psychosocial factors, as well as environmental and dietary factors.[4,6]

Pharmacological therapy for dyspepsia is based on acid-inhibiting drugs, namely proton pump inhibitors (PPIs) and histamine-2 receptor antagonists (H2RAs).[7,8] Prokinetic agents are also administered to accelerate disrupted gastrointestinal motility due to changes in visceral sensitivity.[6,9] The British Society of Gastroenterology (BSG) recommends PPIs as the first-line therapy for dyspepsia with strong recommendations and high-quality evidence. H2 receptor antagonists are also used as first-line treatment, but with weaker recommendations and low-quality evidence.[10] PPIs like omeprazole and lansoprazole, H2RAs like cimetidine, famotidine, ranitidine, and prokinetic agent domperidone are commonly used for dyspepsia therapy in Indonesia (Nabila et al., 2022; Sakaguchi et al., 2012).[11,12] Restricting foods and drugs that can alter gastrointestinal motility is also essential.

The selection of medications for patients who have experienced dyspepsia syndrome requires careful assessment of their use. Non-steroidal anti-inflammatory drugs (NSAIDs) are one effective class of drugs for controlling various inflammatory conditions and alleviating inflammatory symptoms like pain.[13] NSAIDs are widely used; however, the risk of adverse effects on the gastrointestinal tract, especially the gastric mucosa, can occur, warranting limitation or dose reduction of NSAIDs in dyspepsia patients.[14] A meta-analysis demonstrated a significantly higher prevalence of dyspepsia events in NSAID users (OR 1.59, 95% CI 1.27-1.99).[15] Adverse effects such as nausea, vomiting, and gastric irritation can occur with parenteral NSAID administration.[16] This can exacerbate dyspeptic symptoms if administered to dyspeptic patients. A case report is presented involving the use of metamizole, a parenteral NSAID, in a patient with dyspepsia syndrome.

CASE REPORT

A 66-year-old woman was admitted to the hospital with complaints of nausea and breathlessness while walking. The patient was diagnosed with dyspepsia, anemia, and hypertension. She had a history of lung infection and had undergone a blood transfusion one month ago. Using the Visual Analogue Scale (VAS), the patient assessed her pain as a score of 4. Vital signs were blood pressure 117/90 mmHg, heart rate 122 beats per minute, respiratory rate 28 breaths per minute, and body temperature 37.40¬∞C. Laboratory results revealed hemoglobin level of 5.43 g/dL and leukocyte count of 19,800/Cmm. The patient received fluid resuscitation therapy with 0.9% NaCl at a rate of 19 drops per minute, ranitidine injection 2x50 mg, metoclopramide injection 3x10 mg, metamizole injection 3x1 g, and packed red blood cell transfusion. Monitoring on the second and third days of therapy showed a decrease in body temperature to a range of 36-36.70¬∞C. By the third day, the complaints of breathlessness and bloating had improved, and the anemia condition showed improvement with a post-treatment hemoglobin increase of 11.7 g/dL. Pain improvement was also observed with a decrease in VAS scores on the second day (score of 3) and on the third day (score of 2), with no signs of gastrointestinal bleeding. Upon discharge, the patient was prescribed omeprazole 2x20 mg, amlodipine 10 mg, spironolactone 50 mg, nucral syrup 3x15 mL, and rindobion 1x1.

DISCUSSION

Metamizole sodium is classified as a commonly used parenteral NSAID in Indonesia. Metamizole is a pyrazolone derivative with a chemical structure related to amidopyrine. This medication exerts analgesic-antipyretic, spasmolytic, and weak anti-inflammatory effects.[17,18] As a non-selective NSAID, metamizole inhibits the cyclooxygenase (COX) enzymes, namely COX-1 and COX-2, which are

involved in prostaglandin (PG) precursor synthesis.[19] Inhibition of COX-1 results in the suppression of PGE2's gastroprotective function, thus increasing the risk of gastrointestinal disorders like dyspepsia.[18,20] An infrequently but dangerously occurring adverse effect associated with metamizole usage is agranulocytosis. Agranulocytosis is defined as a neutrophil count lower than 0.5 × 109 L-1 (<500 µL-1). In specific patients, metamizole can induce agranulocytosis with an average risk occurrence after one week of usage.[21,22]

Cardiovascular adverse effects of metamizole usage have been reported in systematic reviews and meta-analyses of metamizole's side effects. Administration of parenteral metamizole causes hypotension compared to paracetamol (RR 3.48, 95% CI 1.07-11.27). Meanwhile, compared to other NSAIDs, side effects such as headache, dizziness, and vertigo are most commonly observed (RR 0.75, 95% CI 0.57-0.99).[18]

This case report demonstrates the absence of emerging adverse effects during metamizole usage. Metamizole exhibits good gastric tolerance.[16] Although metamizole strongly inhibits COX-1, very few reports indicate occurrences of duodenal ulcer-related adverse effects. Research conducted by Konijnenbelt et al (2017) revealed that metamizole is the NSAID with the lowest upper gastrointestinal tract side effects compared to other NSAIDs.[23] In line with Konijnenbelt's findings, a meta-analysis conducted by Kotter et al (2015) indicated no difference in side effect occurrences between metamizole and placebo, paracetamol, aspirin, or other NSAIDs. A total of 79 trials involving 4000 patients administered short-term metamizole showed no difference in side effect occurrences and no reports of agranulocytosis.[18] The administration of gastrointestinal protective agents in this case, such as ranitidine, an H2 receptor antagonist, besides alleviating the patient's complaints of nausea and vomiting, can be implemented to prevent gastrointestinal side effects.[24]

CONCLUSION

As a mild analgesic, metamizole exhibits good gastric tolerance. Short-term metamizole therapy is safe for use in patients with dyspepsia syndrome. Administering it concurrently with gastric acid inhibitors like H2 receptor antagonists can also reduce gastrointestinal disturbances.

AUTHORS' CONTRIBUTIONS

Khoirul Anam and Rina Widiyawati collected research data and wrote this journal. Tita Sugesti, M. Hari Pristantiningtyas, Herya Putra Dharma, and Muhammad Muchlis selected the hospital cases that could be used as case reports, and also guided the writing of this journal. Jainuri Erik Pratama, Adji Prayitno Setiadi, and Marisca Evalina Gondokesumo reviewed and supervised the journal. All authors have read and approved the final version of the journal.

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