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Original Research

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An Overview of the Curcumin-Based and Allicin Bioactive Compounds as potential treatment to SARS-CoV-2 with structural bioinformatics tools

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Abstract: The recent outbreak of SARS-CoV-2 across the globe and the absence of a specific cure against the disease lead the scientific community to investigate some alternative indigenous treatments. SARS-CoV-2 is the virus responsible for the coronavirus ailment 2019 (COVID-19). This virus has 4 auxiliary proteins namely the S (spike), E (envelope), M (membrane), and N (nucleocapsid) proteins. The main proteases and RNA dependent RNA polymerase are also essential structures by which the virus replicates and survives. Each of these proteins are structures of the virus that are potential targets for drugs which are leads in the drug discovery process of any drug for the virus. Currently available treatments are not specific to the disease and therefore carry unwanted adverse effects that can be highly dangerous and sometimes fatal. Many of these treatments are supplementary in nature or based on repurposed drugs from other viral outbreaks. Alternatives of conventional drugs are required to control the spread and severity of the disease. Allicin, curcumin and their derivatives have been researched for their antiviral property and shown to have good binding affinity towards SARS-CoV-2 structures essential in their survival, especially the main proteases and RNA dependent RNA polymerases. The structural bioinformatics tools have elicited methods to predict the bioactivity of the natural product-based compounds. Apart from the beneficial medication that they offer, natural products carry along other advantages for the current pandemic situation in terms of supply, logistics, and affordability.

Keywords: Bioactive Compounds; Coronavirus; COVID-19; *In Silico*; SARS-CoV-2.

INTRODUCTION

The outbreak of SARS-CoV-2 that began in Wuhan, China has spread to virtually all parts of the world[1]. Numerous people have been infected and a portion have also died. The status of pandemic has been declared for this virus outbreak with nations that unable to control the spread and mortality of their own population. The discovery and production of vaccines remains the only effective method of reducing deaths and severe cases during the pandemic. Several companies have developed and gotten authorization for use by the general public in the past year including Pfizer, Moderna, Sinovac, Johnson & Johnson among

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others[2]. Despite being able to provide protection against the virus with their own efficacy rates, vaccines are only able to contain infections and minimize symptoms which is not a cure against the disease itself[3].

Antiviral drugs are a class of drugs that can treat and cure viral infections[4]. Until today, there are no antiviral drugs that specifically target SARS-CoV-2 and cures the associated disease. All attempts at using antiviral drugs are repurposed medications that are meant for other infections, of which include Remdesivir that was proven effective and used in the Ebola outbreak[5]. However, repurposed drugs have their own drawbacks against the novel coronavirus in the form of adverse effects that range in severity and forms[6,7]. The costs associated with these medications are also not cheap, especially in a prolonged period of infection. Alternatives that are both safer and more affordable would provide substantial benefit towards the treatment of SARS-CoV-2 where it could reach more people, including those with less financial capabilities and therefore reduce severe cases or even mortality rates[8].

Natural products derived from plants are abundant in countries rich with spices such as Indonesia, Southern China, and India[9]. Local people of such countries have deep inclinations toward the use of traditional herbs and plants as treatments for all sorts of illnesses[10]. When studied further, traditional medications may contain properties that are associated with antiviruses. Some of which may have been used to treat virus infections effectively in the community. In addition to effective treatments, there are other advantages that these natural products bring such as the readily massive supply for the product as well as the significantly lower costs compared to pharmaceutically produced medications such as remdesivir and biosimilars such as plasma convalescent therapy[11]. A favorable alternative may be present in one of these natural products and this review paper aims to identify natural products that have the potential to treat SARS-CoV-2 and can provide an alternative against current pharmaceutical drugs.

OVERVIEW APPROACH

Keywords were selected based on their relevance to the main aim with specific attention to allicin and curcumin compounds and its effects on the SARS-CoV-2 virus, especially for the main proteases and RNA dependent RNA polymerase structures. By using the keywords, such as SARS-CoV-2, Main proteases, RNA dependent RNA polymerase, allicin, curcumin, *In silico* drug design, and Docking, independently or in conjunction with each other in various orders through NCBI, literatures were founded in their Pubmed Central (PMC) database in https://www.ncbi.nlm.nih.gov/pmc/. The journals used for the topic foundation were collected and selected with publication dates ranging between 2020 - 2021. Other supporting literature from outside the range have also been included for its merits in justifying the findings.

RESULTS AND DISCUSSION

SARS-CoV-2 is the virus responsible for the coronavirus ailment 2019 (COVID-19). It has 4 auxiliary proteins namely the S (spike), E (envelope), M (membrane), and N (nucleocapsid) proteins. Each of these proteins are structures of the virus that are potential targets for drugs which are leads in the drug discovery process of any drug. The virus also has two other structures that are highly influential in their replication process, the main protease and RNA-dependent RNA-polymerase (RdRp). Main proteases in beta-coronaviruses are distinct to human proteases which acts as a potential drug target that does not interfere with other human cellular activities. The function of the main proteases of beta-coronaviruses is to exclusively separate polypeptide sequences through cleaving and the section that this occurs is specifically after a glutamine residue.

This substrate specificity is not found in human host-cell proteases[12]. On the other hand, the RNA-dependent RNA-polymerase found in SARS-CoV-2 is crucial in the transcription and replication of the viral genome[13]. Since the process of replication and viral transcription is essential to the virus survival, it is an obvious drug target for many drug developments. Attempts in finding a cure or vaccine have been directed towards the 4 auxiliary proteins and the two important proteins, main proteases and RNA-dependent RNA-polymerase since it is believed that these structures hold key roles in the virus infection process.

Currently the treatment of COVID-19 has relied heavily on the symptoms and complications that the disease brings along. In most cases, the basic treatment includes providing medical support in the form of oxygen and fluid supply and antibiotics for secondary infections. Several vaccines have also been developed and approved for public administration including that from Pfizer, Moderna and AstraZeneca. However, a vaccine does not cure an infected person rather it prevents severe symptomatic cases from occurring. A cure ideally needs to have the property to inhibit and cease the replication process of the virus within the body. No such medication has been discovered for COVID-19. The WHO has supported clinical trials for the use of drugs against the disease by using repurposed drugs that are meant for other illnesses including that of remdesivir, chloroquine, hydroxychloroquine, and dexamethasone. As these drugs are not specifically developed for COVID-19, there are adverse effects that need concern. Remdesivir is administered intravenously and was used previously in the Ebola virus outbreak. It can inhibit virus replication however results from clinical trials have shown that the faster recovery experienced by patients was not significant enough to be considered effective. The drug is also currently undergoing an Adaptive COVID-19 Treatment Trial 3 or ACTT3 with Rebif. Chloroquine and hydroxychloroquine are malarial drugs that have no significant observed benefit towards COVID-19. In addition, the drug also has concerns of inducing cardiac problems in infected patients. Dexamethasone is a glucocorticosteroid meant to treat inflammation that is also used in cancer treatment and prevention[14]. Together with the use of ventilators or oxygen supplementation, dexamethasone has been observed to increase mortality rates among patients[15].

Medications derived or based on natural products are promising in terms of its supply, safety, and effectiveness. It has also been used by communities and passed down for generations as folk medicine. Additionally, natural products are often present in massive supply and are readily available to large populations and possibly without the need of production. Commonly used natural products for illnesses are endophytes and medicinal plants in which some may contain antiviral properties against various viruses. A medicinal plant called theaflavin has been observed to be able to inhibit RdRp and suppress COVID-19[16]. Likewise, allicin and curcumin have promising properties against the virus that is shown by their high binding affinity to both the main proteases and RdRp[17]. Allicin and curcumin are compounds that are present in common kitchen ingredients known as garlic and ginger, respectively. The commonality of these products provides a potential that exceeds manufactured drugs where it can reach people in a wider range of economic categories with ease compared to costly patented drugs. The table 1 briefly describes the medicinal properties for both the compounds.

There has been research and clinical trials that are conducted using both compounds and their derivatives. Most of the research has been done through means of *in silico* methods, especially molecular docking. A research study has identified the potential use of organosulfur compounds, which includes allicin, and flavonoids for their immunomodulatory effects. Immunomodulatory compounds can stimulate or inhibit the body's immune system against diseases and infections. The effects have supported the study's conclusion of organosulfur

and flavonoid's ability to reduce viral infections against SARS-CoV-2[18]. Another research has identified another potential drug candidate through *in silico* molecular docking targeting the SARS-CoV-2 spike protein or also known as the S auxiliary protein. The identified compound is a curcumin derivative, called Bis-demethoxy curcumin, which was observed to have a high binding affinity towards the viral spike protein[19]. This spike protein is essential in the virus infection process when entering a host and inhibiting such mechanisms would hinder the virus survivability. Despite the research showing positive results, it has not been proven in real-life situations and therefore, clinical trials would be required to validate the findings and assess its significance as a drug candidate. Some drugs that are based on curcumin and its derivatives are in the process of clinical trial research. One of these clinical trial researches is for the oral spray "ArtemiC®", which is based on curcumin and artemisinin compounds. The research was conducted in collaboration between MGC Pharmaceuticals and a Swiss firm, Micelle Technology and it has progressed to complete Phase II of clinical trials with diagnosed COVID-19 patients located in India and Israel[20]. Another study conducted by the Isfahan University of Medical Sciences in Isfahan, Iran has gained approval for clinical trials and will commence its recruitment process. Their drug candidate is a co-supplementation of curcumin and piperine which aims to determine the efficacy on the recovery process on COVID-19 diagnosed patients.

Table 1. Description of the medicinal properties for allicin and curcumin compounds.

No.	Allicin	Curcumin
1	Functional food, well known for its immunomodulatory, antimicrobial, antitumor properties, etc.	Curcumin can address a wide scope of potential applications, such as pain management.
2	Able to decrease the concentration of leptin.	Capable of boosting natural immunity and protective defense.
3	Decreases the levels of leptin, leptin receptor, and PPAR-γ.	Good tolerability and safety profiles have appeared in clinical trials.

In order to develop the treatment of coronavirus, a new conventional drug is needed, that drug is Remdesivir. In the latest research on conventional drug, remdesivir, which is a nucleotide analogue prodrug that perturbs viral replication, has been evaluated in clinical trials to thwart the Ebola threat in 2014[21]. Among the candidate therapies, remdesivir has shown efficiency in both in vitro and in vivo against coronavirus, however a much-needed clarity surrounding the repurposing of the approved drugs and experimental agents against coronavirus is needed. In the research, there are two proposed for the alternative treatment, the natural treatment, and the conventional drug. The natural treatment is based on the plant-based treatment. The components used in the natural treatment are garlic and turmeric. Garlic and turmeric have been alleged as an effective cure against the coronavirus disease. Garlic contains the sulfur phytochemicals that provide anti-inflammatory, antitumor, substantial immunomodulatory, and cardioprotective features[18]. The components inside the garlic such as S-allyl cysteine sulfoxide (alliin), ajoenes (E- and Z-ajoene), vinyldithiins (2-vinyl-(4H) -1,3-dithiin, 3-vinyl-(4H)-1,2-dithiin), and diallyl (di and tri) sulfide allow the garlic to counter back the virus threats. In the latest research, researchers have found the structure of the the main protease of the SARS COVID-19, a serine-type Mpro (chymotrypsin-like protease (3CLpro)) protease with the kind of amino acids (such as Thr24, Thr26, and Asn119) near the active site. Since the protease is responsible for virus replication and because of proteolytic maturation of the covid, the infection rate might have decreased due to the hindering the cleavage of the viral polyprotein. As for the other natural treatment, turmeric has been used in the old/traditional medical way and an integral part of Asian cooking. Turmeric

(curcumin) managed to prevent influenza A-virus injury by blocking the nuclear factor kB signalling and inhibiting the inflammatory cytokines. The role of turmeric in repressing the inflammatory process might be helped in preventing the Covid disease[22]. Drugs derived from the natural resources as bioactive antiviral compounds have more potential to cure coronavirus infection as compared with other synthetic drugs with less side effects. Finally, the conventional drug, Remdesivir. Remdesivir is an antiviral drug, used to de-accelerate the RNA viral infections of SARS-COV2. It was reported by researchers on the successful recovery of COVID-19 patients by using remdesivir[23]. Nevertheless, treating severe coronavirus patients with remdesivir has not shown good responses.

The structural bioinformatics tools might assist to find the favorable natural product leads to inhibit the SARS-CoV-2 virus[24]. It would require two essential instruments, namely the natural product database and the necessary software[25,26,27,28]. The pipeline is comprises on these steps: Sequence analysis, protein structure analysis, QSAR analysis, Protein Docking, ADMETOX, and molecular dynamics simulation[29,30]. The protein-ligand complex as the result of the pipeline is shown in the figure 1. It elicited the attachment of the ligand into the cavity of the protein based on the most favorable binding energy. The tools are easy to use, straightforward, and could assist wet laboratory research accordingly[31].

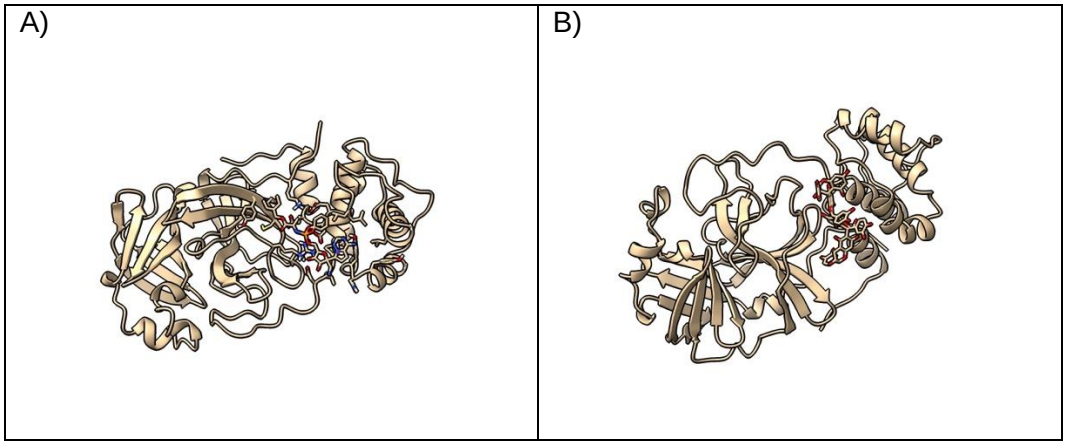

Figure 1. The 3D visualization of 3CL-protease SARS-CoV-2 protein complex with a) remdesivir b) Chemspider compound ID: 17337318 from the SARS-CoV-2 virtual screening research[32,33].
Note: Images were depicted with ChimeraX software and reproduced with the permission of the corresponding author on behalf of others[34].

For the future prospect, plant-based medicine might be very valuable. Regarding the market, the plant-based medicine might be low cost compared to the conventional drugs. Allicin and Curcumin can be easily manufactured and made available due to its natural raw material. In addition, the nature of allicin and curcumin were have well immunomodulatory, antimicrobial, anti-inflammatory, antimutagenic, antitumor properties, it makes Allicin and curcumin can be developed further for other viruses in the Coronavirus family and may be repurposed for treatment against other viruses. According to researchers, by using the pharmacoinformatic and hypothetical studies on allicin and curcumin, with in-vitro (In vitro assessment of protease inhibitory properties) and in-silico (Molecular docking and molecular dynamics) techniques, stated that the allicin and curcumin were revealed to occupy the binding site comprising of GLY-143, SER-144, CYS-145, and HIS-172[35]. By that, it means that curcumin has appreciable binding affinities cathepsin K, COVID-19 main protease, and SARS-CoV 3C-like protease which are all target proteases involved in viral entry. Thus, the binding of allicin and curcumin could influence the conformational dynamics

encoded in the structures of the proteases and subsequently their respective functional properties and innate capability to potentiate the pathogenesis of coronavirus. This observation could substantiate use of phytochemicals as therapeutic agents based on their target specificities and potencies[36,37,38]. However, this potential medicine should be most applicable to countries with a large supply of spices such as India and Indonesia. Other countries would benefit as well. Another benefits, by this proposed treatment, some things that indeed to be taken care more such as method of disease containment while awaiting vaccine rollout, minimize casualties, help recovery of patients resulting in immune system development, might not require trained practitioners to administer, distribution can be faster as requirements are less (i.e., freezer and security), quicker treatment for remote areas and with minimize cost already observed well. Nevertheless, in this paper, based on the molecular docking result, it is shown that allicin and curcumin could have potential to become the medicine of COVID-19, further clinical experiments were needed to elaborate and validate the statement more, and to develop the competent medicine for the future.

CONCLUSION

The COVID-19 has become a recognized and concerning global pandemic which has affected whole nations around the world, an unorthodox alternative solution for a cure is needed to slow down the virus threat whilst pharmaceutical companies continue their efforts. Scientists all over the globe have been racing to find the cure as soon as possible so cases and casualties will be minimized. Research on natural compounds has identified 2 potential alternatives, curcumin-based and allicin which has been observed through in silico methods for their high binding affinity towards SARS-CoV-2 structures and antiviral properties. The potential for both compounds can extend beyond its medicinal benefits and into other factors of concern such as logistics, supply, and safety. Countries with an abundance of spices would primarily benefit from this discovery, if proven effective, though it does not limit other countries from reaping the benefits. Likewise of conventional medicine, natural products may also be capable of treating other concerning viruses in the larger betacoronavirus family or even beyond. Clinical trials are highly required in the validation of these findings to identify certain obstructions of their benefits and would likely play an important role for natural products to proceed further against SARS-CoV-2 and subsequently COVID-19.

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

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Analysis of time exposure to DNA touch quality on face shield using STR CODIS – TH01 and D18S51

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Abstract: A number of countries, including Indonesia, have taken preventive measures and made efforts to break the chain of the spread of COVID-19. The impact of government policies is felt in the economic sector, which causes many people to lose their jobs but is required to meet the needs of daily life. This affects the increase in crime rates during the pandemic, which coincides with the government's policy to implement health protocols by using face shields outside the home. This allows the discovery of evidence in the form of a face shield to be identified through DNA touch. This type of research is experimental analytic with a time-series design. This study aims to analyze the effect of exposure to the environmental temperature range of 28.2 ^{o}C - 29.3 ^{o}C and humidity range of 88 - 94.2% on the quality of DNA touch on the face shield by giving the 1st, 7th, and 14th-day duration treatment using STR CODIS. TH01 and D18S5. The result is an effect of prolonged exposure time on DNA quality, as evidenced by the Anova test with $p < 0.05$.

Keywords: Crime; DNA Touch; DNA Quality; Face Shield.

INTRODUCTION

The government has taken preventive measures and efforts to break the COVID-19 transmission chain, some of them are social activity restrictions such as working from home, studying from home, maintaining distance, using personal protective equipment (masks and face shields), and washing hands.[1] Government's policies that are enforced might trigger an economic crisis, which causes many people to lose their jobs.[2,3] Mobility restrictions and changes lead to increased crime rates because many people have lost their jobs but still have to make their ends meet.[4] Crimes during the pandemic include theft, looting minimarkets, robbery, and mugging.[5] The increase in more time spent at home during the pandemic also triggers violent crimes such as domestic violence and child abuse.[4] Another crime that takes advantage of the pandemic is when the government and security forces are focusing on handling Covid-19, namely terrorism. It is easier for terrorists to carry out their acts of terror, such as the suicide bombing that occurred at the Makasar Cathedral Church in March 2021.[5]

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These crimes occurred during the pandemic period, which coincided with the issuance of a government policy to implement a health protocol by using a face shield when outside the home in the form of a face shield, this made it possible to find evidence in the form of a face shield at the crime scene or its surroundings. Identification can be made with face shield evidence by examining DNA in the form of DNA touch originating from skin and sweat epithelium.[6,7] DNA touch, which is DNA transfer due to contact from the perpetrator or victim with surrounding objects that are at the crime scene (TKP), can increase the potential for trace evidence and make it easier for investigators to relate it to the facts of the incident and prove the perpetrator or victim to avoid proof errors.[8,9] A Previous study has analyzed DNA quality on exposure to high temperatures ranging from 500^{o}C - 750^{o}C and on the effect of prolonged exposure on DNA quality to determine gender.[10,11,12]

The type of face shield used in this study is a face shield that has a sponge on the forehead area which is in direct contact with the skin, making it a DNA touch storage area, and a rubber strap that surrounds the head that puts enough pressure to the head, so the amount of DNA stored is affected significantly.[6] A study on identifying evidence using DNA touch on a face shield has not yet been carried out, while one of the potential pieces of evidence during a pandemic that can be found at a crime scene or its surroundings can be a face shield. Findings of evidence are not always found at the time of the incident but can also be found a few days after the incident. Evidence in the form of DNA, especially DNA touch, is easy to degrade and decrease in quality, one of which is caused by temperature. Therefore, the purpose of this study is to determine the effect of environmental temperature on the quality of DNA touch on a face shield that is still effective, which can be used as forensic identification material in determining perpetrators and victims by utilizing traces of evidence in the form of DNA touch.

MATERIAL AND METHOD

The study population was 15 student community volunteers. Volunteers who were used as the study population had met several research criteria such as being male, aged 15-30 years, being active outdoors for 3 hours, and being willing to fill out informed consent. The sample size was calculated using the Cochran and Cox method with SD = 13, the lowest mean = 120.17, and the highest mean = 144.67 obtained 7 repetitions for each sample treatment group. So the required sample is 21 samples obtained from the results of the repetition calculation multiplied by the number of treatments (7 x 3 = 21 samples). Sampling was carried out by random sampling; Fifteen volunteers were then randomly selected by asking them to draw a paper containing numbers from 1 to 7, where people who picked the paper that contained the numbers were selected as respondents.

The DNA touch sample collection was obtained by asking the respondent to use a new face shield that had not been opened from the wrapper, and then the face shield was used for 3 hours while doing outdoor activities. The samples were then taken to the Human Genetic Institute of Tropical Disease Laboratory, Universitas Airlangga, for cutting. The cuts were made in 3 parts for each face shield sample. The total number of samples was 21 pieces of a face shield, divided into 3 groups according to different exposure times at ambient temperature. Group I consisted of 7 samples exposed to ambient temperature for one day, Group II consisted of 7, which were exposed to ambient temperature for seven days, and Group III consisted of 7 exposed to ambient temperature for 14 days. We selected 1, 7, and 14 days as exposure time because one-day exposure is correlated to the beginning of the crime scene identification process by the investigator (first day), the 7th day is the maximum limit for investigators to

conduct a crime scene examination, and the 14th day is the limit for submitting case files to the public prosecutor.[13,14]

Group I DNA samples was extracted using DNAzol with the initial steps: The DNA extraction stage used the DNAzol method by immersing the face shield pieces in 20 cc sterile distilled water in a 50 cc conical tube sonicated for 15 minutes. The sonicated results were transferred to another tube and centrifuged at 12000 rpm for 10 minutes to collect the pellets. The resulting pellet was added with 1000 l of phenol and 200 l of chloroform and then incubated for 2 hours. The sample was centrifuged at 8000 rpm for 10 minutes, added isopropanol ratio (1:1), and incubated for 1 hour. The sample was centrifuged again at 12000 rpm for 10 minutes to obtain DNA on the pellet. The pellets were then washed with 70% ethanol and then centrifuged. The supernatant from the centrifuge was discarded by inverting the tube and placing it in an upright position until the ethanol evaporated and dried, then 50 l sterile distilled water was added and stored at 4oC. The same was done for the samples on the 7th and 14th days. After all the samples were extracted, it was continued to determine the content and purity of the DNA using a UV-Vis spectrophotometer at a wavelength of 260 – 280 nm. Then, DNA amplification was carried out by PCR using the TH01 and D18S51 loci, and the last step was electrophoresis using acrylamide gel to see the DNA band against the TH01 (152 – 195 bp) and D18S51 (286 – 366 bp) loci.

The obtained data was twenty-one DNA content from the spectrophotometer result, then statistically analyzed using SPSS software version 23. The study data was first tested with the Shapiro Wilk normality test with α = 5% and homogeneity test, where a p-value of > 0.05 revealed that the obtained data were normally distributed and homogeneous respectively. The next step was to perform the statistical test using the One Way ANOVA Test.

RESULTS AND DISCUSSION

The results of DNA level measurements (Table 1) were conducted using a spectrophotometer at a wavelength of 260/280 nm. The table shows different mean DNA touch levels and purity on faces shields on each exposure time (1, 7, and 14 days) and the mean ambient temperature and humidity. The minimum requirement for DNA purity to have proceeded to DNA amplification is 1.8 – 2, while the Short Tandem Repeat (STR) examination requires a minimum DNA level of 1 – 25 ng. The results of DNA level and purity measurement of this study had reached the minimum required DNA.[15]

Table 1. The Results of DNA Touch Level Measurement on Face Shield

Time Exposure	Sample Number	The average amount of DNA (X±SD) (µg/ml)	Average Purity of DNA	Average Ambient Temperature (°C)	Average Humidity (%)
Day 1	7	1778±529.30	1.29	28.6	90
Day 7	7	856±258.55	1.22	28.9	89
Day 14	7	1153±487.58	1.24	28.7	89.5

Table 2. ANOVA Statistical Test Results

	Sum of Squares	Df	Mean Square	F	p-value
Between Groups	3100808.667	2	1550404.333	7.954	0.003
Within Groups	3508540.000	18	194918.889		
Total	6609348.667	20			

The data in table 2 show differences in DNA quality which was affected by exposure time (1, 7, and 14 days). The difference in DNA quality level could be seen in table 1, while the fluctuations that occurred could be seen in figure 1. Figure 1 provides an overview of fluctuations in DNA levels at different exposure

times. The decrease in DNA level occurred on day 7 and then increased on day 14.

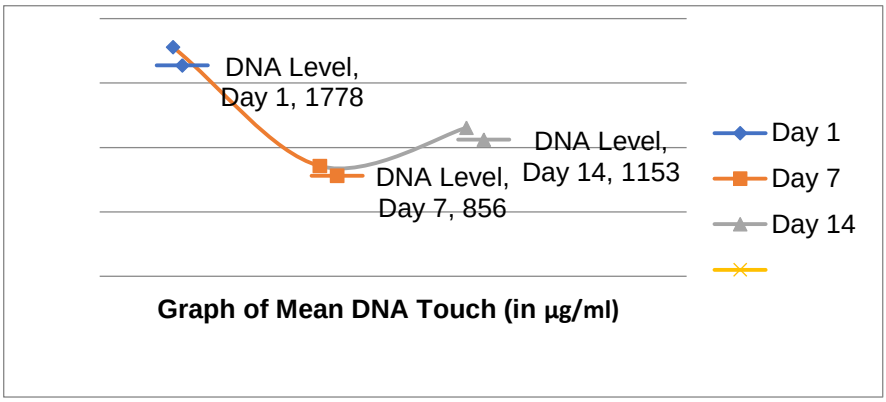
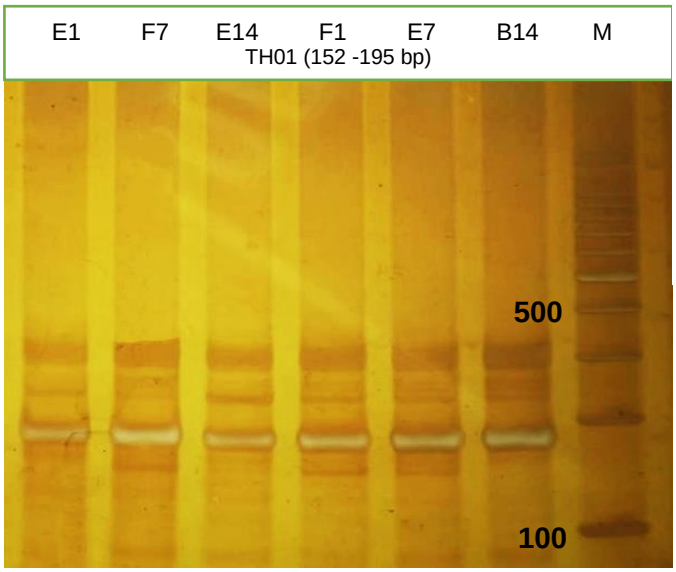


Figure 1. Graph of Mean DNA Touch Level on Face Shield Based on Different Exposure Days

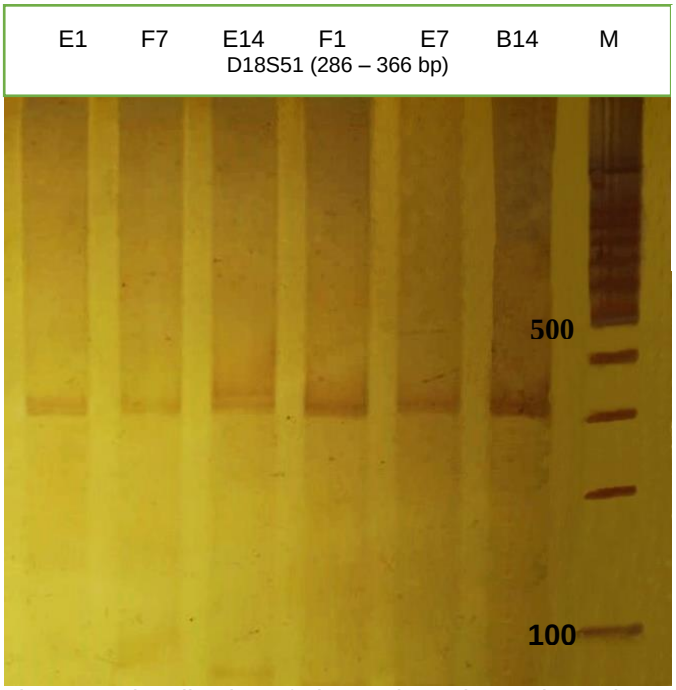
Figure 2 is a visualized image using acrylamide gel at the Short Tandem Repeat Combined DNA Index System (STR CODIS) TH01 (119 – 155 bp) locus of DNA touch samples at the highest and lowest levels, while figure 3 is the result of visualization of STR CODIS D18S51 (286 – 366 bp) at the highest and lowest levels. The visualization of STR CODIS TH01 and D18S51 are the finding of bands in all samples. This indicates that the selected loci is a potential locus for the respondents in accordance with the recommendations of the FBI (Federal Bureau Investigation) and is an illustration of the success of the amplification process. Good visualization during electrophoresis indicates that the purity and content of DNA obtained are adequate to be used as a DNA examination material. In addition, the DNA obtained should not be degraded, if it undergoes degradation, it must be kept to a minimum because it causes the primer not to attach during the annealing process to the target DNA to be amplified.[11] The difference in the fluctuation of DNA touch levels from the results of the study on the length of time of exposure still gives good results in DNA visualization; this proves that the sample in this study produced good levels and purity of DNA.[10,16]



Description:

E1 : Day 1 Sample (highest DNA level)
F7 : Day 7 Sample (highest DNA level)
E14 : Day 14 Sample (highest DNA level)
F1 : Day 1 Sample (lowest DNA level)
E7 : Day 7 Sample (lowest DNA level)
B14 : Day 14 Sample (lowest DNA level)
M : *Marker ladder* 100 bp

Figure 2. Visualization of electrophoresis results at the TH01 locus (152 - 195 bp) on day 1, day 7, and day 14



Description:

E1 : Day 1 Sample (highest DNA level)
F7 : Day 7 Sample (highest DNA level)
E14 : Day 14 Sample (highest DNA level)
F1 : Day 1 Sample (lowest DNA level)
E7 : Day 7 Sample (lowest DNA level)
B14 : Day 14 Sample (lowest DNA level)
M : *Marker ladder* 100 bp

Figure 3. Visualization of electrophoresis results at the D18S51 locus (286 – 366 bp) on day 1, day 7, and day 14.

Factors that can influence differences in DNA quality and fluctuations are due to DNA touch derived from skin epithelial cells (corneocytes), sweat, protein, free DNA, and contamination from the environment. Touch DNA derived from corneocytes is considered to have little DNA content and is easily fragmented, but skin epithelial cells still contain residual DNA that can provide value for identification using short tandem repeats (STR).[7] The difference in DNA touch level was influenced by the tendency of a person's ability to leave DNA touch, the surface used as attachment location, and the exposure time of DNA to external factors.[7,8] Exposure time, temperature, humidity, UV ray, and microorganism are factors that trigger DNA degradation, which could affect the difference in DNA quality.

DNA degradation is influenced by environmental factors (endogenous factors), including humidity, temperature, UV light, and microorganisms. The DNA degradation affects the primer attachment process to target DNA, which will be duplicated in the annealing stage, significantly affecting the DNA level produced during amplification.[11,17,18] Dry storage condition has a relatively low success rate compared to humid condition for STR analysis process.[9] This difference was seen in the results of DNA touch levels (table 1), where the highest DNA level occurred at the lowest mean temperature and highest humidity (Group I) and then decreased due to an increase in temperature and humidity on the 7th day, and then the DNA level was increased again on the 14th day following a decrease in temperature and an increase in humidity.

The increase in temperature was related to UV radiation in sunlight which causes DNA damage.[19] Other supporting factors are pressure and the type of surface where the DNA touch was attached. The pressure produced by the face shield strap was influenced by the elasticity of the strap, which was correlated to the respondent's head size. Pressure could increase DNA transfer on the skin surface, which affects the amount of DNA stored.[6,20] Sponge, used as the face shield surface, is a porous surface and might also affect the amount of DNA due to partial migration into the substrate.[20,21]

CONCLUSION

The conclusion from the results of the analysis in this study is that there is an effect of time exposure in the time range (1, 7, and 14 days) with environmental temperature on the quality of DNA touch on the face shield using STR CODIS – TH01 and D18S51. Detection of the visualization of the CODIS – TH01 and D18S51 STR PCR results showed bands on gel electrophoresis on days 1, 7, and 14.

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 results from the author's research and has never been published in other journals.

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Green synthesis of silver nanoparticles from papaya seed extracts with alkaloid content for antibacterial application

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Abstract: This research aims to develop a silver nanoparticle (AgNPs) using several plant extracts for antibacterial application. The papaya seed extract has been fractioned by n-hexane, ethyl acetate, water, and ethanol. The n-hexane fraction was the only fraction that succeeded in synthesizing AgNPs. The characterization methods showed AgNPs marked at 430 nm with UV-Vis and 1640 cm^{-1} with FTIR. SEM observed the aggregation of spherical AgNPs at the 200 nm scale. The particle size of 91.3 nm was measured with PSA that confirmed the nanoscale of the synthesized material. All fractions contained alkaloid compound, and ethyl acetate fraction showed a group of indole with specific wavenumber at 2623 cm^{-1}, 1737 cm^{-1}, and 1237 cm^{-1} representing N-H, C=O, C-N, respectively. All fractions at every concentration (25%, 50%, 75%, 100%) have been tested and showed the medium effect on bacterial growth inhibition. Among all fractions, the AgNPs n-hexane fraction has the highest bacterial effect, which was indicated by mean values of inhibition zone 7.2 mm against *S.aureus*, as well as 6.6 mm against *E.coli*. ANOVA analysis showed that AgNPs n-hexane fraction has a significant inhibition zone compared to other fractions against *S.aureus* (*p=0.002*), but not significant to *E.coli* (*p=0.128*). The insignificant results on *E.coli* because of gram-negative bacteria's biophysical characteristics, such as membrane cell wall and flagellin. This research emphasized that AgNPs could be synthesized via a green process of nucleation by using plant extract that effectively inhibits the growth of *S.aureus* and *E.coli*. Further studies on the mechanism of the antibacterial effect at the molecular level might be investigated soon.

Keywords: Silver Nanoparticle, Papaya seed, n-Hexane, Alkaloid, S.aureus.

INTRODUCTION

The decrease of the potential of the antibiotic against bacteria, especially Staphylococcus Aureus and Escherichia Coli, has triggered the development of new antibiotic agents[1]. The research on herbal extracts has been extensively reported for decades. However, the current progress on using organic compounds for the green synthesis of nanoparticles is still highlighted worldwide.

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One of the most widely investigated nanoparticles in health and medical applications is silver nanoparticles (AgNPs). Moreover, AgNPs have been reported to cause acute toxicity and damage the structure of bacterial cells, so that silver nanoparticles were proposed as a potential antimicrobial agent[2].

A natural product that can be antibiotic is the papaya plant[3] because of bioactive compounds such as alkaloids that play the role of antibacterial effect. Today, alkaloid compounds draw the attention of researchers on antibacterial activity. Alkaloids have different levels of antibacterial effect, depending on the place and origin of the alkaloids obtained[4]. There are differences in antibacterial activity in gram-positive and gram-negative bacteria, whereas the efficacy depends on the extraction solvent used in the extraction and isolation[5]. Research by Juliantina et al., 2009[6] reported the mechanism of alkaloids as an antibacterial by interfering with the peptidoglycan components in bacterial cells. The bacterial cell wall defects and causes cell death[7].

This research reports the synthesis of silver nanoparticles (AgNPs) using plant extracts followed by antibacterial tests. Before synthesizing nanoparticles, papaya seeds were extracted by graded fractions of water, ethanol, ethyl acetate, and n-hexane. Then the silver nanoparticles were synthesized by a nucleation process supported by plant extract. The characteristics of the nanoparticles were analyzed using UV-Vis, FTIR, SEM, and PSA. All fractions and AgNPs were tested for antibacterial activity using disc diffusion by calculating the inhibition zone diameter. Data obtained were evaluated by ANOVA Analysis.

MATERIAL AND METHOD

The papaya seeds (Carica papaya)[8] were collected from the traditional market, washed and dried, then identified in the herbarium of the University of North Sumatra. Solvents used are aqua dest, ethanol 96%, ethyl acetate, and n-hexane. Escherichia Coli, Staphylococcus Aureus, Nutrient Broth (NB), Mayer reagent, Dragendorff, DMSO 10%, Tween 80, and Liberman Burchad reagent have been used for antibacterial tests.

The instruments used for AgNPs synthesis are heating mantle (electrothermal), soxhlet apparatus, oven (Memmert), microwave (LG), analytical balance (Denver Instruments), 50 mesh sieve, magnetic stirrer (Daihan LabTech), UV-Vis Spectrophotometer (Shimadzu 1601), Particle Size Analysis (PSA) (Malvern 1.20), Scanning Electron Microscope (SEM) and FTIR Spectrophotometer.

About 1.7kg of dried and roughly crushed papaya seeds were macerated by ethanol with a ratio of 1:6. Samples were macerated for three days with stirring one time per day. The crude extract yield was about 48.1924 g (2.83%). About 5 g crude extract dissolved in 80 ml of aquadest at 70oC then moved into a separating funnel. Repeatedly, the graded fractionation was conducted three times by 80 ml of each n-hexane, ethyl acetate, and ethanol.

The synthesis of AgNPs was carried out by mixing 10 ml of AgNO3 solution (1 mMol) and 200uL of n-hexane fraction. The mixture was stirred for about 30 min until homogeneous. The formation of AgNPs was evaluated by a UV-Vis spectrophotometer (Bakir, 2011). The characterization of AgNPs was analyzed by FTIR (Nessa, 2010), SEM [9], and PSA (Manual Book LB-550).

The Muller Hinton Agar (MHA) media was poured aseptically and solidified. A bacterial suspension was taken by cotton swab and then scratched on the surface of the MHA media. Divided into four quadrants and labeled outside the petri dish. A paper disc with AgNPs was placed on the marked part then Incubated for 24. The clear zone was measured around the paper disc.

Statistical analysis has been employed to determine the normality of data obtained using Kolmogorov Smirnov and the homogeneity by Levene methods. Then one-way ANOVA was applied to evaluate the significances of each sample

RESULTS AND DISCUSSION

3.1 Synthesis of Silver Nanoparticles (AgNPs)

The n-hexane fraction was the only fraction that succeeded in synthesizing AgNPs. We could not get any AgNPs formed in water, ethanol, and ethyl acetate fractions. The content of flavonoid compounds in the n-hexane fraction might affect the nucleation process of the nanoparticles since the flavonoid compound has been known as natural reducing agents and role in the nucleation process. As a bio-reducing agent, flavonoid converted Ag^{+} to Ag^{0}. As a capping agent, flavonoid stabilized the nano size of the synthesized nanoparticles[10]. Several studies have reported a significant effect in the synthesis of nanoparticles on changes in shape, size, and morphology depending on pH, temperature, extract concentration, metal salt concentration, and reaction time [11,12,13].

3.2 UV-vis spectra analysis

All fractions were measured by UV-vis spectra, as shown in Figure 1. The literature [14] described that the wavelength of alkaloids ranged from 203 nm to 285 nm. Figure 1(b) showed absorbance at 240 nm, and 250 nm indicated a group of alkaloids named indole [15]. Figure 1(e) showed absorbance at 280 nm of AgNPs synthesized with n-hexane fraction, this indicated carbonyl which usually ranged from 270 nm to 300 nm[15] . Our results showed that AgNPs in a colloidal system have contributed to a strong absorbance ranging from 400 nm to 500 nm, which is a typical absorbance of AgNPs[16] .

3.3 FTIR spectra analysis

In general, strong absorbances at 3745 cm^{-1} and 3318 cm^{-1} indicated a stretching vibration of hydroxyl groups. The peak at 1640 cm^{-1} of Figure 2(d) represented stretching vibration of C-N amines or amines aliphatics, whereas 1406 cm^{-1} indicated amides[17] . Alkaloid compound was indicated at 2623 cm^{-1}, 1737 cm^{-1}, and 1237 cm^{-1} of N-H, C=O, and C-N, respectively[18]. Another study on the characterization of AgNPs and papaya extract by (Jain 2011)[19] showed absorbances at 1697 cm^{-1}, 1618 cm^{-1}, 1514 cm^{-1}, 1332 cm^{-1}, and 1226 cm^{-1} that indicated vibrations of -C-C, C-O, -C-C- aromatics, C-O ether, C-O polyols such as hydroxy flavone and catechin. In our study, especially in ethanol and ethyl acetate fractions, we found specific absorbance at 1244 cm^{-1} and 1237 cm^{-1} that represented polyol groups that role the bioreduction of Ag^{+} to Ag^{0}. In contrast, the polyol was oxidized to an unsaturated carbonyl that caused vibration at 1640 cm^{-1}.

3.4 SEM Images analysis

SEM images showed a spherical morphology with a scale of 200 nm, which indicates a high density of AgNPs in Figure 3(a). At the same time, Figure 3(b,c,d) showed that AgNPs were evenly distributed and aggregated at different magnifications. A study by (Balavijayalakshmi and Ramalakshmi 2017)[8] explained that aggregation was made of a high concentration of plant extracts, and the agglomeration may cause destabilization of AgNPs. UV-Vis data also supported these phenomena showing that absorbance at 400 nm indicated spherical nanoparticles [9].

3.5 Particle Size Analysis

The Polydispersity Index (PI) in figure 4 showed a broad distribution range of the sample, more than one peak, and the particle size varies (heterogeneous). The PSA data showed that the particle size of AgNPs is 91.3nm; this result has strongly confirmed the definitive nanoscale of the synthesized materials that should be ranged from 1 nm to 100 nm [20].

3.6 Antibacterial Analysis

The antibacterial effect of each fraction and AgNPs have been tested against *S. aureus* and *E.Coli* with the variation of concentration 25%, 50%, 75%, and 100%. This measurement parameter is the mean diameter of the inhibition zone.

Table 1. Antibacterial effects on *S. aureus.*

Bacteria	Sample (Fraction)	Concentration (%)	Inhibition Zone (mm)			Mean	Mean of each fraction	SD	*p*-value
			Test-1	Test-2	Test-3				
Staphylococcus aureus	Water	25	6.9	6.2	6.7	6.6	6.620	0.236	p= 0.002 < 0.05
		50	7.6	6.4	6.9	6.9			
		75	6.4	6.3	6.7	6.4			
		100	6.5	6.2	6.7	6.4			
	Ethanol	25	6.4	6.1	6.5	6.3	6.715	0.315	
		50	7.0	6.2	6.9	6.7			
		75	6.9	6.2	7.1	6.7			
		100	7.9	6.2	7.2	7.1			
	Ethyl Acetate	25	6.1	6.2	6.2	6.1	6.370	0.143	
		50	6.4	6.2	6.6	6.4			
		75	6.3	6.5	6.6	6.4			
		100	6.3	6.9	6.2	6.4			
	AgNPs	25	6.9	6.9	7.0	6.9	7.205	0.219	
		50	7.0	7.3	7.1	7.1			
		75	7.2	7.4	7.5	7.3			
		100	7.6	7.0	7.6	7.4			

In the water fraction of papaya seed extract, antibacterial effects against *S. aureus* were found at all concentrations, namely 25%, 50%, 70%, and 100%. The highest effect was at a concentration of 50%, where the inhibition zone was 6.96 mm. The result of this study was in line with (Kusumawati's 2020)[21] reported that the water fraction of papaya seeds has antibacterial effects against *S. aureus* with an inhibition zone of 8.43 mm to 12.98 mm. This effect is due to metabolite compounds, namely flavonoids, alkaloids, carbonyls, and terpenoids.

The ethanol fraction of papaya seed extract has antibacterial effects at all concentrations. The highest effect was at a concentration of 75%, and the inhibition zone was 6.73 mm. Research by (Roni, Maesaroh, and Marliani, 2019) [22] reported that ethanol fraction of papaya seed has antibacterial effects against *S. aureus* with an inhibition zone diameter of 12.2 mm. (Kusumawati's 2020) [21] also found that the ethanol fraction of papaya seeds has an antibacterial effect against *S. aureus* with inhibition zone diameter of 5 to 9 mm. This is presumably due to the presence of alkaloids and flavonoid compounds that can damage the bacterial membrane structure, resulting in the growth inhibition of bacteria [4].

The ethyl acetate fraction of papaya seed extract was effective against *S. aureus* at all concentrations. A significant effect was found at a 75% and 100% concentration, with an inhibition zone of 6.46 mm. The research results by (Roni, Maesaroh, and Marliani, 2019) [22] found that the ethyl acetate fraction of papaya

seed extract could act as an antibacterial for *S. aureus* with an inhibition zone of 11.6 mm. However, this study did not clearly explain the mechanism of bacterial inhibition.

The synthesized AgNPs were found to act as an antibacterial against *S. aureus* at all concentrations, and the best seen at 100% concentration, with an average inhibition zone value of 7.4 mm. The obtained AgNPs have the best antibacterial effect among all treatments, with an average inhibition zone of 7.205 mm. The effect of AgNPs on the leakage of membrane proteins and reducing agents of the bacteria by increasing the permeability of the cell membrane of *S. aureus*. The presence of high plasma membrane leakage causes damage to the bacteria, which makes the bacterial structure decompose and causes death [23]. The n-hexane fraction of papaya seed extract has been reported to contain alkaloids, flavonoids, terpenoids, and saponins [24]. Alkaloids can disrupt peptidoglycan and inhibit bacterial topoisomerase enzymes[25]. Flavonoids could inhibit bacterial growth by reducing cell membrane permeability due to protein and membrane complex binding [26]. Terpenoids, the inhibitory ability, involves a reaction that forms a robust polymeric bond with the outer membrane of the bacterial cell wall, thereby causing porin infection[27] .Saponins are thought to cause leakage of proteins and enzymes in [28].

Based on Table 1, that all fractions have antibacterial activity against *S. aureus* at all concentrations with an average inhibition zone of 6.2-7.2 mm. This activity belongs to a medium-strength antibacterial[1]. The AgNPs showed a significant antibacterial effect compared to other fractions with the *p-value* < 0.05.

Table 2. Antibacterial effects on *E.coli.*

Bacteria	Sample (Fraction)	Concentration (%)	Inhibition Zone (mm)			Mean	Mean of each fraction	SD	*p*-value
			Test-1	Test-2	Test-3				
Escherichia coli	Water	25	6.1	6.2	6.3	6.2	6.246	0.062	p= 0.128 > 0.05
		50	6.1	6.2	6.3	6.2			
		75	6.1	6.4	6.3	6.2			
		100	6.2	6.3	6.5	6.3			
	Ethanol	25	6.2	6.4	6.3	6.3	6.590	0.204	
		50	6.6	6.8	6.7	6.7			
		75	6.9	6.6	6.8	6.7			
		100	6.8	6.8	6.2	6.6			
	Ethyl Acetate	25	6.1	6.1	6.2	6.1	6.348	0.228	
		50	6.4	7.0	6.5	6.6			
		75	6.3	6.1	6.2	6.2			
		100	6.3	6.6	6.4	6.4			
	AgNPs	25	6.1	6.1	6.3	6.1	6.623	0.218	
		50	6.2	6.2	6.6	6.3			
		75	6.5	6.2	7.1	6.6			
		100	6.6	6.3	6.9	6.6			

In the water fraction of papaya extract, the highest effect against *E.coli* was found at a concentration of 100%, where the inhibition zone was 6.33 mm. (Jenab's, 2017)[29] found that the water fraction of papaya seed extract had an antibacterial activity with an inhibition zone of 7.13-12.20 mm. (Hidayati, 2019) [24]

also found that highest effect at 100% with an inhibition zone of 6.50 mm. This effect might be due to flavonoids and saponins in the fraction of water.

The ethanol fraction of papaya seed extract showed that all concentrations have antibacterial against *E.coli*. The best seen was at a concentration of 75% with inhibition zone of 6.76 mm. In addition, the ethanol fraction was obtained as the highest antibacterial among all treatments, with inhibition zone of 6.590 mm. These results were supported by (Taufiq 2015) [30] reported that ethanolic papaya extract can inhibit the growth of E. coli which is related to the active compound in each fraction.

The ethyl acetate fraction of papaya seed extract has an antibacterial effect against *E.coli* at all concentrations. The highest was found at a concentration of 50%, with inhibition zone of 6.63 mm. This result was in line with (Roni, Maesaroh, and Marliani, 2019)[22] that the presence of antibacterial activity with inhibition zone was 11.96 mm. They suggested that a terpenoid class can inhibit the growth of *E.coli* bacteria. Similar results showed that the ethyl acetate fraction of papaya seed extract was antibacterial against *E.coli* with an inhibition zone of 11.13-18.13 mm[29]. Moreover, (Hidayati 2019)[24] also mentioned antibacterial activity in the ethyl acetate fraction of papaya seeds with an inhibition zone of 13.83 mm, which was related to alkaloids.

The AgNPs extract has antibacterial against *E.coli* at all concentrations, and the best was found at concentrations of 75% and 100%, with inhibition zone of 6.6 mm. The study by conducting an antibacterial test of AgNPs using dried and mashed papaya seeds without extraction found that the synthesized AgNPs could inhibit the growth of *E. Coli* with an average inhibition zone of 9.1 mm. Several studies reported that various pathogenic organisms were inhibited by effectively biosynthesized AgNPs [31,32,33,34,35].

Based on Table 2 that all fractions have antibacterial activity against *E. coli* at all concentrations with an average inhibition zone of 6.3-7.2 mm, which belong to a medium-strength antibacterial. Unfortunately, the AgNPs did not show a significant antibacterial effect compared to other fractions with the *p-value* > 0.05. Several previous studies also reported insignificant results, which may be related to a similar resistance to AgNPs. It was explained that the large numbers of flagellin in bacteria could repel the AgNPs, which could reduce the antibacterial effectiveness, and test results will be varied [35].

3.7 Mechanism of Antibacterial Effect

Limited studies regarding the AgNPs synthesized by n-hexane fraction for the antibacterial application. However, the inhibition zone in the antibacterial test was known reported that the shape of the nanoparticles influenced it. In the previous discussion, the SEM results showed that AgNPs formed tended to be round or spherical. An explanation by (Kim, 2011)[36] stated that the shape of AgNPs affects its ability as an antibacterial. Triangular AgNPs with lattices on the base plane showed the most potent biocidal properties against bacteria than spherical and rod-shaped nanoparticles. This is because the triangular AgNPs can inhibit the growth of bacteria at a total silver of 1µg. The silver ions will cause the loss of K^+ ions from the bacteria, which will cause a potassium deficit, so that cell membrane leakage occurs, and this does not occur in spherical or rod-shaped nanoparticles.

Nevertheless, the target of silver ions in the plasma or cytoplasmic membrane of bacteria is associated with several enzymes and DNA. When bacterial growth is inhibited, silver ions will be deposited into vacuoles and cell walls such as granules. Silver ions inhibit cell division, damage cell membranes and bacterial cell organelles. As an additional explanation, silver ions can associate with nucleic acids, preventing association with DNA bases rather than with phosphate groups[36].

CONCLUSION

The AgNPs have been successfully synthesized using the n-hexane fraction of papaya seed extract. The colloidal system of AgNPs formed in this study was spherical and tended to be aggregated observed by SEM. UV-vis spectra have indicated the presence of AgNPs at 280 nm and 430 nm. FTIR Spectra also showed a typical absorbance of AgNPs at 1640 cm^{-1}. PSA has detected broad distribution and heterogeneous particle size. All of the papaya seed extract fractions showed the presence of alkaloid metabolites, especially indole alkaloids found in the ethyl acetate fraction. All fractions and AgNPs colloidal system have a medium antibacterial effect against *S. aureus* and *E.Coli* ranging from 6 mm to 7.2 mm. However, AgNPs were found to be more significant against *S. aureus* instead of *E.Coli* that could be explained by the biophysical characteristic of bacteria and the agglomeration of the AgNPs.

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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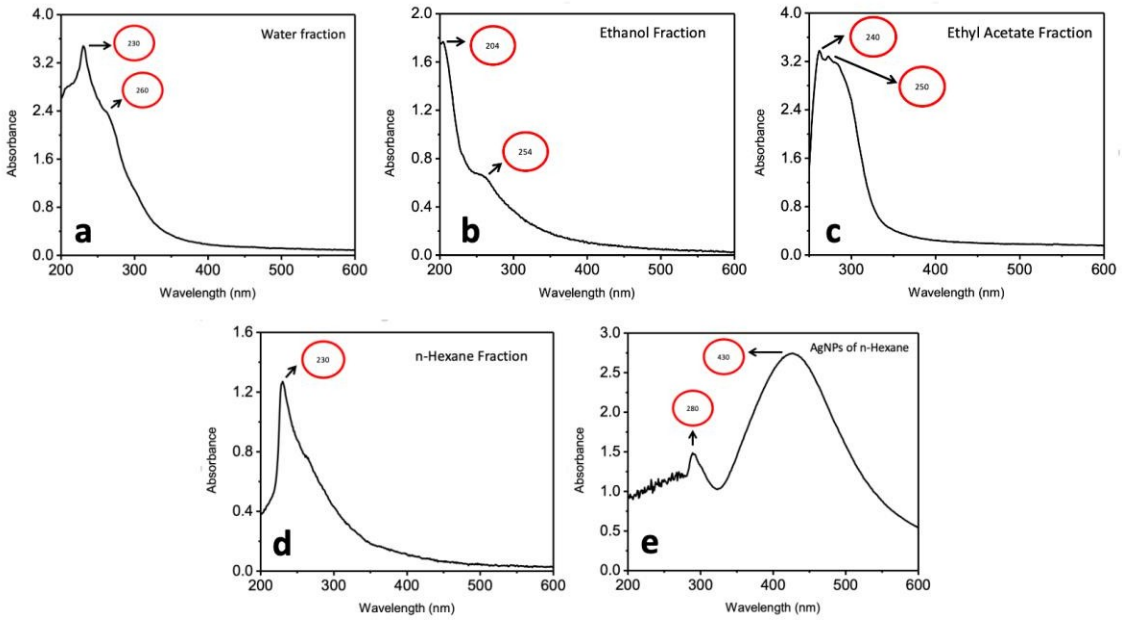

Fig.1 UV-vis data of each fractions; (a) water, (b) ethanol, (c) ethyl acetate, (d) n-hexane fractions, and (e) AgNPs

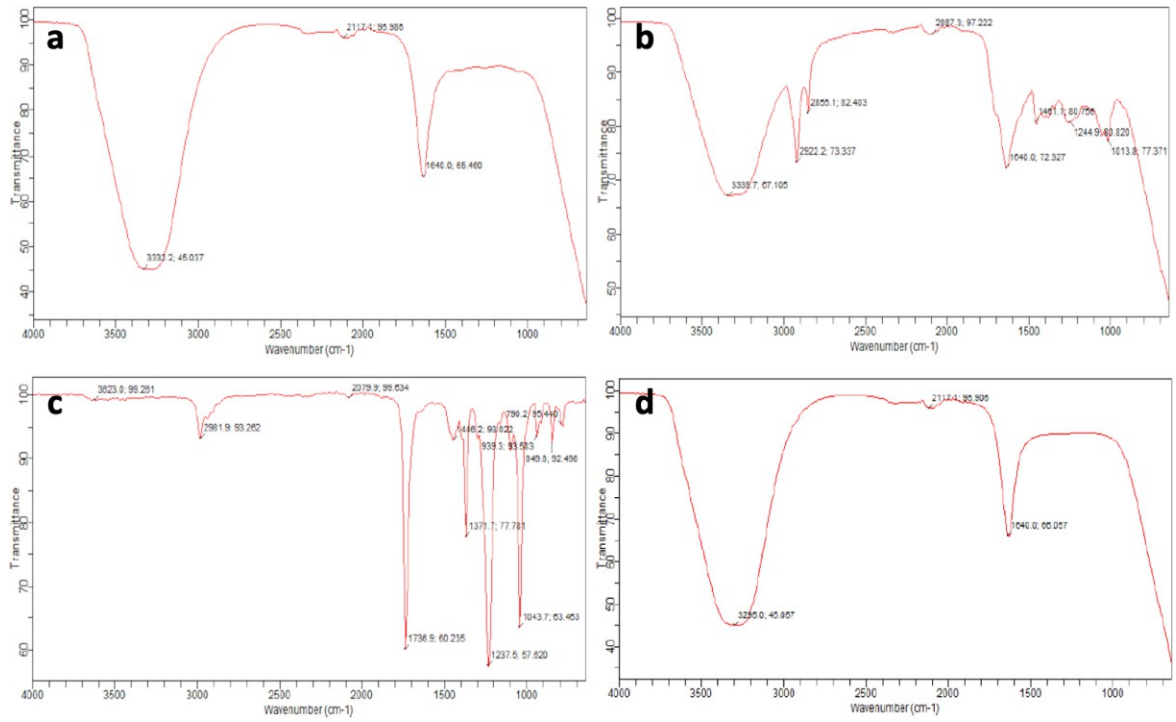

Fig.2 FTIR data of each fractions; (a) water, (b) ethanol, (c) ethyl acetate, and (d) AgNPs.

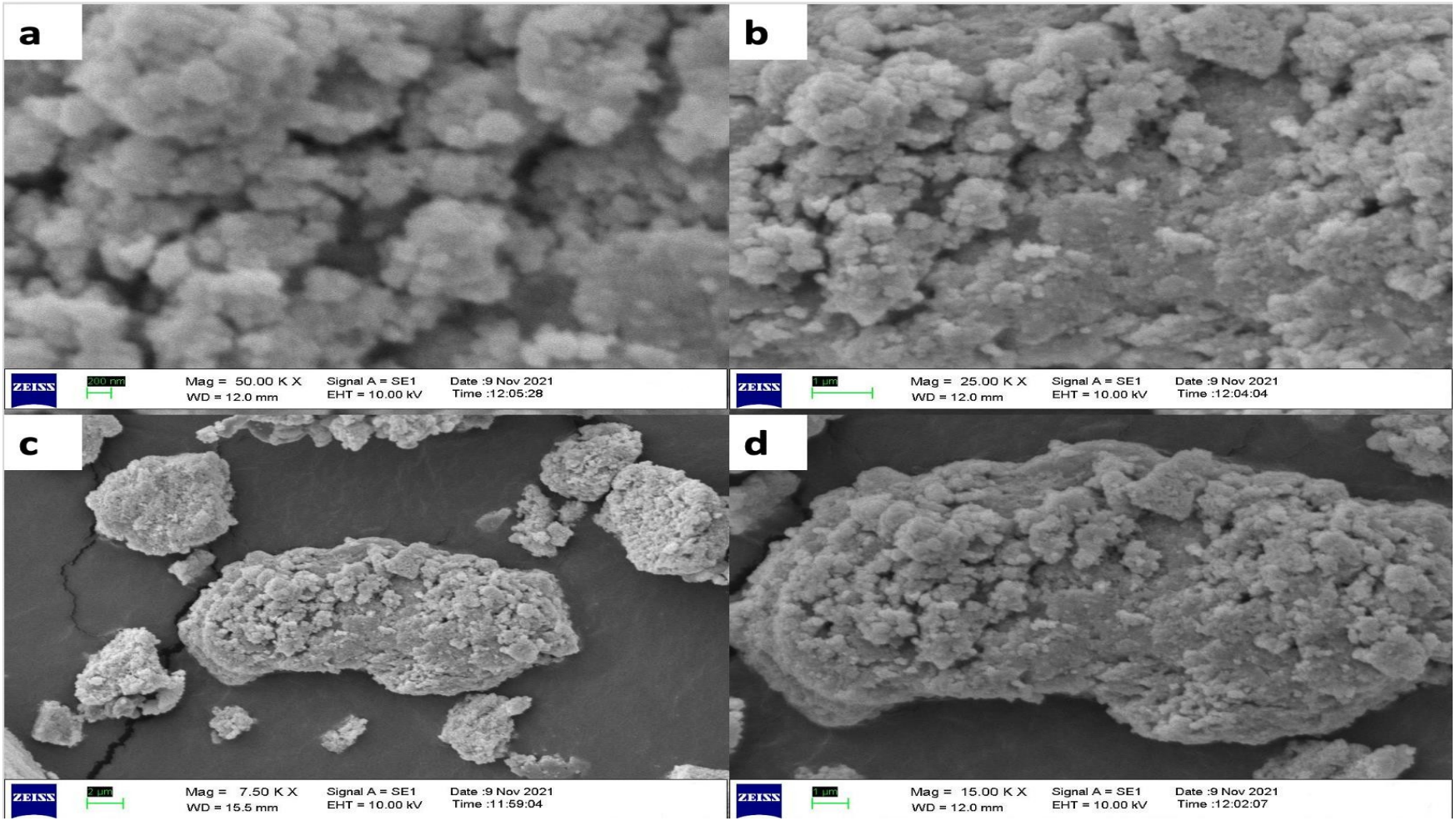

Fig.3 SEM images showed agglomerated and spherical AgN

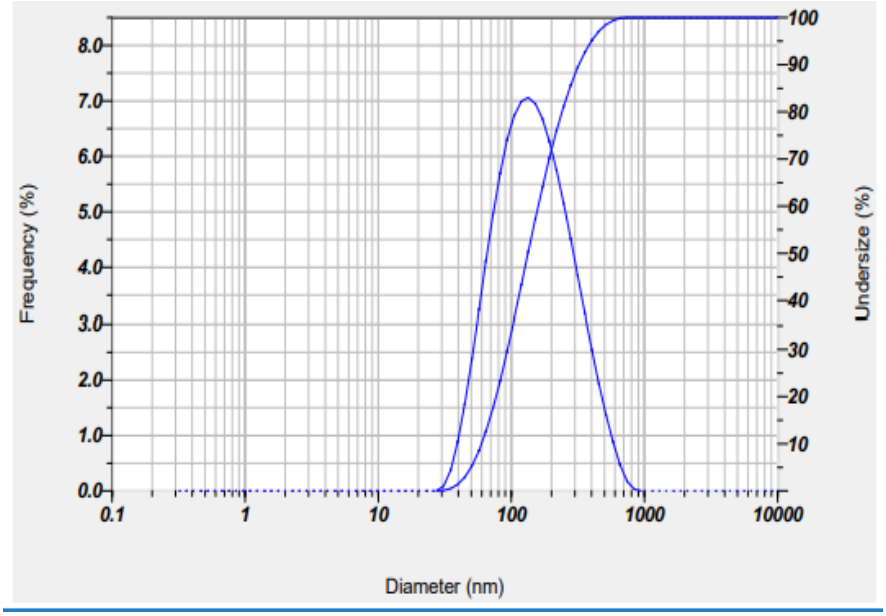

Fig.4 Particle Size Distribution of AgNPs.

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Original Research

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Antituberculosis agents of Lactobacillus plantarum and Pediococcus acidilactici Lactic Acid Bacteria in Breast milk isolates against Mycobacterium tuberculosis

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Abstract: Tuberculosis (TB) is one of the infectious diseases that have become a major problem in Indonesia. This disease is caused by the *Mycobacterium tuberculosis* bacteria. The bacteria are commonly treated with antibiotics. However, the use of irrelevant antibiotics is the most influential factor of antibiotic resistance. Therefore, natural ingredients are required as novel antibiotic agents, some of which are sourced from lactic acid bacteria. This study aims at investigating the antituberculosis activities of *Pediococcus acidilactici* and *Lactobacillus plantarum* isolated from breast milk against *M. tuberculosis*. *Pediococcus acidilactici* and *Lactobacillus plantarum* breast milk isolates were rejuvenated with MRS broth media, and the supernatant was then neutralized to pH 7.0 using a pH meter by adding 1N NaOH solution. Antituberculosis activity test was performed with Lowensten Jensen media to investigate the growth of *M. tuberculosis*. The results of this study showed that *Pediococcus acidilactici* had antituberculosis activity at 48 hours at concentrations of 80% and 90%, while the secondary metabolites of *L. plantarum* had antituberculosis activity at 24 hours and 48 hours at concentrations of 60%, 70%, 80%, 90%. Therefore, this study concludes that *P. acidilactici* and *L. plantarum* bacteria have the potential to be developed as antituberculosis agents.

Keywords: Antituberculosis, Lactic acid bacteria, *Pediococcus acidilactici*, *Lactobacillus plantarum*.

INTRODUCTION

Indonesia is one of the most populous countries in the world, ranking fourth with a population of 273,523,615 people[1]. This large number requires the government to prosper its people because numerous problems are likely to arise, including political, social, economic, and health issues.

One of the prevalent health issues in Indonesia is tuberculosis (TB), an infection caused by *Mycobacterium tuberculosis* bacteria[2], which can trigger symptoms in the respiratory tract. A person suffering from tuberculosis will experience symptoms of continuous cough, fever, and chest pain[3]. This disease can be transmitted through close contact with patients. Droplets resulted from coughing and sneezing are spread by the wind and then stick to other people. Rajni and Laxman[4] reported that this disease is one of the death-causing illnesses that are most difficult to control properly and effectively.

There have been many studies researching tuberculosis and *Mycobacterium tuberculosis*. Palomino and Anandi[5] reviewed the molecular basis

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and mechanism of drug resistance in *Mycobacterium tuberculosis*. Igarashi[6] also investigated the development of antituberculosis drugs derived from natural products. Furthermore, several related studies were conducted to obtain more effective drugs or methods for treating this disease, one of which is the potential of a secondary metabolite as an antimicrobial agent that can inhibit the growth of microbes[7].

Secondary metabolites are antimicrobial peptides produced by bacteria that are effective to treat tuberculosis. These compounds serve as chemical defenses against microorganisms that are resistant to heat. In the pharmaceutical field, these have been widely developed in the food industry and have great potential as antimicrobials that can kill pathogens. Sharma et al[8] reported that secondary metabolites of *Bacillus subtilis* GAS101 can inhibit the growth of *Staphylococcus epidermidis* and *Escherichia coli* in broad ranges of temperature [30-121°C] and pH [2-12]. Aguilar-Perez et al[9] researched the ability of secondary metabolites that have antibacterial activity against *M. tuberculosis*.

Bacteriocins are produced by lactic acid bacteria (LAB). These bacteria are gram-positive, catalase-negative, and rod-shaped, and can perform carbohydrate fermentation[10]. LAB can be found or isolated from a variety of sources, including breast milk. Breast milk is known to contain various bacteria beneficial for babies, particularly those that aid in immune system development. *Staphylococcus*, *Lactobacillus*, *Pseudomonas*, *Enterococcus*, and other bacteria are frequently found in breast milk[11],[12]. *Pediococcus pentosaceus* OZF is isolated from breast milk[13].

Studies on the potential of *L. plantarum* and *P. acidilactici* bacteria as antituberculosis agents isolated from breast milk in inhibiting the growth of *M. tuberculosis* have not been carried out. Therefore, a study that investigates the potential of those bacteria as antimicrobials and their further development into antituberculosis agents that can inhibit the growth of *M. tuberculosis* bacteria is required.

MATERIAL AND METHOD

Rejuvenation of *Lactobacillus plantarum* and *Pediococcus acidilactici* bacteria

L. plantarum and *P. Acicilactici* bacteria isolates were obtained from breast milk[14]. The isolates were then rejuvenated in the medium of MRS agar with 1% $CaCO_3$ and incubated at 35±2ºC for 2-3 days under anaerobic conditions. The clear zone formed around the bacterial colonies was suspected to be LAB.

Isolation of secondary metabolites from *P. acidilactici* and *L. plantarum*

P. acidilactici and *L. plantarum* were cultured in MRS broth. After being cultured on the medium, the bacteria were incubated for 24 hours and then centrifuged to obtain the content of secondary metabolites in LAB. The supernatant produced was saturated with ammonium sulfate to 80% under cold conditions, and the precipitate was filtered and dried. It was then dissolved with potassium phosphate buffer at pH 7 and secondary metabolites with neutral pH were obtained.

The anti-mycobacterium activity of *P. acidilactici* and *L. plantarum*

Antituberculosis activity test of secondary metabolites of *P. acidilactici* and *L. plantarum* isolates from breast milk against *M. tuberculosis* was performed using Lowenstein Jensen (LJ) media. Secondary metabolites of *P. Acidilactici* and *L. Plantarum* with concentrations of 90%, 80%, 70%, and 60% were incubated and then contacted with *M. tuberculosis* for 1 hour, 24 hours, and 48 hours.

RESULTS AND DISCUSSION

Based on the results of the study, isolated microbes of *Lactobacillus plantarum* and *Pediococcus acidilactici* were obtained. *Lactobacillus plantarum* are gram-positive, rod-shaped, convex, and entire, while *Pediococcus acidilactici* are gram-positive, catalase-negative, round-shaped, and non-motile. From the two isolates, secondary metabolites which had been cultured on MRS media were isolated. Further, the secondary metabolites were tested for antimicrobial activity against *M. tuberculosis* as pathogenic bacteria that cause tuberculosis (TB).

The antimicrobial activity test of secondary metabolites of *L. plantarum* and *P. acidilactici* showed different results based on the variations in the contact periods, including 1 hour, 24 hours and 48 hours, as presented in Table 1. These results indicate that the secondary metabolites of each isolate have different abilities in inhibiting the growth of *M. tuberculosis*.

Table 1. The results of antimicrobial activity test of secondary metabolites of *L. plantarum* and *P. acidilactici* with various contact periods with *M. tuberculosis*

Concentration (%)	**Period** (hour)	**Anti-tuberculosis Activity**	
		L. plantarum	***P.acidilactici***
90	1	positive	positive
80		positive	positive
70		positive	positive
60		positive	positive
90	24	negative	positive
80		negative	positive
70		negative	positive
60		negative	positive
90	48	negative	negative
80		negative	negative
70		negative	positive
60		negative	positive

Table 1 demonstrates that secondary metabolite of *L. plantarum* with contact period of 1 hour and concentrations of 60%, 70%, 80%, and 90% show positive results against *M. tuberculosis*. The results exemplify that secondary metabolites that are administered to *M. tuberculosis* are not effective to inhibit the growth of *M. tuberculosis.* With a one-hour initial contact period, the antimicrobial activity of secondary metabolites may not work optimally, allowing *M. tuberculosis* to continue actively growing and reproducing. Figure 1 shows that *M. tuberculosis* is developing rapidly and a large number of yellow bacterial colonies grow on the media.

Positive results were also shown by the secondary metabolite activity of *P. acidilactici* with one hour period and the same concentration variations of 60%, 70%, 80%, and 90% without any inhibitory activity against the growth of *M. tuberculosis* bacteria. Even at a concentration of 90%, the bacteria that cause tuberculosis could continue to grow and reproduce.

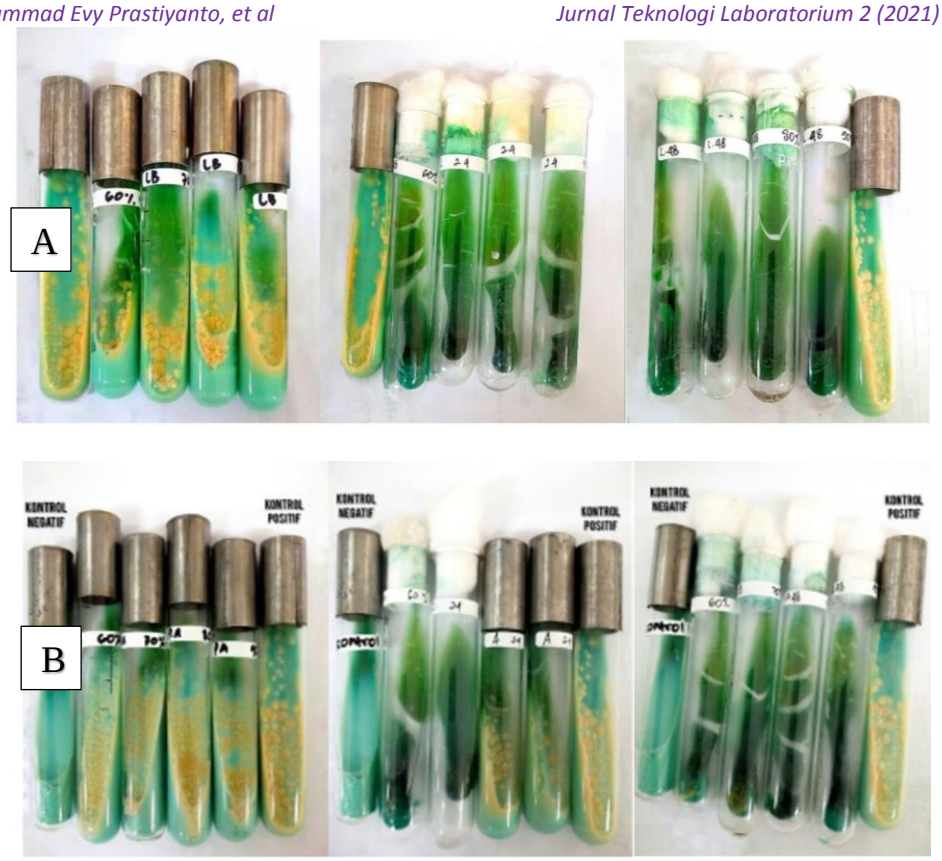

Figure 1. The results of the activity test of secondary metabolites (*L. Plantarum* and *P. acidilactici*) [15] from breast milk isolates against *M. Tuberculosis* with contact periods of 1 hour, 24 hours, and 48 hours

After 24 hours of incubation, significant inhibitory activity was shown by the secondary metabolite of *L. plantarum* against *M. tuberculosis*. All concentration variations (60%, 70%, 80% and 90%) of *L. plantarum* secondary metabolites demonstrated the ability to inhibit *M. tuberculosis*, as indicated by nearly the absence of *M. tuberculosis* bacteria in the growth media.

Further, within the 24 hour incubation period, secondary metabolites of *L. plantarum* were effective in hindering the growth of *M. tuberculosis* even at a concentration of 60%. The study by Lin[15] also reported that secondary metabolites produced by *L. plantarum* showed effective antimicrobial activity against *Vibrio parahaemolyticus*. Moreover, Hasan et al[16] found the activity of secondary metabolites as pathogenic antimicrobials in the isolates from yogurt.

However, different results were shown by secondary metabolites of *P. acidilactici*. With various concentrations (60%, 70%, 80% and 90%) and the same incubation period of 24 hours, the secondary metabolites did not demonstrate any inhibitory activity against *M. tuberculosis*, as summarized in Figure 1. The bacteria causing TB kept growing and reproducing well.

Furthermore, with an incubation period of 48 hours, the secondary metabolite of *L. plantarum* appeared to show better inhibitory (antimicrobial) activity, as proven by no more growth of *M. tuberculosis* on MRS media. In other words, the medium was free from *M. tuberculosis*. Secondary metabolites of *P. acidilactici* with an incubation period of 48 hours also demonstrated the ability to inhibit the growth of *M. tuberculosis*. However, the inhibitory activity only occurred at high concentrations (80% and 90%). Meanwhile, at the concentrations of 60% and 70%, *P. acidilactici* did not show any growth inhibition activity. Gaspar et al. (2018) reported that secondary metabolites can block the growth of urogenital pathogenic bacteria, such as *Gardnerella vaginalis*, *Streptococcus agalactiae* and *Pseudomonas aeruginosa*.

Therefore, these results signify that secondary metabolites are effective in inhibiting the growth of *M. tuberculosis* pathogenic bacteria. In particular, secondary metabolites of *L. plantarum* isolated from breast milk have a better inhibiting ability than *P. acidilactici*. Thus, further research is required to investigate their potential as antituberculosis agents and identify the benefits.

CONCLUSION

Secondary metabolites of *Lactobacillus plantarum* and *Pediococcus acidilactici* can inhibit *M. tuberculosis* bacteria. The secondary metabolites of *L. plantarum* can inhibit the growth of *M. tuberculosis* during the 24 hour incubation period with concentrations of 60%-90%. Meanwhile, the secondary metabolites of *P. acidilactici* demonstrate inhibitory activity at 48 hours of incubation with concentrations of 80% and 90%, respectively. Thus, the secondary metabolites of *L. plantarum* have better antimicrobial ability than *P. acidilactici*. However, both secondary metabolites have the potential as pathogenic antimicrobials to kill *M. tuberculosis*.

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.

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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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In silico study in toxicity parameters of Pigment Derivated Compounds of Monascus sp. mold as a cervical anti-cancer drugs candidate

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Abstract: Toxicity prediction is very important for the development and design of new drugs because computational toxicity estimates are not only faster than the determination of toxic doses in animals, but can also help reduce the number of animal trials. The test uses pkCSM, Protox II, Toxtree, preADMET and T.E.S.T. from the results of research that has been carried out on 57 pigment derivated compounds of Monascus sp. mold, the results of the pkCSM application are 39 test compounds for the Protox-II application there is 1 compound, the Toxtree application produces 1 compound, for the PreADMET application 4 safe compounds are produced, and for the T.E.S.T application produces 1 compound because it fulfills one of the aspects of ICH (International Conference on Harmonization) S9: non-clinical evaluation for anticancer pharmaceutical 2010 and has the potential as a candidate for anticancer drugs.

Keywords: Monascus sp., Toxicity, In silico.

INTRODUCTION

With the existence of modeling predictions by QSAR, it is possible to predict drug toxicity easier and perhaps even before drug synthesis. Quantitative Structure–Activity Relationships (QSAR) has been widely used in toxicology to predict the liability of novel compounds using structural features of known toxicants. During the last 40 years, many QSAR models have been published predicting carcinogenicity[1] and mutagenicity[2]. Toxic effects are generally categorized according to the site of the toxic effect. In some cases, the eff[3]-activity relationship has been widely used in the world to predict toxicity using computers[4,5].

One of the new drug candidate alternatives is the pigment produced by Monascus sp. Monascus sp. is one type of mold used for fermentation of Angkak. One of the metabolites produced by Monascus sp. is a pigment. Monascus sp. pigment research has progressed very rapidly, there are 3 main pigments from Monascus sp. namely yellow, orange and red. From these main pigments, several pigment-derived compounds are found which are widely used as natural coloring agents in the textile, food, pharmaceutical and cosmetic industries[6,7,8,9]. According to research by Singgih[10], 19 pigment derivates from Monascus sp. have been tested to have smaller bond energy value than Genistein as natural

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ligand. Genistein is an anti-cancer drug that has strong potential in the treatment and prevention of breast cancer because it has anti-proliferative properties. Genistein has an antiesterogenic effect, namely the ability to stop the estrogen hormone binding to cells in order to prevent cancer from growing and dividing. Through binding to ERβ through inhibition of the activity of enzymes involved in estrogen metabolism. Monankarin C with the smallest bond-free energy value is -11.08 Kcal / mol, which means the most stable complex conformation, genetic compounds form hydrogen bonds with Arg346, Glu305, and His475. Monankarin C also has the same interaction so that it is expected to have a better affinity than natural ligands.

The compounds with the next lowest free energy are PP-V Monaskorubin and Monaskin, the best compounds as a candidate for breast cancer drugs, namely Monaskin pigments because they have smaller bond energies than natural ligands. However in PP-V, Monascorubrin, Monankarin C, although they have a small bond energy, this compound is seen from the toxicity test of Monarkarin C which is mutagenic, carcinogenic and toxic to reproduction, whereas in PP-V and Monascorubrin it is toxic to reproduction. Many have been shown to have anticancer activity, but their use is limited due to side effects and high toxic effects[11]. Nevertheless, toxicity can be assessed using computational resources (computational algorithms, software, and data) to organize, analyze, model, simulate, visualize, or predict chemical toxicity[12,13,14,15].

MATERIAL AND METHOD

2.1 Materials and Research Tools

The tools used are the HP brand laptop (3D248N5S laptop), Windows 10 operating system, 64 bit operating system x64- bassed processor, AMD A9-9420 RADEON 5R processor, 4.00 GB RAM, 5 COMPUTER CORES 2C + 3G 3.00 GHz. The programs used are Chem Bio Draw Ultra Version 12 (CambridgeSoft), Chem Bio 3D Ultra Version 12 (CambridgeSoft), and T.E.S.T.

Online Tools for Toxicity Test used are PreADMET (https://preadmet.bmdrc.kr), pkCSM online tool (http://biosig.unimelb.edu.au/pkcsm/prediction), ToxTree (http://toxtree.sourceforge.net) and Protox-II (http://tox.charite.de/tox/). The materials used were 57 derivated compounds of Monascus sp.6

2.2 Materials Preparation

Prior to the in silico toxicity test, 57 pigment derivated compounds of Monascus sp., their molecular structures were drawn using Chem Draw Ultra Version 12, stored in *.sdf or *.pd files and then made in *SMILES* form using the Chem Draw application.

2.3 Prediction of Toxicity

Prediction of the toxicity of the pigment derivated compounds of *Monascus* sp. using online tools [16,17,18]Prior to the *in silico* toxicity test, 57 pigment derivated compounds of *Monascus* sp. depiction of 2-D molecular structures with Chem Bio Draw Ultra Version 12, then in the Chem Bio 3D Ultra Version 12 program to create 3-D structures, then saved in the form of * .sdf or * .pd files. Next, the 57 pigment derivated compounds of *Monascus* sp., The structure is converted into the *SMILES* format with the Chem Draw application. pkCSM online tool (http://biosig.unimelb.edu.au/ pkcsm / prediction). To predict the toxicity (LD_{50}) orally on rodent and the classification of toxicity of compounds based on the Globally Harmonized System (GHS), the Protox online tool (http://tox.charite.de/tox/) was used[19]. The toxicity of the test compounds includes prediction of mutagenesis, carcinogenicity and acute toxicity (LD50). Mutagenesis prediction uses ADMET predictor, T.E.S.T, Protox-II (http://tox.charite.de/protox_II/), Toxtree (http://toxtree.sourceforge.net/),and pkCSM (http://biosig.unimelb.edu.au/ pkcsm/).

RESULTS AND DISCUSSION

Based The toxicity of drug impurities is also closely related to their structure. Structure-activity relationships have been widely used to predict toxicity using[4,5]. The determination of toxicity testing criteria is based on a protocol issued by the ICH (International Conference on Harmonization) S9: non-clinical evaluation for pharmaceutical anticancer, 2010, which includes: General toxicity includes MTD (Maximum Tolerated Dose), and LOAEL (Low observed adverse effect level), toxicity to reproduction or fetal development (DTP = Developmental toxicity potency), mutagenicity and carcinogenicity.

There are many tools for predicting the toxicity of a molecule, some of which are commercial in nature, some of which are online web servers and some of which can be downloaded freely. In the prediction of toxicity[20] in silico, the type of toxicity assessment uses computational resources (algorithms, software, and data) to organize, analyze, model, simulate, visualize, or predict chemical toxicity20. In predicting the toxicity of in silico, pigment derivated compounds of Monascus sp. mold was performed using pkCSM, Protox II, Toxtree, preADMET and T.E.S.T. The data generated from this in silico test can be used to design further testers.

The performance of the pkCSM software in the external validation dataset showed 83.8% accuracy in the mutagenicity test. There are several pkCSM end points, LD50, AMES test, maximum daily dose, and hepatotoxicity[17]. Based on our research, the toxicity data obtained from the pkCSM application for liver toxicity (hepatotoxic) contained 15 positive compounds that could cause damage to the liver, for mutagenesis data on the pkCSM application used the AMES method namely mutagenesis validation test method. There are 5 compounds that are mutagen positive.

The prediction of ProTox-II hepatotoxicity has a balanced accuracy of 82.00% on cross validation and 86.00% on external validation. In our research for hepatotoxicity, it is not stated that there are compounds that cause damage to the liver. Drug-induced hepatotoxicity is a significant cause of acute liver failure and one of the main reasons for drug withdrawal from the market.

Drug-induced liver injury (DILI) is a chronic process or rare event. The conceptual mechanism of DILI is direct cell stress, direct mitochondrial damage and specific immune reactions for the carcinogenicity test, 19 compounds were identified that could affect genes and damage normal cells so that they could become cancer cells. For mutagenesis data carried out using this application, it was found that there were no compounds that could cause mutations. For LD50, there are 2 compounds belonging to class II, 24 compounds belonging to class III, 28 compounds belonging to class IV and there are 3 compounds belonging to class VI based on the Globally Harmonized System[21,22.]

Toxtree's performance in the external validation dataset shows 70% accuracy and 78.3% sensitivity in the carcinogenicity test and 78% accuracy for the mutagenicity test. Toxtree represents the end point of various toxicities, namely the Cramer Rules to see from its functional groups, Kroes TTC to estimate the exposure threshold for drug compounds in humans, Benignin Bosa rules for carcinogenicity (genotoxic and non-genotoxic) and mutagenicity in vitro (AMES test)[23,24.]

In the Toxtree test according to Cramer's rules, there is 1 compound belonging to class I, which is the lowest level of toxicity, while 56 other compounds are included in class III, which means that the highest level of toxicity contains a functional group, the substituent ring which is a marker for the compound is toxic and even possible to have toxicity which is significant and it is estimated that this compound is not guaranteed its safety because it is a substance with a chemical structure that does not allow initial assumptions of safety or may even suggest significant toxicity or has reactive functional groups[25]. For the Benignin / Bossa rules parameter, which is to determine which

compounds can cause carcinogenicity and mutagenicity, 57 compounds tested positive for mutagens were obtained. This is intended as an initial stage of mutagenic in vivo screening, molecular functional groups that are known to be related are aromatic polycyclic hydrocarbons and heterocyclic molecules that can form non-covalent interactions with proteins or DNA bonds that have the potential to be genotoxic[26]. PreADMET provides 62.5% accuracy and 52.2% sensitivity for carcinogenicity tests[27]. PreADMET predicts toxicity based on AMES mutagenicity parameters, the actual predictive value is "positive" or "negative"[21].

Mutagenicity testing used the PreADMET application using the AMES method and the results obtained were 21 compounds that were positive for mutagens, and for carcinogenicity (Carcino Rat) there were 9 compounds that were negative for carcinogens. The compounds tested using the T.E.S.T application contained 9 positive mutagens and 7 N/A compounds (Not Applicated) and 41 other compounds were negative mutagenic, in this application there were N/A results because using the FDA method: Predictions for each chemical test were made using a new model corresponding to the chemical most similar to the test compound. Each model is generated at runtime which is the reason these 7 compounds cannot be translated into this application.

The toxicity test carried out with 5 different applications for toxicity parameters, it can be concluded that there are 4 compounds: N-glutaryl Monascurobamine, Monankarin F, Monaphilones A and Monaphilones B, that meet the criteria issued by ICH (International Conference on Harmonization) S9: non-clinical evaluation for pharmaceutical anticancer (2010) because they have a low level of toxicity from the 57 compounds tested. Whereas for testing each application, the best compound results for anticancer drug candidates according to ICH is as follows: for the application of pkCSM, the best compounds that have the potential as anticancer drug candidates are 39 test compounds, for the Protox-II application there is 1 safe compound; Xantomiscin B because it is included in class VI for LD50 and negative for carcinogens and mutagens, the Toxtree application produces 1 safe compound; N-glucosyl rubropuctamine because according to Creamer rules it is categorized as class 1 which means has a low level of toxicity, for the application of PreADMET 4 safe compounds are produced, namely *Monaspyridine* A, *Monaspyridine* B, *Monaspyridine* C, and *Monaspyridine* D, while for the T.E.S.T application produced 1 safe compound, namely Monaphilol A with negative mutagen and LD50 toxicity category including in class V.

CONCLUSION

Research on 57 pigment derivated compounds of *Monascus* sp. mold, the results were obtained for the application of pkCSM, the best compound that has the potential as an anticancer drug candidate are 39 test compounds, for the Protox-II application there is 1 safe compound, namely *Xantomiscin* B, the Toxtree application produces 1 safe compound, namely *N-glucosyl rubropuctamine*, for PreADMET applications 4 Safe compounds which are *Monaspyridine* A, *Monaspyridine* B, *Monaspyridine* C, and *Monaspyridine* D are produced, while for the T.E.S.T application, 1 safe compound is produced, namely *Monaphilol* A because it fulfills one of the aspects of ICH (International Conference on Harmonization) S9: non-clinical evaluation for anticancer pharmaceutical 2010 and has potential as an anticancer drug candidate.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to this work.

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Table 1. Toxicity Prediction Results of 57 Derivated Compounds of Monascus sp. Using pkCSM, Protox II, Toxtree PreADMET, and T.E.S.T

No.	Compounds	pkCSM		Protox-II		ToxTree	PreADMET	T.E.S.T
		Mutagenicity	Hepatotoxicity	Mutagenicity	Hepatotoxicity	Mutagenicity	Mutagenicity	Mutagenicity
1	*N-glucosyl rubropunctamine*	+	+	-	-	+	-	-
2	*N-glucosyl monascurobamine*	-	+	-	-	+	-	-
3	*N-glutaryl Monascurobamine*	-	-	-	-	+	-	-
4	*N-glutaryl Rubropuctamine*	-	-	-	-	+	-	-
5	Red Derivat 1	-	+	-	-	+	-	-
6	Red Derivat 2	-	+	-	-	+	-	+
7	Red Derivat 3	-	-	-	-	+	-	-
8	Red Derivat 4	-	-	-	-	+	-	-
9	Red Derivat 5	-	+	-	-	+	-	-
10	Red Derivat 6	-	+	-	-	+	-	-
11	Red Derivat 7	-	+	-	-	+	+	N/A
12	Red Derivat 8	-	+	-	-	+	+	N/A
13	Isolat MPs 4	-	-	-	-	+	+	N/A
14	Isolat MPs 3	-	-	-	-	+	-	N/A
15	Isolat MPs 2	-	+	-	-	+	+	N/A
16	Isolat MPs 1	-	-	-	-	+	+	N/A
17	New Red Pigment	+	-	-	-	+	-	+
18	Compound 3	-	-	-	-	+	-	+
19	*Monaspyridine A*	-	-	-	-	+	-	-
20	*Monaspyridine B*	-	-	-	-	+	-	-
21	*Monaspyridine C*	-	-	-	-	+	-	-
22	*Monaspyridine D*	-	-	-	-	+	-	-
23	Red Shandong 1	-	+	-	-	+	+	-
24	Red Shandong 2	-	+	-	-	+	-	-
25	PP-V	-	-	-	-	+	+	N/A
26	*Glycyl-rubropuntatin*	-	+	-	-	+	-	-
27	Un Named	-	-	-	-	+	-	+
28	*Xantomanascin A*	-	-	-	-	+	+	-
29	*Xantomanascin B*	-	-	-	-	+	+	-
30	Yellow II	-	-	-	-	+	+	-
31	*Monankarin A-B*	-	+	-	-	+	-	+
32	*Monankarin C-D*	-	+	-	-	+	-	-
33	*Monankarin E*	+	+	-	-	+	-	-
34	*Monankarin F*	-	-	-	-	+	-	-
35	*Monapurones A*	-	-	-	-	+	-	-
36	*Monapurones B*	-	-	-	-	+	+	-
37	*Monapurones C*	-	-	-	-	+	+	-
38	*Monaphilones A*	-	-	-	-	+	-	-
39	*Monaphilones B*	-	-	-	-	+	-	-
40	*Monaphilones C*	-	-	-	-	+	-	-
41	*Monashexonone*	-	-	-	-	+	-	-
42	*Rubropuctin*	-	-	-	-	+	-	-
43	*Monarubrin (Y,BF)*	-	-	-	-	+	-	-
44	*Purpureus One*	-	-	-	-	+	-	-
45	*Monascuspiloin*	-	-	-	-	+	-	-
46	*Monascusone A*	-	-	-	-	+	-	-
47	*Monascusone B*	+	-	-	-	+	+	+
48	FK-17-P2B2	+	-	-	-	+	+	-
49	Y3	-	-	-	-	+	+	-
50	*Monaphilol A*	-	-	-	-	+	+	-
51	*Monaphilol B*	-	-	-	-	+	+	+
52	*Monaphilol C*	-	-	-	-	+	-	-
53	*Monaphilol D*	-	-	-	-	+	-	+
54	*Monasfluor A*	-	-	-	-	+	+	-
55	*Monasfluor B*	-	-	-	-	+	+	-
56	*Monascuskaodione A*	-	-	-	-	+	+	+
57	*Monascuskaodione B*	-	-	-	-	+	+	-

(+) : Positive
(-) : Negative
N/A : Not Applicable

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Anti Depression Potential of Papaya Seed Extracts in Wistar Rat Models: a Study on Body Weights, Blood Glucose, Interleukin-6 and Malondialdehyde Levels with Force Swimming and Tail Suspension Tests

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Abstract: The purpose of this study is to evaluate the potential of papaya seed extract as an antidepressant based on body weight, glucose levels, Interleukin-6, and malondialdehyde levels in treated rats. Force swimming and tail suspension tests have been conducted to measure acute stress and evaluate response behavior. Results showed that the higher concentration of papaya seed extract correlates to higher effectivity on reducing body weight and lowering glucose levels. Dose-response relationships were also observed on the inhibition effect of interleukin-6 and malondialdehyde more effective at higher extract concentrations. The experimental force swimming test and tail suspension test showed the positive effect of extract administration indicated by the shorter immobilization time. Gas chromatography analysis has confirmed the bioactive content in the extract. The main compounds such as; 9-Octadecenoic acid (Z)-methyl ester, Benzyl nitrile, Hexadecanoic acid- methyl ester, and other saponins might be responsible for the antidepressant effects of papaya seed extract. This research encourages further studies on pharmacodynamics, pharmacokinetics, and biomolecules analysis at cellular levels.

Keywords: Antidepression, Papaya seed extract, Saponins, Tail Suspension, Force, Swimming, IL-6, MDA.

INTRODUCTION

Moderate or severe stress can lead to depression[1] and suppress the immune response[2], such as interleukin-6. The IL-6 plays a role in stress reactions and depressive disorders, especially the physical disturbances that accompany depression in the presence of hypothalamic-pituitary-adrenal (HPA) activity[3]. The active transport systems peripherally release cytokines from the blood to the brain. The IL-6 is one of the most investigated cytokines in animal studies of Major Depressive Disorder (MDD). However, genetic studies of IL6 effects in MDD remain controversial. Increased activity of IL-6 causes depression through activation of HPA or by the influence of neurotransmitter metabolism[3,4]. The endocrine and immune systems are integrated through a bidirectional network in which hormones influence immune function, while immune responses are reflected in neuroendocrine changes.They play a role in modulating the response of the HPA at all three levels: hypothalamus, pituitary, and adrenal. Acute effects

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of cytokines are seen on the central nervous system, particularly the hypothalamus[5,6]. Free oxygen radicals increase due to high respiratory oxygen intake and metabolic turnover during stress. During stress, increased energy requirements caused by poor environmental conditions, backbreaking physical work, and psychological trauma (PTSD) require high oxygen intake to meet energy requirements[5]. Prolonged working hours, workload, fatigue, sleep deprivation, psychological trauma resulting oxidative injury indicated by the formation of 8-hydroxy-deoxyguanosine (8-OH-dG) as a biomarker of oxidative DNA damage[5,7].

Herbal medicine as an alternative to cure acute stress has been known for centuries. The active metabolites contained in plant extracts may contribute to the antidepressant effects to recover the symptoms of depression. Bioactive content in plants such as alkaloids, flavonoids, saponins, tannins, and terpenoids have the ability as an antidepressant as good as tricyclic antidepressant drugs, i.e., amitriptyline[8]. Activating neurotrophic factors, such as BDNF will significantly impact neuronal function. Activating neurotransmitter systems, e.g., primarily serotonergic, noradrenergic, and dopaminergic at specific brain regions such as the hippocampus and prefrontal cortex, will activate a long-term chemical communication[8,9].

Several metabolic compounds such as alkaloids, flavonoids, and saponins from papaya seeds have been reported recently. Some studies reported the potential of papaya seed extract as anti-diabetes[10], lowering glucose level[11], hypoglycemic[12], antiobesity[13]. However, a lack of studies reported on the antidepressant effect of papaya seed extracts in rat models. Therefore, here we report an inclusive analysis of the physiological and biomolecular levels of the antidepressant effect of papaya seed extract. This report covers the effect of antidepressants on the body weights, glucose levels, MDA, IL-6 of Wistar rats under treatment with the Forced Swimming Test (FST) and Tail Suspension Test (T.S.). The efficacy of papaya seed extracts has been compared with amitriptyline 0.01 % (w/v) as a positive control.

MATERIAL AND METHOD

2.1. Materials and Tools

Wistar rats 2-3 months, feeding CP 551, amitriptyline 0.01% w/v, aquadest, filter paper, ethanol 96%, papaya seed, IL-6 reagent (EliKine TM Rat IL-6 ELISA Kit) and MDA Bioassay Kit, Glucose ACCU Check, Digital Balancer, Phytochemical tests reagents, and glassware apparatus.

2.1. Methods

Papaya seeds were dried, crushed, weighed 10 g, then heated in an infusion pan, mixed with 100 cc of distilled water. When the temperature reaches 90 ^{o}C let it settle for 15 min, then occasionally stir. Then filter solutions and add enough hot water to the dregs so that the required volume of infusion is 100 ml (10%). Precondition of Wistar rats was provided by feeding, namely CP 551 for one week. Wistar rats were randomly divided into six groups where each group consisted of 5 individuals:

1. Group K = Normal, no stressed, aquadest.
2. Group K- = Negative control, stress, and aquadest.
3. Group K+ = Positive control, stressed, and amitriptyline 0.01% w/v.
4. P1 = Treated with stressed and 10% extract, and amitriptyline 0.01% w/v.
5. P2 = Treated with stressed and 8% extract, and amitriptyline 0.01% w/v.
6. P3 = Treated with stressed and 6% extract, and amitriptyline 0.01% w/v.

The treated groups were placed in a stress inducer illuminated by a 60-watt lamp for 30 min every day. The Force Swimming Test (FST) was conducted by placing the rats in cylindrical tanks partially filled with water, and the immobilization time gets measured[14]. The Tail Suspension Test (TST) was

conducted by hanging the rat tails with tape, and the time of each mouse moving get measured[15].

The blood collection from the heart vessels was conducted by dislocation of cervical vertebral followed by surging the chest cavity to collect 3 ml of blood from the heart. The blood was centrifuged for 10 min at 3000-4000 rpm and let precipitate to get serum layer, then put serum in a microtube and stored at -4 ^{o}C. Blood serum was then analyzed by the ELISA method. To check the IL-6, serum was inserted into the wells given with a Streptavidin-HRP and its reagents. Then incubate for 15 min at room temperature and protect from light. Determine the optical density of each well within 30 min, using a microplate reader set to 450 nm. If wavelength correction is available, then set to 540 nm or 570 nm. To check the MDA, the precipitated protein was resuspended in 2.5 ml of acetic acid and added by 3 ml of thiobarbituric acid (2g/L in 2 mol/L Na_2SO_4). The solution was entirely mixed by vortex then closed and sealed with parafilm. The lid with the tube was slightly perforated with a needle. The entire solution was incubated at a temperature of 950 C for 45 min later, cooled with running water, then centrifuged at 3500 rpm for 10 min. The supernatant formed was then put into a cuvet, and its absorbance was measured at 532 nm.

Data are presented in mean ± standard deviation (mean ± S.D.). Tests for normality and homogeneity of data were carried out. If the data is normally distributed and homogeneous, then One Way Anova was applied. If the distribution data is not normal and not homogeneous, then Kruskal Wallis was applied, and also the Post Hoc test with α ≤ 0,05 was considered significant.

RESULTS AND DISCUSSION

3.1 Phytochemistry and GCMS Analysis

The content in the papaya seed extract (Carica Papaya L.) only consists of saponin groups. Unfortunately, the alkaloids, flavonoids, tannins, steroids, and triterpenoids were indicated negative by a preliminary screening of phytochemistry tests.

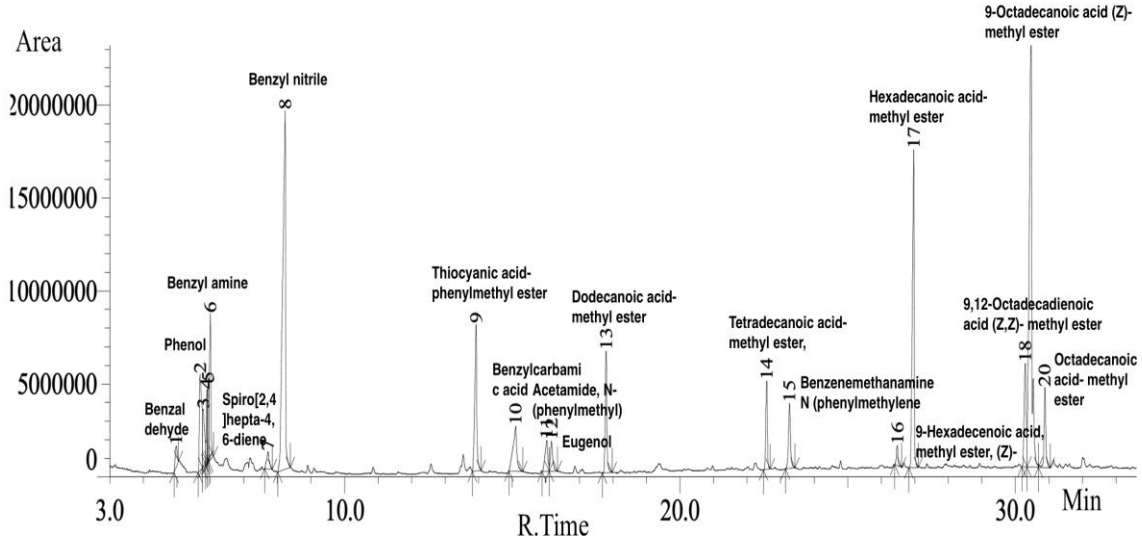

Fig. 1. GCMS Chromatogram of Papaya Seed Extract

The organic compounds in the papaya seeds extract are presented in Figure 1. Based on the peak area (%) of the GCMS data, the main compound was 9-Octadecenoic acid (Z)- methyl ester, a kind of fatty acid methyl ester group, which known as antioxidants[16,5]. Several studies have linked antioxidant effects to phenols and their function as inhibitors of α -glucosides[17,18]. Secondly, Benzyl Nitrile was a precursor for various drugs with potential recreational use[1]. The third primary compound was Hexadecanoic acid-methyl ester, which has

antioxidant and anti-inflammatory compound activity[19,20,21]. Generally, plant phenolics are considered vital components of the human diet and exhibit remarkable antioxidant activity and other health benefits[22]. Even though at a small quantity, eugenol was very well known as a component of clove essential oil and exhibited antibacterial, analgesic, anti-inflammatory, and antioxidant properties[23].

3.2 Body Weight Analysis

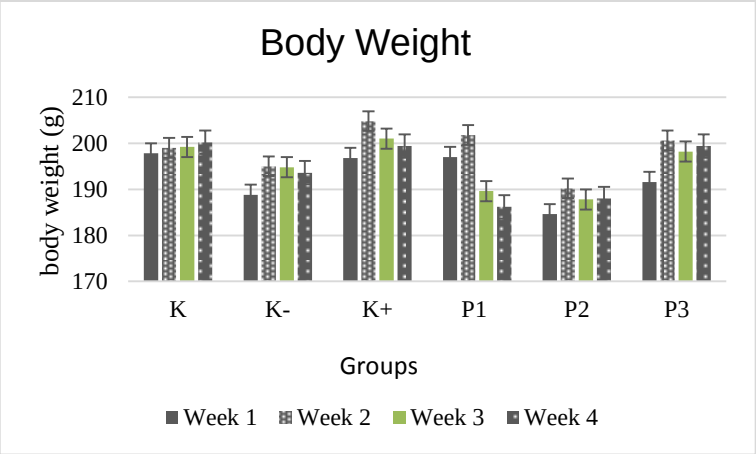

Fig. 2. Body weight of all groups during four weeks of treatment.

Figure 2 shows the differences in body weights of all groups. The body weight decreased significantly in the P1 (186.20 ± 32.136) compared to other groups. Meanwhile, in group K (200.20 ± 47.199), P2 (188.00 ± 23.335) and P3 (199.40 ± 24.966) experienced an increase. There was a decrease for the K+ group (199.40 ± 12.985) but not significant. There was an increase in body weight in the 3rd and 4th weeks of the P2 and P3 groups. However, the weight loss in the P1 group due to the highest concentration of papaya seeds extract at 10%. Where the possibility of the number of active compounds in the content has a more significant effect, based on these data, we can conclude that the higher concentration of papaya seeds extract correlate to higher effectivity on reducing body weight in rat models.

In addition, a specific treatment will reduce the hypothalamic NPY expression and serum leptin in high-fat diet mice[24]. A study showed that chronic stress on repeated Force Swimming Tests reduced body weight and calorie efficiency[25], where the pancreatic lipase enzyme shows an inhibitory effect[13]. While stressed, corticotropin hormone (CRH) will be released from neurons in the parvocellular division[26]. The corticotropin can reduce appetite[23]. Neuropeptide Y also stimulates the inhibition of incoming food (22,23). Overall, stress could have an impact on food intake[25].

3.3 Glucose Levels Analysis

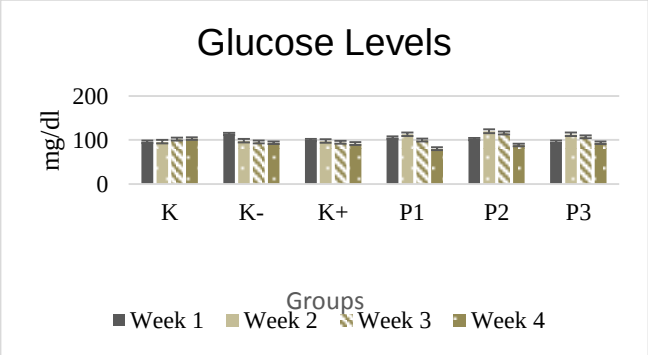

Fig. 3. Glucose levels of all groups during 4 weeks of treatment.

Figure 3 showed a significant effect of papaya seed extract on reducing blood sugar levels of group P1 (80.20 mg/dL ± 15.057). This effect of P1 was better than the P2 (88.80 mg/dL ± 10.86) and P3 (93.60 mg/dL ± 12.661). Papaya seeds contain saponin compounds that have similar activities to insulin. Based on these data, we can conclude that the higher concentration of papaya seed extract correlates to lower glucose levels in the rat.

Saponin could inhibit lipolysis and increase glucose uptake by adipose cells[27]. This study proposed saponins to reduce blood sugar levels with an antihyperglycemic mechanism by stimulating insulin release in pancreatic β-cells[28]. This result was in line with some studies that papaya seed extract could lower blood sugar levels[29,30]. The active substance in papaya seeds may have a hypoglycemic effect that stimulates insulin release from pancreatic beta cells and somatostatin release by suppressing glucagon secretion[10]. In addition, the antioxidant could reduce blood glucose levels by improving pancreatic function (by regenerating cells) to increase insulin production[11]. The main compound, 9-Octadecenoic acid (Z)-, methyl ester, might affect and inhibit the α -glucosidase enzyme activity and minimize the blood glucose level[31].

3.4 Analysis of Interleukin-6

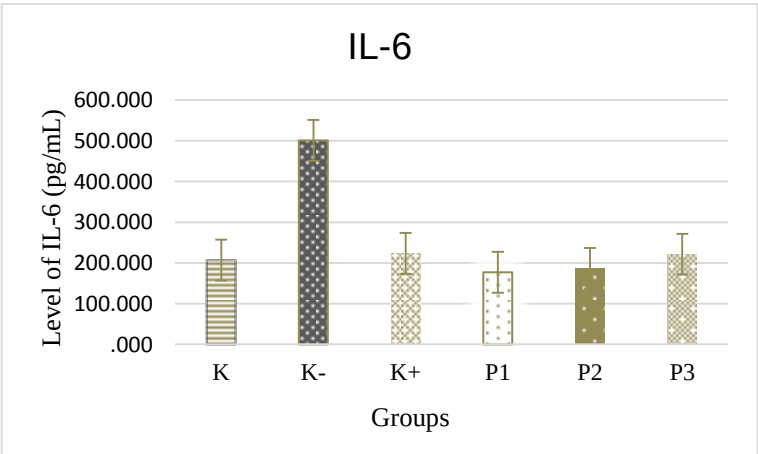

Fig. 4. Level of IL-6 of all groups

Based on the Post Hoc test, the average group was divided into two different groups. The first group is the group of the same average that consists of group P1. The second group consisted of K, K-, K+, P2, and P3. It can be seen that the lowest average of IL-6 levels was P1 (177.3075 pg/ml) and the highest average was K- (500.9739 pg/ml). It was found that the p-value was 0.0489 or

<0.05, which indicated a significant difference in IL-6 between the treatment groups. Based on these data, we can conclude that the higher concentration of papaya seed extract might correlate to lower levels of IL-6.

A study by Nukina et al. (2008) has tested whether stress induces an increase in plasma IL-6 in mice. Plasma IL-6 concentrations increased after one hour of restraint stress and, after that, gradually decreased, indicating that restraint stress could increase plasma IL-6 levels. Further studies found that the induction of increased plasma IL-6 levels by restraint stress was independent of the gut microflora, the primary source of the increase being the liver during stress[32,13]. An increase in IL6 mRNA expression and a fourfold increase in circulating IL-6 levels in the rat hypothalamus was found upon application of restraint stress (3,45). A recent study using prolonged restraint stress in mice also found increased circulating expression of IL 6 mRNA[3,33].

Regarding the metabolite content in the papaya seed extract, we expect that the saponin groups affect T lymphocyte cells in producing cytokinins, thereby suppressing the release of IL-6 activity. While stressed, the IL-6 activity increased, whereas added by plant extract, the IL-6 decreased. In addition, 9-Octadecenoic acid (Z)-, methyl ester may affect IL-6 and play a role in immune cells. However, the process is quite complex, so that more in-depth research is needed to find out. They serve as energy sources and structural components of cell membranes, as signaling molecules and precursors for synthesizing eicosanoids and similar mediators. Another research showed that the localization and organization of fatty acids into different cellular assemblages directly influences the behavior of several proteins involved in immune cell activation, including those related to T cell response, antigen presentation, and fatty acid-derived intermediates as inflammatory agents[34].

3.5 Analysis of Malondialdehyde (MDA)

Figure 5 showed a significantly low MDA level in the P1 group compared to P2 and P3. The positive control (K+) was higher than the P1, with a mean value of 2.2996 ng/ml. Based on the Post Hoc test, it was obtained that the p-value was 0.0464 or < 0.05. This indicated a significant difference in MDA between the treatment groups. Based on these data, we can conclude that the higher concentration of papaya seeds extract might correlate to lower levels of MDA.

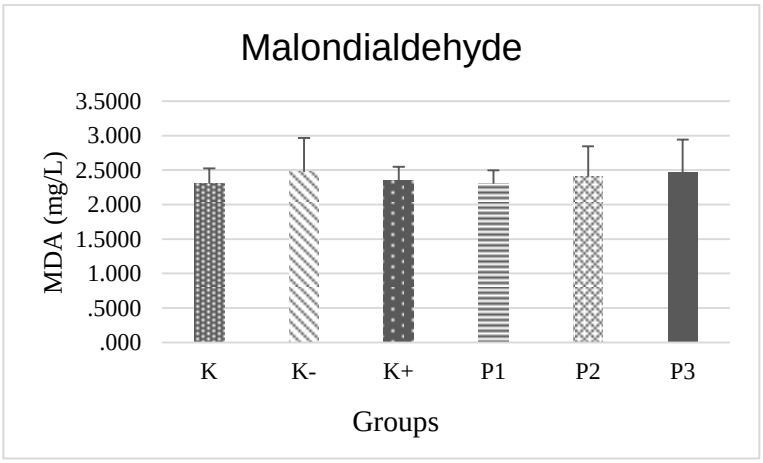

Fig. 5. Level of MDA of all groups

In our study, the compound of 9-Octadecenoic acid (Z)- methyl ester was expected to entrap the free radicals so that MDA lowered. In contrast, the increase of MDA levels was in line with oxidative stress in the body[35]. Papaya seeds have been shown to reduce blood glucose levels and contain flavonoids,

alkaloids, and tannins as a source of antioxidants that can entrap free radicals. A study reported the effect of giving papaya seed extract on blood plasma MDA levels in alloxan-induced rats[36]. MDA production was caused by free radicals in the plasma membrane, which causes inflammation. Another study on binahong extract reported an increase in MDA levels at the time of administration caused by the saponin content of binahong. However, this contrasts with another result that showed a decrease in MDA levels in high-dose ethanol extract of binahong leaves, and the causative factors were not known certainly[37].

3.6 Analysis of Forced Swimming Test (FST)

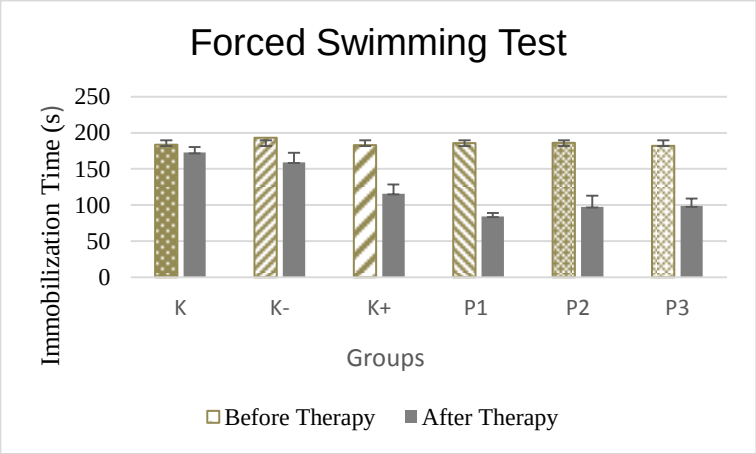

Fig. 6. Forced Swimming Test (FST) of all groups at before and after therapy

The Force Swimming Test showed that the immobilization time in rats decreased significantly in the P1 compared to the P2 and P3 groups. The group was divided into three different groups. The first group was the same average group consisting of P1, P2, and P3. The second group had an average length of FST which consisted of P3 and K+. The third group had the same average length of FST immobilization time consisting of groups K- and K. It was seen that the lowest average FST immobilization was P1=84.20 s and P2=93.60 s. Based on these data, we can conclude that the higher concentration of papaya seeds extract might reduce the immobilization time of FST.

In this study, a group of saponins were found in papaya seeds and might affect neurobehavior. This can be seen from the behavioral test through the FST, where the immobilization time in rats decreased during the FST examination. This occurs because of the involvement of noradrenergic activity[12,35]. Nevertheless, it does not affect the activity of MAO-A and MAO-B[35,38]. In addition, saponins also affect the performance of p-regulation through the BDNF signaling pathway. Where plasma cortisol was decreased, spinal dendritic density was increased, and hippocampal neurogenesis was induced by UCMS[31,35]. Meanwhile, the 5-HT, DA, NE, and 5-HIAA also increased, and the immobilization time decreased[31,38].

3.7 Analysis of Tail Suspension Test (TST)

The Tail Suspension Test showed a significant decrease of immobilization time in group P1 (63.20 s ± 7.791) compared to groups P2 (73.20 s ± 4.658) and

P3 (79.20 s ± 3,633). There was a difference in the negative control group (146.80 s ± 8,167) and the positive control group, where the positive control group (91.20 s ± 5,718) experienced a decrease in TST time more than the negative control group. Based on the Anova test, the calculated F-value was 164,081 with a Sig-value of 0.000. Based on these data, we can conclude that the higher concentration of papaya seeds extract might reduce the immobilization time of TST.

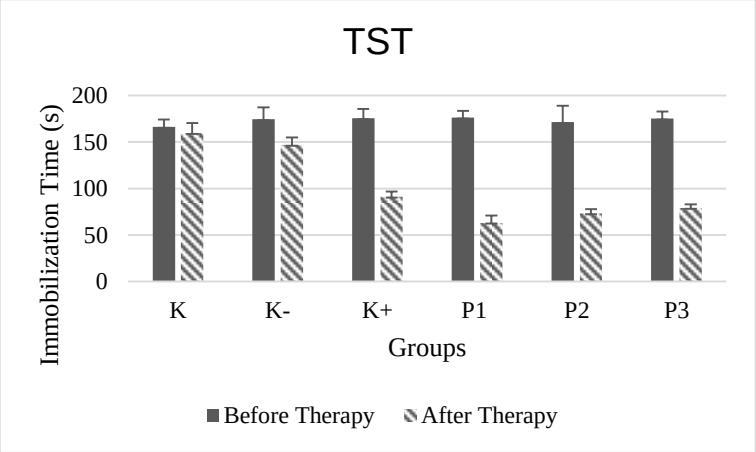

Fig. 7. Tail Suspension Test (TST) of all groups at before and after therapy

Some studies reported that saponin compounds could reduce the immobilization time; meanwhile, the level of 5-HT, DA, and N.E. and 5-HIAA were increased[31,38]. However, there was no effect on MAO-A and MAO-B activities reported[12,38]. Plasma cortisol was decreased, spinal dendritic density was increased, and hippocampal neurogenesis was induced by the UCMS[31,12].

3.7 Proposed Mechanism of Antidepressant

Groups of saponins have been reported to reduce body weight by 20 to 30% by reducing the hypothalamic NPY expression[24]. Neuropeptide Y stimulates the inhibition of incoming food[39,24]. Saponins have insulin-like activity, and they could inhibit lipolysis and increase glucose uptake by adipose cells[29], causing a decrease in blood glucose levels. Saponin compounds also affect neurobehavior. This effect can be seen from the behavioral test through the FST and TST, where the immobilization time in rats decreased during examinations. Some studies reported involvement of noradrenergic activity[38,40]. Here we proposed the mechanism that major compounds and saponin groups contained in papaya seed extract would activate the immune cells to lower IL-6, including those associated with T cell responses. Saponins groups were also suspected of capturing free radicals so that MDA decreased

CONCLUSION

The papaya seeds extract showed potential effects as antidepressants compared to amitriptyline in rat models. The higher concentration of extract might correlate to higher effectivity on reducing body weight, glucose level, IL-6, and MDA, and limited immobilization time of FST and TST. The phytochemical analysis confirmed the main compounds are 9-Octadecenoic acid (Z)-methyl ester, Benzyl nitrile, Hexadecanoic acid- methyl ester, and other saponins contained in the extract might affect the α -glucosidase enzyme to minimize the blood glucose. Additionally, the fatty acids may influence the behavior of several proteins involved in immune cell activation. Further studies on the immune

response, enzymatic, hormonal, hypothalamic-pituitary-adrenal, and other neurotrophic factors are also well encouraged.

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

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Decreasing the SGPT level of male wistar rats induced by gentamicin with purslane ethanol extract

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Abstract:
Antibiotics from the aminoglycoside group, such as gentamicin, are frequently used for the infection therapy of gram-negative bacteria including Salmonella typhi. Some studies show that gentamicin can cause hepatotoxicity and increase the level of SGPT (serum glutamic pyruvic transaminase). The purpose of this research is to figure out the decreasing SGPT level of male Wistar rats induced by gentamicin with the purslane ethanol extract. This research employed a post-test only control group design, utilizing 25 male Wistar rats divided into 5 groups. The normal control group (NorG) was without any treatment, while the positive control (PG) was intraperitoneally injected with gentamicin 60 mg/kg of rat body weight. The treatment groups consisted of P1, P2, and P3 were intraperitoneally injected with gentamicin at the dosage of 60 mg/kg of rat body weight for 7 days and then administered with purslane ethanol extract respectively at the dosage of 200, 300, and 400 mg/kg of rat body weight per oral for 7 days. The analysis on the SGPT level was conducted with the IFCC modification method using chemistry analyzer. One way ANOVA test shows that there were significant differences in SGPT levels among groups. LSD post hoc test shows that purslane ethanol extract at the dosage of 400 mg/kg of rat body weight significantly decreased the SGPT level ($p<0.05$) when compared to the positive control group. The administration of common purslane ethanol extract at the dosage of 200, 300, and 400 mg/kg of rat body weight can decrease the SGPT level of male Wistar rats induced by gentamicin.

Keywords: SGPT, Purslane ethanol extract, Gentamicin.

INTRODUCTION

Antibiotics from the aminoglycoside group, such as gentamicin, are frequently used for the infection therapy of gram-negative bacteria including Salmonella typhi. Some studies show that gentamicin can cause hepatotoxicity and increase the level of SGPT (serum glutamic pyruvic transaminase).[1] SGPT or ALAT (alanine aminotransferase) is an enzyme specifically produced by liver cells, in which SGPT is released when the liver is in damaged condition. One medicine causing the significantly increasing SGPT level is gentamicin.[2, 3] To decrease the SGPT level, the administration of high antioxidant substances, such as purslane plant.[4, 5]

Studies on the utilization of common purslane extract have been conducted by researchers throughout the world, including in Indonesia. The research conducted by Ahangarpour *et al.* (2018) shows that the common purslane extract has a protective effect on the pancreas by decreasing hypoglycemic activity and insulin resistance.[6] The research conducted in Indonesia by Saptaningtyas et al. (2020) also shows that common purslane

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ethanol extract can improve the level of high-sensitivity CRP (Hs-CRP) with the total score of male Wistar rats' renal tubular degeneration induced by gentamicin is equal to that in normal condition.[7]

The increasing SGPT in the male Wistar rats' serum induced by gentamicin indicates an abnormality in their liver. Oxidative stress is a key mechanism responsible for liver damage resulting from the administration of gentamicin which results in the increasing level of SGPT.[8, 9] The antioxidants contained in purslane extract have an important role against oxidative stress and minimizing the negative impacts of oxidative stress. High antioxidant activity in common purslane extract has been investigated since containing gallotannin, omega-3 fatty acid, ascorbic acid, tocopherol, kaempferol, quercetin, and apigenin.[10] Gentamicin induces increasing oxidative stress and free-radical production and suppresses the antioxidant defense system in the liver. The increasing lipid peroxidation results in lipid membrane damage and causes hepatoxic necrosis triggering the increasing level of SGPT. The antioxidant substances contained in common purslane extract suppress the lipid peroxidation and increase the antioxidant defense system in the liver by increasing the glutathione content and improving the superoxidase dismutase activity.[2, 4, 11, 12]

The purpose of this research is to figure out the effect of purslane ethanol extract on SGPT level of male Wistar rats induced by gentamicin. The utilization of purslane extract in decreasing the SGPT level has never been reported before. Thus, it is expected that the results of this research may become a candidad for phytotherapy against the liver damage caused by gentamicin.

MATERIAL AND METHOD

Time and Place of Research

The research was conducted after obtaining ethical approval from the Ethics Committee of FK UNISSULA (Decree no. 285/ VII/2018/Bioethics Committee).This research was conducted from April to September 2020. The place of this research was carried out at clinical pathology laboratory, Medical Laboratory Technology, Universitas Muhammadiyah Semarang.

Research Design

This research used a completely randomized with post-test only control group design. 25 samples of male Wistar rats aged 8-12 weeks ah the body weight of 150-200 grams. The samples were divided into 5 treatment groups respectively consisting of 5 male Wistar rats. The control group was the normal group (NorG) which was only treated with distilled water, while the positive control group (PG) was a group intraperitoneally injected only with gentamicin at the dosage of 60 mg/kg of rat body weight for 7 days. The treatment groups respectively consisted of P1, P2, and P3 intraperitoneally injected with gentamicin at the dosage of 60 mg/kg of rat body weight for 7 days and then administered with purslane ethanol extract at the dosage of respectively 200, 300, and 400 mg/kg of rat body weight per oral for 7 days.

Materials and Tools

Materials and tools needed in this research are purslane, ethanol (96%, MERCK), male Wistar rats' serum, gentamicin, SGPT reagent (Diasys), rotary evaporator, erlenmeyer, tube test, and chemistry analyzer (Mindray BA-88A).

Research Procedures

Purslane Extraction

Purslane plants were washed thoroughly and dried in an oven at 550 C, then grind them well. One liter of 96% ethanol was added to 100 grams of grinded purslane, mixed, and soaked overnight. The supernatant of the 96% ethanol purslane was taken to rotary evaporator to be evaporate until we get the purslane extract.

Research termination

The research termination was performed on day 8 for NorG and day 15 for PG, P1, P2, and P3 by taking their blood through the orbital sinusitis of Wistar rats' eyes. The obtained blood was collected in the red vacutainer, waited until frozen, and then centrifuged to take the serum.

SGPT and Statisitical analysis

The serum was then analyzed based on their SGPT level with the modification method of IFCC (International Federation of Clinical Chemistry and Laboratory Medicine) using chemistry analyzer in Unit/liter (U/L). The data resulted from the examination of SGPT level were the analyzed using SPSS with One way ANOVA and LSD post hoc test ($p<0.05$.

RESULTS AND DISCUSSION

The data obtained from the mean results of SGPT level examination on the influence of common purslane ethanol extract on male Wistar rats induced by gentamicin were presented in Figure 1.

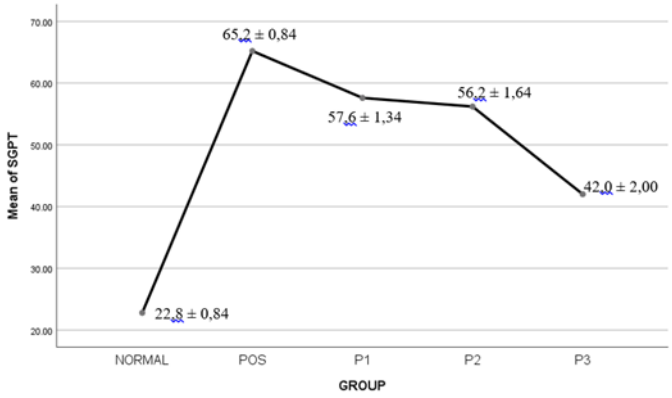

Figure 1. Chart of SGPT Level (Mean ± SD)

Based on Figure 1, it can be seen that the highest mean SGPT level was in positive group (PG), while the lowest was in NorG. The increasing SGPT level significantly happened in PG. The highest decreasing mean SGPT level was in P3 respectively followed by P2 and P1. The decrease was not yet equal with the average SGPT level in NorG, however, has reached the reference value of SGPT level, that is, 18-45 U/L.[13] The results of ANOVA test show that the value of $p < 0.05$ so that it was considered having a significant difference. Meanwhile, post hoc test was then performed to know the differences among groups.

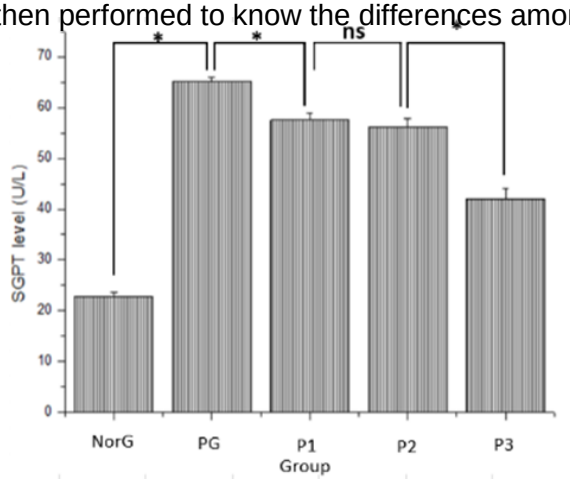

Figure 2. POSTHOC Test on SGPT Level

Figure 2 show that purslane ethanol extract was able to decrease the SGPT level of male Wistar rats induced by gentamicin. SGPT is enzyme catalyzing the amino group changes to form the oxaloacetic metabolism in liver. SGPT can be found in the cytosol of hepatocytes. When liver injury occurs, SGPT is released from injured liver cells and causes a significant increase in serum SGPT activity. The increasing SGPT level may occur due to the use of certain medicine, such as gentamicin.[14, 15] Gentamicin at the dosage of 60 mg/kg of rat body weight induced intraperitoneally causes the increasing SGPT level when compared with that in normal control group. The increasing SGPT level of male Wistar rats induced by gentamicin was in accordance with the result of research previously conducted by Aboubakr in 2016 and Mishra in 2018.[9, 11] Gentamicin is antibiotics from aminoglycoside group which may result in hepatotoxicity. Hepatotoxicity or liver damage has been confirmed with the increasing SGPT level and eventually causing the mitochondrial disfunction. Gentamicin enter the cell by endocytosis or kation channel. Endocytosis transfer gentamicin into endoplasmic reticulum and lysosome, causing cathepsin release, and trigger cell death.[16, 17] Gentamicin increases the production of superoxide anion, hydrogen peroxide, and hydroxyl radical produced by mitochondria. The formed free radicals cause the peroxidase of phospholipid membrane, DNA damage, and protein denaturation.[9, 11, 18]

Gentamicin is covalently related to intracellular protein which possibly decreases the ATP and actin disorder may occur. The destruction of actin fibril on the surface of hepatocyte causes the inflaming cells and breaking cell membranes. Toxin in hepatocyte induces the oxidative stress of cell organelles, such as endoplasmic reticulum and mitochondria possibly causing necrosis or apoptosis.[19] Hepatotoxic metabolite oxidates the thiol protein group and results in reactive oxygen species (ROS). The increasing production of ROS influences the permeabilities of mitochondrial membranes which also then influences the ATP synthesis and expulsion of protein between membranes then triggering necrosis or apoptosis.[20, 21, 22] The research results show that purslane ethanol extract at the dosage of respectively 200, 300, and 400 mg/kg of rat body weight influenced the SGPT level of male Wistar rats induced by gentamicin. Purslane ethanol extract at the dosage of 400 mg/kg of rat body weight could significantly decrease the SGPT level. Some studies have shown the utilization of purslane extract as antioxidant, anti-inflammation, antitumor and anti-bacteria.[23, 24, 25, 26, 27] The chemical content of common purslane ethanol extract includes alkaloid, flavonoid, terpenoid, vitamin, and mineral. The antioxidant content in purslane ethanol extract against free radical formed by reacting with the free radicals stabilizes free radicals and changes free radicals into non-reactive compounds.[28, 29] The antioxidant binds electrons from free radicals and inhibits the chain reaction from the formation of free radicals. Flavonoid and alkaloid in antioxidant react as antioxidant donating hydrogen atoms to free radicals. Vitamin and mineral have the function as cell maintainer by increasing phagocytosis and suppress the occurring inflammation.[9, 20, 30]

CONCLUSION

The administration of purslane ethanol extract at the dosage of 200, 300, and 400 mg/kg of rat body weight can decrease the SGPT level of male Wistar rats induced by gentamicin.

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

OPEN ACCESS

The need for purine examination on fisherman: Case study in Tafure of North Ternate of North Moluccas

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Abstract:

The need for purine check on Fisherman is important because when the fisherman excess consuming highly purine contained in the food in a long rime periode will cause purine metabolism disorder and can lead to purine deposition in joint tissue and sorouding, and finally will become gout arthritis. The uncontrolled of Gout Arthritis will lead to kidney stone, kidney function impaired, and end with kidney failure. Research aims was to analyzes purine effect toward body metabolism functions. Research types using is analytic research with design one sample group to analyzes purine effect toward body metabolism functions on fishermans in Tafure of North Ternate of Moluccas. Population is whole member fishermans groups in Tafure sub district with sex men as amount 55 peoples. Data analyzes use one samples t-Test, research results precentage in the form frequency distribution table with explaining and narration. Research results showed that respondents characteristic most of the attain the age of 26-45 years as much as 29 respondents (53%), had been BMI normal category as amount 46 respondents (84%). Uric acid levels normal is 32 respondents (60%), abnormal as amount 23 repondents (40%), and food consumption habitually high purine. Statistic test results using one samples t-Test showed that age mean 44.49+Std.D 11.460 p0.000<p0.05, BMI mean 22.62_Std.D 2.468 p0.000<p0.05 and uric acid levels mean 6.69+Std.D 1.275 p0.000<p0.05. That's mean there is influencing between aged and BMI with increasing uric acid levels that food consumption high purine influencing uric acid levels on fishermans in sub district Tafure North Ternate District.

Keywords: Uric Acid Levels, Fishermans, Ternate.

INTRODUCTION

In the Covid-19 pandemic era need communities food endurance to body immune maintain with food consumption is contain nutriens is enough to body protection on exposure covid 19 virus attack. Also to with fishermans is to be in North Ternate District. Fisherman is person who living by sea results livehood and living in vilages in beach or coast.[1]

Many risk factors is caused by occured uric acid increased on blood the other gender, obese, alcohol drinking food consumption could be caused happened uric acid increased. Uric acid disease or called with gouthy arthritis happened on male, start from puberty age until to ages maximum 40-50 years, while on female, uric acid precentage happened begin after going to menopause phase. Increased incidence uric acid while in the developed country or developing country as more as highest particulary on males is age 40-50 years. Uric acid levels on males increased as going on with ages added by persons[2].

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In Indonesian uric acid disease 35% happened on males bellow ages 34 years.[3] Gouthy Arthritis and rheumatoid arthritis be at rank third after arhtrocis and rheumatoid arthritis, Rheumatic disease patients in Indonesian approximally almost 80% population with aged 40 years or more[4]. Uric acid levels normal depend on aged and gender, according to World Health Organization (WHO) uric acid levels normal adult females i.e 2,4-6,0 mg/dl and adult males i.e. 3,0-7,0 mg/dl. When more this levels category by had been Hyperuricemia. Hyperuricemia condition very potence to leading happened gouthy arthritis attack.

North Ternate District has coast environment as tourisme object has interested with it coast tourisme. Futhermore, communities economy wheel also develop with sea fish processed products from fishermans. This research to be done in North Ternate District with consideration fisherman communities habitually pattern in beach coast which consumption fish as main menu and also as bisnis area to livehood for families.[5]

MATERIAL AND METHOD

After aquired examination result of uric acid, then to be continued with using statistic test with one samples t-test, and furthermore research result precentage in to frequency distribution tables included explanation and narration. This research has been got approval or ethical approval information letter by Health Research Ethic Commision no.496/KEPK/IX/2019.

RESULTS AND DISCUSSION

Research results showed that from 61 fisherman peoples has been in Tafure Sub District North Ternate District who approved to participation within this research as a mount 55 respondents. Research results could be precentage in the below:

Table 1. Respondents Frequency Distribution Based On Aged, BMI, and Uric Acid Fisherman in Tafure Sub District North Ternate District

No	Variables	f	%
1	Aged Category:		
	a. Adolescence (12-25 years)	3	5
		29	53
	b. Pre- Adult (26-45 years)	20	36
	c. Adult (46-55 years)	3	5
	d. Elder (>65 years)		
2	BMI Category:		
	a. Normal	46	84
	b. Overweight	5	9
	c. Obese	4	7
3	Uric Acid Levels:		
	a. Normal (3,5-,7 mg/dl)	32	60
	b. Abnormal (>7 mg/dl)	23	40
	Total = 55 respondents		

Based on data by table 1 showed that most of the respondents on the adults males aged group as amount 29 respondents (53%). BMI category be in the range normal limit as amount 46 respondents (84%) and uric acid levels fisherman be in normal range as amount 32 respondents (60%).

Table 2. Analyzed Results Bivariate On Aged, BMI and Uric Acid Variables Fisherman in Tafure District North Ternate District.

Variables	Mean	Std Deviation	t	p-Value	95% Confidence Interval of the Difference	
					Lower	Upper
Age	44.59	11.46	28.792	0.000	41.39	47.59
BMI	23.62	2.49	70.964	0.000	6.35	7.04
Uric Acid Levels	6.69	1.27	38.927	0.000	22.95	24.28
N=55						

Samples types which using to uric acid examination on the this research using capiller blood. Uric acid blood levels examination on research samples to be done as quantitative using examination tools (Easy Touch GCU Test), because this examination not need time so long, efficiency and could used whenever and ever. This test performed with strip test methods using with capillar blood samples, uric acid measured on fishermans performed on patient condition was fasting for 10-12 hours, furthermore appear measured on the screen with value mg/dl.

Based on the research results get found highest uric acid (>7mg/dl) as amount 23 respondents (40%) and uric acid levels normal as amount 32 respondents (60%). The Highest and lowerest uric acid levels influenced by many factors like as aged, gender, high purine food consumption habitually, BMI, and kidney dysfunction. When occurred increasing uric acid blood levels, then would be caused occurred hyperuricemia. Gouthy arthritis or uric acid is frequently disease get found and distribution in whole world. Gouthy arthritis or usually called with uric acid is result end from purine catabolism (break out). Purine is one of chemical structure group formed dyoxynucleoacid (DNA). Purine group included is adenosine and guanosin. When DNA is destroyed purine would be catabolism. Prevalence uric acid patients was highest in Indonesia at be coast area population and is most highest in Manado-Minahasa ethnic as amount 29,2%[6].

Research results showed that uric acid levels on table 2 with category normal most of on adults aged (26-45 years) i.e. as amount 45%, whereas uric acid category was highest most of on elder group (46-65 years) as amount 27%. Uric acid diseases or usually called with gouthy arthritis as a diseases who attack old elder group especially males gender. This disease frequently caused disorders on one cartilage examples most frequently on one thumb base, although could be attack more of one cartilage. This disease frequent attack old elder and rarely get found on person who aged old below 60 years with means most many on aged 65-75 years, and more and more frequently get found with aged added[7].

Old elder groups appear cells degeneration caused by aging process who could be resulted on organ weakness, physical retardation, appear of many more diseases like as uric acid levels increased who could be lead happened disease as kidney stone, gout, and rheumatic[8]. Uric acid called to gouth arthritis included a degenerative disease who cartilage attack, and most frequent get found in communities included experienced by old elder [9].

Research results based on BMI category on table 1, dominantly respondents on normal category as amount 84%, whereas on overweight category 9% and obese as amount 7%. Most of respondents with normal BMI category caused by heavy physical activity to be performed by fishermans everyday when shipping so avoided from risk factors obese. This research

suitable by research results by Fiktor I. Boleu in[10], who revealed that hyperuricemia prevalence (with more body weight) on ethnics in Halmahera very highest category. Most respondents with BMI in the overweight and obese category possessed uric acid levels was highest enough. Frequently overweight related to uric acid levels and as one of risk factor occured gouth on asymptomatis hyperuricemia. It is relationship with hyperuricemia incidence which appropriate with overweight more severe. BMI more much then as more and more body weight heavy, so more much highest permeate plasma uric acid in to artilage space[10].

Purine food consumption highest habitually and excessive consumption could be caused uric acid levels increased, where purine amount in to body would be get through normal range. Foods and highest purine resources e.g. meat, fish and fooding and vegetables, like as peanuts, asparagus, cabbage flower, spinach and mushrooms[10]. Fooding resources contain highest purine between 0,5-0,75 mg/dl purine was consumption[11]. Based on research result as uric acid levels with normal dan highest category wholes 100 percent possessed purine high consumption habitually. It is caused uric acid is not only caused by purine high food consumption, but other many risk factors like as aged, and physically activity.

Based on research results to be done that uric acid levels on fishermans had been increased although not all had been uric acid increased. Purine highest consumption influenced uric acid increased suitable with addition aged old and addition body weight catcher fish in Tafuret North Ternate District.

CONCLUSION

Uric acid levels on catcher fishermans had been increased although not all had been uric acid increased. Purine highest consumption influenced uric acid increased suitable with addition aged old and addition body weight catcher fish in Tafuret North Ternate District.

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

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Binahong leaf extract activity in the 8th day of wound healing infected with Staphylococcus aureus towards collagen tissue

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Abstract:
Infectious wound treatment that isn't dosed properly might have negative effects including bacterial resistance, hence plant-based solutions like binahong (Anredera cordifolia (Ten.) Steenis) leaf extract is needed. Alkaloids, saponins, and flavonoids found in binahong leaf act as anti-inflammatory, antiseptic, increase fibroblast cells, and increase collagen production throughout the healing process and scar tissue creation. The goal of the study was to determine the thickness of collagen tissue using Masson's Trichrome staining on the 8th day of wound healing infected with S. aureus after administering binahong leaf extract. The research method used a completely randomized design with 4 research groups, normal control, negative control, treatment 25% (P1), and 50% (P2) concentrations of binahong leaf extract. The thickness was measured in 5 fields of view at 400x magnification with a score of 0-4. The results showed that the P2 group had the same average collagen thickness as the normal control group, which was 50% in each field of view (score 3). While the average thickness of the P1 group was 25% in each field of view (score 2). These results indicate that the administration of binahong leaf extract at 25% and 50% concentrations can stimulate the formation of collagen on S. aureus-infected wounds.

Keywords: Collagen thickness, Wound healing, S. aureus infection, Masson's Trichrome.

INTRODUCTION

Due to the general morphology of the skin on the outside of the body, it is frequently subjected to skin friction, so that it often suffers from injuries, whether caused by disease, wounds, or physical trauma[1]. Wounds are caused by sharp objects such as knives that damage the anatomy of the skin tissue which is characterized by the edges of the wound in the form of straight and regular lines. Wounds that are not treated can cause effects such as loss of tissue substance, bacterial contamination, and lead to complications such as infection[2]. Infection can occur when microorganisms enter the body and cause trauma or damage[3]. *Staphylococcus aureus* which has alpha hemolysis and a toxin that can cause necrosis of the skin is one of the bacteria that caused the infection[4]. Infection by *S. aureus* is characterized by tissue damage with purulent abscess[5].

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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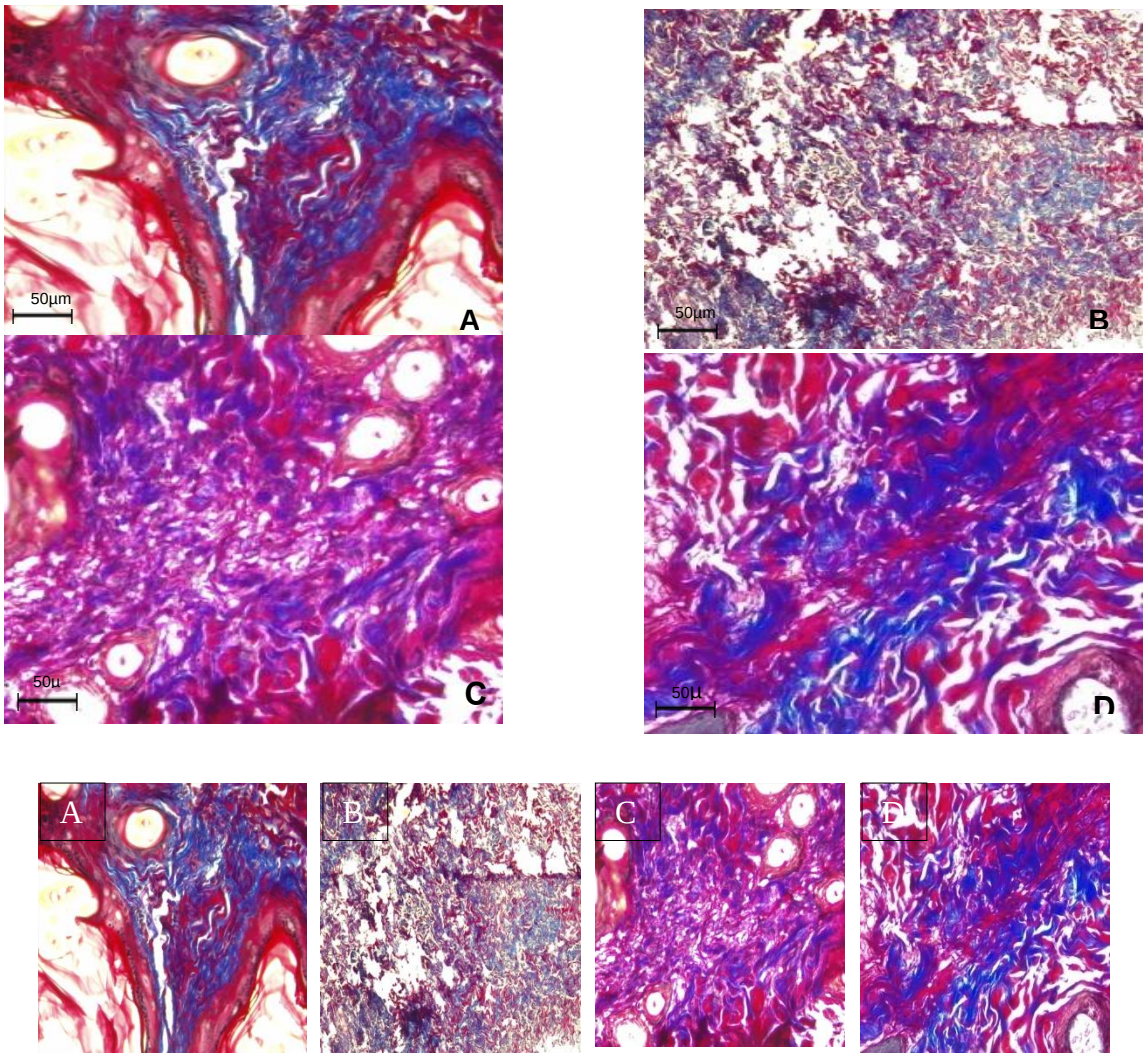

Figure 1. Identification of collagen (blue color) by Masson's Trichrome staining of skin tissue in the KN group with a score of 3 (A); K- score 0 (B); P1 score 2 (C); P2 score 3 (D) (400x)

Table 3. Average Score of Collagen Thickness Measurements in Each Treatment Group

Treatment Group	Collagen Thickness Mean Score
Normal Control Group (KN)	3,00[a]
Negative Control Group (K-)	0,00[b]
Treatment Group 1 (P1)	2,00[c]
Treatment Group 2 (P2)	3,00[a]

Different letters in each value in the same column indicate a significant difference (P<0.05) in the Maan Whitney test.