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Antioxidant Activity of Sago (Metroxylon sagu Rottb) Pith Waste

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A B S T R A C T

Sago pith waste contains flavonoid and tannin compounds. Flavonoids and tannins are well-known phytochemical compounds that act as natural antioxidants that can inhibit free radicals. This study aimed to determine the antioxidant activity of sago (Metroxylon sagu rottb) pith waste extract. Sago pith waste was obtained from Jayapura Regency and extracted by maceration method using 1500 ml of ethanol 70% as a solvent for 5 days. Furthermore, phytochemical screening was carried out on the thick extract. 2,2-Diphenyl-1-picrylhydrazy (DPPH) assay was used to determine antioxidant activity from ethanol extract of sago pith waste with variation in concentration as follows: 100 ppm, 200 ppm, 300 ppm, 400 ppm, and 500 ppm. The study's results found that the secondary metabolite content of the ethanol extract of sago pith waste were flavonoids, phenolic compounds and tannins. From the antioxidant activity test results, the inhibition percentages of the extracts were 30.98%, 40.66%, 51.15%, 65.90%, and 70%, respectively. The IC50 value of the sago pith waste ethanol extract was found to be 283.11 ppm, which was categorised as weak activity of antioxidants. It can be concluded that sago pulp extract has the potential as a source of natural antioxidants.
Keywords: Antioxidant, DPPH, Sago pith waste

INTRODUCTION

Sago is a carbohydrate-producing agricultural plant widely found in Southeast Asia and has been used as a local food by the community as a substitute for rice (1). In Indonesia, Papua has the largest sago plantations with an area of 4,749,424 Ha. Sago can be processed into various food products, such as sago flour(2). Sago starch has the potential as a raw material for making capsules (edible film) to replace gelatine. Dry sago starch is the best raw material that meets the quality standards of SNI 06-3735-1995 in terms of organoleptic colour, odour, water content, ash content and heavy metal content(3). In addition, research by Suwarda et al. (4) found that edible film from sago acetate starch was relatively more stable when stored at low or high temperatures, had good mechanical properties and was resistant to water vapour so that it could be used as a packaging material in humid environments.

Processing sago into food products usually produces sago pith waste, which is generally just thrown away or burned. So far, sago waste has only been used as animal feed(5). Sago pith waste contains primary metabolites such as carbohydrates, water, protein and cellulose fibre(1). Meanwhile, the secondary metabolites in sago pith waste are phenolics, flavonoids and condensed tannins(6). From the potential content of phytochemical compounds, sago pith waste can be used as a source of antioxidants.

Antioxidants are compounds that can be used to slow down or inhibit the oxidation process (7).
Antioxidants can inhibit the activity of free radicals by donating one electron to free radical compounds(8). The most important benefit is maintaining the integrity and function of lipid membranes, cell proteins, and nucleic acids and controlling signal transduction and gene expression in the immune system. Antioxidants in the body reduce and eliminate free radicals. The antioxidant system that complements the immune system can inhibit the reactivity of free radicals(9). The development of antioxidants from natural ingredients is often done in the form of semisolid preparations such as lotions and creams as one of cosmetic ingredients.

A study by Momuat et al..(10) reported that the total antioxidants, total phenolics and total flavonoids were higher in ethanol extract from flour of dry sago baruk compared to flour from fresh sago baruk extract. On the other hand, flour of fresh sago baruk extract had a slightly higher antioxidant activity compared to flour of dry sago barok extract with the antioxidant activity were 83.08% and 81.49%, respectively.

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Another research by Ginting(6) entitled Antioxidant Activity of Water and Ethanol Extracts from Sago Bark (Arenga microcarpha) showed that the water extract and ethanol extract have a total phenolic content of 150.31 and 88.92µg/mL. While the total condensed tannins for water extract and ethanol extract, respectively, are 39.91 and 39.74 µg/mL. Ethanol extract and water extract with concentrations of 56.23 µg/mL and 1949.84 µg/mL have antioxidant activity in the very strong category with IC50 values of 1.75 ppm and 3.29 ppm.

In the area around Lake Sentani, Jayapura district, where sago pith waste was taken in this study, had high productivity of Sago. Some sago plants around lake Sentani have a fairly high starch content, such as phara, yebha, osukul, and folo(11). Based on the researcher's initial survey, around Lake Sentani, especially in the Nendali village area, sago pith waste has few benefits and is simply thrown away. So, researchers are interested in studying the antioxidant activity of sago pith waste

MATERIALS AND METHODS

This research was laboratory experimental research to test and observe the antioxidant activity of sago (Metroxylon sagu rottb) pith waste. The research was conducted at the Pharmacognosy Laboratory of the Jayapura Ministry of Health Polytechnic, the Chemistry Laboratory of the Chemistry Department of Cendrawasih University and the Pharmacy Department of Pharmacy, Cendrawasih University. The research was conducted in May – June 2021.

The object of this study was sago (Metroxylon sagu rottb) pith waste as a sample taken from Nendali village, Jayapura Regency, Papua Province, was 3 kg.

Sample Preparation

Sago (Metroxylon sagu rotted) pith waste from sago processing was taken and then air-dried for 1 x 24 hours at room temperature, then dry sorting was carried out, after which the sago (Metroxylon sagu rotb) pith waste was ground using a blender grinder (12). The grinding was carried out until the sago pith waste became smooth at the lowest speed, and then the grinding results were sieved to obtain sago pulp that was the same size.

Extract Making

Sago (Metroxylon sago rottb) pith waste extraction in this study was carried out using the maceration method. The sago (Metroxylon sagu rottb) pith waste was weighed for 300 grams and then macerated using 1500 ml of 70% ethanol 70% as the solvent (1:5b/v). Soaking was done for 24 hours, at room temperature, until the colour of the solvent changed from initially clear to brown, which matched the colour of sago (Metroxylon sagu rottb) pith waste. Stirring was done 2 times a day, and after the filtering process, the residue was soaked again with the solvent, and the filtrate was collected. This method was repeated for the 2nd and 3rd days. The filtrate results were evaporated using a vacuum rotary evaporator at a temperature of 70 C to separate the solvent from the filtrate; then, a water bath was used until a thick extract with less water content was obtained.

Flavonoid Examination

A total of 3 ml of extract solution was added with Magnesium powder, 2 ml of HCl and 2 ml of amyl alcohol, then shaken vigorously until 2 layers were formed, either it was red, orange or yellow, which was shown the presence of amyl alcohol layer(13).

Condensed tannin examination

1 mL of extract was reacted with iron (III) chloride (FeCl3) solution. If a greenish-black or bluish-black colour is formed, it indicates the presence of condensed tannins(13).

Examination of phenolic compounds

1 mL of solution was put into a test tube, then 2 drops of 1% iron (III) chloride solution was added. If the results are positive, the solution changes colour to green or blue-green(13)

Antioxidant activity testing

Determination of antioxidant activity using the DPPH method: the initial step was to make a 0.1 mm DPPH solution, with 2 mg of DPPH powder dissolved using methanol pa to a volume of 50 mL in a 50 mL measuring flask then placed in a bottle covered with aluminium foil(14).

Before conducting the antioxidant test, the liquid extract was centrifuged at 3000 rpm for 1 hour. The antioxidant test was carried out after obtaining the sago pith waste extract sediment from the centrifugation results. A total of 0.025 grams of sago pith waste powder was dissolved with ethanol in a 50 ml measuring flask to make a stock solution of 500 (mg/l). This stock solution was used to prepare solutions with variance of concentration as follows: 100 ppm, 200 ppm, 300 ppm, 400 ppm, and 500 ppm.

The procedure for making a positive control solution was 0.002 grams of vitamin C powder dissolved with ethanol in a 50 ml measuring flask to make a stock solution of 20 mg/L. This solution was used as a stock solution to make solutions of 2 ppm, 4 ppm, 6 ppm, 8 ppm, and 10 ppm. The test was carried out by pipetting 1 ml of sample solution from various concentrations (100 ppm, 200 ppm, 300 ppm, 400 ppm, and 500 ppm) and then adding 3 ml of DPPH to each. Furthermore, the mixed solutions were incubated at a temperature of 37 C in a dark room. The absorbance was measured at 517 nm wavelength.

The data obtained from the antioxidant activity test of sago pith waste was first calculated for the percentage of antioxidant activity using the formula below:

Antioxidant Activity (%) = (Ab-As) / Abx 100%, where Ab is the Blank absorbance and As is the sample absorbance

After getting the inhibitory percentages from each sample concentration, a linear regression calculation (X, Y) was carried out to obtain the IC50 (Inhibition Concentration 50) value. X was the concentration, and Y was the percentage of antioxidant activity (%). To obtain the IC50 value, the Microsoft Excel program was used to determine the linear regression and the IC50 data was obtained:

$$y = a + bx$$

Where y is the response variable, b is the slope coefficient, x is the predictor variable, and a is the y-intercept of the line. The lowest IC50 value indicates the highest antioxidant activity.(14)

RESULTS AND DISCUSSION

This study's samples were taken from sago processing facilities in the Nendali village area, East Sentani District, Jayapura Regency. In this village, sago processing uses a grater machine, and the grated results are filtered and soaked in a storage tank to separate the starch from the pith waste. The sago starch produced is usually sold or consumed by the residents of Nendali village themselves as a source of carbohydrates; the remaining or unused sago pith waste is used as an object in this study. Jayapura has two types of sago: thorny sago or Metroxylon rumphii Mart and non-thorny sago or Metroxylon sagu Rottb(15). This study used a type of sago without spines.

Results of Ethanol Extract from Sago Pith Waste

In this study, sago pith waste was made into powder form to expand the surface areas so in the extraction process, it can accelerate the extraction of metabolite compounds by the solvent. The extraction method was a maceration process with the aim of extracting efficacious substances that are heat resistant or not heat resistant(16). The extraction process was carried out by soaking the sago pith waste with 70% ethanol to draw all the chemical components in the sago pith waste. The principle of this method is diffusion on the cell wall by the solvent that will enter the cell containing the active substances so that the substances will dissolve in the solvent and come out due to the difference in saturation or the concentration equilibrium inside and outside the cell wall. The filtrate obtained was brownish because the sago pith waste used was brownish in colour due to enzymatic activity during storage, where the polyphenol compound in the sago was oxidised into quinone and became a polymer that formed a brown colour (6).

The filtrate obtained was then concentrated using a rotary evaporator to evaporate the solvent below its boiling point to obtain a thick or liquid extract. The liquid extract obtained was then centrifuged to separate the filtrate from the sediment. Centrifugation is the separation of particles based on their particle weight; particles with a higher density than the solvent will sink to the bottom (6,10).

The results of sago pith waste extraction with 70% ethanol using the maceration method can be seen in Table 1:

Table 1. Sago pith waste extract yield

Powder weight (g)	Extract weight (g)	% Yield
300	0.036	0.012

Based on Table 1, the brown sago pith waste liquid extract was 0.036 g, yielding 0.012%. The yield compares the extract obtained from the initial simplicias (17). The higher the yield value, the more extract is produced. The quality of the extract produced is usually inversely proportional to the amount of yield produced(16,18).

Phytochemical Compound Identification Results

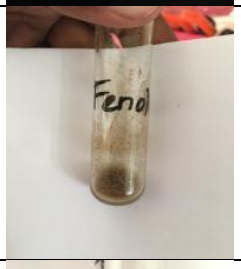
The sago pulp extract produced was then phytochemically screened to determine secondary metabolite compounds. This study conducted tests to determine the presence of flavonoids, phenolics, and tannins, which are natural antioxidants from plants. Identification of flavonoids was carried out by adding magnesium powder, hydrochloric acid, and amyl alcohol, which showed positive results in the form of a yellow colour that was attracted to amyl alcohol. There were condensed flavonoids, phenolics and tannins as a result of phytochemical compounds identifying process in the ethanol extract of sago pith waste, as can be seen in Table 2.

…A colour change occurs in flavonoid identification because the flavonoid compound is reduced by adding magnesium powder and hydrochloric acid. Phenolic identification was made by adding Iron Chloride (III) / FeCl3 to the liquid extract, and a blackish-blue colour was formed. The reaction between the phenol group and FeCl3 causes the formation of the blackish-blue colour. Furthermore, the test for the presence of tannins was done by adding FeCl3, and positive results were obtained, with a greenish-black colour change caused by the reaction between FeCl3 and one of the hydroxyl groups in the tannin compound(13).

Antioxidant Test Results

…The antioxidant activity of sago pith waste extract was measured to determine the ability of secondary metabolite compounds from sago pith waste to reduce free radicals from DPPH. Ethanol extract of sago pith waste that has been added with DPPH can be seen in Figure 1, and the test results are in Table 3.

Table 2. Results of Phytochemical Compounds in Sago Pith Waste

Class of compounds	Colour parameters	Results	Picture	Conclusions
Flavonoid	Shown in red, orange and yellow	Formation of yellow colour		+
Phenolic	Shown in green–blue	Blackish blue color		+
Condensed tannins	Shown as greenish-black or bluish-black	Greenish black colour		+

A.

B.

Figure 1. Antioxidant activity test
A. Extract before the addition of DPPH; B. Extract after the addition of DPPH

Table 3. Antioxidant Activity of Sago Pith Waste Ethanol Extract and Vitamin C

Sample	Concentration (ppm)	% Inhibition	IC50
Sago Pith Waste Extract	100	30.98%	283.11 ppm
	200	40.66%	
	300	51.15%	
	400	65.90%	
	500	70.00%	
Vitamin C	2	14.26%	9.55 ppm
	4	18.52%	
	6	25.57%	
	8	40.98%	
	10	56.23%	

The table above showed that the IC50 for sago pulp extract was 283.11 ppm and had weak activity for antioxidants. Vitamin C was used as the positive control. Vitamin C had an IC50 of 9.55 ppm, and therefore, it can be concluded that Vitamin C had strong antioxidant activities. An antioxidant activity test was done by reacting DPPH free radicals with sago pith waste extract. This method's working principle of antioxidants is the capture of free radicals by donating H atoms to DPPH radicals, as shown in Figure 2 (19). DPPH solution is easily oxidised, indicated by a change in colour from purple to pale yellow. This indicates that DPPH is in a paired state (stable condition)(6,7,10,19).

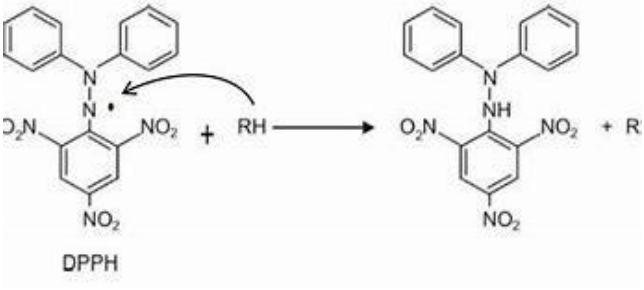


Figure 2. Mechanism of DPPH free radical scavenging

It is known that the higher the concentration, the higher the percentage of inhibition of sago pith waste, which means that the inhibitory power against DPPH radicals increases with increasing concentration. The highest concentration, 500 ppm, had a percentage inhibition value of 70%, so sago pith waste ethanol extract has great potential to counteract DPPH free radicals. Sago pith waste ethanol extract can release hydrogen atoms on the violet-coloured DPPH radical and convert it into diphenylpicrylhydrazine, a yellow-coloured non-radical substance (10.19).

From the percentage of inhibition obtained, a comparison curve was made to compare the percentage of inhibition of sago pith waste ethanol extract against the concentration of sago pith waste extract. From this curve, a linear regression equation was calculated to determine the IC50 value(6). The IC50 value of sago pulp extract was 283.11 ppm, classified as a weak antioxidant, and the IC50 value for the positive control of vitamin C was 9.55 ppm, classified as a strong antioxidant. The ability of a compound to ward off free radicals is inversely proportional to the IC50 value, where the smaller the IC50 value of a compound, the stronger its antioxidant activity(9).

The activity of an extract in counteracting DPPH free radicals depends on the ratio of flavonoid, phenolic and condensed tannin compounds(6,10). The DPPH free radical scavenging effect increases with the increase in the amount of extract. Flavonoid compounds work as antioxidants by donating their hydrogen atoms to free radicals to form stable radicals with low energy levels, which come from compounds that have lost their hydrogen atoms. In the aromatic ring structure, a resonance process will occur to form a more stable antioxidant radical. As a result, it will be difficult to experience other radical reactions. While tannin compounds function as secondary antioxidants because tannins can chelate iron ions and slow down the oxidation process(9,10).

Vitamin C is used as a positive control because it is a natural antioxidant that is easy to obtain, cheap, and easy to consume from nature. Vitamin C has the property of being easily soluble in water; however, the oxidation process, heat and alkali can easily damage vitamin C. Vitamin C is mainly found in vegetables and fruits. As an antioxidant, Vitamin C works by binding to Oxygen so that it does not support oxidation reactions (oxygen scavenger)(20).

CONCLUSION

Sago pith waste ethanol extract has weak antioxidant activity, with secondary metabolites found in the extract flavonoids, phenolics and condensed tannins.

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CONFLICT OF INTEREST

There are no conflicts of interest in research or publication that could influence the research results.

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Self-Medication Knowledge, Attitude, and Practice of Health Science Students in Indonesia: A Cross Sectional Study

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A B S T R A C T

Self-medication is a term to describe an act of using medication, whether traditional or synthetic, for self-treatment. This study was carried out to determine the pattern, attitude, and knowledge of self-medication among health science major students in Bangkalan, Indonesia. This cross-sectional study was conducted from May to June 2023. Data was obtained through self-administered questionnaire and the results expressed as percentages.This study enrolled 204 students from different majors, i.e. pharmacy (39.5%), nurse (33%), midwifery (17.9%), medical record science (9.1%), and others (3%). All of the participants have practiced self-medication in the last six month, at least once. The most common reason for self-medication were the mildness of the diseases (50%), the urgency to relieve symptoms fast (13%), and previous medical knowledge (13%). Multivitamin (42.2%) and analgesics (35.8%) were the most frequent used drugs for self-medication. The students tend to have positive attitude favoring self-medication. The majority (72.55%) of students demonstrated good level knowledge of self-medication. An effort to raise public awareness of the disadvantage of self-medication and the responsible way to practice it should continuously be made. The pharmacist should actively contributed in raising public awareness and more education should be given to the students regarding the risk of self-medication, self-medication errors can cause unwanted side effects such as overdose, therapeutic errors, drug interactions, resistance, and can aggravate the disease and cause new diseases. A pharmacist is at the forefront of providing self-medication services in pharmacies and hospitals and pharmacists play an important role in strengthening the knowledge and attitudes of the community regarding the use of a drug to be consumed, the delivery of information that will have an impact on the quality of life of the community.

Keywords: health science students; Indonesia;self-medication;self-treatment

INTRODUCTION

Traditionally, self-medication has been described as an act of using a drug, herbs, or home remedies based on one's own initiative without consulting a medical practitioners, to treat a disease they self-diagnosed (1). Self-medication includes purchasing medicine without physician's prescription, using old prescription to acquire medicine, sharing medication or seeking advice from friends and family, and taking the leftover medicines stored at home (2). Despite of its advantage in easing the patient to obtain their medication, self-medication also possess healthcare concern, such as wrong diagnosis that lead to wrong treatment, antimicrobial resistance, dangerous drug interaction, and delay in diagnosis and treatment of a major illness (3).

Self-medication practices, in particular, is more prevalent in developing countries, in which access to healthcare and medical cost play an important role in encouraging people to self-medicated (2,4). On the other hand, antibiotic resistance continue to be the global issue that need to be addressed. Thus, inappropriate self-medication that generated irrational use of medicine is becoming the major concern of healthcare professional. In order to fully achieve the benefit of self-medication, i.e. treat minor diseases, save time and money for medical expenses, a responsible practice of self-medication is mandatory.

Self-medication by patients should be implemented based on adequate knowledge and attitudes to avoid misuse of drugs. If the wrong drug is chosen or the dose is exceeded, it will cause poisoning. will cause poisoning. Therefore, medication must be in the right dose and at the right time. time of use. Pharmacies must provide information about medicines, including

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information services for prescription drugs, over-the-counter drugs, and herbal medicines. Pharmacists should provide information about drugs used for self-medication. There is also a need for socialisation of self-medication to improve the attitude and understanding of participants regarding the appropriate use of drugs for self-medication (5). A person must have the right knowledge and attitude to be able to perform appropriate and relevant self-medication so as to minimise the adverse effects of medication errors.

Self-medication is known to be a common practice among university students. As a constituent of community that reflected the highly educated group, the university students are expected to have a better understanding of appropriate use of medicine and self-medication practice. In addition, the students majoring in health science will be the professional that provide healthcare information to the community in a few years. Therefore, it is important to assess the general knowledge of self-medication in the university students, especially in health science major. This study is aimed to determine the pattern and knowledge of self-medication among health science major students in STIKes Ngudia Husada Madura, Indonesia.

MATERIAL AND METHODS

A cross-sectional, anonymous, questionnaire based study was conducted at STIKes Ngudia Husada Madura, Indonesia from May to June 2023. A self-administered questionnaire were used to collect data from medical students, from the first year to the fourth year. The instrument was developed by adapting and integrating similar study question from previous study. The questionnaire containing both open and closed ended question regarding basic demographic information and self-medication pattern and knowledge. Student's consent to participate in the study was taken prior to collecting the data. The sampling method uses the stratified random sampling method where the formula is a sampling technique that divides the population into several strata or small groups based on certain characteristics relevant to the research. The number of samples obtained was 204 participants were included in this study. The obtained results were imported to Microsoft Excel, analyzed, and reported as percentages. The study protocol was reviewed and approved by STIKes Ngudia Husada Madura research and ethics committee (ID Number: 193/KEPK/STIKES-NHM/EC/IV/2023).

RESULTS AND DISCUSSION

a. Participants demographic

The total of 204 respondents consisted of 153 (75%) female students and the rest 51 (25%) were males. The majority (73%) of participants aged between 17-19 years old, while 21.1% and 5.9% of the participants represented the age of 20-22 years old and 23-25 years old, respectively. This research was attended by several health departments, namely pharmacy (39.5%), nurse (33%), midwifery (17.9%), medical record science (9.1%), and others (3%). The demographic characteristics are depicted in table 1.

Table 1. Demographic data

Group	Subgroup	Frequency (n=204)	Percentage (%)
Gender	Female	153	75,0
	Male	51	25,0
Age	17-19 y.o	149	73,0
	20-22 y.o	43	21.1
	23-25 y.o	12	5.9
Study Major	Pharmacy	77	39.5
	Nursing	59	33,0
	Midwifery	35	17.9
	Medical Record Science	18	9.1
	Others	6	3,0
Year of Study	First year	30	14.7
	Second year	71	34.8
	Third year	98	48,0
	Fourth year	5	2.5

b. Self-medication practice of the participants

Out of 204 participants, 27.9% reported that they practice self-medication once in the last six month. A number of 22.5% students stated that the frequency the self-medicated was twice in the last six months, while the rest 49.6% of the participants reported they practice self-medication three times or more in the last 6 months.

The following chart demonstrated the students' reasons in practicing self-medication (Figure 1). The most common (50%) reason is the mildness of the symptoms they experienced. The other reasons that encourage the students to practice self-medication were they already have medical knowledge regarding the symptoms (13%), needed fast relieve of the symptoms (13%), have medicine stocks at home (11%), cost or money consideration (7%), living far from health facility (4%), and lack of time to visit the physician (2%).

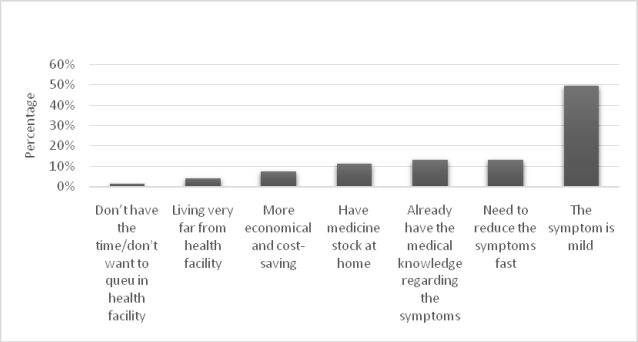

Figure 1. Common causes of practicing self-medication among students

The diseases reported by the students who practiced self-medication were considered minor (Figure 2). The most frequent minor ailment that the students tried to ease with self-medication is fever (56.9%), following by pain (36.30%), common cold (33.3%), and dyspepsia (31.40%). Another minor health problems that the participants decided to self-medicate were sore throat (14.20%), acne (13.70%), diarrhea (12.30%), allergy (9.30%), eye problems (6.90%), fungal infection on skin (5.40%), and constipation (2.90%).

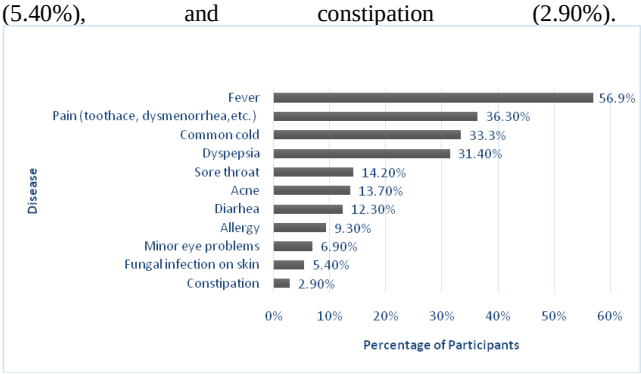

Figure 2. Minor ailments frequently eased with self-medication

The most commonly used drugs in self-medication practice is multivitamin or health supplement (42.2%), analgesics (35.8%), and antipyretics (29.40%), as presented in Figure 3. Other than the aforementioned drugs were eye drops (19.60%), gastrointestinal drugs (13.20%), oral rehydration solution (6.90%), and antimicrobial (5.90%). The least taken drug without prescription was cough and cold medicine (5.40%).

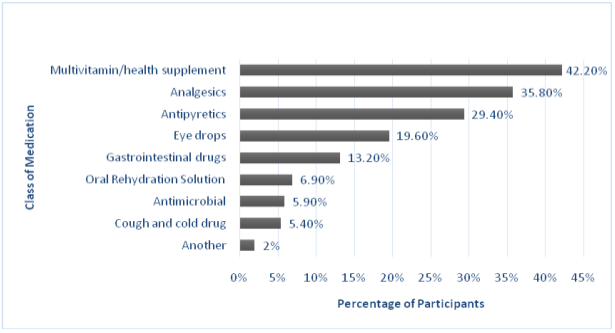

Figure 3. Common medication used when self-medicating among students

The major consideration the participant taken into account while choosing the drug to self-medicate is their previous experience in using the drug (48.5%), while the dosage form of the drug is the least (8.3%) factor the participant considered, as demonstrated in Table 2.

Table 2. Students' consideration in choosing drugs in self-medication practice.

Consideration	Number of Participants (n = 204)	Percentage (%)
Previous experience using drugs	99	48.5
Brand	49	24
Price	38	18.6
Dosage form	17	8.3
Others	1	0.5

The participants reported that the major source of their knowledge in self-medication practice was gained through class or lecturer (35.8%) or by following by old prescription (20.1%). The students received less information from family or friends (17.2%), pharmacist recommendation (11.8%), internet (9.8%), and research articles (4.9%). The common source of participants' self-medication knowledge was presented in Table 3.

Table 3. Source of knowledge regarding self-medication practice

Source	Number of Participants (n = 204)	Percentage (%)
Academic knowledge	73	35.8
Old prescription	41	20.1
Family/friends	35	17.2
Pharmacist recommendation	24	11.8

Internet	20	9.8
Research articles	10	4.9
Others	1	0.5

When asked about the influence of medical knowledge on their attitude toward self-medication, more than half (54.4%) prefer to consult the physician first before deciding to use the medicine and 12.7% felt more confident in practicing rational self-medication (Table 4). On the contrary, 24% felt that it encourage them to be more cautious, 5.9% were anxious of the side effect or potential medication error, and 2.9% were thought that they don't recommend self-medication.

Table 4. Influence of knowledge on self-medication practice among students

Influence of knowledge on self-medication practice	Number of Participants (n = 204)	Percentage (%)
I think it is better to consult the doctor first, before deciding to use the medicine.	111	54.4
I feel confident in doing a rational and appropriate practice of self-medication.	26	12.7
I need to be cautious in doing self-medication	49	24
I don't want to practice self-medication in fear of experiencing side effects or medication error.	12	5.9
I don't recommend my family and my friends to practice self-medication.	6	2.9

c. Participant attitude of self-medication

To examine the attitude of participants toward self-medication practice, the participants were given several statements with 5-point scale answer (Table 5). The highest mean score was 3.69 out of 5-point scale for item "As a medical science student, I encourage my family, friends, and the people around me to practice self-medication", followed by 3.31 for item "Self-medication is considered safe and would not causing an adverse effect". The lowest mean score of agreement was 3.2 out of 5-point scale for item "Over the counter drugs are as effective as the prescription drug". The overall mean score for attitude toward self-medication was 3.40 out of 5-point scale, with an SD of 0.21.

Table 5. The attitude of health science major students toward self-medication

Attitude toward self-medication	Strongly agree	Agree	Neutral	Disagree	Strongly disagree
As a medical science student, I encourage my family, friends, and the people around me to practice self-medication.	45 (22.1%)	87 (42.6%)	37 (18.1%)	34 (16.7%)	1 (0.5%)
Over the counter drugs are as effective as the prescription drug.	19 (9.3%)	72 (35.3%)	51 (25%)	55 (27%)	7 (3.4%)
Self-medication is considered safe and would not causing an adverse effect	26 (12.7%)	71 (34.8%)	53 (26%)	48 (23.5%)	6 (2.9%)

d. Student knowledge of self-medication

More than half (79.9%) of the students were aware that using or purchasing the old prescription is considered self-medication. A total of 151 (74%) students knew that the maximum daily dose of paracetamol is 4 gram. Nearly all (92.2%) of the students had good knowledge about the proper way to use antacid. We also asked the students about their understanding of the term 'three times daily' as in drug administration. Surprisingly, more than half (79.9%) of the students agree that it is means that they should intake their medicine at breakfast, lunch, and dinner. This meant that the majority of students held misunderstanding about drug administration interval. On the other hand, more than half (71.6%) of the students were aware that not all medicine need to administered after meals. A total of 117 (57.4%) students knew that oral antibiotics must be purchased with prescription. The majority (83.3%) of students recognized the importance of using analgesics after meal to prevent the occurrence of drug side effects.

The total score of self-medication knowledge range from 0 to 7; a good level of knowledge was considered for total score of 5, 6, and 7 out of 7, and poor level of knowledge was considered for a total score of 0, 1, 2 3, and 4 out of 7. The result revealed that 56 (27.45%) students from a total of 204 students had poor knowledge regarding self-medication. On the other hand, 148 student which represented 72.55% of the sample, had good knowledge of self-medication, indicated by their total score of 5 and above.

The total of 204 respondents consisted of 153 (75%) female students and 51 (25%) male students. The majority of respondents were female due to the lack of interest of male students in pursuing education in the health sector compared to female students, especially in the Bangkalan area. The majority (73%) of participants were between 17-19 years old, while 21.1% and 5.9% of participants represented 20-22 years old and 23-25 years old respectively. The age range of 17-19 years has a higher percentage compared to other ages, this is due to the fact that the introduction of education from an early age is a motivation to learn early. The demographic characteristics are depicted in table 1.

Self-medication appeared to be a common practice among university students. Our study showed that 29.7% participant have practiced self-medication at least once in the last 6 months (Table 6). According to the previous studies, the prevalence of self-medication tend to high in people with a background in health sciences (6,7). This can be due to various factors, such as higher clinical knowledge, better access to the internet, and perceptiveness that self-medication iscost-effective (8). In a study on pharmacy and medical students in Iran, the researchers found that knowledge was the predominant factor that influence the tendency of self-medication (9).

Table 6. Frequency of self-medication in the last 6 month

Frequency of Self-Medication	Students (n=204)	Percentage (%)
Once	57	27.9
2 times	46	22.5
3 times	39	19.1
4 times	20	9.8
5 times	34	16.7
>5 times	8	3.9

The interesting finding of this study is the most widely used medication among the students is multivitamin (42.20%). This followed by analgesics (35.8%) and antipyretics (29.4%) as the most frequently used drug in self-medication practices among the students (figure 3). Our findings were different from the majority of similar studies, which reported analgesics and antipyretics as the most common drugs used during self-medication (10-12). This may be explained by the massive advertisement of multivitamin as an immune booster during Covid-19. Due to their busy schedule, it is well known that university students have an unhealthy living habits (lack of sleep, poor diet, etc.) which further encouraged them to take vitamin supplements to maintain or improve their health. On the other hand, we found that the percentage of self-medication using antibiotics among our students is lower (5.90%) than that reported on similar study. In contrast, a similar study in India reported the percentage of antibiotics usage during self-medication was 34.9% (n= 488), while studies in Afghanistan and Ethiopia reported that the percentage of participants used antibiotics without prescription was 21.3% and 26.5%, respectively (13-15). While it was worth to note that our students has greater awareness of antimicrobial resistance, the small number of students that used antibiotics in their self-medication practice still call for continuous education and a rigorous regulation regarding antibiotics use.

This study reveals that the most common minor ailments in which the students self-medicated were fever, pain (headache, toothache, dysmenorrhea, etc.), common cold, and dyspepsia (figure 2). The reason that frequently justified the students to practice self-medication was the mildness of the illness, followed by the urgency to reduce symptoms fast and having sufficientknowledge about their disease and its treatment (figure 1). Similar reasons were concluded in other studies (16,17).

When the students were asked about the source of information for self-medication, the current study participants indicated medical knowledge and previous prescription as the main source (table 4). On the other hand, only 11.8% students utilized the pharmacist's pharmaceutical service to assist them in their self-medication practices. This finding is concerning, since the risk of drug-related issues may be high if people receive inadequate information from healthcare professional, especially the pharmacist, regarding their medication (18). Therefore, active participations of pharmacists are needed to increasing public awareness and to assist people in practicing self-medication.

In order to practice self-medication correctly, one must have adequate knowledge about the appropriate way of drug usage. To assess the participant basic knowledge of self-medication, we asked several questions related to medication usage (Table 7). The result showed that the majority (72.55%) of participants had good knowledge of self-medication. But interestingly, more than half of the students (79.9%) seems to have a misunderstanding about the correct instruction of drug administration. Therefore, it is worth to take this into account as one of important topic in education or campaign to improve knowledge about self-medication. Interestingly, due to their positive experience in practicing self-medication, the students tend to recommend their family and friends to self-medicated. They also regarded self-medication as a safe practice and would not causing dangerous medical consequences. This may be due to the medical knowledge they acquired which made them overconfident in regard of self-medication practice. Therefore, it

is important for students to provide good education to their family and friends about proper self-medication, so that self-medication carried out by their family and friends does not result in medication errors.

Table 7. Basic self-medication knowledge of the participants

Question/Statement	True (n = 204)	False (n = 204)
Using drugs that have previously been prescribed by a doctor for symptoms similar to what you are feeling now, is one of self-medication practices.	163 (79.9%)	41 (20.1%)
Maximum daily dose of Paracetamol is 4 gram.	151 (74%)	53 (26%)
Antacid tablet need to be chewed before swallowed to get the optimal effects.	188 (92.2%)	16 (7.8%)
If the medicine label says 3x a day, it means the medicine is used at breakfast, lunch and dinner	163 (79.9%)	41 (20.1%)
All medicine should be used after meal.	58 (28.4%)	146 (71.6%)
All oral antibiotics can't be purchased without prescription.	117 (57.4%)	87 (42.6%)
Analgesics should be used after meals to avoid the side effects in gastrointestinal.	170 (83.3%)	34 (16.7%)

The fact that the students were aware of the risk of self-medication but believe that they have sufficient knowledge to remain safe, means that the students need more education about the risk of self-medication. Insufficient knowledge about self-medication practice (i.e. the dose, time of intake, possible side effect, etc.) may be the risk factors of the occurrence of serious side effects (antibiotic resistance, skin problem, and hypersensitivity reactions)[1]. Awareness campaign and continuous education about self-medication are highly recommended to ensure that rational practice of self-medication will be achieved and possible complication or adverse effect could be prevented. As well as the importance of the pharmacist's role in providing information and education related to self-medication on how to use drugs properly and correctly and the side effects caused when there is an error in drug selection.

The limitations of the study only focus on one campus, this is due to lack of access and time while conducting research, this can be overcome by expanding or increasing the research sample involving several departments on the health campus. Where in this study there are health departments that are also included, namely medical records, which in fact in the world of service will be lacking and almost do not provide services on drug use information to the public

CONCLUSION

Despite of the cost-effectiveness of self-medication, but the inappropriate practice can bring serious consequences. An effort to raise public awareness of the disadvantage of self-medication and the responsible way to practice it should continuously be made. Pharmacist as the reliable component of healthcare system need to make an active contribution to promote better health care practices. Furthermore, the health science students, as future healthcare professionals, should be educated more about the potential risks of inappropriate self-medication.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Molecular Docking Study of 3-Amino-2-Phenylquinazoline-4(3H)-One Derivative as A Potential COX-2 Selective Analgesic Candidate

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A B S T R A C T

Background:
Quinazoline is a group of alkaloid compounds found in several plant and animal families, such as plants in the Rutaceae family. Non-selective COX-2 inhibitors, while effective analgesics, may also inhibit COX-1 in the gastrointestinal tract, potentially disrupting protective mucus production. This research aims to assess the potential of the derivative compound 3-Amino-2-Phenylquinazoline-4(3H)-One as an analgesic agent through molecular docking. The selection of test compounds was conducted using the Topliss Tree method. The potency of the compounds was assessed based on rerank scores and interactions with amino acids in COX-2 (PDB ID 1PXX) and COX-1 (PDB ID 1EQG). The findings suggest that compound 14cpq may exhibit selective COX-2 inhibitory activity. This is supported by its lower rerank score with COX-2 (-85.2374 arb. units) compared to COX-1 (-63.9889 arb. units), as well as its interactions with amino acids Ser1530 and Met1522 within the COX-2 binding site, similar to sodium diclofenac. Furthermore, 14cpq displays distinct interaction patterns with COX-1 compared to ibuprofen, reinforcing its potential selectivity for COX-2. However, further research is required to ascertain the effectiveness of these compound as selective COX-2 analgesics.

Keywords: *3-amino-2-phenylquinazoline-4(3H)-one derivatives; analgesic; molecular docking*

INTRODUCTION

Analgesics are pharmacological agents employed to alleviate pain. These agents act on both the central and peripheral nervous systems. Analgesics are categorized into two main types: opioid analgesics and non-opioid analgesics (NSAIDs) (1). NSAIDs function by reducing the production of inflammatory mediators through the inhibition of cyclooxygenase (COX) enzymes. Non-selective COX-2 NSAIDs, in particular, can inhibit COX-1 in the gastrointestinal tract, potentially disrupting mucus secretion in the stomach and leading to gastrointestinal irritation or ulceration. One widely used non-opioid analgesic available on the market is Sodium Diclofenac (2).

The analgesic activity of Sodium Diclofenac is characterized as non-selective, with a notable inhibitory effect on the COX-2 enzyme compared to COX-1. This activity is attributed to the competition between phenylacetic acid (the chemical structure of Sodium Diclofenac) and arachidonic acid for binding to the COX enzyme (3). Despite its greater inhibition of COX-2, Sodium Diclofenac is associated with a risk of gastrointestinal adverse effects (4). Therefore, the development of COX-2 selective analgesics is essential to mitigate these adverse effects and enhance therapeutic safety.

Quinazoline is a class of alkaloids commonly found in various plant families, including the Rutaceae family (5). Chemically, quinazoline is characterized by a structure that includes a benzene ring fused to a pyrimidine ring (6). This heterocyclic structure is crucial for the biological activity of quinazoline, and it has been extensively studied for the development of biologically active compounds (7). Pharmacological activities of quinazoline derivatives that have been identified include antimalarial (8), antibacterial (9), antiviral (10), and analgesic (11) properties.

In silico research can be conducted using molecular docking computational models. Molecular docking enables the prediction of interactions between compounds and target proteins. The outcomes of molecular docking are typically represented by rerank scores, which indicate the binding affinity and specificity of a compound for a target protein (12). This study aims to evaluate the pharmacological potential of the derivative compound 3-amino-2-phenylquinazoline-4(3H)-one as a selective COX-2 analgesic through molecular docking simulations, comparing its activity to that of Sodium Diclofenac.

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MATERIALS AND METHODS

Protein Target Selection

The target proteins used in this study had resolutions ranging from 1.0 to 3.0 Å and were selected from the Protein Data Bank (www.rcsb.org). Two proteins were utilized: COX-2 (PDB ID: 1PXX) and COX-1 (PDB ID: 1EQG). The 1PXX protein represents the active site of COX-2 from *Mus musculus* with a resolution of 2.9 Å, while the 1EQG protein corresponds to the COX-1 complex from *Ovis aries* with a resolution of 2.61 Å. Protein 1PXX is bound to the natural ligand Sodium Diclofenac, whereas protein 1EQG is bound to natural ligan Ibuprofen.

Protein Target Preparation

The structure of the COX-2 protein (PDB ID 1PXX) and the COX-1 protein (PDB ID 1EQG) was downloaded from the Protein Data Bank (www.rscb.org) and saved in Protein Data Bank (.pdb) format. The protein was then imported into Molegro Virtual Docking version 5 for preparation and removal of unused molecules. Specifically, chain B of the protein and natural ligand (DIF_1701[B] for COX-2 and IBP_1701[B] for COX-1) were selected, while the cofactor and water molecules were excluded. The prepared protein was then ready for the molecular docking phase.

Ligand Preparation

The selection of ligands for testing was based on the Topliss Tree method, incorporating substituents into the phenyl structure of the 3-amino-2-phenylquinazoline-4(3H)-one compound. The derivatives of 3-amino-2-phenylquinazoline-4(3H)-one were prepared using ChemDraw Professional version 16.0 and Chem3D version 16.0. The 2D structures of the derivatives were drawn using ChemDraw and saved in ChemDraw Exchange (.cdx) format. These 2D structures were then imported into Chem3D and converted to 3D forms. In Chem3D, the 3D structures of the 3-amino-2-phenylquinazoline-4(3H)-one derivatives were energy-minimized to prepare them for molecular docking simulations (13). The structures were subsequently saved in .sdf format and were ready for use in molecular docking.

Molecular Docking Method Validation

Docking validation was performed using Molegro Virtual Docking version 5 with the prepared protein structure. The protein and the natural ligand (DIF_1701[B] for COX-2 protein and IBP_1701[B] for COX-1 protein) were subjected to docking simulations with a grid resolution of 0.3 Å. The validation of the docking method was assessed by evaluating the Root Mean Square Deviation (RMSD) values. The RMSD values were obtained from the molecular docking results and used to evaluate the accuracy of the docking simulations.

Molecular Docking

Docking simulations were conducted using Molegro Virtual Docking version 5. Prior to molecular docking, binding cavities were identified based on expanded van der Waals surface criteria, a cavity volume range of 10 - 10,000, and a grid resolution of 0.8 Å. Among the five detected binding cavities, the one occupied by the natural ligand (DIF_1701[B] for COX-2 protein and IBP_1701[B] for COX-1 protein) was selected for further molecular docking analysis. Subsequently, the prepared derivatives of 3-amino-2-phenylquinazoline-4(3H)-one were docked with the protein. The molecular docking process was carried out with a grid resolution of 0.3 Å and a binding site radius of 15 Å. Evaluation of the ligands included internal energy scores (ES), internal hydrogen bonds (H-bonds), and Sp2-Sp2 torsions. For each compound, the pose with the lowest rerank score was selected for interaction visualization with the target protein. Interaction visualization was performed using the ligand map feature in Molegro Virtual Docking version 5. The rerank scores and ligand interactions with amino acids were compared for evaluation.

RESULTS AND DISCUSSION

Root Mean Square Deviation (RMSD) is a metric used to assess the accuracy of molecular docking methods by quantifying the deviation between the positions of non-hydrogen atoms in the ligand and the target protein. An RMSD value of less than 2.0 Å indicates a high level of agreement between the docking results and the experimentally determined structures available in the Protein Data Bank (PDB). In this study, the RMSD value obtained for the molecular docking method was 1.4028 Å for COX-2 protein and 0 Å for COX-1 protein, suggesting that the method provides accurate docking results and is suitable for molecular docking applications (14).

The derivatives of 3-amino-2-phenylquinazoline-4(3H)-one selected for testing were identified using the Topliss Tree method. This approach involves selecting more potent compounds by applying substitutions to the phenyl ring (15). Using this method, a total of 21 compounds were identified as ligands for the study. **Figure 1** illustrates the Topliss Tree diagram and the ligands that were tested.

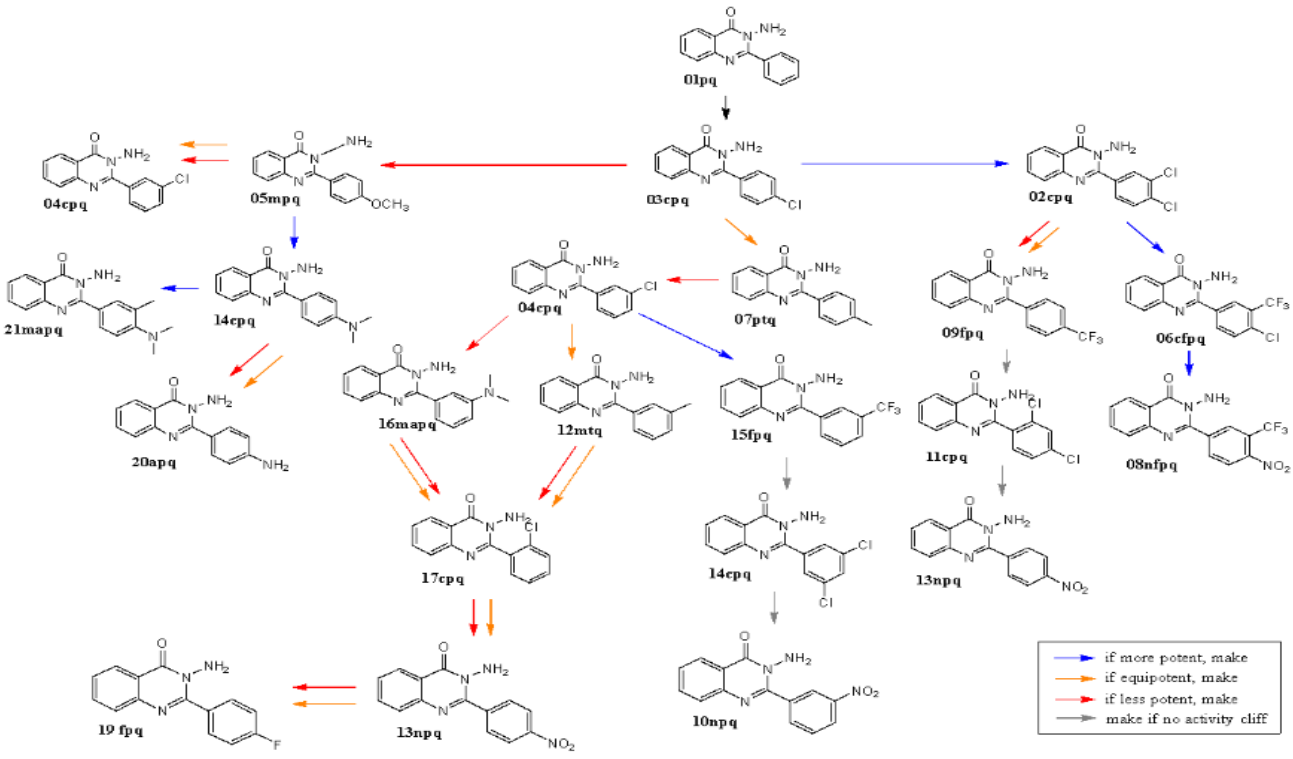

Figure 1. Topliss Tree Diagram of 3-Amino-2-Phenylquinazoline-4(3H)-One Derivatives

The results of molecular docking for the 3-amino-2-phenylquinazoline-4(3H)-one derivatives as potential selective COX-2 analgesics are presented in terms of rerank scores and ligand-protein interactions. A lower rerank score indicates a higher affinity of the ligand for the protein (16). Out of the 21 derivatives assessed, 9 compounds exhibited rerank scores that were lower than that of Sodium Diclofenac (-79.007). These compounds include 15fpq (-87.8871), 09fpq (-87.1458), 06cfpq (-86.2818), 14cpq (-85.2374), 08nfpq (-83.9039), 11cpq (-83.5682), 16mapq (-82.1640), 10npq (-79.8635), and 21mampq (-79.0522). Comprehensive molecular docking results are detailed in **Table 1**.

Table 1. Molecular Docking Results of 3-Amino-2-Phenylquinazoline-4(3H)-One Derivatives Against COX-2 Protein

Compounds	Rerank Score (Arbitrary units)	Interaction		
		Hydrogen bond	Steric Interaction	Electrostatic Interaction
01pq	-73,0055	-	Val 1523, Phe 1518	-
02cpq	-74,7511	-	Val 1523, Trp 1387, Leu 1384	-
03cpq	-73,2909	Ser 1530	Met 1522, Trp 1387	-
04cpq	-75,8820	Ser 1530	Leu 1384	-
05mpq	-77,7079	-	Phe 1518, Val 1523, Trp 1387	-
06cfpq	-86,2818	-	Leu 1531	-
07ptq	-73,2542	Ser 1530	Trp 1387	-
08nfpq	-83,9039	Tyr 1385, Tyr 1355, Ser 1530	Ser 1530, Trp 1387, Gly 1526, Ser 1353, Val 1523, Leu 1352, Phe 1518	-
09fpq	-87,1458	-	Val 1523, Leu 1352	-

10npq	-79,8635	Phe 1518	Val 1523, Ser 1353	-
11cpq	-83,5682	-	Val 1523, Phe 1518, Leu 1384	-
12mtq	-76,3502	Ser 1530	Leu 1384	-
13npq	-78,7911	Tyr 1385	Tyr 1355, Ser 1353, Leu 1352, Gln 1192, Phe 1518, Val 1523	-
14cpq	-85,2374	Ser 1530	Tyr 1385, Met 1522, His 1090	-
15fpq	-87,8871	-	Phe 1518, Val 1523	-
16mapq	-82,1640	-	Phe 1518, Val 1523	-
17cpq	-76,0704	Ser 1530	-	-
18mapq	-75,1403	Tyr 1355	Val 1523, Ser 1353, Phe 1518	-
19fpq	-75,6048	Ser 1530	Trp 1387	-
20apq	-75,8635	Ser 1530	Met 1522	-
21mampq	-79,0522	Tyr 1355	Tyr 1352, Val 1523, Phe 1518	-
Sodium Diclofenac	-79,0070	Tyr 1385, Ser 1530	Tyr 1355, Leu 1352, Met 1522	His 1386
	Similarity of interactions with amino acid **Tyr 1385** through **Hydrogen Bonds**			
	Similarity of interactions with amino acid **Ser 1530** through **Hydrogen Bonds**			
	Similarity of interactions with amino acid **Tyr 1355** through **Steric Interactions**			
	Similarity of interactions with amino acid **Leu 1352** through **Steric Interactions**			
	Similarity of interactions with amino acid **Met 1522** through **Steric Interactions**			

Ligand interactions with amino acids in a protein are crucial for the biological activity of a compound. Among the nine compounds with lower rerank scores than sodium diclofenac, only three compounds (08nfpq, 09fpq, and 14cpq) exhibited similar interactions with the COX-2 protein compared to the natural ligand (sodium diclofenac). In the inhibition of COX-2 protein, the amino acids Tyr 1385 and Ser 1530 are particularly significant for analgesic activity. These amino acids are located at the active site and can bind with the carboxylate groups of carboxylic acid-containing NSAIDs, leading to COX-2 inhibition (17). The interaction of Sodium Diclofenac with these amino acids is illustrated in **Figure 2.** Among the 9 compounds with rerank scores lower than that of Sodium Diclofenac, only 2 were found to interact with the amino acids Tyr 1385 and Ser 1530. These compounds are 08nfpq and 14cpq. The interactions of these compounds with the amino acids in the COX-2 protein are depicted in **Figures 3a and 3b**.

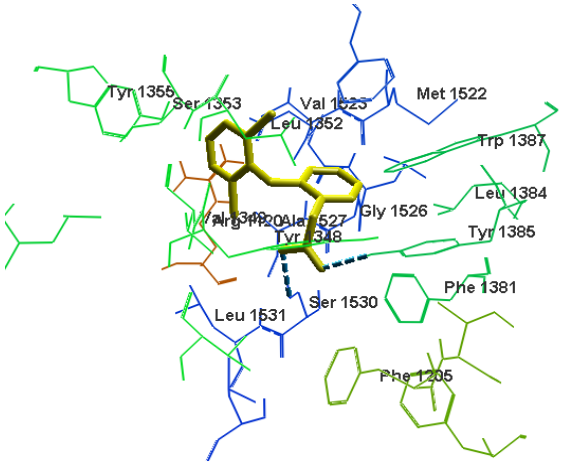

Figure 2. Visualization of Sodium Diclofenac Interactions with Protein 1PXX.
Sodium Diclofenac interacts with Tyr 1385 and Ser 1530 through hydrogen bonds

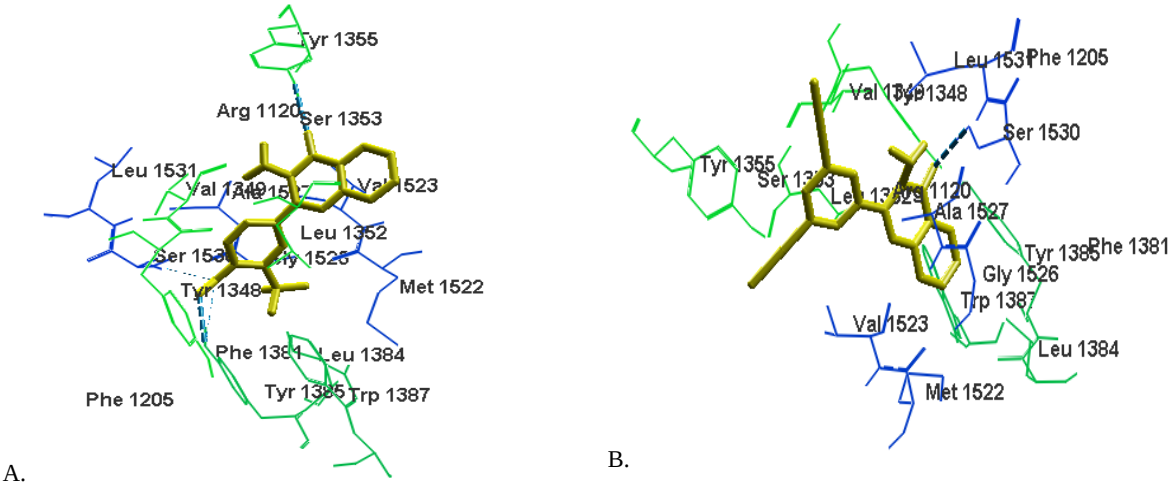

Figure 3. Visualization of Amino Acid Interactions in the COX-2 Protein with Compounds 08nfpq (A) and 14cpq (B). Compound 08nfpq interacts with Tyr 1385 and Ser 1530 via hydrogen bonds, and with Ser 1530 through steric interactions. Compound 14cpq interacts with Ser 1530 through hydrogen bonds and with Tyr 1385 through steric interactions.

Compounds 08nfpq and 14cpq interact with amino acids in COX-2 through hydrogen bonds and steric interactions, similar with Sodium Diclofenac. The hydrogen bonds between the ligands and the amino acids (08nfpq with Ser 1530 and Tyr 1385; 14cpq with Ser 1530) are the most significant. These hydrogen bonds strengthen the ligand's affinity for the amino acids by forming bonds between hydrogen atoms and electronegative atoms (18). In the case of compound 08nfpq, the carbonyl oxygen atom interacts with the hydrogen atoms of the amino acids Tyr 1385 and Ser 1530. Similarly, in compound 14cpq, the carbonyl group engages in interactions with the amino acid Ser 1530.

The interactions of both compounds with amino acids are further enhanced by steric interactions. Steric interactions are related to the influence of the ligand's volume, including its minimal and maximal dimensions, on the binding site. Ligands with bulky substituents can impact the orientation of the ligand, allowing for optimal binding within the binding site and potentially resulting in improved activity (19). Steric interaction similarities with Sodium Diclofenac were observed in compound 14cpq. Specifically, 14cpq interacts with the amino acid Met1522, which is also involved in the interaction with Sodium Diclofenac.

Table 2. Molecular Docking Results of 08nfpq and 14cpq Compounds Against COX-1 Protein

Compounds	Rerank Score (Arbitrary units)	Interaction		
		Hydrogen bond	Steric Interaction	Electrostatic Interaction
08nfpq	-107,635	-	Ala 202, **Thr 206**, Leu 390, His 388, **Phe 210**, **Asn 382**	-
14cpq	-63,9889	-	Ser 353, Phe 518, Ala 527, Arg 120, Met 113, Val 349	-
Ibuprofen	-95,4991	Thr 206	Tyr 385, His 207, **Phe 210**, **Thr 206**, **Asn 382**	His 207
	Similarity of interactions with amino acid **Thr 206** through **Steric Interactions**			
	Similarity of interactions with amino acid **Phe 210** through **Steric Interactions**			
	Similarity of interactions with amino acid **Asn 382** through **Steric Interactions**			

Docking results presented in **Table 2** suggest that compound 08nfpq has the potential to interact with the COX-1 protein. This is supported by its similarities to the natural ligand of COX-1 l (Ibuprofen) particularly in forming steric interactions with amino acids Thr206, Phe210, and Asn382. The lower rerank score of 08nfpq compared to ibuprofen indicates a stronger affinity for COX-1. Furthermore, the rerank score of 08nfpq is lower for COX-1 than for COX-2, highlighting its lack of selectivity for COX-2 and a stronger inhibitory effect on COX-1.

In contrast to compound 08nfpq, compound 14cpq exhibits a lower potential to interact with COX-1. Molecular docking results indicate that 14cpq lacks the same amino acid interactions as ibuprofen. This finding suggests that 14cpq is less likely to inhibit COX-1. The higher rerank score of 14cpq compared to ibuprofen indicates a lower affinity for the COX-1 protein. Additionally, the affinity of 14cpq for the COX-2 protein is stronger than its affinity for COX-1 based on its rerank score. Therefore, compound 14cpq has the potential to be developed as a COX-2 selective analgesic.

Among the 21 derivatives of 3-amino-2-phenylquinazoline-4(3H)-one tested, only 1 compounds, namely, were predicted to selectively exhibit COX-2 enzyme inhibition activity. Compound 14cpq exhibits a higher affinity for the COX-2 protein compared to its affinity for COX-1. Furthermore, compound 14cpq shares binding similarities with diclofenac sodium, specifically interacting with the amino acids Ser1530 and Met1522 in the COX-2 protein, but does not exhibit binding similarities with ibuprofen in the COX-1 protein. Consequently, compounds 14cpq warrant further investigation to assess their efficacy as selective COX-2 analgesics.

CONCLUSION

Molecular docking effectively predicted the potential biological activity of 3-amino-2-phenylquinazoline-4(3H)-one derivatives as selective COX-2 analgesics. Among the 21 compounds analyzed, 14cpq exhibited promising COX-2 selective analgesic activity, as evidenced by its lower rerank score with COX-2 (-85.2374 arb. units) compared to COX-1 (-63.9889 arb. units). Additionally, 14cpq formed interactions with amino acids Ser1530 and Met1522 within the COX-2 binding site, similar to sodium diclofenac, while displaying distinct interaction patterns with COX-1 compared to ibuprofen. However, further studies are required to evaluate the efficacy of both 14cpq as selective COX-2 inhibitors.

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CONFLICT OF INTEREST

The author declares that there are no conflicts of interest related to the research presented in this article.

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The Effect of Ethanol Extract of Phaleria macrocarpa Fruit Combined with Deferiprone on Peripheral Blood Counts in Iron-Overloaded Rats

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A B S T R A C T

Iron overload poses health risks due to its role in reactive oxygen species (ROS) formation.This condition is commonly found in patients with thalassemia due to ineffective erythropoiesis and repeated blood transfusions. Previous studies have shown that excess iron can cause damage, including to the hematological system. Meanwhile, iron chelation therapy with deferiprone, a standard chelator for managing iron overload, is also known to have hematological side effects. We evaluated the efficacy of ethanol extract of *Phaleria macrocarpa* Fruit (PM) against deferiprone-induced alterations in hematological parameters in iron-overloaded rats. Six groups were studied: control, iron-overloaded (IO), deferiprone (D), PM, and two combination groups (DPM-1 and DPM-2). Hematological parameters were assessed at baseline (week-3) and post-treatment (week-8), including total white blood cell count (WBC), lymphocytes (LYM), granulocytes (GRAN), platelet count (PLT), red blood cell count (RBC), hemoglobin (Hb), mean corpuscular volume (MCV, mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). Iron overload induced mild changes, with significant increases in MCV, alongside non-significant increasing trend in LYM and decreasing trends in other parameters. Deferiprone led to significant decreases in RBC and Hb, with non-significant increases in MCV and non-significant decreases in other parameters. PM group showed significant decreased in PLT, RBC, and Hb, and a significant increase in MCV and MCH, accompanied by non-significant increase in MCHC and non-significant decreasing trends in WBC, LYM, and GRAN. Combination treatment of ethanol extract of *Phaleria macrocarpa* fruit with deferiprone at usual dose (DPM-1) resulted in significant changes, including decreases in GRAN, RBC, Hb and MCHC and increasing MCV, accompanied by non-significant increase in MCH and non-significant decrease in other parameters. The parameter changes are less pronounced in the DPM-2 group, where the dose of deferiprone is lower, compared to the DPM-1 group. PM alone exhibited minimal effects on hematological parameters compared to deferiprone (except for PLT), indicating the need for further research to elucidate the specific cellular and molecular pathways influenced by these treatments to support the use of PM as adjunct therapy in patients with iron overload.

Keywords: deferiprone, hematology, iron chelator, iron overload, *Phaleria macrocarpa*

INTRODUCTION

Iron, a transition metal, serves as a crucial micronutrient for humans. Similar to many other metals, iron exhibits various oxidation states, with ferrous (Fe2+) and ferric (Fe3+) being the most common. Within the human body, iron plays a vital role in regulating essential biological processes, including various redox reactions, cell proliferation, and DNA synthesis (1). Additionally, iron functions as an integral component of hemoglobin and myoglobin, facilitating oxygen binding and transportation (2). Despite its importance, excess iron (iron overload) can lead to the formation of reactive oxygen species (ROS), resulting in damage to DNA, proteins, and lipids (3).

Thalassemia stands as the most prevalent cause of secondary iron overload (4). In Indonesia, approximately 3-10% of the total population carries the β-thalassemia gene, and 2.6-11.0% carry the α-thalassemia gene. With a birth rate of around 20% annually and a population of 200 million, an estimated 2,500 new cases of β-thalassemia major are predicted each year (5). In thalassemia cases, mutations result in decreased synthesis of α/β hemoglobin chains, rendering erythropoiesis ineffective. This leads to increased iron absorption in the intestines as a response to enhanced erythropoiesis. Iron excess in thalassemia patients is also exacerbated by regular blood transfusions performed to correct anemia caused by ineffective erythropoiesis (6).

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Several studies indicate that iron overload exerts suppressive effects on hematopoiesis. The impairment of hematopoiesis is attributed to the damage inflicted on hematopoietic cells and the hematopoietic microenvironment. Chai et al.'s research on mice revealed that increased reactive oxygen species (ROS) resulting from iron overload reduced the clonogenic function of hematopoietic stem and progenitor cells (HSPCs), as evidenced by a decrease in hematopoietic colony-forming counts (7). Zhou et al.'s study demonstrated that induced iron overload in mice through four weeks of iron-dextran administration led to a decrease in hemoglobin (Hb), platelet count, and white blood cell (WBC) count. Iron overload in this study resulted in reduced viability and proliferation activity of bone marrow mononuclear cells (8).

In efforts to mitigate iron overload, thalassemia patients receive iron chelators (9). Presently, commonly used iron chelators encompass deferoxamine (DFO), deferiprone (DFP), and deferasirox (DFX). In Indonesia, the accessibility and affordability of these medications pose challenges, with a majority of patients (63.7%) being administered DFP (10,11). However, the principal drawback associated with the clinical application of DFP is its hematologic adverse events, including neutropenia, agranulocytosis, and thrombocytopenia (12).

Research conducted by Kittipoom et al. revealed that 2.8% of thalassemia patients treated with deferiprone experienced agranulocytosis (13). Another study by Panigrahi et al. identified cytopenia as the second most frequent adverse effect, occurring in 7.2% of patients. Within this study, 4.5% experienced neutropenia/agranulocytosis leading to therapy discontinuation, while three patients encountered transient mild leucopenia or thrombocytopenia, allowing for the continued use of DFP treatment (14). Hu et al.'s research indicated that deferiprone exhibits toxicity towards hematopoietic stem and progenitor cells (HPSC) (15). Although relatively rare, hematological abnormalities resulting from deferiprone administration, particularly agranulocytosis, can have fatal consequences, as reported by Mainou et al.(16). The adverse effects on the hematological system necessitate regular monitoring of the complete blood count, with a particular focus on neutrophil levels. Tricta et al. emphasized the importance of weekly monitoring of the absolute neutrophil count (ANC) for all deferiprone users to reduce the risk of developing agranulocytosis and its potential complications. Additionally, discontinuation of therapy is recommended at the first sign of infection or neutropenia (ANC < 1.5 × 109/L), and rechallenge is to be avoided. However, it's acknowledged that this monitoring protocol may pose a burden for some patients (17).

Given the identified challenges with current iron chelators, particularly their hematological side effects, exploring alternative drugs, especially those derived from domestic sources, becomes imperative. *Phaleria macrocarpa* emerges as a promising natural resource proven to reduce iron levels in both serum and organs. The extract from *Phaleria macrocarpa* fruit contains various beneficial compounds, among which mangiferin has demonstrated its ability to decrease iron levels in the plasma of iron-overloaded rats by up to 60% and enhance its excretion in urine (18). Research by Muruganandan and Gupta highlighted the protective effects of mangiferin against cyclophosphamide-induced erythrocytopenia and leucopenia in rats. In vitro studies demonstrated that mangiferin enhanced the survival of lymphocytes exposed to H2O2 (19). Previous research findings indicate that besides mangiferin, extracts of *Phaleria macrocarpa* fruit also contain various other compounds such as quercetin, naringin (20), naringenin, and epigallocatechin-3-gallate (21), all of which have been demonstrated to possess antioxidant effects and are capable of reducing oxidative stress in various cells or organs (22-27).

Verna et al. compared the effects of deferiprone, mangiferin, and ethanol extract of *Phaleria macrocarpa* fruit extract administered individually to rats with iron overload condition. Their study demonstrated that the administration of 100mg/kgBW ethanol extract of *Phaleria macrocarpa* fruit significantly lowered plasma iron levels(28). Hypothesizing that the administration of ethanol extract of *Phaleria macrocarpa* fruit at 100 mg/kgBW can improve hematologic profiles in iron-overloaded rats by reducing body iron levels and counteracting free radicals through its antioxidant capabilities, we also anticipate that combining ethanol extract of *Phaleria macrocarpa* fruit with deferiprone may mitigate the hematological side effects induced by deferiprone. To investigate this hypothesis, we generated iron-overloaded rats and analyzed peripheral blood parameters in these rats.

MATERIALS AND METHODS

Ethical Approval

Ethical approval for this research had been obtained from the Ethics Committee of the Faculty of Medicine, University of Indonesia-Cipto Mangunkusumo Hospital (No.KET-964/UN2.F1/ETIK/PPM.00.02/2022)

Experimental Animals and Sample Size

Animals used in this study were healthy, male Sprague-Dawley rats of ± 8 weeks old with an average body weight of 200-250 grams. The animals were obtained from the Indonesian Food and Drug Authority (BPOM) Jakarta. Maintenance and treatment of experimental animals was carried out at Animal Research facilities, Indonesia Medical Education and Research Institute (IMERI), Faculty of Medicine, Universitas Indonesia. Hematological analysis was carried out in Pharmacokinetic Laboratory, Faculty of Medicine, Universitas Indonesia.

The sample size was determined using Federer's equation for an experiment with six treatment groups (refer to the Study Design and Groups section). According to the calculation, a minimum of four rats per group was required. To account for potential rat mortality during the study, each group's sample size was increased to five rats, resulting in a total of 30 rats.

Study Design and Groups

The research conducted was an in-vivo experimental research with a parallel design. Animals were randomly divided into 6 treatment groups:

1. Normal group (N): normal rats and with no intervention.
2. Iron overload (IO) group: iron-overloaded rats treated with aquadest (negative control group).
3. Defereriprone group (D): iron-overloaded rats treated with deferiprone equivalent to the usual dose in humans (462.5 mg/kg body weight of mice; positive control group).
4. *Phaleria macrocarpa* extract (PM) group: iron-overloaded rats treated with ethanol extract of *Phaleria macrocarpa* fruit (100 mg/kg body weight).
5. *Phaleria macrocarpa* extract+ deferiprone without dose reduction (DPM-1): iron-overloaded rats treated with the combination of ethanol extract of *Phaleria macrocarpa* fruit (100 mg/kg body weight) and deferiprone (462.5 mg/kgBW).
6. *Phaleria macrocarpa* extract+ deferiprone with dose reduction (DPM-2): iron-overloaded rats treated with the combination of ethanol extract of *Phaleria macrocarpa* fruit (100 mg/kg body weight) and deferiprone of half the usual dose (231.25 mg/kg rat weight).

The deferiprone dose for rats (462.5 mg/kg BW) was determined by converting the human dose (75 mg/kg BW) using the human equivalent dose (HED) formula[29]. The PM extract dose (100 mg/kg BW) was selected based on Verna's research, which demonstrated significant reductions in plasma iron levels at this dosage[28].

Plant Extraction

Extraction of *Phaleria macrocarpa* fruit was conducted following the method employed by Verna. The maceration technique was utilized, using 70% ethanol as the solvent to yield an extract with higher flavonoid and phenolic content. A total of 1000.05 grams of dried *Phaleria macrocarpa* fruits were macerated with 70% ethanol at the Balai Penelitian Tanaman Rempah dan Obat (Balittro) Bogor, resulting in an ethanol extract yield of 34.59% (crude extract). Subsequently, concentration was performed at the Laboratory of the Center for Biofarmaka Studies, IPB, using a rotary evaporator for 6 hours, resulting in 81.5833 grams of concentrated ethanol extract (28). The concentrated extract was then diluted with distilled water to achieve a solution with a concentration of 50 mg/mL.

Iron-Overload Induction and Treatment

Iron overload induction for the IO, D, PM, DPM-1, and DPM-2 groups involved intraperitoneal injections of 0.3 mL iron dextran (containing 15 mg Fe) twice a week for three weeks. This method, based on previous research, has been proven to elevate plasma iron levels up to 40 times and ferritin levels up to 10 times higher than the normal group (29,30). After the three-week induction period, the experimental treatments were administered to the respective groups until the 8th week, while iron overload induction persisted until the completion of the study at the 8th week (30).

For the DPM-1 and DPM-2 groups, the test substance was prepared by mixing ethanol extract of *Phaleria macrocarpa* fruit and deferiprone in a microtube, vortexed, and then administered to the experimental animals. The volume of the test substance given to the rats was adjusted to ensure the dosage corresponded to their body weight. All test substances were administered orally once a day using a gastric tube, beginning from the 3rd week until the 8th week of the study.

Hematological Analysis

Blood samples were collected in the 3rd and 8th weeks of the study. Prior to the procedure, rats were anesthetized with ketamine/xylazine. Once anesthesia was achieved, blood was collected from the retro-orbital sinus of the eye using a hematocrit tube inserted and pressed at a 45° angle at the outer corner of the eyes. The blood was then collected up to 1.5 mL in a lithium heparin-containing vacutainer. Subsequently, the blood samples were analyzed using a hematological analyzer (Onetech Med, Model No.A9).

The following hematological parameters were examined: total white blood cell count (WBC) in x10^3/µl, lymphocytes (LYM) in x10^3/µl, granulocytes (GRAN) in x10^3/µl, platelet count (PLT) in x10^3/µl, red blood cell count (RBC) in x10^6/µl, hemoglobin (Hb) in g/dL, mean corpuscular volume (MCV) in femtoliters (fL), mean corpuscular hemoglobin (MCH) in picograms (pg), and mean corpuscular hemoglobin concentration (MCHC) in g/dL.

Statistical Analysis

Data analysis was performed using SPSS version 26. The mean differences among treatment groups (a total of 6 groups) for each parameter at week 3 (baseline) were assessed using one-way ANOVA for normally distributed and homogenous data, Welch's ANOVA for normally distributed but non-homogenous data, and Kruskal-Wallis for non-normally distributed data. On the other hand, the mean differences between week 8 and week 3 for each treatment group (week 8 vs. week 3) were analyzed using dependent t-tests for normally distributed data and Wilcoxon tests for non-normally distributed data. All graphs were generated using the GraphPad Prism program (GraphPad Software, Inc., San Diego, CA).

RESULTS AND DISCUSSION

Peripheral Blood Counts in Iron-Overloaded Mice after 3 Weeks of Induction

To investigate the effect of iron overload on the hematopoietic system, we examined peripheral blood hematological parameters in all iron-overload groups (IO, D, PM, DPM-1, and DPM-2) and the control group. The results are presented in Table 1. Statistical tests indicated that the induction of iron overload for 3 weeks did not cause significant changes in hematological parameters in the control, IO, D, PM, DPM-1, and

DPM-2 groups ($p > 0.05$). Most values were within the normal range or slightly below or above the normal values. One sample from the control group was excluded due to sample lysis and inability to evaluate, resulting in a sample size of only 4 in the control group.

Table 1. Hematological parameters at 3rd week (mean±standard deviation)

Parameter	C	IO	D	PM	DPM-1	DPM-2	p-value
WBC ($x10^3$/µl)	12,85 ± 3,86	12,76 ± 3,19	12,32 ± 2,54	14,98 ± 2,32	12,76 ± 5,07	13,54 ± 4,61	0,892
LYM ($x10^3$/µl)	6,55 ± 1,85	5,78 ± 1,78	6,02 ± 2,47	7,98 ± 1,02	6,80 ± 3,88	6,88 ± 3,94	0,531
GRAN ($x10^3$/µl)	4,20 ± 1,61	4,70 ± 0,90	4,50 ± 0,96	4,78 ± 1,22	3,94 ± 0,70	4,70 ± 0,66	0,765
PLT ($x10^3$/µl)	1167,50± 490,09	925,60± 437,33	868,60± 686,00	1235,40± 86,19	1171,40± 377,75	926,40± 218,42	0,205
RBC ($x10^6$/µl)	8,61 ± 1,23	8,25 ± 1,02	8,25 ± 1,84	9,15 ± 0,35	8,95 ± 0,92	8,63 ± 0,92	0,756
Hb (g/dL)	17,83 ± 2,76	17,40 ± 1,72	18,64 ± 2,50	18,90 ± 0,73	18,98 ± 1,50	18,40 ± 1,42	0,733
MCH (pg)	20,65 ± 0,49	21,14 ± 1,00	23,06 ± 3,09	20,60 ± 0,43	21,22 ± 0,61	21,30 ± 0,77	0,296
MCHC (g/dl)	33,80 ± 1,16	33,66 ± 2,06	34,88 ± 3,43	32,02 ± 0,81	33,22 ± 1,32	33,22 ± 2,43	0,209
MCV (fL)	61,20 ± 1,80	62,88 ± 1,31	65,98 ± 3,62	64,46 ± 1,82	63,92 ± 1,60	64,46 ± 2,51	0,077

Peripheral Blood Counts after Treatment

Various studies indicate that deferiprone, as an iron-chelator, has side effects on the hematologic system. To investigate (1) the effects of deferiprone on the hematologic system in iron-overload conditions, (2) the potential of the ethanol extract of *Phaleria macrocarpa* fruit to improve hematologic profiles in iron-overload conditions, and (3) the potential of ethanol extract of *Phaleria macrocarpa* fruit to reduce the side effects of deferiprone in iron overload conditions, we examined hematological parameters of peripheral blood after 5 weeks of treatment across all treatment groups and compared the changes between weeks 3 and 8 in each group. The results can be seen in Figure 1. One sample from the control group was excluded due to sample lysis and the inability to evaluate, resulting in a sample size of only 4 in the control group.

As expected, rats that were not induced with iron overload and did not receive any treatment in the control group (C) did not show significant changes in hematologic parameters between week 8 and week 3. Meanwhile, the iron-overloaded group without any treatment (IO) exhibited a significant increase in MCV at week 8 compared to week 3. Although not significant, there was a trend of decrease in other hematological parameters, namely WBC, GRAN, PLT, RBC, Hb, MCH, and MCHC, accompanied by an increase in lymphocytes.

The administration of deferiprone alone to iron-overloaded rats resulted in a significant decrease in RBC and Hb at week 8 compared to week 3. In other hematological parameters, although not significant, there was a trend of decrease in WBC, LYM, GRAN, PLT, MCH, and MCHC, and a slight increase in MCV. On the other hand, the administration of ethanol extract of *Phaleria macrocarpa* fruit to iron-overloaded rats led to a significant decrease in PLT, RBC, and Hb with significant increase in MCH and MCV at week 8 compared to week 3. Although not significant, other parameters showed a decreasing trend, including WBC, LYM, and GRAN, while MCHC exhibited a slight increase.

The combination of ethanol extract of *Phaleria macrocarpa* fruit and deferiprone at usual dose resulted in a significant decrease in GRAN, RBC, Hb, and MCHC, as well as a significant increase in MCV at week 8 compared to week 3. Although not significant, other parameters showed a decreasing trend, including WBC, LYM, PLT, and a slight increase in MCH. When the dose of deferiprone was reduced to half of the usual dose, the combination with ethanol extract of *Phaleria macrocarpa* fruit caused a significant decrease in GRAN, PLT, RBC, and Hb, as well as a significant increase in MCV at week 8 compared to week 3. Although not significant, there were slight increase in MCH and slight decrease in WBC, LYM and MCHC.

Several studies suggest that iron overload has a suppressive effect on hematopoiesis. However, in this study, the 3-week injection of iron dextran may not have been sufficient to induce significant changes in peripheral blood counts. Chai's study concludes that damage resulting from iron overload occurs gradually [7]. Okabe et al.'s research showed that in a model injected with iron for 4 weeks, peripheral blood counts were also not remarkably changed. Although platelet levels significantly increased, they remained within normal limits. Meanwhile, WBC, RBC, and Hb did not differ significantly between the IO and control groups in the same study (31).

In this study, following an initial induction period of 3 weeks, we proceeded with the induction process while simultaneously commencing therapy, which extended until the 8th week. For clarity of understanding, we will discuss the effects of therapy administration based on treatment groups.

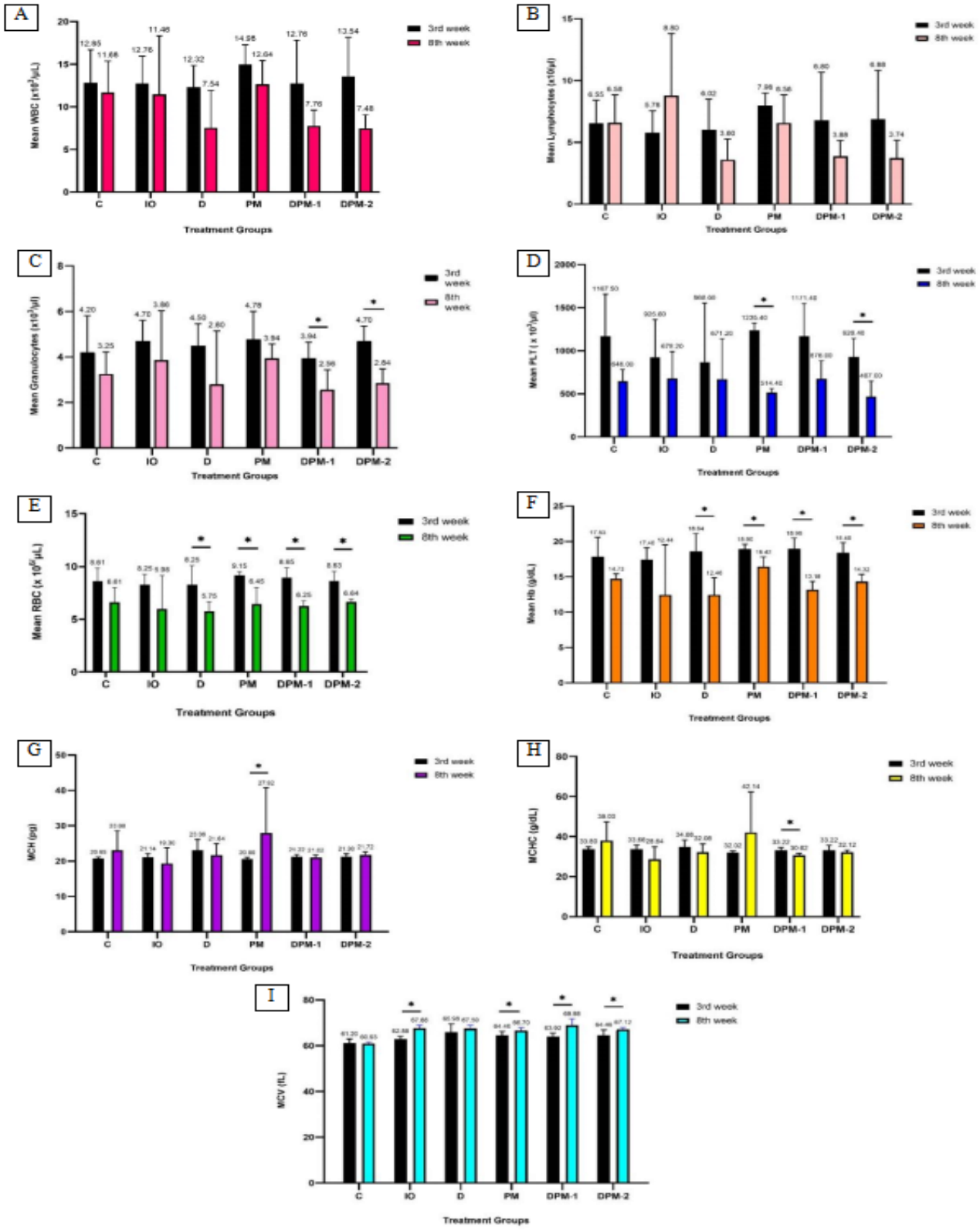
A
Mean WBC (x10³/µL)
12.85
11.66
12.76
11.46
12.32
7.54
14.95
12.64
12.76
7.76
13.54
7.48
3rd week
8th week
C
IO
D
PM
DPM-1
DPM-2
Treatment Groups
B
Mean Lymphocytes (x10/µl)
6.55
6.58
5.78
9.80
6.02
3.60
7.96
6.56
6.80
3.88
6.88
3.74
3rd week
8th week
Treatment Groups
C
Mean Granulocytes (x10³/µl)
4.20
3.25
4.70
3.86
4.50
2.60
4.78
3.94
3.94
2.56
4.70
2.84
3rd week
8th week
Treatment Groups
D
Mean PLT (x 10³/µl)
1167.50
646.00
905.60
679.20
868.00
671.20
1235.40
514.40
1171.40
676.00
928.40
487.60
3rd week
8th week
Treatment Groups
E
Mean RBC (x 10⁶/µL)
8.61
8.61
8.25
8.98
8.25
5.75
9.15
8.45
8.85
8.25
8.83
8.64
3rd week
8th week
Treatment Groups
F
Mean Hb (g/dL)
17.63
14.73
17.48
12.44
18.94
12.46
18.90
16.47
18.99
13.18
18.48
14.32
3rd week
8th week
Treatment Groups
G
MCH (pg)
30.88
31.99
21.14
19.90
23.39
21.64
30.88
37.92
21.33
21.40
21.30
21.72
3rd week
8th week
Treatment Groups
H
MCHC (g/dL)
33.80
38.03
33.86
28.84
34.89
32.08
32.02
42.14
33.22
30.82
33.22
32.12
3rd week
8th week
Treatment Groups
I
MCV (fL)
61.20
60.93
62.98
67.66
65.99
67.50
64.48
66.52
63.90
68.86
64.48
67.12
3rd week
8th week
Treatment Groups

Figure 1. Changes in hematological parameters from week 3 to week 8 in all treatment groups (*p<0.05)

Iron-overloaded group without treatment (IO). The significant increase in MCV in iron-overloaded rats in this study is supported by Sadek et al.'s research, which also showed a significant increase in MCV in mice induced with ferric hydroxide polymaltose complex for 4 weeks. An elevated MCV indicates an increase in red blood cell size. However, in their study, the increase was not limited to MCV but also observed in RBC, Hb, HCT, and MCH, indicating a possible compensatory mechanism to safely sequester excess iron, such as increasing the size and concentration of hemoglobin within red blood cells (MCH) (32,33). However, in this study, other hematologic parameters experienced a decrease, suggesting a potential damage to hematopoietic progenitor cells that leads to the decrease in those parameters (except for MCV and lymphocytes).

In Zhou et al.'s study, iron-overload induced by iron-dextran for 4 weeks in mice led to a decrease in Hb, platelet count, and WBC. Iron-overload conditions resulted in reduced viability and proliferation activity of bone marrow mononuclear cells, mediated by the downregulation of SIRT3 expression and activity. SIRT3 regulates SOD2 (an antioxidant found exclusively in mitochondria) activity by modulating the acetylation level of SOD2. Reduced SIRT3 expression and activity led to increased acetylation of SOD2, reducing its activity and causing an increase in free radicals in mitochondria. This, in turn, led to mROS-dependent autophagic cell death and bone marrow damage (8).

Taoka et al.'s investigation demonstrated that in situations of iron overload, the development of erythroid burst-forming unit colonies and the maturation of erythroblasts were significantly inhibited. These effects were alleviated by iron chelation with deferoxamine (DFO). Furthermore, excessive iron burden induced apoptosis in immature erythroblasts by increasing intracellular reactive oxygen species (ROS) levels (34). Additionally, the high ratio of polyunsaturated fatty acids to total lipids in erythrocytes and erythrocyte membranes suggests susceptibility to lipid peroxidation. Moreover, red blood cells are particularly prone to lipid peroxidation due to their continuous exposure to high oxygen tension and the presence of elevated iron ion concentrations (35).

Despite the lack of remarkable influence on peripheral blood counts, the observed decreasing trend in this study could also be attributed to changes in the microenvironment due to iron overload. Okabe et al.'s research demonstrated that iron overload conditions disturb the hematopoietic microenvironment, marked by a decrease in mRNA levels of various cytokines, chemokines, and adhesion molecules involved in hematopoiesis, such as CXCL12, VCAM-1, IGF-1, and stem cell factor (SCF), by up to 1/20 compared to normal controls. Additionally, there was a reduction in erythropoietin protein levels and thrombopoietin mRNA. Thrombopoietin, produced in the liver, plays a role in platelet production. The decrease in thrombopoietin levels may be caused by iron accumulation in the liver, leading to impaired thrombopoietin production (31).

Meanwhile, in this study, there was an increase in lymphocytes. This finding is supported by Chai et al.'s research, which demonstrated an increase in the percentage of lymphocytes in iron-overloaded mice compared to the normal mouse group[7]. A study by de Souza Aquino et al. also showed an increase in lymphocytes in rats induced with FeSO4 administration. The rise in lymphocytes likely occurs in response to the injury caused by excess FeSO4 administered to the rats, as lymphocytes serve as a primary defense mechanism against the harmful effects of excess iron (36). This is mediated by the ability of lymphocytes to uptake non-transferrin-bound iron (NTBI). Furthermore, the iron is likely stored in ferritin, consistent with evidence demonstrating that T lymphocytes can synthesize H-ferritin (37).

Deferiprone-treated group (D). Changes in hematological parameters in this group may occur as a result of both iron-overload conditions and the side effects of deferiprone. As previously explained, iron-overload conditions can lead to damage to hematopoietic progenitor cells, both white blood cells and red blood cells, and disrupt proteins necessary for platelet production, resulting in a decrease in related parameters (8,31,34). Meanwhile, the increase in mean corpuscular volume (MCV) could represent a compensatory mechanism aimed at safely sequestering excess iron (32,33).

In addition to iron-overload conditions, changes in hematological parameters in this group may be attributed to the side effects of deferiprone. Hu et al.'s study demonstrated that deferiprone is toxic to hematopoietic stem and progenitor cells (HPSC) (15). Another study by Vlachaki et al. indicated that the addition of serum from patients treated with deferiprone to progenitor cell cultures of the granulocytic lineage resulted in maturation arrest in those cell cultures. The research showed a decrease in the number of progenitor cell colonies of the granulocytic lineage (38). Therefore, there is a possibility that damage to progenitor cells caused by deferiprone may contribute to the decline in hematological parameters in the deferiprone-treated group.

***Phaleria macrocarpa*-treated group (PM).** The administration of ethanol extract of *Phaleria macrocarpa* fruit to iron-overloaded rats resulted in a significant decrease in PLT by 58,36%. The decrease in platelet count observed in this group is the most significant compared to other groups, including the untreated iron-overloaded (IO) group. This decline in platelet count may be attributed to the induction of iron-overload and the side effects of the extract on platelets. It is possible that there are other compounds within the extract that synergistically contribute to the reduction in platelet count. Based on research by Hendra et al., *Phaleria macrocarpa* fruit contains naringin

(20). A study by Li et al. regarding the toxicity of naringin showed that although not significant, there was a trend of decreased platelet count in male beagle dogs administered naringenin for 3 and 6 months (39). Additionally, the ethanol extract of *Phaleria macrocarpa* fruit also contains naringenin (21), which has been demonstrated to decrease platelet counts in diabetic rats (40).

Meanwhile, compared to other groups, only this group demonstrated concurrent increases in mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular volume (MCV). Additionally, the decrease in hemoglobin (Hb) in this group was also the smallest compared to other groups. As previously discussed, the simultaneous increase in MCH, MCV, and MCHC under conditions of iron-overload suggests the possibility of compensatory mechanism to safely sequester excess iron, one of which is by increasing the size and concentration of hemoglobin in red blood cells (32,33). Therefore, it is plausible that the active compounds present in the ethanol extract of *Phaleria macrocarpa* fruit may aid in optimizing this compensatory mechanism.

When compared to the untreated iron-overloaded (IO) group, the decreases in WBC, GRAN, RBC do not differ significantly. Thus, it can be assumed that the reduction in these parameters in this group is likely attributable to the condition of iron overload rather than the side effects of the administration of the ethanol extract of *Phaleria macrocarpa* fruit. The extract of *Phaleria macrocarpa* fruit is known to have a protective effects on both white blood cells and erythrocytes. Research conducted by Muruganandan and Gupta has underscored the protective properties of mangiferin against cyclophosphamide-induced leucopenia and erythrocytopenia in rats (19). Rodriguez's investigation demonstrated that mangiferin enhanced erythrocyte resistance to H2O2-induced reactive oxygen species production (35). The research conducted by Hazalin et al. indicates that the extract of *Phaleria macrocarpa* fruit possesses high antioxidant capacity and is capable of providing protective effects against oxidative stress (41).

Specifically, regarding lymphocytes (LYM), a decrease was observed in this group, whereas a significant increase was noted in the untreated iron-overloaded (IO) group. Intriguingly, mangiferin compounds present in the extract are known to enhance the survival of lymphocytes exposed to H2O2 (19). Therefore, the decline in lymphocytes in this context might be attributed to other constituents within the extract. The ethanol extract of *Phaleria macrocarpa* fruit contains epigallocatechin-gallate (EGCG), which is recognized for its suppressive effects on lymphocytes. Findings from Munkyong et al.'s study indicated that compared to mice fed a control diet, those fed with 0.3% EGCG exhibited reduced lymphocyte proliferation and inhibited T cell division and cell cycle progression (42).

When compared to deferiprone, the decrease in white blood cell count (WBC), lymphocytes (LYM), and granulocytes (GRAN) in this group was considerably smaller. Therefore, it can be inferred that the adverse effects of the ethanol extract of *Phaleria macrocarpa* fruit on WBC, LYM, and GRAN are not as pronounced as those of deferiprone.

***Phaleria macrocarpa*+Deferiprone-treated group (DPM-1 and DPM-2).** Based on the results, the magnitude of parameter changes in the DPM-2 group appears smaller compared to the DPM-1 group, except for white blood cell count (WBC) and platelet count (PLT). For instance, the decrease in granulocytes in the DPM-1 group was 46.44%, whereas in the DPM-2 group, it was 39.57%. This difference may be attributed to the fact that in the DPM-2 group, the dose of deferiprone was reduced by half from the standard dosage. As previously explained, deferiprone is recognized for its toxicity to hematopoietic stem and progenitor cells (HPSC) (15) and has been shown to decrease the number of progenitor cell colonies of the granulocytic lineage (38).

Additionally, a noteworthy discovery in this study is that the combination of deferiprone with the ethanol extract of *Phaleria macrocarpa* fruit (DPM-1 and DPM-2) resulted in a significant reduction in granulocytes, whereas when administered separately, the decrease in both parameters was not significant. When examined individually, the administration of deferiprone alone led to a decrease in granulocytes by 37.78%, while the administration of the ethanol extract of *Phaleria macrocarpa* fruit resulted in a decrease of 17.57%. The decrease in granulocytes in the PM group was equivalent to that in the IO group. This suggests that the reduction in granulocytes in the DPM-1 and DPM-2 groups may be more attributable to the condition of iron overload and the side effects of deferiprone, and the addition of the extract may not have been sufficient to ameliorate this condition. This notion is further supported by the data from the DPM-2 group, where a reduction in the dose of deferiprone led to a smaller decrease in granulocytes compared to the DPM-1 group

CONCLUSION

As a conclusion, this study highlights the hematologic modulatory properties of the ethanol extract derived from *Phaleria macrocarpa* fruit. Both deferiprone and the extract were found to induce alterations in various hematologic parameters when administered independently. Additionally, combining deferiprone with the extract led to further modifications, potentially indicative of dose-dependent effects from deferiprone. Further research is needed to elucidate the mechanism of action of the combination of *Phaleria macrocarpa* fruit extract and deferiprone on the hematologic system, thereby allowing for more conclusive conclusions to be drawn, including the possibility of interactions.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose**.**

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Detection of Chili Powder Adulteration with Rhodamine B in Traditional Markets of Singosari District Using Fourier Transform Infrared (FT-IR) Spectroscopy and Chemometrics

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ABSTRACT

Introduction: Chili powder (Capsicum annum L.) is a spice that has many benefits as a cooking ingredient, a natural red dye, and a traditional medicinal ingredient. The high demand for chili, the short shelf life, and the fluctuations in chili prices have led to the adulteration of chili powder with economic motives, namely with Rhodamin B. Based on RI regulation No. 239/Men.Kes/Per/85, that Rhodamin B is a dangerous color substance and is prohibited for use in drugs, food, and cosmetics.

Objectives: This study aims to determine whether there are adulterated chili powder products in the Traditional Market of Singosari District, Malang Regency.

Methods: This study used Fourier Transform Infrared (FT-IR) Spectroscopy and Chemometrics in data processing and used KLT Densitometeic to determine capsaicin levels in chili powder. Data processing was performed using multivariate calibration namely PLS (Partial last Square) and OPLS-DA (Orthogonal Partial Least Squares Discriminant Analysis). The samples used in this study were 10 market samples, control chili powder samples, and mixed samples of chili powder with Rhodamin B synthetic dye with a concentration of 0-50% (b/b).

Results: From the results of the study, it was found that the capsaicin content in chili powder was 624.77 µg/g, then the results of OPLS-DA processing of market samples 1 to 7 were estimated to be adulterated chili powder and samples 8 to 10 were estimated to be pure chili powder. Furthermore, market samples 1 to 7 were tested using PLS. From the PLS results, the best calibration model was obtained at wavenumbers 1800-1180 cm-1 where the calibration R^2 value was 0.9989; RMSEC value was 0.789; R^2 validation of 0.9968; RMSEP of 1.93. PLS results show that in samples 1 to 4 and sample 6 it is estimated that there is a Rhodamine B as an adulterant while in samples 5 and 7 no Rhodamine B was detected in the chili powder, possibly the material added is not from synthetic dyes.

Conclusion: So it is concluded that there are pure and adulterated chili powders in the Singosari Traditional Market and the FTIR and chemometrics were successful detect the pure and adulterated chili powders.

Keyword: Chili Powder, Rhodamine B, Chemometrics, FT-IR

INTRODUCTION

Red chili (*Capsicum annum L.*) is one type of chili in Indonesia and is an important spice with a high level of consumption, as seen from data from the Central Statistics Agency (BPS) which recorded consumption reaching 636.56 thousand tonnes in 2022 (1). However, red chilies have a short shelf life so many are processed into chili powder (2). Chili powder is useful as a spicy enhancer, flavoring ingredient, natural dye, traditional medicine, and industrial raw material for livestock (3). In addition, chili powder also has health benefits due to its capsaicin content, which is antioxidant, antimicrobial, anticancer, and analgesic 4). Capsaicin has neurological effects, a significant reduction in total, myocardial, and aortic cholesterol serum levels, and pain relief (3). The many benefits of chili powder and its high consumption have led to the adulteration of chili powder for economic motives.

Food adulteration is adding illegal substances either intentionally or unintentionally to food as an imitation of the adulterated product so that it does not comply with established official standards (5). In this case, many adulteration cases occur, namely the adulteration of chili powder with rhodamine B. Rhodamine B is a synthetic color that is declared as a hazardous substance and prohibited from being used as a mixture of drugs, food, and cosmetics according to the Indonesian Minister of Health Regulation No. 239/Men.Kes/Per/85(6). Rhodamine B has carcinogenicity, reproductive and developmental toxicity, neurotoxicity, and chronic toxicity in

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humans and animals (7). However, due to its intensive color, it is easily available, and when used as a mixture of chili powder rhodamine B does not provide a significant color difference and does not mask the distinctive aroma of chili.The detection of chili powder adulteration is very challenging due to the similarity of the colour of chili and Rhodamin B , so an analytical tool is needed to detect this. Therefore, the combination of analytical tools FTIR and chemometrics could be an attractive choice because this method was efficient, easy-to-use, fast, and cheap analysis (8,9,10,11). In addition, Fourier transform infrared (FTIR) spectroscopy combined with Partial Least Squares (PLS) chemometrics and Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA) are most often used for the detection of adulteration products. The OPLS-DA was used for classifying samples (8,9,12,13) while Partial Least Squares (PLS) was used to measure the level of adulterants (8,14).

MATERIALS AND METHODS

This type of research is Descriptive Observational to detect whether chili powder sold in Singosari Market, Singosari District, Malang Regency, East Java Province contains Rhodamin B as an adulterant using Fourier Transform InfraRed (FT-IR) spectroscopy and chemometrics methods.

Sampling

The population in this study was all chili powder Illegal chili powder product and sold in the Singosari traditional market, Singosari District, Malang Regency, East Java Province. Considerations for the selection of the study population were based on data from the Central Statistics Agency (BPS) on the types of traditional markets managed by local governments, after selection, a market survey was conducted with traders selling chilli powder without BPOM registration and finally one traditional market was found, namely Singosari Market. The samples of this study were 10 samples of illegal Chili Powder product not registered by BPOM from the traditional market of Singosari District, Malang Regency, East Java. The sampling technique of the research object for laboratory tests uses the Saturated Sampling technique where sampling is based on when all members of the population are used as samples (15,16). The criteria for sampling and selection of samples were divided into inclusion criteria, namely chili powder that was not registered with BPOM and claims of chili powder statements from traders, while the exclusion criteria were chilli powder that had BPOM registration numbers and was obtained other than from Singosari Traditional Market.

Materials

The materials used in adulteration detection are red chili porder obtained from the traditional market of Singosari District, Malang Regency, East Java Province, then determined and powdered at the Materia Medika Batu; 10 samples of market chili powder obtained at the traditional market of Singosari District, Rhodamin B (Technical Grade) (Duta Jaya Chemical Store), Acetone (EMSURE®).

Sample Preparation

Pure Chili Powder : Pure chili powder was made from chili fruit that has been determined and powdered at UPT Materia Medika Batu,

Mixed Sample : The mixed sample was a mixture of Rhodamine B and pure chili powder with a concentration range of 0-50% (w/w). The mixture samples was homogenized using vortex.

FTIR analysis

The FTIR instrument used was Qatar-S Single Bounce Diamond ATR with attenuated total reflectance (ATR) sample handling (Shimadzu, Japan), the sample area was cleaned using acetone, the basic spectrum (background) was scanned before taking measurements on the sample, the sample to be scanned was prepared, the sample was placed on the ATR crystal, the sample was measured in 32 scans and at a separation power (resolution) of 16 cm-1, the scan was carried out at a wavelength of 4000-400 cm^(-1) and replaced the sample 3 times with a replication of scanning 3 times (18,19). After being scanned, the ATR was cleaned using acetone, then dried with a tissue paper.

Data Processing

Data were processed using SIMCA UMETRICS for OPLS-DA, and then data were processed using TQ Analyst for PLS. data were entered into the software (numerical and nominal data), optimization was carried out, and score plots were obtained which illustrated the OPLS-DA clustering, and R square in PLS.

TLC Densitometry

Weighed 100 mg of sample (pure chili powder), put into a 2 mL microtube. Added 1 mL of ethanol. Vortexed for 30 seconds, sonicated for 60 minutes. Maceration for 24 hours at room temperature. Sample was vortexed and centrifuged. 10 µl of supernatant was dispensed on a silica gel 60 F254 plate, with capsaicin standards included. Inserted into a chamber containing saturated mobile phase toluene-chloroform-aceton (45:25:30). Expanded to the limit, remove and dry. Densitized at a wavelength of 228 nm. Rf. 0.60. Calculated capsaicin content.

RESULTS AND DISCUSSION

3.1 Determination

Determination of chili was carried out before the chili powder-making stage to obtain and type of chili used in the study. Determination of chili fruit was carried out at UPT Laboratorium Herbal Materia Medica, Batu. The determination of the big red chili plant is as follows:

Kingdom : Plantae
Division : Spermatophyta
Sub division : Angiosperms
Class : Dycotyledonae
Nation : Solanales
Family : Solanaceae
Genus : Capsicum
Type : *Capsicum annum L.*
Regional Names : Chili, big chili. (17)

3.2 TLC Densitometry

Table 1. TLC Densitometry Chili Powder

Sample	Sample Spotting Volume	Final add of sample	Number of spottings in sample	Area	Average	Capsaicin standard in dried sample (CODEX, 2011) (µg/g)
	(µl)	(mL)	(µg)		µg/g	
Chili Powder	10,0	1,0	1045,00	3382,03	624,77	134 – 1.333
	10,0	1.0	1042,00	3350,64		

In this Densitometric KLT, the stationary phase used or the plate used is silica gel 60 F254, then the mobile phase used is toluene: chloroform: acetone in a ratio of 45:25:30, then the Densito spot capsaicin is measured at a wavelength of 228 nm. So the Rf value of 0.60 was obtained and the standard curve results were obtained with the equation y = 3458x + 1111.9 with a correlation coefficient (R^2) of 0.9867. By Table 1, two densitometric KLTs were carried out on the same sample and the results obtained were 628.22 µg/g and 621.31 µg/g respectively with the average value of capsaicin compound levels in the control chili powder sample of 624.77 µg/g. Meanwhile, the capsaicin standard based on CODEX STAN 307-2011 is 134 – 1.333 µg/g. So the results of the determination of capsaicin levels in the control chili powder sample are by the standard capsaicin levels in the literature.

3.3 FTIR spectra analysis

Table 2. Typical FTIR band assignments of Chili Powder and Capsaicin

No	Chili Powder		Capsaicin		References
	Wavenumbers (cm^{-1})	vibration of functional group	Wavenumbers (cm^{-1})	vibration of functional group	
1	3316,98	O-H stretching vibration	3400-2400	-OH	(18)
2	2911,86	C-H stretching vibration	3000–2850	C-H	(18)
3	1725,96	C=O carbonyl vibration	1850-1650	C=O	(18)
4	1619,31	Overlap of C=C stretching vibrations on the benzene ring and N-H group bending vibrations	1680-1600	C=C	(18)
5			1640-1550	N-H	
6	1411,57	C-O-H Bending vibration	1440–1220	C-O-H	(18)
7	1031,99	C-O group stretching vibrations	1300-1000	C-O	(18)

In this study, the analysis of chili powder samples was carried out using Shimadzu Fourier Transform Infrared Spectroscopy, QATR-S SINGLE BOUNCE DIAMOND ATR at a wavelength of 4000-400 cm^{-1} with a resolution of 16 cm^{-1} and 32 times scanning. In Table 2, the reading of the FTIR spectra shows that the typical absorption in the chili powder sample is a capsaicin compound with the presence of -OH, -NH, -CH, C=C, C=C, and C-O-H groups.

Table 3. Typical FTIR band of Rhodamine B

No	Functional groups	Wave Numbers (cm^{-1})	Rhodamine B		References
			Wave Numbers (cm^{-1})	Description of functional group	
1	O-H	3400–2400	2556	Carboxylic acid O-H stretching vibrations overlap C-H vibrations	(18)
2	C-H				
3	N-H	Sekitar 1550	1559,58	Combination of N-H bending and C-N stretching vibrations in secondary amides	(18)
4	C-N				
5	C-O-H	1440–1220	1420,21	C-O-H bending vibrations	(18)
6	C-O	1320 –1210	1299,28	Carboxylic acid C-O stretching vibrations	(18)
7	C-Cl	730-550	627,57	C-Cl stretching vibrations	(18)

In this study, the analysis of synthetic dye samples using Shimadzu Fourier Transform Infrared Spectroscopy, QATR-S SINGLE BOUNCE DIAMOND ATR at wavelengths of 4000-400 cm^{-1} with a resolution of 16 cm^{-1} and 32 times scanning.

The reading of the spectrum in Table 3 shows that the typical absorption in the Rhodamin B sample is found in the C-O carboxylic acid group, N-H secondary amide, and C-N.

3.4 Sample Overlay

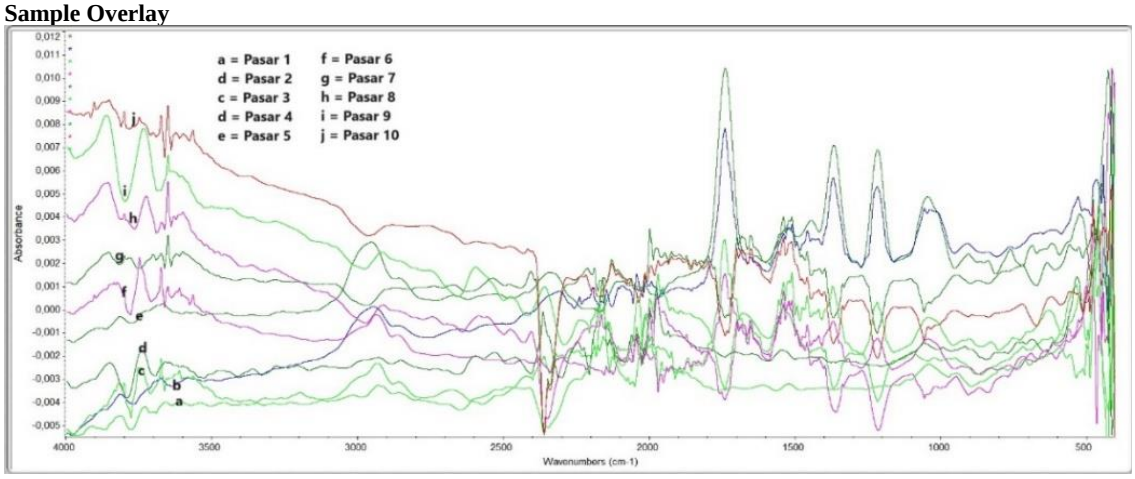

Figure 1. Overlay of Market Sample spectra

Figure 1 shows the results of overlaying market samples 1-10. All samples overlap and the spectra of all samples are almost the same.

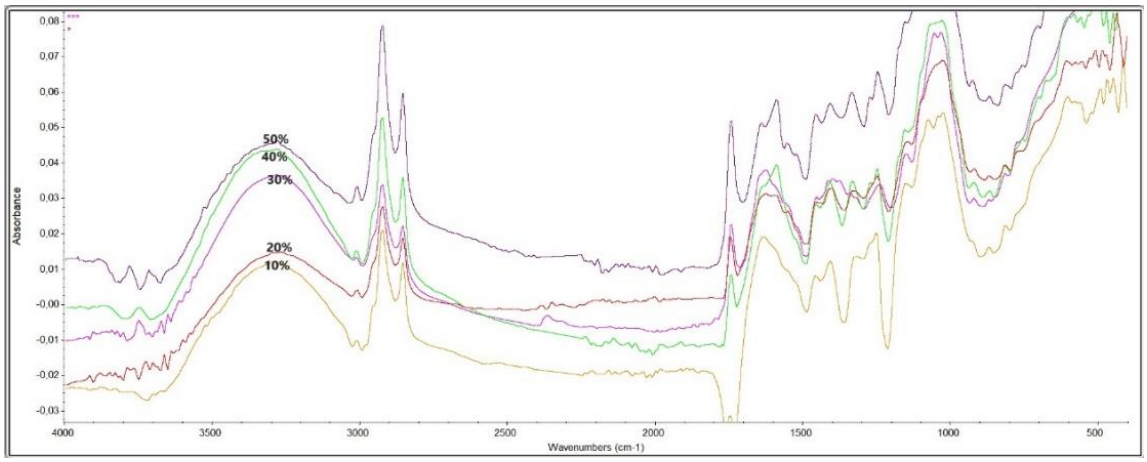

Figure 2. Overlay spectra of mixed samples

Figure 2 shows an increase as the concentration of the dye mixed with chili powder increases and obtained in the wavelength region 1800-1180 cm^{-1}.

3.5 OPLS-DA

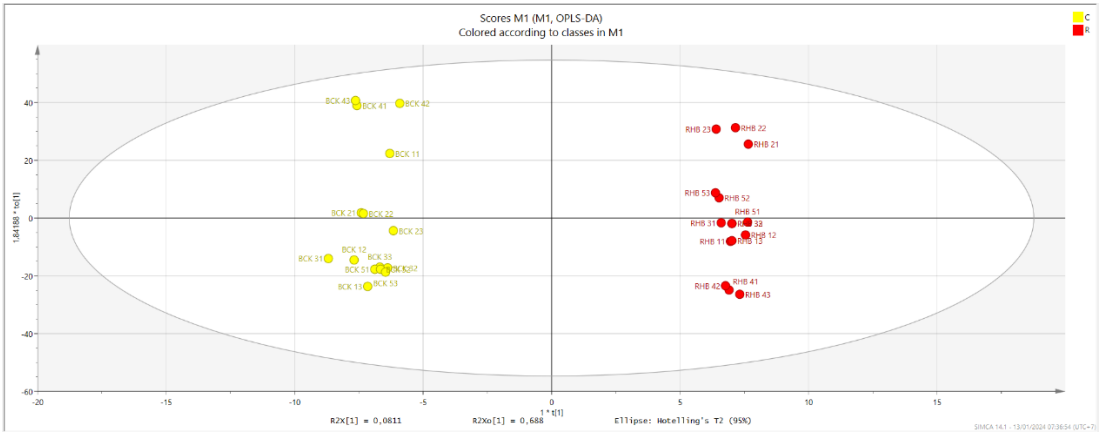

Figure 3. OPLS-DA Plot Score of Chili Powder and Rhodamine B Samples

Description: Yellow (Pure chili powder), Red (Rhodamine B)

Figure 3 is the result of OPLS-DA processing of chili powder and Rhodamine B samples. It was found that OPLS-DA was able to classify the two samples well. Furthermore, the OPLS-DA results were validated by removing the group information and changing the sample name on 1/3 of the total number of samples for validation and 2/3 of the total number of samples for calibration (8,19). Table 3 shows that OPLS-DA is able to group samples according to their class with a 100% correctness rate.

Tabel 3. OPLS-DA Validation of Chili Powder and Rhodamine B Samples

Model	Number of samples	Calibration		Validation	
		Number of samples	*Correct*	Number of samples	*Correct*
Chili Powder	15	10	100%	5	100%
Rhodamin B	15	10	100%	5	100%
Total	30	20	100%	10	100%

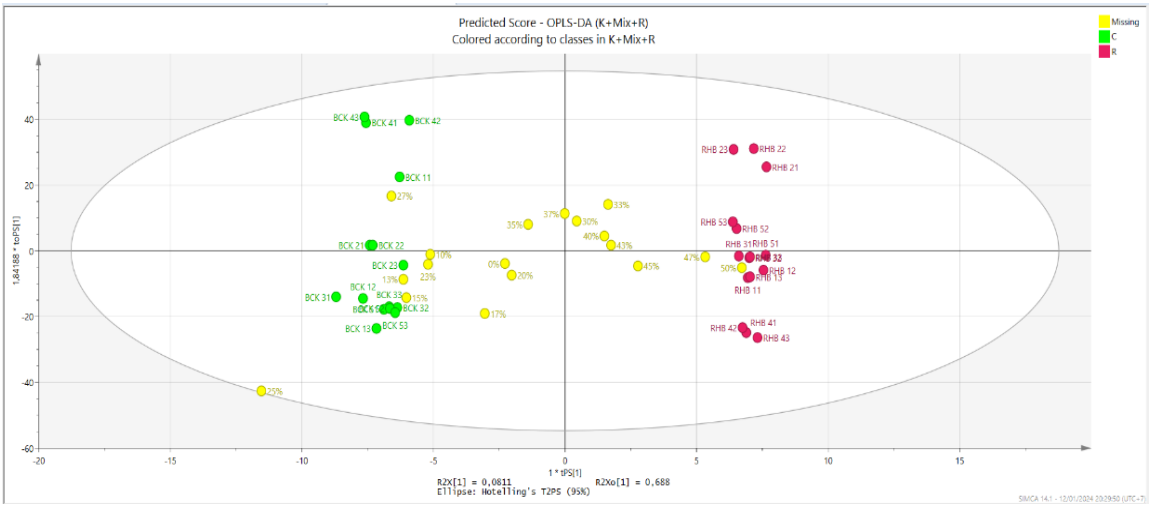

Figure 4. OPLS-DA prediction of mixed samples

Notes: Green (pure chili powder), Yellow (mixed sample), Red (Rhodamine B).

The prediction of mixed samples between chili powder and Rhodamine B using OPLS-DA, based on Figure 4, it is predicted that in mixed samples with high concentrations of 47% (w/w) and 50% (w/w) there is a true mixture detected because the location of the grouping is in the Rhodamine B group.

Table 4. Market Sample Prediction with OPLS-DA

Sample Number	Number of Replications	Chili	Rhodamin B	Conclusion
1	9	2	7	Impure
2	9	0	9	Impure
3	9	0	9	Impure
4	9	0	9	Impure
5	9	7	2	Impure
6	9	0	9	Impure
7	9	6	3	Impure
8	9	9	0	Pure Chili
9	9	9	0	Pure Chili
10	9	9	0	Pure Chili

3.6 PLS

The wavelength used in FTIR Spectroscopy is 4000-400 which then requires optimization as in table 5 to achieve the most optimal value with the results of the R^2 value (Calibration and Validation) > 0.99 and the smallest RMSEC & RMSEP value, so that it is obtained in the following table The most optimal wavelength that results in R^2 > 0.99 and the smallest RMSEC & RMSEP value to get the best prediction results (Siregar et al, 2018). At 1800-1180 cm^{-1} wavelength where the calibration R^2 value is 0.9989; RMSEC value 0.789; R^2 validation 0.9968; RMSEP 1.93.

Table 5. Quality parameters of the PLS models for quantification of Chili powder adulteration with Rhodamine B, using different sets of wavenumber

Multivariate Calibration	wavenumber (cm^{-1})	Calibration		Validation	
		R^2	RMSEC	R^2	RMSEP
PLS	4000-400	0,9755	3,680	0,8900	6,90
	3800-2800	0,8545	8,670	0,8627	11,0
	1800-1180	**0,9989**	**0,789**	**0,9968**	**1,93**
	1600-600	0,9987	0,861	0,9963	2,73

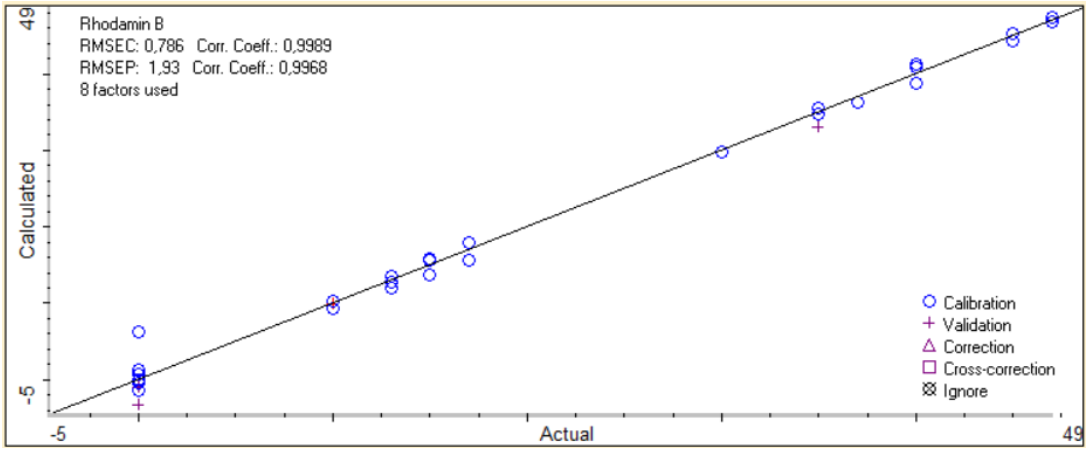

Figure 6. PLS Validation of Mixed Samples of Chilli Powder and Rhodamine B at wavelengths of 1800-1180 cm^{-1}

After multivariate calibration using TQ analysis, validation of the selected wavelengths was carried out using the Leave One Out cross-validation technique. Validation is done in PLS by dividing the data set into calibration data and validation data. Validation data consists of only one observation data, while calibration data uses all observation data except one 8,19).

After multivariate calibration using the PLS method, the model developed from the PLS method was used for the prediction of Rhodamine B content in market samples 1-10. It was found that the model developed from the PLS method at a wavelength of 1800-1180 value to get the best prediction results (22). At 1800-1180 cm^{-1} was able to predict the counterfeit content of market samples well. Based on the results of the prediction of market samples by PLS, it was found that each of the market samples was predicted to have counterfeiters with the levels listed in Table 6.

Table 6. Prediction Results of Market Sample with PLS

Sample Name	Rhodamine B content (%b/b)			$\bar{X}$ and SD (% w/w)	Conclusion
	1	2	3		
Sample 1	0,64	0,98	1,69	1,103 ± 0,44	Adulterated with Rhodamine B
Sample 2	12.06	11,67	13,27	12,47 ± 0,52	Adulterated with Rhodamine B
Sample 3	8,08	8,99	9,30	8,79 ± 0,52	Adulterated with Rhodamine B
Sample 4	20,43	20,66	21,43	20.84 ± 0,43	Adulterated with Rhodamine B
Sample 5	-1,22	-1,47	-1,14	-1,28 ± 0,14	Adulterated with other material
Sample 6	11,13	10,46	6,86	9,48 ± 1,88	Adulterated with Rhodamine B
Sample 7	-2,28	-0,78	-0,59	-1,22 ± 0,76	Adulterated with other material

DISCUSSION

Determination of chili powder was carried out before the Capsaicin content analysis stage with the aim of obtaining the identity of the sample to be used for research and ensuring that the sample used was really a red chili. The sample of red chili fruit used in this study is a chili fruit of the species *Capsicum annum L*. The characteristics of the chili used in this study are, elongated conical fruit, straight or bent, hanging, shiny smooth

surface, 1-2 cm in diameter, 4-17 cm long, short-stemmed, red. Seeds are flat, approximately 4 mm in diameter, young yellow after old brown (17).

Calculation of capsaicin content in control chili powder samples was conducted at the Integrated Research and Testing Laboratory of Gadjah Mada University, Yogyakarta. Calculation of capsaicin levels was carried out by KLT Densitometry method. The use of the Densitometry method in determining compound levels is considered to have high specificity, the results obtained are reliable, the work is relatively easy and fast and the amount of solvent needed is relatively small (20). The results of capsaicin compound levels in the control chilli powder sample were 624.77 µg/g. Meanwhile, the capsaicin standard based on CODEX STAN 307-2011 is 134 - 1.333 µg/g which is categorized as medium spicy. So the results of the determination of capsaicin content in the control chili powder sample are in accordance with the standard capsaicin content in the literature. The capsaicin content in chilies varies according to the type of chilli plant, the level of maturity at the time of harvesting, processing and storage, as well as the conditions of the growing area (region, climate, season).

Tests using FTIR spectroscopy on Rhodamine B samples and control chili powder. Both samples were tested with the same treatment. The samples of chili powder and Rhodamine B dye have quite distinctive differences, namely the shape of the spectrum that does not overlap and the difference in the absorption area of 3500-1500 cm^{-1}. It was found that the typical absorption in the Rhodamin B sample was found in the C-O carboxylic acid group, N-H secondary amide, and C-N. While in chilli powder that typical absorption is found in the O-H group, and C=O carbonyl.

The results of infrared spectrophotometer (IR) analysis of chili powder shows an absorption band in the wave number region of 3316.98 cm^{-1} indicating the presence of stretching vibrations of the -OH group with medium absorption intensity and a widened band shape. Then the band that appears at a wave number of 2911.86 cm^{-1} is a stretching vibration of the C-H group. The appearance of an absorption band at 1725.96 cm^{-1} indicates the presence of stretching vibrations of the C=O carbonyl group. Furthermore, there is an absorption at a wave number of 1619.31 cm^{-1} indicating the overlapping of the C=C group on the benzene ring and the vibration of the N-H group. There is also a sharp absorption band at wave number 1031.99 cm^{-1} which is the stretching vibration of C-O-H group. Based on the infrared spectrophotometer results, it is suspected that chili powder does contain a capsaicin compound with the presence of -OH, -NH, -CH, C=C, C=O, and C-O-H groups (6,25). From the results of infrared spectrophotometer (IR) analysis of Rhodamine B, it shows that the typical absorption in Rhodamine B samples is found in the C-O group of carboxylic acids, N-H secondary amides, and C-N. This shows that Rhodamine B used as a counterfeiter in this study is really a Rhodamine B compound.

Furthermore, mixing between chili powder and Rhodamine B was carried out. The mixture was made in the concentration range of 0-50% (w/w), after weighing the sample was mixed using a vortex to be mixed perfectly. After conducting analysis using FTIR on all samples, namely control chili powder samples, market chili powder samples, mixed chili powder samples, and Rhodamine B, all samples were processed by grouping to determine the presence of counterfeiters.

Clustering of samples was carried out using OPLS-DA multivariate calibration. OPLS-DA is a supervised clustering method that uses data that has been labelled or the sample group is already known. OPLS-DA clustering is based on the separation between components that are relevant for distinguishing groups (discriminant components) and components that are not relevant (orthogonal components) (19,27).

Before being used to predict the presence of counterfeiting in market samples, OPLS-DA was first validated by removing the names and eliminating the groups of 1/3 of the total samples used and then predicted 1/3 of the total samples whether they belonged to the group according to their own sample type. This treatment was carried out on chili powder and Rhodamine B samples so that 1/3 of the total samples were validated and 2/3 of the total samples were calibrated (8,11). It was found that the samples whose names were removed and the groups were correctly entered the group according to the reality of the sample type itself and the results were 100% correct in table 3. Therefore, OPLS-DA is ready to be used to predict market samples. OPLS-DA is able to separate chili powder and Rhodamine B samples well, and can be used for prediction on market samples whether there is adulteration with Rhodamine B or not. It was found that market samples 1-7 were not pure chili powder (other ingredients were added) and market samples 8-10 were predicted to be pure chili powder.

In the next stage of analysis of mixed samples and market samples using PLS, the FTIR spectra of each mixture were processed with the PLS multivariate calibration method. The selection of optimal conditions is carried out to produce the best calibration model, the selection is based on the wavelength region that produces the highest R^2 (Coefficient of determination) value for both calibration and validation to show how good the regression model is, the lowest RMSEC (Root Mean Square Error of Calibration) and RMSEP (Root Mean Square Error of Prediction) values to show the smallest prediction and calibration errors23). In the PLS analysis, the best wavelength used was the 1800-1180 cm^{-1} wavelength where the R^2 calibration value of 0.9989 was obtained; RMSEC value of 0.789; R^2 validation of 0.9968; RMSEP of 1.93.

Next, validation of the model that has been developed is carried out using a cross-validation technique using the leave-one-out technique. In this technique, one of the calibration samples (eg a 10% sample) is removed, then this sample is modeled with the remaining samples. Next, the predicted value of the 10% sample from the remaining sample calibration model is calculated. This is done continuously until the samples are removed one by one and modeled with the remaining calibration samples (8,11). After validation, we continue to predict the level of counterfeiting in market samples 1 to 7 because in the OPLS-DA processing results it was detected that there was counterfeiting in these samples so we will continue detecting the level of counterfeiting with PLS by entering the market sample data and

setting the actual number and then the level of each sample. the market will be predicted by PLS as shown in the results of table 5.7. The results obtained from samples 1, 2, 3, 4, and sample 6 are estimated to contain Rhodamine B adulterants, while samples 5 and 7 are estimated to be adulterated with Unknown ingredients other than Rhodamine B.

According to Minister of Health Regulation (PERMENKES) no. 239/Menkes/Per/V/1985 designated Rhodamine B as one of 30 dangerous coloring substances and is prohibited from being used in medicine, food and cosmetics. This shows that samples 1,2,3,4 and 6 do not comply with regulations in Indonesia. Meanwhile, according to research conducted by Novita, it shows that the toxic effect of Rhodamine B is present at a concentration of 0.2% for a period of 21 days with histopathological studies. Therefore 0.2% can be considered the minimum level of Rhodamine B that causes health hazards (24). So the levels of Rhodamine B in market samples 1,2,3,4 and 6 exceed the minimum concentration limit which causes health hazards.

Negative impacts due to consumption of Rhodamine B will appear if the dye is consumed long term. Rhodamine B can also cause acute effects if 500 mg is ingested, which is the toxic dose. The possible toxic effect is gastrointestinal irritation (25). Long-term use of Rhodamine B in food can cause liver dysfunction or cancer. Because Rhodamine B is very toxic and it has also been reported that Rhodamine B has carcinogenicity, reproductive and developmental toxicity, neurotoxicity, and chronic toxicity in humans and animals (26).

The limitations of this study include the following:

1.Sampling was only carried out in February 2024 in one traditional market in Malang Regency, so that it can only describe conditions at one time because market conditions can change.

2.The chili fruit species used in this study is only Capsicum annum, so it can only detect one chili species while other chili species cannot be detected.

3.The type of counterfeiter used in this study is only Rhodamine B, so other types of counterfeiters cannot be detected.

CONCLUSION

Based on the results of research and discussion that has been done in this study, it can be concluded that the FTIR Spectroscopy method combined with Chemometrics for multivariate calibration using PLS produces a good calibration model with a calibration value of 0.9989; RMSEC value of 0.789; validation 0.9968; RMSEP 1.93. The OPLS-DA method can classify samples well and can detect counterfeiting in chili powder samples at Singosari Traditional Market, Singosari District, Malang Regency. The samples of chili powder sold in Singosari Traditional market, Singosari Sub-district, Malang Regency contained pure chili powder (market samples 8,9,10) and there were 7 falsified samples (market samples 1 to 7) based on the results of OPLS-DA clustering chemometrics.

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CONFLICT OF INTEREST

There is no conflict of interest in this study.

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Anti-Aging Potential and Quercetin Determination of Melastoma malabathricum L. Leaves Extract

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A B S T R A C T

Background: *Melastoma malabathricum* L. is a wild shrub that traditionally used in South-East Asia as wound healing. This plant extracts contain phenolic, flavonoid and tannin. The leaves extract also has many pharmacological activity however the anti-aging activity of the leaves extract has not been studied. **Methods**: The leaves of *M. malabathricum* were macerated and refluxed using chloroform and ethanol consecutively using. Quercetin content of the leaves extract was determined by HPLC. Anti-aging activity was evaluated by anti-elastase, and anti collagenase inhibitor activity of the leaves extract was assessedby a fluorometric method. **Results:** The Yield Percentage of the *M. malabathricum* leaves extract was 16.53% w/w containing 9.2 mg/g quercetin.. The leaves extract of *M. malabathricum* L. possed anti-elastase and anti collagenase activity with IC_{50} 80.39±2.36 ppm and 63.3±3.32 ppm, respectively. **Conclusions:** *M. Malabathricum* leaves extract was promising as a raw material for anti-aging cosmetic

Keywords: *Melastoma malabathricum* L.; anti-aging; anti-elastase; anti collagenase; quercetin

INTRODUCTION

Melastoma malabathricum L. (Melastomataceae) is a wild shrub that widely grows in South-East Asia. It is traditionally used as herbal medicine in Malaysia, India, China, and Indonesia. All parts of this plant has been used as ethnomedicine to treat diarrhea, dysentery, toothache, and stomachach. The roots are commonly used to alleviate pain from mouth ulcers in children, while the stems are frequently employed to treat various skin diseases. Powdered leaves and roots can be used to accelerate the healing of wounds or chickenpox . The leaves are traditionally used to treat wounds, acne, and dark spots on the skin (1). Dayak Tribe in Kalimantan, Indonesia, also used the leaves as wound healing(2).

Ethanol extract of *M. malabathricum* leaves has demonstrated wound healing and antiviral activities against herpes simplex virus type I. The leaf extract significantly helps fasten wound healing by reducing bleeding time, improving scar tissue formation, and diminishing acne (3,4). Additionally, the leaf extracts exhibit pharmacological activities such as antibacterial (3,5), antiproliverative(6), antioxidant(7), and antiinflammatory(6,8). The powdered leaves demonstratedastringent properties that contribute on dysentery treatment, relieve hemorrhoid pain, and heal wounds (6). Phytochemical analysis reveals that the leaves contain flavonoids, triterpenes, tannins, saponins, steroids, glycosides, and phenolic compounds (1). Flavonoids, triterpenes, and tannins contribute to the anti-inflammatory activity of the leaves (3,4). Notably, the leaf extract contains quercetin, quercitrin, and kaempferol-3-O-(2',6'-di-O-p-trans-coumaroyl)-β-glucoside (3,4).

Despite its broad pharmacological activity, there is still a lack of studies about an anti-aging activity of the leaves extract. *M. malabathricum* leave has potent wound healing properties which improve collagenation, enhances epithelization and promotes wound closure(5). High flavonoid and tannins, including ellagitannin and kaempferol-3-O-β-D-glucoside (astragalin), were responsible for the wound healing activity(3,4). The bioactive compounds of the leaves extract also increase the proliferation rates of human skin fibroblast(4). The previous study stated that glucoside also stimulated collagen formation in human skin fibroblast(5). Moreover, Ellagic acid derivative showed inhibitor activity on elastase release of human neutrophil(9). A mixture of extracts from three melastoma species (*M. malabathricum*, *M. decemfidum*, and *M. hirta*) has been tested for anti-elastase and anti-collagenase activities (10).

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Therefore, This study aims to evaluates the anti-aging activity of *M. malabathricum* leaves extract through *in vitro* anti-elastase and anti collagenase assay.

MATERIALS AND METHODS

Materials

Quercetin (Sigma Aldrich, Singapore), Phosphatidylcholine (Phospholipon 90G and P 30 Lipoid ®, Germany), Neutrophil Elastase Inhibitor Assay Kit (Abcam, UK), Collagenase Inhibitor Assay Kit (Abcam, UK), Carbomer (Carbopol Ultrez 30), female *Sprague Dawley* rats (Agricultural, IPB, Indonesia). The use of animal in this study was approved by ethics committee from Cipto Mangunkusumo Hospital, Faculty of Medicine, Universitas Indonesia (No. 0103/UN2.F1/ETIK/2018). All solvent used in this study was an analytical grade or higher.

Methods

- **Plant Materials and Extract Preparation**

The leaves of *M. malabathricum* were collected from the hill in Samboja, Kutai Kertanegara, East Kalimantan, Indonesia and authenticated in Indonesian Science Institution (Lembaga Ilmu Pengetahuan Indonesia, LIPI. The leaves were air dried under a black cloth, then powdered using a grinder. The leaves powder was stored in an airtight container protected from light until further use.

The extraction method was adapted from Savic (2016)(11)and Michel (2014)(12). The leaves powder (350 g) was pre-extracted with chloroform (1:10 w/v, 3x24h) and then air dried to omit chloroform residue. The dried leaves powder was refluxed with ethanol (1:10 w/v, 3x2h) to obtain ethanol extract. The extract was then concentrated with a vacuum rotary evaporator (Buchi Rotavapor R-100, Japan) to obtained dry extract. The dried extract was stored in airtight container and kept in the refrigerator until further use. Yield of extraction was calculated by the formula:

$$Percentage\ yield\ \left(\%\frac{w}{w}\right) = \frac{(weight\ of\ dried\ extract)}{(weight\ of\ sample\ powder)} \times 100$$

- **Determination of quercetin content**

Quercetin content, as a marker constituent, was determined by HPLC method(13). HPLC analysis (Shimadzu LC-20AD, Japan) was performed with reversed phase column (Hypersil Gold C18, 150x4.6 mm, 5 µm diameter particles) and mobile phase consists of acetonitrile:2% acetic acid in water(40:60, v/v). The mobile phase and samples were filtered through a 0.45 µm nylon membrane filter (Whatman, USA) and degassed ultrasonically for 10 min before use. Samples were analyzed with an injection volume of 20 µL, the flow rate of 1 mL/min, and detection at 370 nm using a diode array detector (DAD). Calibration curves of quercetin were established by plotting the areas of peaks against eight concentration of quercetin standards (500-3.90625 µg/mL). This calibration curve was used to determine quercetin concentration of the extract.

- **Anti Elastase activity *in vitro* assay**

Antielastase activity was evaluated fluorometrically as protocol provided in the kit. Briefly, five concentrations of the extracts were prepared as sample tests. A fifty µL diluted Neutrophil Elastase (NE) solution was mixed with a 25 µL sample tests (or assay buffer for enzyme control) in 96-well black microplate (Thermo, USA). The mixture was incubated at 37°C for 5 min, and a 25 µL substrate was mixed to each sample. The kinetics of the enzyme activity was measured fluorometrically (RFU) at Ex/Em 400/505 nm (Glomax ver.3) from 0 min to 30 min. The calculation of percentage inhibition as the following formula.

$$Inhibition\ (\%) = \frac{(RFU_{enzim\ control} - RFU_{sample})}{RFU_{enzim\ control}} \times 100$$

The results were expressed as mean±SD of duplo experiments. Five concentration of extract sample and % inhibition of elastase was plotted for the IC_{50} values using analysis of probit (SPSS Inc.).

- **Anti-Collagenase activity *in vitro* assay**

Anti-collagenase activity was performed fluorometrically according to the protocol provided in the kit. Briefly, five concentrations of extracts in water were prepared as sample tests. A one µL sample test (or assay buffer for enzyme control) was mixed with a 5 µL diluted collagenase and 44 µL assay buffer in 96-well black microplate. The mixture was incubated for 15 minutes at room temperature. A 50 µL substrate was added to each well. The fluorescences (RFU) were measured immediately at Ex/Em 490/520 nm on a microplate reader in kinetic mode for 45 minutes at 37°C protected from light. The relative inhibition was calculated as the following formula:

$$Relative\ Inhibition\ (\%) = \frac{(RFU_{enzim\ control} - RFU_{sample})}{RFU_{enzim\ control}} \times 100$$

The experiment was done in duplo and the results were expressed as mean±SD. The IC_{50} values were calculated from plotting five concentration of the extract samples versus % inhibition of collagenase using analysis of probit (SPSS Inc.).

3 Result and Discussion

Result

Percentage yield and quercetin content of the *M. malabathricum* leaves extract

The yield of extraction was 16.53% w/w. Equation of calibration curve of quercetin standard was y = 82094x + 11933,

R^2 = 0,9984. The quercetin content of the dried extract was 9.20 ±0.81 mg/g extract.

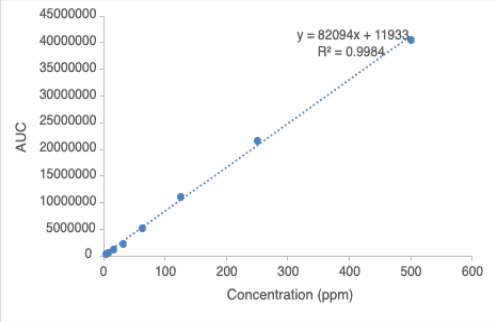
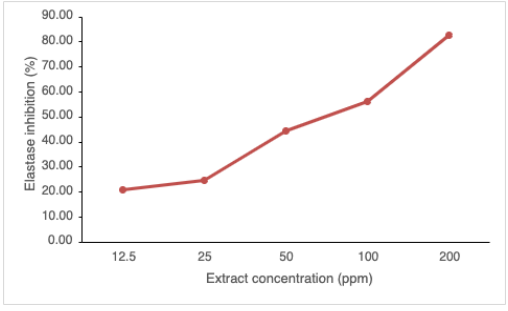


Figure 1. Calibration Curve of Quercetin Standard

Anti-aging activity of the extract

Anti-aging activity was determined *in vitro* by elastase inhibitor and collagenase inhibitor activity of the extract. Probit analysis of the sample concentration versus % inhibition activity shown that extract has potent inhibition activity against elastase and collagenase with IC_{50} 80.39±2.36 ppm and 63.29±3.32 ppm, respectively.



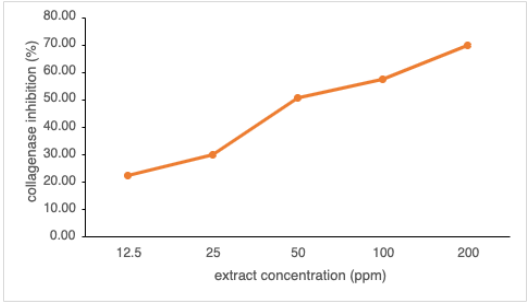


Figure 2.

Discussion

Extraction method affects the phytoconstituents in the extract. In this study, *M. malabathricum* leaves were pre-extracted with chloroform to removed non-essentials content, such as chlorophyll which can interfere with biological activity and physical appearance of the extract (12). After defatting with chloroform, the dried powder leaves were refluxed with 96% ethanol as this organic solvent is best to extract the marker constituent of the extract, i.e., quercetin (11). Our previous study showed that pre-extraction process with chloroform did not influence the quercetin content of *M. malabathricum* leaves extract (MLE) (14). However, quercetin content of the MLE in this study was higher than the previous study in which the quercetin content of *M. malabathricum* from a different location in Malaysia ranged between 0.1-1.5 mg/g extract (15). The chemical composition of plants can be influenced by environmental factors. Variations in soil properties, nutrient content, and climatic conditions in different growing locations can cause plants of the same species to exhibit differences in their chemical constituents or active compounds (16).

The present study investigated the anti-elastase and anti-collagenase activities of the MLE, highlighting its potential in anti-aging applications. The findings revealed that MLE has the IC_{50} -which is less than 100 ppm for both anti-elastase and anti-collagenase activity. It concludes that MLE exhibits high inhibitory effect on both elastase and collagenase enzymes, which are crucial in skin aging process. Elastase and collagenase are enzymes that degrade elastin and collagen, respectively, contributing to skin elasticity and firmness loss, leading to the formation of wrinkles and sagging skin (17). The inhibition of these enzymes is a strategy for maintaining skin structure and function, thereby reducing the visible signs of aging (18). Another study analyzing anti-elastase and anti-collagenase activity of seaweed extracts used IC50 values within the range of 6.25–100 µg/mL. These ranges demonstrate high potency and are consistent with benchmarks in enzyme inhibition studies (19).

Matrix metalloproteinases (MMPs), including collagenases (e.g., MMP-1, MMP-8) and elastase, play critical roles in the degradation and remodeling of the extracellular matrix (ECM). Collagenases degrade fibrillar collagen into smaller fragments, while elastase targets elastin, a protein essential for maintaining skin elasticity and structural integrity. Under normal physiological conditions, these enzymes are tightly regulated; however, their overexpression contributes to pathological processes such as skin aging (19).

The high anti-elastase and anti-collagenase activity observed in MLE align with its traditional use in wound healing, providing a scientific basis for its effectiveness (2). this activity of MLE is attributed to its high phenolic and flavonoid contents. Phenolic is known to have anti-aging activity against elastase and hyaluronidase (20). Total phenolic content of MLE was range between 145-222 mg GAE/g, supporting its potency (14). Previous studies have shown that flavonoids can inhibit MMPs, including elastase and collagenase. This inhibition occurs through mechanisms such as binding to the active sites of these enzymes and reducing oxidative stress, which regulates MMP expression. By protecting ECM components, flavonoids

demonstrate anti-inflammatory, anti-aging, and therapeutic potential in preventing ECM degradation-related conditions (19). The MLE contained flavonoid compounds e.g., Quercetin, quercitrin, and kaempferol-3-O-(2",6"-di-O-p-trans-coumaroyl)-β-glucoside (3,4). The marker constituent, quercetin, was known to have anti-elastase and anti-collagenase (21–23). Besides that, kaempferol and rutin which is also contained in the extract(15,23,24) have the anti-collagenase activity(21,25).

4. Conclusion

In conclusion, the anti-elastase and anti-collagenase activities of *M. malabathricum* leaves extract showed its potential as an effective natural agent for skin aging prevention. The presence of bioactive compounds, such as phenols and flavonoids, supports its traditional use in wound healing. Quercetin, as a flavonol compound, support the mechanism of action of the extract. This study provides a foundation for further exploration of M. malabathricum as a valuable ingredient in anti-aging products.

5. CONFLICT OF INTEREST

There is no conflict of interest in this study.

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Increasing The Digital Health Competency of Salatiga City Pharmacists in The Era of Digital Health Transformation

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A B S T R A C T

Background: The Indonesia Ministry of Health has established six pillars of health transformation, one of which is health technology transformation. Pharmacist as a professional health care must have digital health competency to contribute to the success of digital health transformation in Indonesia.
Objective: This research aims to analyze and improve the digital health competency of Salatiga city pharmacists.
Method: This research is a quasi-experimental research with a one-group pretest-posttest design. The number of participants was 30 pharmacists from the city of Salatiga. Digital health competency levels were measured before and after training using the Public Health Informatics Competencies for Primary Health Care (PHIC4PHC) questionnaire instrument. Competency levels are presented as ordinal data so that the Wilcoxon test is used to determine differences before and after intervention.
Result: Participants in this study consisted of 63% pharmacists at pharmacies, 20% Primary Clinic pharmacists, and 17% Community Health Center pharmacists. Participants were aged between 26 and 47 years old with a period of pharmacist practice between 4 and 22 years. Analysis of respondents' digital health competency level before training obtained results of 2% basic level, 27% understanding level, 43% fluent level, and 23% expert level. Meanwhile, after 2 weeks of respondents applying the results of digital health training, there was an increase in respondents' competency to 30% fluent level and 70% expert level. The pharmacist's health competency increased significantly (p-value< 0.05). 20 participants increased their competency level, and 10 participants did not change their competency level.
Conclusion: Digital health training has been proven to significantly increase the digital health competency of Salatiga city pharmacists.
Keywords: e-Health, Telepharmacy, Informatics, Pharmacists, competence

INTRODUCTION

The Indonesian Ministry of Health has developed six pillars of health transformation to achieve advanced Indonesia, namely through the transformation of Primary Services, Referral Services, Health Resilience Systems, Health Financing Systems, Health Human Resources, and Health Technology (1). The digital technology transformation of the health sector will have an impact on at least five things. First, improve the quality of health services. Second, make it easier to access health services. Third, increase the added value of the health sector economy with an orientation towards domestic products. Fourth, accelerate the achievement of government priority programs in the health sector. Fifth, increase the competency of health human resources while ensuring their distribution evenly throughout the country (1).
The digitalization of healthcare has many potential benefits including reducing turnaround times, medication errors, and adverse drug events; better resource allocation; advancing preventative care, and enabling greater adherence to clinical guidelines (2,3). Despite the many benefits of digital health, the adoption of digital tools and technologies in healthcare has been slow in many countries, including the United States, Europe, and Australia (4,5). Poor digital health competency of health professionals was found to be the most common barrier to the adoption of digital health services (5). It should be emphasized that increasing digital Health competencies can lead to increased adoption of new digital tools and technologies among Health workers, because to increase the digitalization of health services, health professionals have been recognized as a key factor in the digital transformation of the health services sector. Therefore, they must be equipped with digital health competencies, ranging from basic skills (e.g. computers, and tablets) to more complex skills, such as teaching patients about the safe and appropriate use of data sources and digital technologies (6).

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Digitalization in healthcare has changed rapidly over the past decades, and online information and mobile phone applications play an increasingly large role in healthcare. Along with these changes, the skills to search for, select, assess, and apply online health information and healthcare-related digital applications are becoming increasingly important (7). This skill is called digital health literacy or e-health literacy (8). Digital health literacy is using information and communication technology to support health and health services (9).

Digital health literacy is a collection of six basic skills, namely traditional literacy, health literacy, information literacy, scientific literacy, media literacy, and computer literacy (8). On the other hand, digital health literacy not only requires the ability to search for health-related information, understand the information, and apply it appropriately but also demonstrates advanced technologies involving patient empowerment and engagement, information sharing, and social networking (10,11). Health professionals need to have health digitalization competencies because health professionals must be able to identify and use reliable sources of health information from the internet and other relevant information sources to make evidence-based medical decisions and to improve the delivery of health services to patients (8,12–14).

Digital competence is one of the key competencies for continuous learning both for personal life and for professional work (15). Pharmacists as health professionals must continuously update their knowledge and skills so they can keep up with developments with the latest trends in issues related to drug management and therapy. The Accreditation Council for Pharmacy Education defines Continuing Professional Development as “a lifelong process of active participation in learning activities that assists individuals in developing and maintaining ongoing competency, enhances their ability of professional practice, and supports the achievement of career goals." Pharmacists must develop competency in providing centered care to patients; work as part of an interdisciplinary team; practice evidence-based medicine and focus on quality improvement (16).

Community pharmacists have an important role in improving public health, but advances in telehealth and digital technology mean that community pharmacists are changing the methods they use to serve customers and patients (17). Canadian community pharmacists frequently use digital health in their practice and recognize the benefits of this technology (18). Digital health will continue to be a key driver of practice transformation and improved quality of care (18).

Based on the background above, this study aimed to identify and analyze pharmacists' digital health competencies to support the achievement of Indonesia's health transformation, especially in the health digitalization transformation pillar.

MATERIALS AND METHODS

Methods

This research is a quasi-experimental research with a one-group pretest-posttest design. Researchers chose to use a quasi-experimental method because this method is similar to the experimental method but is more flexible in that it does not use random assignment (19). In this study, researchers were unable to carry out random assignment due to limited research subjects. The criteria for participants in this study were pharmacists who were still actively practicing in primary health care facilities such as public health centers, community pharmacies, or primary clinics; willing to become a research participant; and have practice experience minimum of 1 year as a pharmacist. Ethical approval is carried out at the Ngudi Waluyo University Ethics Committee for human research subjects with interventions, so informed consent is required as a form of participant consent in this research. This study was conducted in January 2024. The selection of pharmacists as respondents was determined using non-random method with an accidental sampling technique, where respondents involved in this research voluntarily agreed to become participants. The selection of participants was carried out through announcements in the WhatsApp group of the Indonesian Pharmacists Association of Salatiga City. Within 2 weeks, 30 pharmacists were found who were willing to be involved in the research. The number of subjects in this research meets the minimum sample rules for quantitative research, namely 30 subjects (20).

The level of digital health competency was measured before and after training. The training is carried out in accordance with the indicators to be measured, including knowledge and skills about health information systems, general computer skills, office and network application skills, knowledge about security and legality, skills for access, management, integration and evaluation of health information. The level of digital health competency was measured using the Public Health Informatics Competencies for Primary Health Care (PHIC4PHC) questionnaire instrument which consists of a total of 42 statements. This questionnaire is designed to measure the level of Health informatics competency of Health workers in primary healthcare facilities. The validity of this questionnaire has been tested for the population in Indonesia (21). The questionnaire measures: knowledge of health information systems with 8 statements, skills in using health information systems with 3 statements, general computer use skills with 10 statements, skills in using office applications with 9 statements, skills in using internet networks, knowledge of data security and validity, Health information access skills, Health information management skills, Health information integration skills, and Health information evaluation skills each with 2 statements. Each statement was selected using a Likert scale with answer choices from STS= Strongly disagree; TS=Disagree; ATS=Between agree and disagree; S=Agree; SS= Strongly agree. A positive statement is worth 5 if you strongly agree and 1 if you strongly disagree. A negative statement is worth 5 if you strongly disagree and 1 if you strongly agree. The total score of the 42 statements is then analyzed using the following formula to determine the level of competency:

PHIC4PHC = (((Q1 + Q2 + Q3 + Q4 + Q5…. + Q42)/42)-1)*50/4

The index values are then categorized as follows:

0-25 = ‘Basic ’

>25 to 33 ='Literacy' = understand
>33 to 42 = 'Fluency'
>42 = 'Mastery' = expert

The results of measuring differences in competency levels before and after training used non-parametric statistics with the Wilcoxon test using SPSS ver.26. Expert is coded 4, Fluent is coded 3, Understanding is coded 2 and Basic is coded 1 (22).

RESULTS AND DISCUSSION

This research has been approved by the Research Ethics Commission of Ngudi Waluyo University with number: 0112/KEP/EC/UNW/2024 dated 19 January 2024. This research was conducted on 30 participants with data on the characteristics of various respondents and reviewed in terms of the type of professional practice place, gender, age, and length of practice experience listed in Table 1.

Table 1. Participant Demographics

Category	Information	Frequency (n)	%
Place of Professional Practice	Community Pharmacy	19	63
	Primary Clinics	6	20
	Public Health Center	5	17
Gender	Male	3	10
	Female	27	90
Age (Years) (Mean±SD = 35,6±6,1)	26 – 35	15	50
	36 – 45	14	47
	46 – 55	1	3
Length of practice experience (Years) (Mean±SD = 12,5±5,5)	1-10	10	33
	11 – 20	18	60
	>20	2	7

The characteristics of the participants in this study, in terms of gender, are more dominant, namely female respondents. This is by data from the Indonesian Pharmacists Association, Salatiga City Branch Managers, where pharmacists are dominated by women. Respondents' ages varied, most were in the range of 26-35 year. The two characteristics of the pharmacist profession in this study are in line with research conducted by Ebtavanny, 2023 where female pharmacists dominate as research respondents at 84% and the age of most respondents is in the range of 26-35 years (23).

The results of filling in the PHIC4PHC questionnaire which consists of 42 statements were analyzed to determine the competency level of pharmacists before and after training. Figure 1 shows an overview of participants' digital health competency levels before the training, with results of 7% basic level, 27% understanding level, 43% fluent level, and 23% expert level. Meanwhile, after 2 weeks of respondents applying the results of digital health training, there was an increase in respondents' competency to 30% fluent level and 70% expert level.

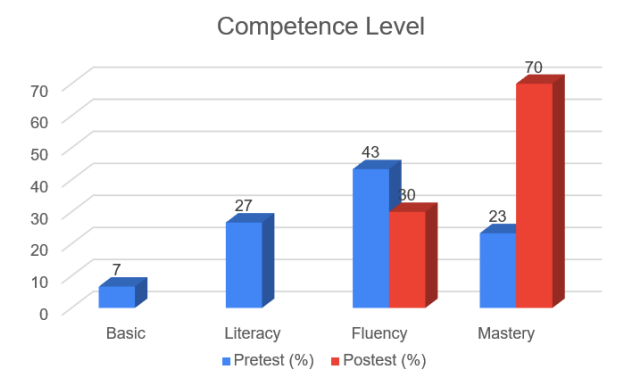

Figure 1. Digital health competency level of Salatiga City pharmacists before and after training

The average pretest score of 36.32 shows that the digital health competency of Salatiga city pharmacists is at the Current Level. The digital health competency level of Salatiga city pharmacists increased to expert with a posttest score of 43.42 after receiving training and carrying out pharmaceutical services by utilizing digital health technology. Salatiga City pharmacists are expected to be able to apply digital health technology in daily pharmacy practice to improve performance benchmarks for health service workers, including service delivery, diagnosis, clinical management, prescription-related practices, patient follow-up, and data management. Moreover, digital health technology can improve interprofessional communication, compliance with clinical protocol standards, and the personal skills and

competencies of healthcare workers. Health worker performance improves with the use of correlated digital Health technology to optimize communication skills; reliable and fast access to data; development of professional expertise and skills; increased productivity, efficacy, and accuracy; improving service quality; reduced time commitment to professional activities; and advance the acquisition of knowledge (24).

The results of statistical tests using the Wilcoxon test using SPSS ver.26 showed that the level of digital health competency of participants before and after training was significantly different (Asymp. Sig 0.00 < 0.05). With 20 participants experiencing an increase in their competency level and 10 participants maintaining their competency level. These findings are in line with research by Kanfe, 2022 (25). found significant factors that determine the knowledge and attitudes of health workers towards the use of digital health information systems. Efforts to provide adequate training, adequate resources, skills related to the use of digital health information systems, increased motivation and feedback will help improve and achieve the expected knowledge and attitudes that support the use of digital health information systems to improve the quality of health services.

Analysis of the level of digital health competency of pharmacists in the city of Salatiga according to the division of indicators in the questionnaire. The digital health competency indicators measured using the PHIC4PHC questionnaire consist of 4 general categories and 10 indicators. Based on research results, the average digital health competency level of pharmacists before training had 9 indicators in the fluent category and 1 in the understanding category, namely in the skills category for using health information systems. After training, the pharmacist's digital health competency level in all indicators is in the expert category as can be seen in table 2.

Table 2. Analysis of the competence level for each category

Main Categories	Indicator	Competence Level on	
		Pretest	**Posttest**
1. **Cognitive Skills**	*Health information system knowledge*	Fluency	Mastery
	Health information system skills	Literacy	Mastery
2. Technical Proficiency	*General computer skills*	Fluency	Mastery
	Office application skills	Fluency	Mastery
	Network skills	Fluency	Mastery
3. Ethical Skills	*Security and Legal Knowledge*	Fluency	Mastery
4. Health Information Literacy	*Health information access*	Fluency	Mastery
	Health information management	Fluency	Mastery
	Health information integration	Fluency	Mastery
	Health information evaluation	Fluency	Mastery

Cognitive proficiency includes knowledge and skills in health information systems. The cognitive skills of pharmacists in the city of Salatiga before receiving digital health training were at a fluent and understanding level and increased to an expert level after receiving the training. WHO has identified that the problem of incomplete reporting is enormous and is related to a lack of knowledge and attitudes among health workers characterized by a lack of analytical skills, training, and lack of initiative in using information (26,27). Understanding the attitudes and knowledge of health service providers towards the use of digital health information systems is important for the provision of effective and efficient health services (28). The use of digital health information systems is expected to improve the quality of health services, but the lack of positive attitudes and adequate knowledge is one of the main factors that hinder the use of digital health information systems among health service providers (28–30). Determining the attitudes and knowledge of healthcare providers will also help in understanding the impact of DHIS on the workload and quality of clinical healthcare services (28,31). The use of health information systems in Sub-Saharan Africa is too low due to low knowledge and attitudes of Health service providers (28). The level of digital health competency of Salatiga City pharmacists is already at the expert level, so it is hoped that pharmacists will be able to support the digitalization of health transformation.

The technical skills of health workers include computer usage skills, office application operating skills, and internet networking skills. The digital health technical skills of Salatiga city pharmacists before receiving training were at a fluent level and increased to expert after training. Computer literacy can be described as the computer-related knowledge necessary to acquire, communicate, process, and understand the basic knowledge necessary to make appropriate health decisions (32). Health workers must be skilled in the use of information communication and technology (ICT) and must continue to be developed (33). The adoption of digital health technologies will depend largely on users' computer skills. Given the enormous data requirements and increasing patient care, there is an urgent

need for all healthcare professionals to have technical computer proficiency to meet the need for better healthcare through electronic healthcare tools, which are growing rapidly across the world (34–36). Since most of the study respondents were of active age and had many years of work experience, they should be encouraged to self-direct computer appreciation training and more on-the-job training. This research is in line with the results of Sibiya's research, 2023 that there is a need for ongoing training and retraining of existing staff, and computer literacy should be emphasized as part of the requirements for future employment. Computer operating skills and knowledge are big determining factors in the adoption of digital health technologies (37).

Salatiga city pharmacists' health information technology ethical skills were initially at a fluent level and after receiving the training they became expert levels. Ethical skills in the use of digital health technology relate to health workers' knowledge of security and law. The healthcare sector can benefit greatly from developments in digital technology. The development of digital health applications must guarantee the privacy and safety of patients and the data collected (38). The topic of security and legal data in health information technology has received a lot of attention. To prevent potentially slowing factors in the development and implementation of digital health, we need to do the following: privacy and independence; consent and comfort; clinical research and routine clinical data; responsibility and standardization; and privacy and solidarity (38). Pharmacists must be able to sort legal data and secure patient data in the information system used.

Health information literacy includes access, management, integration, and evaluation of health information. The competency of Salatiga city pharmacists in the health information literacy component before the training was at a fluent level and increased to expert after the training. Health literacy is essential to enable healthcare providers to integrate evidence-based knowledge into their professional practice (39,40). Health literacy is also important to ensure the health and well-being of health service providers themselves, as well as those around them (for example the patients they care for) (41).

Analysis of the level of digital health competency of pharmacists in the city of Salatiga in this research is still limited to a framework of 10 indicators, but several other frameworks can measure the digital competency of health workers from other indicators. The digital capabilities of the pharmaceutical workforce can be further tailored to pharmacy practice through the inclusion of specific examples of digital technologies and digital skills relevant to the pharmaceutical field (42). Numerous studies have shown that health professionals' lack of health literacy contributes to poor health outcomes (higher mortality rates and poorer overall health status), health disparities, and increased costs (43–45). The increasingly complex and fragmented healthcare system and growing patient demands for self-care, care coordination, and system navigation require increasingly strengthened health literacy (45).

Pharmacy's growing role in collaborative healthcare teams increasingly relies on a variety of digital health technologies and digital literacy. Increasing digital health competency can be obtained through training. In Indonesia, there is no digital health training scheme, especially for the clinical role of pharmacists. Limited evidence was found regarding positive digital literacy training experiences. Pharmacists want or need more digital literacy training. The recommended core competencies for pharmacy informatics are based on digital literacy and so can be a starting point for further research, which should be expanded to include all pharmacy staff. Along with the growing role of pharmacists (46).

In launching the US Government's Digital Strategy in 2012, President Obama stated, 'I want us to ask ourselves every day, how can we use technology to make a real difference in people's lives' (47). As the role of pharmacy in health services continues to grow, it is necessary to increase pharmacists' digital skills, most recently from the UK Academy of Medical Royal Colleges which emphasized the need to 'increase professional skills in the field of digital health technology so that the significant benefits that can be generated by technology can be realized' (48). Digitalization has not yet been fully implemented in clinical practice, and several factors have been identified as possible barriers, including the competence of healthcare professionals (49). Increasing the healthcare digitalization competence of healthcare professionals has been recognized as a key factor in the digital transformation of the healthcare sector (6).

The limitations of this study are the small population size and low willingness of subjects to become samples, so it can only use non-probability sampling. Participants only practiced in community pharmacies, primary health care facilities and public health centers. There is no participants that practiced in hospitals. In the future, it is hoped that it can reach a larger number of subjects so that it can capture a broader picture of pharmacists' digital literacy competencies.

CONCLUSION

The digital health competency level of pharmacists in the city of Salatiga has increased significantly after participating in pharmacy digitalization training and is ready to support digital health transformation in pharmaceutical services in particular.

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CONFLICT OF INTEREST

There is no conflict of interest in the research that has been carried out.

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Formulation Physical Characteristic of Hard Candy Lozenge of Citrus Limon Essential Oil on Various Types of Sugar Free Candy Base (Isomalt, Mannitol, Sorbitol)

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A B S T R A C T

Citrus limon is a natural ingredient that has potential as an anti-anxiety. The dopamine-enhancing effect on the blood brain barrier due to consuming citrus limon essential oil can help relieve stress, fatigue, dizziness, and anxiety. This study aims to formulate citrus limon essential oil hard candy lozenge with various types of sugar free candy bases and evaluate its effect on the physical characteristic of the lozenge. Previously, this study was preceded by identifying the D-Limonene compounds as an active material on the citrus limon essential oil using the GC-MS method. There are three formulas used in this study, including F1 (isomalt), F2 (mannitol), and F3 (sorbitol). Physical characteristic tests carried out include organoleptic, weight variations, dimension, hardness, friability, dissolved pH, and dissolve time. The identification test of citrus limon essential oil using GC-MS showed the presence of D-Limonene compound. Thus, it can be concluded that the raw material of the oil was in accordance with the established quality. The results of the physical characteristics of the three formulas in the tablet dimension test, hardness, and the dissolved pH met the requirements. In addition, the organoleptic test of F1 and F3 yields better results than F2 (murky white color and rough texture). The evaluation of weight variation, friability, and dissolve time of F1 and F3 met specifications, while F2 did not meet specifications. Therefore, the sugar free candy base that can be developed into a hard candy lozenge in this study was by using isomalt (F1) and sorbitol (F3).

Keywords: Citrus Limon, Sugar Free, Hard Candy, Lozenge

INTRODUCTION

Citrus limon is a natural ingredient that has potential as an anti-anxiety. The dopamine-enhancing effect on the blood brain barrier due to consuming citrus limon essential oil can help relieve stress, fatigue, dizziness, and anxiety (1). The constituent components of citrus limon are D-Limonene (47,24-55,23%), geranial, and neral. The D-Limonene compound is the main content of citrus limon which can provide sedative and calming effects, making it a good choice for anxiety (2). Patients suffering from anxiety, depression, or other psychotic-related disorders often have difficulty swallowing tablets. In such cases, buccal preparations, such as lozenges, would be an effective solution to ensure that patients receive a good and efficient treatment regimen (3).

Lozenge is a solid dosage form that is intended to dissolve in the mouth or pharynx (4). Lozenge is classified into 4 types according to its composition and appearance, including chewy lozenge, compressed lozenge, soft lozenge, and hard candy lozenge (5). Hard candy lozenge consists of a mixture of sugar and other carbohydrates that have a candy-like shape (6). The advantages of hard candy lozenge are its sweet taste, smooth texture, and it dissolves slowly in the mouth (5).

There are several methods that can be used in the process of making lozenge, including direct compression, wet granulation, heat-congealing, and melting-molding (7). Melting-molding method is carried out by melting the candy base at its melting point (145-156ºC), then mixed with other excipients, and finally poured into the mold (6). These excipients such as binders, sweeteners, flavors, acidulants (8).

Based on the excipient of candy base, hard candy lozenges are divided into two types, namely sugar base and sugar-free candy base (6). In this study, sugar free candy base was used because it can be consumed by patients without experiencing side effects caused by sugar, such as tooth decay and increase blood glucose. There are 3 types of sugar free candy base used in this study,

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including isomalt (Formula 1), mannitol (Formula 2), and sorbitol (Formula 3).

Isomalt is a substitute for sucrose that has similar characteristics to sucrose. Isomalt has a sweetness level of about 0,45-0,6 times compared to sucrose (=1,0) (9). Mannitol has a sweet taste equivalent to glucose and half of sucrose (10). Sorbitol is an additive that is often used as a filler in solid dosage forms because it has a sweet taste (50-60% sweetness level of sucrose) (10). The three bases can give a cool sensation in the mouth and are proven not to increase blood glucose levels, so those are safe for diabetics. In addition, it also does not cause tooth decay because it does not increase or decrease the pH of the mouth after consumption (9). However, because all three bases still have a fairly low level of sweetness when compared to sucrose, so in this study stevia used as an additional sweetener which has a sweetness level 300 times that of sucrose (11). Othe additional excipients in this study were gum arabica as a binder, citric acid as an acidulant, and peppermint as a flavoring.

The difference in the type of bases used in this study is expected to affect the physical characteristic of the lozenge tablets. The physical characteristic of the lozenge tablets was carried out by determining the organoleptic, weight variation, dimensions (diameter), hardness, friability, dissolving pH, and dissolving time. In addition, the identification of the D-Limonene compound in the raw material of citrus limon essential oil was previously carried out using GC-MS method. The results of the 3 formulas will be analyzed statistically using the one-way Anova method followed by the post-hoc method.

MATERIAL AND METHODS

The materials used in this study were citrus limon essential oil food grade (Rumah Atsiri Indonesia, Karanganyar), isomalt p.g (Beneo, Germany), mannitol p.g (Qingdao Bright Moon Seaweed Group, China), sorbitol p.g (Ueno Fine Chemical Industry, Thailand), gum acacia p.g (Merck, Germany), stevia food grade (Soho Nootropics, China), citric acid p.g (Merck, Germany), Peppermint food grade (Rumah Atsiri Indonesia, Karanganyar).
The tools and instruments used in this study were GC-MS QP2010 SE (Shimadzu, Japan), friability tester (Erweka, Germany), hardness tester TBH 125 (Erweka, Germany), pH meter Laqua 1100 (Horiba, Japan), Hanson Phase One Disintegration Tester (Hanson, USA), Scout digital scales (OHAUS, USA), hotplate magnetic stirrer GuardianTM 3000 (OHAUS, USA), caliper with an accuracy of 0,05 mm (Tricle Brand, China), refrigerator, molds, and other glassware,

Identification of Citrus Limon Essential Oil by GC-MS Method

The first test began by identifying the D-Limonene compound in citrus limon essential oil using GC-MS, a modification of the method from Hojjati *et al* (12). Helium was used as a carrier gas with a flow rate of 0,9 mL/min with a split ratio of 1:20. Furthermore, the injector and detector were set at 230^{o}C and 280^{o}C, respectively. Then, 2 µL of essential oil was injected and run for 27 minutes. The content of essential oil will be identified based on the retention time of GC-MS and a comparison of similarity index, comparison of mass spectra with the library WILEY7, WILEY8, NIST (NIST08, NIST08s)-national institute of standards and technology.

Formula

There are 3 formulas (**Table 1**), each using a different sugar free candy base. The weight of each hard candy lozenge was made at 1,5 grams.

Table 1. Composition of different sugar free candy base

NO	COMPONENT	FUNCTION	(%)		
			F1	F2	F3
1	Citrus limon essential oil	Active pharmaceutical ingredient	4	4	4
2	Isomalt*	Sugar free candy base	90	-	-
3	Mannitol*	Sugar free candy base	-	90	-
4	Sorbitol*	Sugar free candy base	-	-	90
5	Gum acacia	Binder	5	5	5
6	Stevia	Sweetener	0,45	0,45	0,45
7	Citric acid	Acidulant	0,45	0,45	0,45
8	Peppermint	Flavor	0,1	0,1	0,1

*Sugar free candy base was mixed with distilled water at 1/3 of its total weight.

Lozenge specification

The hard candy lozenge that has been made was expected to meet the specifications as in **Table 2** below.

Table 2. Lozenge specification

No	Testing	Specification

1	Organoleptic	- Color: clear yellow - Odor: lemon mint - Taste: sweet lemon mint - Texture: smooth - Shape: cube
2	Weight variation	1,5 grams ± 2% (1,47-1,53 grams) (4,6)
3	Dimensions (diameter)	11,25 mm ± 5%
4	Hardness	4-8 kP (13)
5	Friability	< 1% (14)
6	Dissolving pH	3,8 – 7,0 (5)
7	Dissolving time	5-10 min (300-600 second) (5)

Procedure for Making Hard Candy Lozenge

The manufacturing process begins by dissolving the sugar free candy base into distilled water (1/3 of the weight of the bases) and heating it while stirring evenly until it reaches the desired melting point (isomalt = 155ºC; mannitol = 165ºC; sorbitol 145ºC). After the bases melted and the water had reduced, the other excipients, such as gum acacia, stevia, and citric acid were added and stirred until homogeneous. Citrus limon and peppermint were added when the mixture is slightly warm and stirred again until homogeneous. The mixture was then poured into molds and put into a refrigerator (2-8ºC) until it becomes solid form (4,15).

Physical Characterization

Organoleptic Test

Organoleptic tests were carried out by identifying color, odor, taste, texture, and shape. The aim was to determine the differences in physical organoleptic properties of hard candy lozenge in each formula.

Weight Variation Test

The weight variation test was conducted by weighing 10 lozenge one by one using a Scout digital scale (OHAUS, USA) and then calculating the average weight and standard deviation (15). The specifications of the tablet weight for hard candy lozenge are 1,5-4,5 grams (4,6). The weight of the lozenge used in this study was 1,5 grams ± 2% (1,47-1,53 grams).

Dimension (Diameter) Test

This test was conducted by measuring the diameter of 10 lozenges using a caliper (accuracy level 0,05 mm) (15). The diameter of the hard candy lozenge used in this study was 11,25 mm ± 5%.

Hardness Test

Hardness test was conducted on 10 hard candy lozenges using a TBH 125 Hardness tester (Erweka, Germany) (4). The hardness results were expressed in kilopond (kP) with test requirements of 4-8 kP (13).

Friability Test

This test was conducted to determine the resistance of hard candy lozenge to friction and shock during the manufacturing and distribution process using friability tester (Erweka, Germany). The test was conducted in accordance with the provisions in the USP. The 10 hard candy lozenges were cleaned one by one using a soft brush and then the initial weight of the sample was weight (w_o). Furthermore, the sample was inserted into the device at a speed of 25 rpm for 4 minutes. After that, the sample was removed and cleaned again using a soft brush and then weighed (w_1) to determine the percentage of friability (14). This test was conducted 3 times in replication. The equation used to calculate the %friability can be seen as follows:

$$\%\ Friability = \left(\frac{(w0 - w1)}{w0}\right) \times 100\%$$

***w_0 = initial weight; w_1 = final weight**

Dissolving pH Test

This test was carried out using a Laqua 1100 pH meter (Horiba, Japan). The test began by dissolving 1 hard candy lozenge into 200 mL of distilled water, then the pH was measured 3 times in replication (16). The pH requirements for this lozenge are 3,8-7,0 (5).

Dissolving Time Test

The dissolving time test was conducted by inserting 6 hard candy lozenges into each basket tube of the disintegration tester instrument. The solvent used was distilled water at 37ºC. The dissolving time of the hard candy lozenge was marked by the complete dissolution of the lozenge in the solvent media (15). Then, the time was recorded in seconds. This dissolving time test was carried out 3 times in replication. The ideal dissolving time for the hard candy lozenges is 5-10 minutes (5).

RESULTS AND DISCUSSION

Results of Citrus Limon Essential Oil Using GC-MS Method

The results of the identification test of citrus limon essential oil using the GC-MS method can be seen in **Table 3**.

Table 3. Identification test of citrus limon essential oil using the GC-MS method

Peak #	Rt (min)	Compound	BasePeak (m/z)	Similarity %	Area
5	10,474	*1R-alpha-Pinene*	93	97	2671457
6	11,406	*L-beta--Pinene.*	93	96	10876375
7	**12,478**	***D-Limonene***	**68**	**94**	**27246829**

8	12,807	*1.4-Cyclohexadiene.*	93	96	5926192
9	13,216	*Cyclohexene.*	93	97	379650
10	14,617	*2-Isopropyl-5-Methylcyclohexanol*	71	96	220410
11	15,608	*Cis-Citral*	41	97	2007662
12	16,034	*2,6-Octadienal,3,7-Dimethyl*	69	97	2821241
13	17,194	*2,6-Octadien-1-ol,3,7-Dimethyl-,Acetate,(Z)-*	69	97	825031
14	17,440	*2,6-Octadien-1-ol,*	69	97	825592
15	19,284	*Beta-bisabolen*	69	95	415692

According to the literature, analysis of citrus limon essential oil using GC-MS, obtained the findings of the presence of D-Limonene content which has a molecular formula of $C_{10}H_{16}$ with a retention time of 7,01 minutes and a percentage area of 96,79% (17). **Table 3** showed that D-Limonene was the main component of citrus limon. This compound was proven by the results of the GC-MS chromatogram at peak number 7 at a retention time of 12,478 min, the resulting MS fragment pattern showed the D-Limonene compound with a base peak of m/z 68 (**Figure 1**). This was in accordance with the fragment pattern in the NIST Chemistry WebBook (18). Based on these results, it showed that citrus limon essential oil in this study contains D-Limonene compounds that are in accordance with CoA and comparative literature. Thus, it could be concluded that the essential oil has met the specified quality.

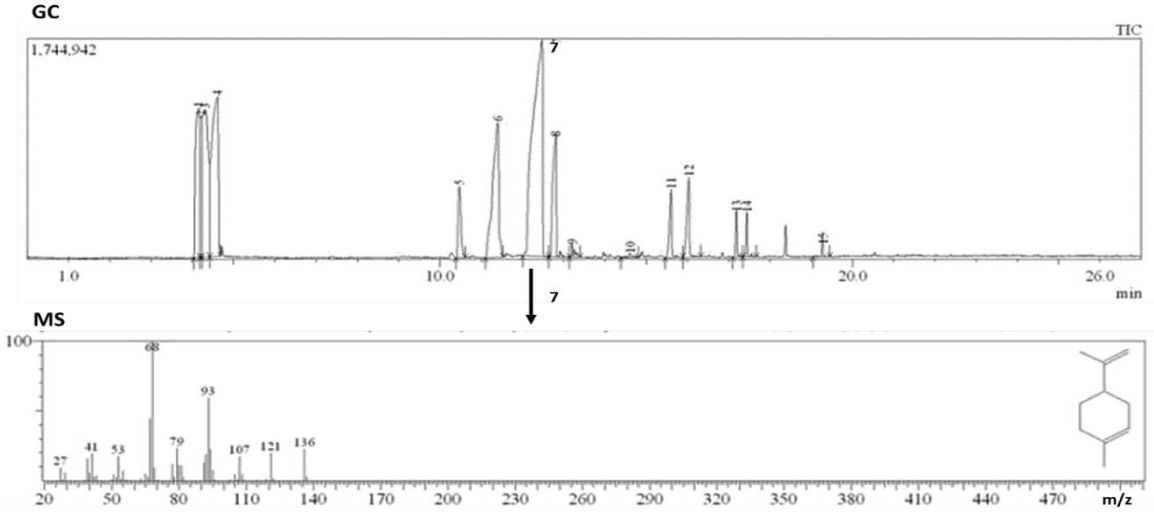

Figure 1. Chromatogram of citrus limon and MS compound D-Limonene (peak number 7)

Results of Physical Characterization of Hard Candy Lozenge

Organoleptic

The results of organoleptic observations in each formula (F1, F2, F3) are shown in **Figure 2**. F1 (isomalt) and F3 (sorbitol) gave a sweeter taste compared to F2 (mannitol). This showed that the addition of stevia with the same concentration in F2 still does not provide a good enough sweet taste. In addition, this was also because the level of sweetness of mannitol was only 50% of the sweetness of sucrose (10). F1 and F3 gave a clear yellowish color, while F2 gave a cloudy white color. The cloudy white color in F2 was caused by mannitol not being able to melt completely when heated and not being able to dissolve completely in water (low water solubility of 216 mg/mL at 25ºC) (19).

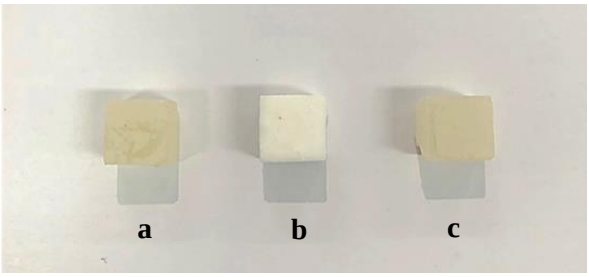

Figure 2. Organoleptic of hard candy lozenge (a) F1 isomalt, (b) F2 mannitol, (c) F3 sorbitol

Weight Variation

Table 4 showed that the weight of hard candy lozenge F1 and F3 were 1,50 $\pm$ 0,01 grams dan 1,49 $\pm$ 0,02 grams, respectively. Both formulas have an average weight that was in accordance with the desired specifications, which was in the range of 1,47-1,53 grams. Meanwhile, F2 showed that the average weight did not meet the specification range. This was due to the difficulty

in the process of pouring the melted lozenge into the mold, mannitol could not melt perfectly during heating so that air cavities were formed in the mold during the process. Therefore, this would have an impact on the results of the hard candy lozenge which was easily fragile when removed from the mold.

Dimension (Diameter)

Table 4 showed that 3 formulas have provided a lozenge diameter that has met the specifications, F1, F2, F3 were 11,27 ± 0,01 mm; 11,23 ± 0,01 mm; dan 11,26 ± 0,01 mm, respectively. This proves that melting method has been able to provide a good and reproducible hard candy lozenge dimension result.

Hardness and Friability

Hardness and friability testing on hard candy lozenge aims to observe the resistance of lozenge to impacts and friction during process, storage, shipping, or before use by patients (4). **Table 4** showed that F2 has the lowest hardness compared to F1 and F3. The same thing is also shown in the result of friability that F2 has the highest friability percentage value (above 1%) (14). This can be associated with the characteristics of the sugar base used, mannitol, which is difficult to melt and dissolve in water during the heating process. So that, the hard candy lozenge has cavities or hollow shape and tends to be cloudy white because mannitol cannot mix very well with other excipients (19).

Dissolving pH

The pH of 3 formulas have met the specifications, F1, F2, F3 were 4,01 ± 0,07; 3,90 ± 0,01; and 4,04 ± 0,05; respectively (5). In addition, the 3 formulas did not show any significant difference in pH between formulas (sig. p > 0,05). The pH of 3 formulas tend to acidic due to the addition of acidulant (citric acid) to the formula. This citric acid is intended to add a sour taste that can balance the sweet taste of the candy (20).

Dissolving Time

Dissolving time testing was conducted to determine the time required for the hard candy lozenge to completely dissolve in distilled water. **Table 6** showed that F1 and F3 provide dissolving times of 594 seconds ± 5 dan 571 seconds ± 13, respectively. These both formulas have met the desired specifications, which are in the range between 300-600 seconds (5). Meanwhile, F2 provides the longest dissolving time 1382 seconds ± 226. This is due to the low solubility of mannitol in water (216 mg/mL at 25ºC) which causes the dissolving time of mannitol as the base of F2 to be very long (19).

Table 4. The physical characterization results of citrus limon hard candy lozenges

Physical Characterization	Results		
	F1	F2	F3
Weight variation	1,50 grams ± 0,01	1,15 grams ± 0,05	1,49 grams ± 0,02
Dimension (diameter)*	11,27 mm ± 0,01	11,23 mm ± 0,01	11,26 mm ± 0,01
Hardness*	8,0 kP ± 0,16	6,0 kP ± 0,20	7,1 kP ± 0,25
Friability	0,09% ± 0,16	1,23% ± 0,58	0,43% ± 0,22
Dissolving pH*	4,01 ± 0,07	3,90 ± 0,01	4,04 ± 0,05
Dissolving time	594 seconds ± 5	1382 seconds ± 226	571 seconds ± 13

*The physical characterization of the 3 formulas has met the requirements.

CONCLUSION

In this study, it can be concluded that the results of the identification test of citrus limon essential oil using GC-MS showed the presence of D-Limonene compound, so this essential oil was in accordance with the specified quality. In addition, the difference in the type of sugar free candy base can affect the physical characteristics of citrus limon lozenge. Isomalt (F1) and sorbitol (F3) can be developed into hard candy lozenge because they provide good physical quality.

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CONFLICT OF INTEREST

The data published in this manuscript has not constitute a conflict of interest to any party.

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Formulation and Evaluation of Bioactive Composite Hydrogel Nanochitosan from Siwalan Fruit Shell (Borassus flabellifer) against Enterococcus faecalis

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A B S T R A C T

Pulp capping is one of the leading treatment methods for reversible pulpitis to maintain pulp vitality. Common pulp capping failures, such as CaOH (calcium hydroxide) and ZOE (zinc oxide eugenol), often result from contamination by microleakage and *Enterococcus faecalis* bacteria. Siwalan (*Borassus flabellifer*) shell, containing chitosan, has antibacterial properties and supports tissue regeneration. This research aims to create a bioactive composite hydrogel with nanochitosan extract from a siwalan shell and evaluate its quality and antibacterial effectiveness against *Enterococcus faecalis*. The nanochitosan extract was prepared through deproteination, demineralization, and deacetylation, followed by FTIR and SEM analysis. The final formulation was evaluated to match the quality standard of the hydrogel. The study used an experimental post-test-only group design with three treatment groups (0.5%, 1%, and 1.5% siwalan shell nanochitosan extract) and two control groups. The antibacterial test was conducted using the tube dilution method, and data were analyzed with One-way ANOVA and Tukey HSD tests. The hydrogel's Minimum Bactericidal Concentration (MBC) was 0.5%. The results of statistical tests show that the calculated F value (37.185) is greater than the F table (2.8661) with a sig value of 0.000 (0.000 < 0.05). Thus, the bioactive composite hydrogel nanochitosan extract from the siwalan shell effectively inhibits the growth *of Enterococcus faecalis* and meets the quality parameters for pulp capping material.

Keywords: *Borassus flabellifer* Shell, Nanochitosan, Bioactive Composite Hydrogel, *Enterococcus faecalis*

INTRODUCTION

The oral cavity is a complex and dynamic environment comprising various surfaces and conditions that foster microbial growth. These conditions, including mucosal and dental surfaces, warm temperatures, high humidity, and nutrient-rich surroundings, contribute to the onset of oral diseases such as dental caries. Dental caries is a bacterial infection that affects the hard tissues of teeth through a slow, degenerative process. According to the 2018 Basic Health Research Data (RISKESDAS), around 88.8% of the Indonesian population is affected by caries, making it one of the top six health issues in the country and signaling a significant public health concern (1). Several factors, such as poor dietary habits, inadequate oral hygiene, and limited access to dental care services, drive the high incidence of caries. Bacteria, particularly *Enterococcus faecalis*, play a central role in the development of caries. This bacterium is not only a pivotal contributor to caries but is also resistant to antibiotics, complicating treatment and potentially leading to further infections if left unchecked. While a combination of antimicrobial and remineralizing agents and antibiotics can help protect and heal pulp tissue, antibiotics present challenges. Over-reliance on antibiotics in caries treatment may fail (2).

Indonesia is recognized as a global biodiversity hotspot, offering substantial potential for various fields, including dental caries management. One plant with considerable potential is the siwalan (*Borassus flabellifer*), a palm species widely found in coastal and tropical areas. Known for its versatility, nearly every part of the siwalan plant is valuable. A notable feature is the siwalan shell, often considered waste, yet it contains 33.34% chitosan (3). Chitosan is well-known for its antibacterial properties and role in promoting tissue regeneration. In its nano form, chitosan exhibits enhanced antimicrobial effects, excellent biocompatibility, and increased efficiency as a drug delivery system. Utilizing siwalan shells for nanochitosan production converts waste into valuable material, significantly amplifying chitosan's bioactivity. This increase in bioactivity makes nanochitosan more effective in promoting the proliferation of odontoblast-like cells and facilitates

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the binding of transforming growth factor-beta 1 (TGF-β1), accelerating the formation of reparative dentin. Consequently, this contributes to faster tooth repair and regeneration (4).

Bioactive composite hydrogels are advanced materials designed to replicate the structure and function of natural tissues. These hydrogels consist of tissue fibbers combined with macromolecules, allowing them to mimic the properties of biological tissues. When formulated with nanochitosan, bioactive composite hydrogels can demonstrate ideal characteristics for use as pulp-capping agents in dental treatments. Their bactericidal or bacteriostatic properties and ability to stimulate reparative dentin formation make them particularly effective for treating dental caries. Developing a bioactive composite hydrogel using nanochitosan derived from siwalan shells offers a promising strategy for dental caries management. This formulation has shown potential as an antimicrobial agent effective against *Enterococcus faecalis*, a primary bacterium involved in caries, thus providing a novel solution for preventing and treating this dental condition (2).

The primary aim of this study is to formulate and evaluate the effectiveness of a bioactive composite hydrogel that contains nanochitosan extracted from siwalan shells against *Enterococcus faecalis*. The effectiveness of the hydrogel was evaluated through antimicrobial tests, exploring its mechanism of action and material stability to ensure its reliability and efficacy. This study seeks to validate the bioactive composite hydrogel's effectiveness in treating dental caries and contributes to developing nanotechnology-based solutions in dental care. The research promotes sustainability by utilizing waste materials such as siwalan shells while offering an alternative, innovative solution to the high prevalence of dental caries in Indonesia.

MATERIALS AND METHODS

The research was conducted at the Pharmacy and Biochemistry Laboratory of the Faculty of Medicine, as well as the Dentistry Faculty Laboratory, Brawijaya University from May to August 2024.

Materials

Siwalan fruit from Tuban Regency, Indonesia is used as raw material for making chitosan extract, The materials used for nanochitosan preparation consist of NaOH 2N, HCI 2N, NaOH 60%, STPP, acetone, and NaOCI, Furthermore, the hydrogel product was made with three concentrations.

Tabel 1. Formulation of Composite Hydrogel Nanochitosan

Materials	**0.5 % (g/100 ml) Concentration**	**1 % (g/100 ml) Concentration**	**1.5 % (g/100 ml) Concentration**
Nanochitosan	0.5	1.0	1.5
HA	-	1.0	1.5
Acetic acid 1%	0.5	1.0	1.5
Carbopol	1.0	1.0	1.5
Propylene Glycol	10.0	10.0	10.0
Glycerin	5.0	5.0	5.0
Methyl Paraben	0.18	0.18	0.18
Propyl Paraben	0.02	0.02	0.02
TEA	1.5	1.5	2.25
Aquadest	Add 100	Add 100	Add 100

Methods

Preparation and Isolation of Siwalan Shell

The preparation was started by selecting the fresh siwalan fruit shell and performing wet sortation. The shell fruit is chopped and dried in direct sunlight for 3 hours. The dried shell was ground using a blender. The isolation process involves several stages. The first stage is deproteinization, which starts by weighing the powder of the siwalan fruit shell and mixing it with 2N NaOH at a ratio of 1:5 (w/v). The dilution was heated at 70℃ for two hours, stirred and cooled for ± 30 minutes, and then filtered. The resulting residue was washed using distilled water and dried at 60℃ for four hours (6). The demineralization stage begins by mixing the deproteinization result with 2N HCl at a ratio of 1:5 w/v.

The mixture was left for two days at room temperature. The sample was heated at 75°C for one hour while stirred and filtered. The residue was washed and dried as before for two hours. The deacetylation stage begins by mixing the demineralization result with 60% NaOH at a ratio of 1:1 w/v. Then, the sample was heated at 90°C for 3 hours while stirred. The mixture was filtered, washed, and dried (5, 6). The depigmentation stage is carried out by adding acetone and NaOCl to the deacetylation residue at a ratio of 1:2 (w/v), then leaving for one day and drying for three hours (6). Chitosan characterization was carried out by determining the functional groups of the material extracted through the last stage of isolation, namely deacetylation, using Fourier Transform Infrared (FTIR) spectroscopy analysis. In the FTIR Spectroscopy analysis, the chitosan sample was placed on an FTIR plate and compared with the FTIR results of pure chitosan standards and library match results (8).

Nanoparticles Preparations

The chitosan extract was dissolved in 300 mL of 1% acetic acid. Then, 60 mL of 0.84% tripolyphosphate solution was dripped while stirring at a speed of 1200 rpm. The suspension was lyophilized (7). Chitosan nanoparticles were analyzed using Scanning Electron Microscopy (SEM). SEM determines the shape and size of chitosan particles.

Composite Hydrogel Nanochitosan Preparations

The process begins with dissolving the nanoparticles in 1% acetic acid. Prepare 70 grams of hot aquadest at 98-100°C, then add methylparaben and propylparaben while stirring at 500 rpm. Propylene glycol was added, the temperature was decreased to 38-42°C, and the dissolved extract and HA were added alternately. Add glycerin and stir at 1000 rpm. Disperse carbopol 940, then add triethanolamine until a gel with a watery consistency is formed (19). Evaluation of finished products includes organoleptic, homogeneity, pH, viscosity, spreading, and adhesion tests.

Hydrogel Quality Evaluation

The evaluation of hydrogel preparation consists of several stages. First, organoleptic observations were carried out on the product's color, shape, and odor. Furthermore, the hydrogel should be semisolid, watery, homogeneous in color, and have a characteristic extract odor. Second, a homogeneity test was performed by placing the hydrogel between two glass slides and checking for coarse particles. Third, pH was measured using a calibrated pH meter, aiming for an alkaline pH suitable for reparative dentinogenesis. Fourth, viscosity was measured using a Rion viscometer with spindle number 2. Fifth, spreadability was evaluated by measuring the diameter of the spread with varying weights. Sixth, adhesion was tested by measuring the time the hydrogel remained stuck between two glass slides under pressure (9).

Antibacterial Activity Test

The study used three treatment groups, each of which was a concentration of bioactive composite hydrogel nanochitosan extract of siwalan shell of 0.5%, 1%, 1.5%, a positive control group (CaOH paste), and a negative control group (aquadest) with each repetition five times. The method used was tube dilution. *Enterococcus faecalis* was incubated for 24 hours at 37°C. Incubation was conducted in an anaerobic test tube; then, the colony was inserted into BHI-B (Brain Heart Infusion Broth) until the amount obtained was comparable to Brown III solution with a concentration of 10^8 CFU/ml. The solution was diluted again until it reached 10^6 CFU/ml.

Five sterile test tubes were prepared. Each tube was filled with distilled water, CaOH, bioactive composite hydrogel nanochitosan formulation of 0.5%, 1%, and 1.5% siwalan shell extract as much as 1 ml, then added 1 ml of each bacterial suspension. All tubes were incubated in anaerobic tubes for 24 hours at 37°C. The antibacterial power of the test material was determined by measuring the turbidity of the tube using white paper with black lines. In contrast, the control tube was used as a comparison. After that, each tube was given a level based on its clarity.

After determining the antibacterial power, each material was taken using an ose, scratched on BHI-A (Brain Heart Infusion Agar), and incubated in anaerobic tubes for 24 hours at 37°C. The bacterial colonies that grew were counted using a colony counter. Antibacterial activity was determined by calculating the OI (original inoculum), which is bacteria with a concentration of 10^6CFU/ml cultured in agar media before incubation (21).

Statistical Test

The bacterial colony data were analyzed using SPSS 25. If a normal statistical distribution and the same or homogeneous data variance ($p > 0.05$) were obtained, then one ANOVA analysis was continued to determine the significance of the influence of the results of each treatment. A Post Hoc Tukey HSD test determined the treatment group with the most significant difference in reducing *Enterococcus faecalis*.

RESULTS AND DISCUSSION

Isolation of Chitosan from Siwalan Shell

The first steps of raw material preparation include sorting, washing, drying, and grinding. This process aims to extract the necessary part of the siwalan fruit, specifically the shell, and remove impurities that may affect the isolation results. Next is the deproteinization process, which involves removing the protein from the siwalan shell. Demineralization aims to eliminate the minerals from the siwalan shell, resulting in a chitin product. During deacetylation, the acetyl groups in chitin are degraded and produce chitosan, identified as the primary process of chitosan isolation. The isolation process is carried out to obtain purer chitosan. The depigmentation or color separation stage is conducted to remove pigments from the isolated chitosan product (6). The yield results obtained step by step in this experiment are summarized in **Table 2.**

Table 2. Table of Chitosan Isolation Results

Stage	Dry weigh	Yield (w/w)	Color
Initial mass: 35 gram			
Deproteinization	34	97.1%	Brown
Demineralization	32	94.1%	Brown
Deacetylation	25	78.1%	Brown
Depigmentation	20	80%	Brown
Used 6 gram			
Nanoparticle Synthesis	5	83.3%	Brown

The table shows that the highest yield is obtained during deproteinization, while the lowest occurs during deacetylation. The yield decreases as the processing stages advance, producing chitosan with 80% acetyl removal and brown coloration. This

brown color, produced during deacetylation, does not meet the desired white chitosan specification, indicating a successful depigmentation stage. The incomplete depigmentation process accounts for this discrepancy. According to the quality standards set by Protan Laboratories Inc. (1987), chitosan should be white. The suboptimal soaking time during the depigmentation stage likely contributed to the color issue. A proper depigmentation process, which produces white chitosan powder, typically involves acetone and additional bleaching agents such as sodium hypochlorite (NaOCl) (6). In this case, using acetone alone was insufficient to remove all pigments, and adding NaOCl would be necessary to achieve the desired whiteness.

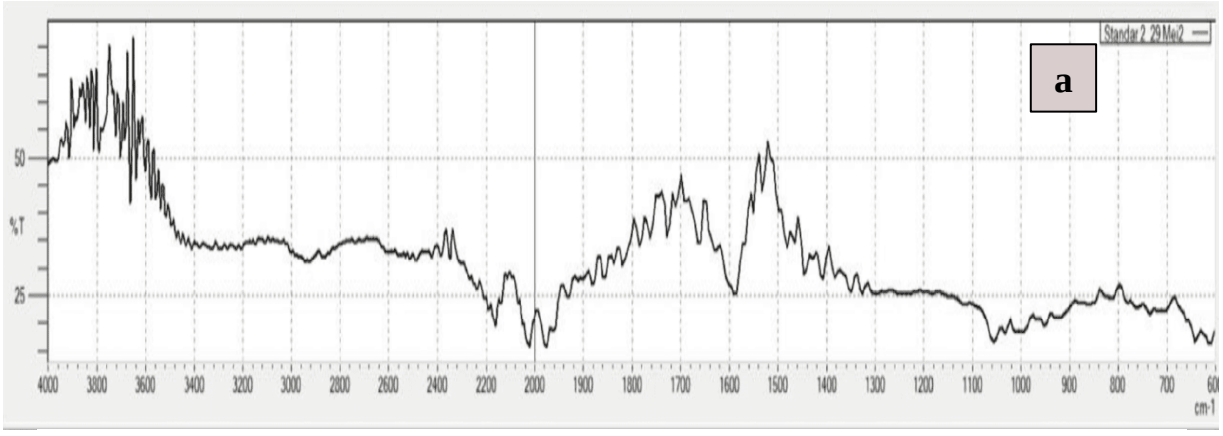
A comparison with previous research on chitosan isolation from green mussel shells involved a deproteinization step using a 3.5% (w/v) NaOH solution at a ratio of 1:10 (w/v) (5). The demineralization phase was performed using 1N HCl with a 1:10 (w/v) ratio followed by deacetylation using 40% (w/v) NaOH at 1:15 (w/v). Another study focused on chitosan isolation from vannamei shrimp shells, the depigmentation stage was conducted by adding acetone at a 1:2 (w/v) ratio (6). The results from studies on chitosan isolation from green mussel shells are presented in **Table 3**

Table 3. Table of Chitosan Isolation Result from Green Mussel Shells

Stage	Yield (w/w)	Color
Deproteinization	92.452%	Cream
Demineralization	46.477%	Grey
Deacetylation	81.33%	White
Depigmentation	76.038%	Brownish White

FTIR Analysis

A.



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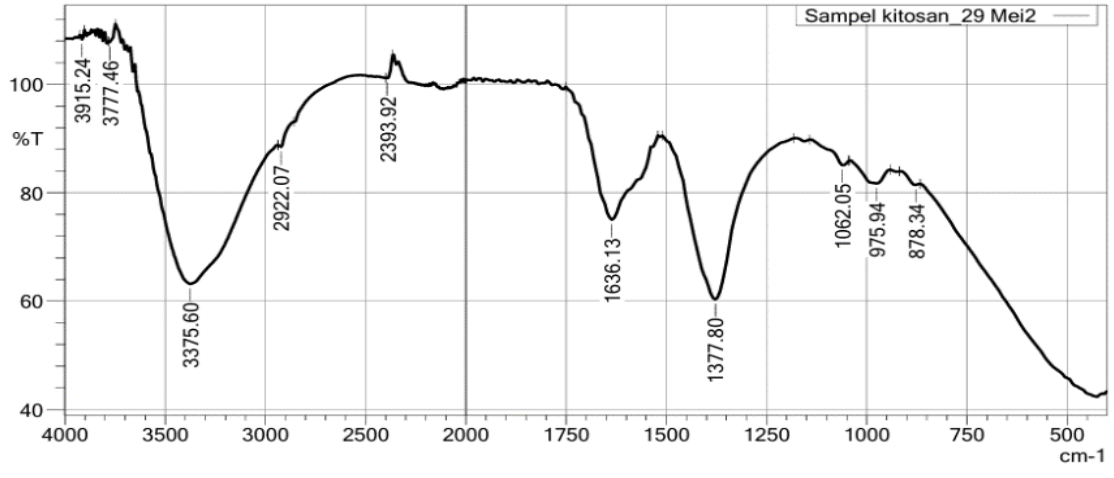


Figure 1. (A) FTIR spectrum of standard chitosan; (B) FTIR spectrum of isolated chitosan sample of siwalan fruit shell

Chitosan characterization was conducted using FTIR instrumentation by analyzing the functional groups of the material isolated from the siwalan shell, comparing it with the FTIR spectrum of standard chitosan, and matching the sample spectrum with a library. Based on the FTIR spectrum in the figure above, the produced chitosan sample meets the specifications regarding the functional groups present in chitosan (8). Compared with the standard and library, the functional groups of the chitosan sample show a high degree of similarity. This is evidenced by comparing functional groups at specific wavelengths we conducted on the FTIR chromatogram results, as shown in **Table 4.**

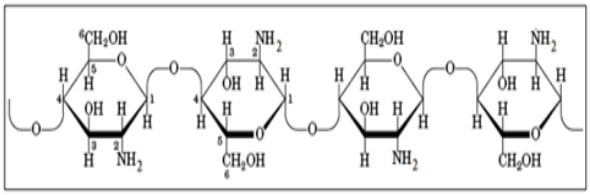


Figure 2. Molecular structure of chitosan

The functional groups in the molecular structure of chitosan, as shown in **Figure 2**, include amine groups (-NH_2) and hydroxyl groups (-OH). The amine group is primarily found on the second carbon atom (C-2) within the D-glucosamine units of chitosan, while the hydroxyl groups are located at the C-3 and C-6 positions. From the FTIR spectrum data above, absorption bands can be observed, indicating vibrations of the functional groups present in chitosan. The characterization results of the functional groups found in chitosan can be seen in **Table 4 below**.

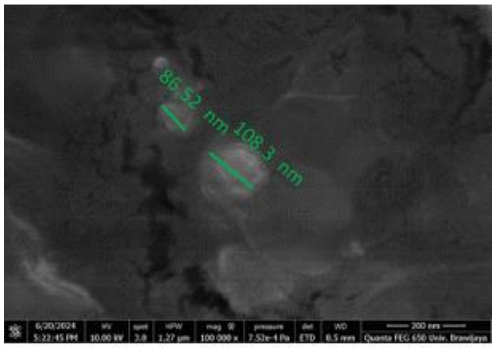
Table 4. Functional Groups of Chitosan

Functional Groups	Compound	Vibration Type	Wave Number (cm-1)		Range of Wave Number (cm-1) (Arsyi *et al.*, 2018)
			Chitosan Sample	Chitosan Standard	
C-H	Saturated Alkane	*bending*	3777.46	3811.90	3000-3850
O-H and -NH	Phenol and Amine	*stretching*	3375.60	3639.68	3650-3200
C – H aliphatic	Alkane	*stretching*	2922.07	2801.50	2800-2925
C-H	Aromatic Ring	*stretching*	2393.92	2013.58	3300-2700
C = O	Carbonyl	*bending*	1636.13	1666.30	1740-1560
C – H	Aliphatic	*bending*	1377.80	1382.13	1465-1370
C-N	Amine	*stretching*	1062.05	1045.42	1350-1000

SEM Analysis

Dentin consists of microscopic dentinal tubules with a diameter of approximately 2-4 μm. The size and reactivity of nanoparticles allow the material to penetrate further into the dentinal tubules, with the potential for decontamination, remineralization, and sensitivity reduction (12). SEM observations showed chitosan nanoparticles with spherical morphology (nanospheres) ranging in size from 39.0 nm to 108.3 nm, enabling good penetration into the dentinal tubules, in comparison to previous research, which utilized nano-hydroxyapatite material for in vitro study on human third molars. The same testing method was used, namely SEM particle size analysis, which indicated a size of 50 nm (17). The result indicates that the result of the chitosan isolation nanoparticle sample is appropriate for its intended application, specifically for use in dentin.

a.



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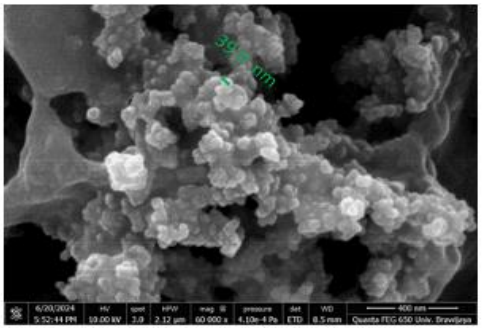


c.

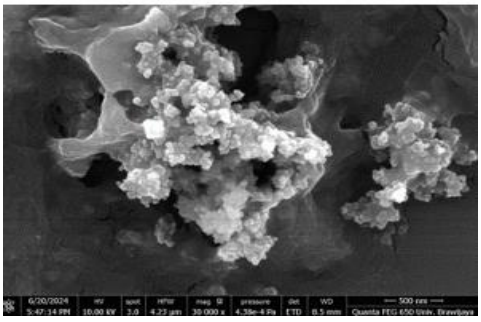


Figure 3. (a) Chitosan nanoparticles at 100,000x magnification with particle size of 86.52 nm and 108.3 nm; (b) Chitosan nanoparticles at 60,000x magnification with particle size of 39 nm; (c) Chitosan nanoparticles at 30,000x magnification

Evaluation Results of the Preparation Organoleptic Test

Table 5. Organoleptic Test Result

Formula	Odor	Color	Form
K 0.5%	Distinctive smell	Clear dark brown	Semi-solid, watery
K 1%	Distinctive smell	Turbid light brown	Semi-solid, watery
K 1.5%	Distinctive smell	Clear dark brown	Semi-solid, watery

Observations of organoleptic characteristics conducted visually revealed that the preparations exhibited identical odor, color, and form among all concentrations, except for the color at 0.5%. Therefore, each preparation is similar to the criteria due to the absence of a strong odor (13) characterized as good specification.

Homogeneity Test

Table 6. Homogeneity Test Result

Formula	Homogeneity
K 0.5%	✓
K 1%	✓
K 1.5%	✓

The formulas have a homogenous structure and soft texture, which indicates that the mixing process was successful. It can be concluded that the preparation meets good specifications as it is free from clumped particles or coarse grains (22).

pH Test

Table 7. pH Test Result

Formula	pH
K 0.5%	8.10
K 1%	8.14
K 1.5%	8.12

Alkaline conditions might promote the release of bonds between the dentin collagen and growth factors, leading to the release of TGF-ß1 and other bioactive molecules initially trapped within the dentin. The alkalinity in pulp capping materials and the presence of TGF-ß1 have been proven beneficial in reparative dentinogenesis (10). Based on previous research examining the chemical and physical properties of pulp capping agent formulations, the alkaline pH observed ranged between 8 and 12.4. Thus, the pH of the prepared hydrogel formulation falls within the optimal pH range.

Viscosity Test

Table 8. Viscosity Test Result

Formula	Viscosity (dPa.s)
K 0.5%	120
K 1%	140
K 1.5%	120

The viscosity results fulfill the specification range, confirming its suitability for safe and effective use. Hydrogels' optimal viscosity range is between 50 dPa.s and 400 dPa.s. Compared to

previous research, four hydrogel formulations containing the active ingredient sodium diclofenac were reported to have viscosity values ranging from 260 to 380 dPa.s (20).

Spreadability Test

Table 9. Spreadabilty Test Result

Formula	Weight of 50 g (cm)	Weight of 100 g (cm)	Weight of 200 g (cm)	Weight of 500 g (cm)
K 0.5%	4.5	4.7	5.1	6.1
K 1%	4	4.3	4.7	5.3
K 1.5%	4.6	4.9	5.2	6

The spreadability results rise as the weight of weights increases and meets the specified range of 5-7 cm. Therefore, it can be concluded that the preparation can be effectively used (16). The evaluation of spreading ability in hydrogel formulations is crucial for determining the extent to which the formulation can disperse over the exposed dentine surface. Optimal spreading assures that the formulation is distributed and effective in delivering therapeutic benefits, such as improved absorption of active ingredients and enhanced local concentration at the target site, namely dentinal tubules. Furthermore, optimal spreading contributes to user comfort and satisfaction, as it can influence the ease of application and the sensory properties of the hydrogel (14).

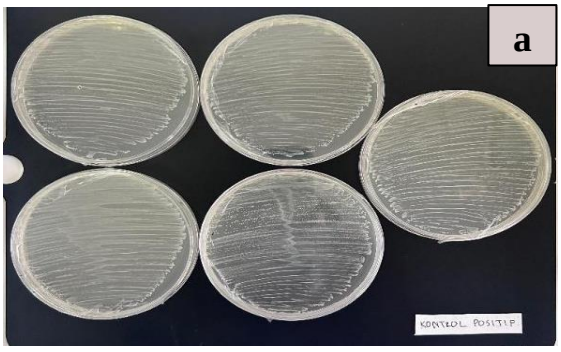
Adhesive Strength Test

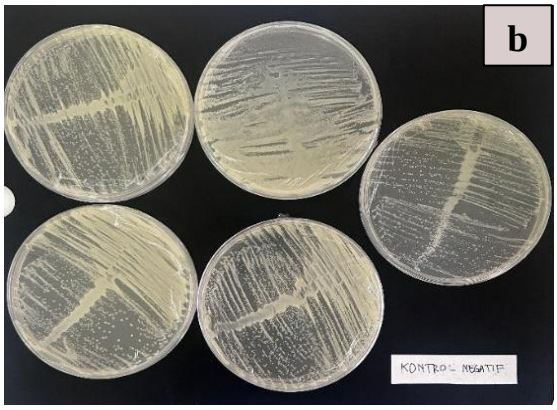
Table 10. Adhesive Strength Test Result

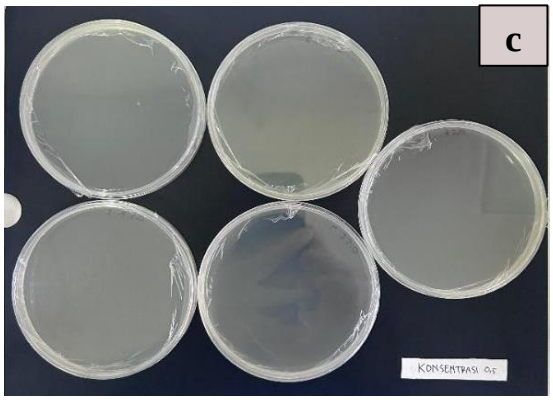
Formula	Time (seconds)
K 0.5%	7.13
K 1%	6.42
K 1.5%	4.69

The adhesive strength test results indicate that as the concentration of the preparation increases, its adhesive strength decreases. However, the results still fall within the range of good adhesive strength for hydrogel preparations. The requirement for adhesive strength in gel formulations is at least 4 seconds (14).

Result of Antibacterial Activity Test







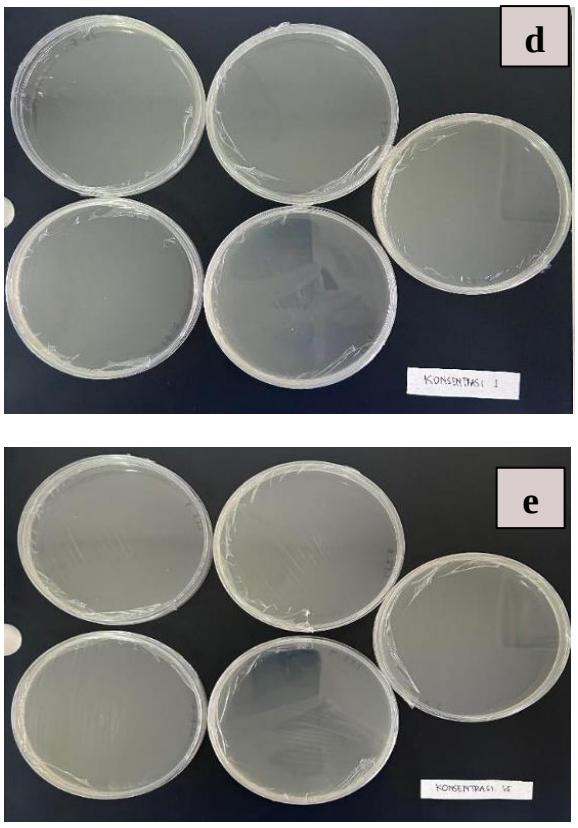


Figure 4. (a) Positive control antibacterial test; (b) Negative control antibacterial test; (c) Antibacterial test of concentration 0.5; (d) Antibacterial test of concentration 1; (e) Antibacterial test of concentration 1.5

The antibacterial activity test was determined based on the turbidity of tubes before and after incubation, followed by turbidity level assessment. The research findings indicate that there was no change in turbidity of the bioactive composite hydrogel tubes containing nanochitosan extract from siwalan fruit shell at concentrations of 0.5%, 1%, and 1.5% after incubation. From the test results, it was found that the bioactive composite hydrogel containing nanochitosan extract from siwalan fruit shell at concentrations of 0.5%, 1%, and 1.5% could inhibit the activity of *Enterococcus faecalis* bacteria.

The minimum bactericidal concentration (MBC) of the bioactive composite hydrogel containing nanochitosan extract from siwalan fruit shell against *Enterococcus faecalis* was determined when colony growth was 0 or less than 0.1% of the initial inoculum (OI) (21). It was found that there was no bacterial colony growth in the test material at a concentration of 0.5%. According to the criteria,

The bioactive composite hydrogel containing nanochitosan extract from the siwalan fruit shell at a concentration of 0.5% represents the MBC. This finding is consistent with previous research, which reported that the ethanol extract of siwalan fruit shell showed activity against *Streptococcus mutans* in MIC and MBC tests at a concentration of 7.813 mg/mL (21). Another study mentioned that chitosan isolated from siwalan fruit shell showed MIC activity against *E. coli* at concentrations below 1% (3).

Table 11. Colonies Growth and Turbidity Level of Each Formulation

Treatment	Number of Colonies					Average	Turbidity
	I	II	III	IV	V		
Control (-)	636	1452	872	1208	844	1002.4	Turbid (++)
Control (+)	648	960	452	472	652	636.8	Turbid (++)
K 0.5%	0	0	0	0	0	0	Clear (-)
K 1%	0	0	0	0	0	0	Clear (-)
K 1.5%	0	0	0	0	0	0	Clear (-)

Based on the one-way ANOVA analysis, the research data shows that the calculated F value exceeds the tabulated F value. The calculated F value in this study is 37.185, with a tabulated F value of 2.8661 and a significance value of 0.000 (0.000 < 0.05), indicating rejection of the null hypothesis (H0) and acceptance of the alternative hypothesis (H1). In other words, the treatment

of bioactive composite hydrogel containing nanochitosan extract from siwalan fruit shell significantly influences the antibacterial effectiveness against *Enterococcus faecalis* growth. This significant antibacterial capability is attributed to the active substance reaction from siwalan shell extract, specifically optimized into nanochitosan. The antimicrobial mechanism of chitosan against bacteria can be explained through two theories. The first theory states that the amino functional groups on chitosan can bind to bacterial cell walls, causing leakage of intracellular constituents and resulting in bacterial lysis. The second theory suggests that chitosan begins its action by damaging bacterial cell walls, subsequently binding to intracellular components, blocking mRNA, and inhibiting protein synthesis (17).

Based on Pos Hoc Tukey HSD analysis, it is shown that the first treatment using Formula 1 (0.5%), positive control (CaOH 1.0 g), and negative control (distilled water) have significantly different levels with values of 0.00 CFU/mL, 636.80 CFU/mL, and 1002.40 CFU/mL. The second treatment using Formula 2 (1.0%), positive control (CaOH 1.0 g), and negative control (distilled water) also show significantly different levels with values of 0.00 CFU/mL, 636.80 CFU/mL, and 1002.40 CFU/mL. The third treatment using Formula 3 (1.5%), positive control (CaOH 1.0 g), and negative control (distilled water) similarly exhibit significantly different levels with values of 0.00 CFU/mL, 636.80 CFU/mL, and 1002.40 CFU/mL. Therefore, based on these research findings, it can be explained that the concentrations in Formula 1, Formula 2, and Formula 3 have equal antibacterial effectiveness against *Enterococcus faecalis* growth, which is significantly better compared to the antibacterial effectiveness of the standard pulp capping material CaOH.

CONCLUSION

The bioactive composite hydrogel containing nanochitosan extract from siwalan fruit shell has been proven to have nanoparticle sizes ranging from 39.0 nm to 108.3 nm. It has met all quality standards for hydrogel formulations, as confirmed by organoleptic, homogeneity, pH, viscosity, spreadability, and adhesiveness evaluations, thus making it suitable for use as a pulp capping agent. The bioactive composite hydrogel containing nanochitosan extract from siwalan fruit shell can be formulated as a hydrogel preparation with concentrations of 0.5%, 1%, and 1.5%. The Minimum Bactericidal Concentration (MBC) of the bioactive composite hydrogel containing nanochitosan extract from siwalan fruit shell against *Enterococcus faecalis* is found at a concentration of 0.5%. The antibacterial activity of all concentrations against *Enterococcus faecalis* growth is the same, which is much better than the antibacterial activity of the standard pulp capping material.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest to disclose regarding this research.

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Testing of Antibacterial Activity and Characterization of Chemical Compounds Composing Essential Oil From Lemo Cuco Fruit Skin (Citrus macroptera Mountrous)

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A B S T R A C T

Lemo Cuco (*Citrus macroptera Mountrous*) is one of the plant species of Rutaceae grown in the districts of Bone and Sinjai, South Sulawesi. This fruit is commonly used for food as scent, cough reliever and as fishy and meaty deodorizing. The peel has a special scent indicating presence of essensial oil components. This study aims to evaluate antibacterial activities and and to characterize compounds in ethanol extract dan n-hexane extract Lemo Cuco (*Citrus macroptera Mountrous*). This study used the soxhlet extraction, and phytochemical test and the characterization of components in extracts with GC-MS and antibacterial activities using the disc diffusion method. The results obtained from the study were testing the antibacterial activities of Lemo Cuco (*Citrus macroptera Mountrous*) peel extract against the *Staphylococcus aureus* and *Salmonella typhi* bacteria. Inhibition of ethanol and n-hexane extract against the bacteria S. aureus included weak, medium, and strong category according to the concentrations (20%, 10%, 5%, 2.5% and 1.25%), whereas inhibitory against *S. typhi* bacteria in n-heksane included weak, medium, and strong category, whereas ethanol extract included medium and weak category, even not active. Based on the results of phytochemical identification of the Lemo Cuco (*Citrus macroptera Mountrous*), peel extract indicated existence of flavonoids, phenolic, steroids, terpenoids, alkaloids and saponins compound. n-heksane exctract contained special fraction saponins, whereas ethanol exctract was nothing. Characterization using GC-MS indicated existence of monoterpenoid and sesquiterpenoid compound. Therefore, the n-hexane extract from the skin of the lemo cuco has potential as an antibacterial.
Keywords: antibacterial acitivity, lemo cuco, essensial oil, *Salmonella typhi*, *Staphylococcus aureus*

INTRODUCTION

Indonesia has abundant natural wealth and biodiversity and is located on the equator with a tropical climate. The diversity of existing plants can be utilized both traditionally and modernly with the isolation of natural compounds in the form of secondary metabolite compounds (1). These secondary metabolite compounds vary greatly in type and quantity in each plant (2). Secondary metabolite compounds can be obtained from parts of the plant starting from the fruit, skin, leaves, stems, and roots (3), in the form of active compounds such as flavonoids, alkaloids (4), saponins, steroids and terpenoids (5). One of the terpenoid compounds is essential oil which can be obtained from several species such as *Compositae, Matricariae, pinaceae, Labiatae, Rutaceae* and etc (6).

One of the *Rutaceae* species found in the citrus family such as lime (*Citrus aurantium* L.), Pontianak orange (*Citrus nobilis* Lour.), Sunkist orange (*Citrus sinensis* L. Osbeck), kaffir lime (*Citrus hystrix* DC) and lemon (*Citrus limon* L.). In the Bugis tribe, especially in the Sinjai and Bone areas, there is a type of orange whose morphology resembles a lemon, the community knows it as "Lemo Cuco". This orange has a distinctive aroma that is commonly used for cooking as a flavoring, cough suppressant and as a fishy odor remover in fish and meat.

The use of orange fruit and leaves has been known by the community since ancient times as a traditional medicine. The leaves are usually used to overcome fatigue and as a food flavoring. While the fruit skin is used as a medicine for boils, internal heat, dermatitis, breast inflammation, scaly skin and peeling skin (7), making cakes and sweets (2). Orange peel contains essential oils that can be extracted so that it has a high selling value (8), which is widely used as a fragrance, soap and

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cosmetics industry (9). Several studies on citrus have been reported, such as research on lime peel extract (*Citrus aurantium*) which can reduce dental plaque with alkaloid and flavonoid content (10), Pontianak orange peel (*Citrus nobilis* Lour.) with limonene content against subterranean termites (11) and Sunkist orange peel (*Citrus sinensis* L. Osbeck) as a natural styrofoam solvent (12). Other studies have also revealed that lemon peel extract (*Citrus limon* L.) and kaffir lime peel (*Citrus hystrix* DC) have antibacterial activity (13). Research by (10) and (14), revealed that orange peel extract with 70% ethanol solvent has higher effectiveness against microorganism tests than methanol, acetone and dichloromethane solvents. Ethyl acetate extract and kaffir lime peel oil are more potent against *Staphylococcus aureus* than *Escherichia coli* (15). Research by (16) stated that the rutaceae family has high antibacterial activity against 20 serotypes of *Salmonella*. Research by (17) using 100% kaffir lime juice has optimal inhibitory power on the growth of *Salmonella typhi*. This is in accordance with research by (18) that kaffir limes peel has stronger antimicrobial power compared to kaffir lime fruit. The peel of the kaffir lime fruit contains essential oils that have antibacterial effects (19) (20), antifungal (21) (22), antioxidants (23) (24), and fresheners. The antibacterial effect is obtained due to the presence of citronella compounds in it (25). Research by (26) and (27) reported that kaffir lime leaves contain tannins, steroids, triterpenoids, and essential oils of 1-1.5% with a citronellal content of 64.15%, beta citronellal 10.17% and lonalol 5.31%. Other studies such as those reported by (8) revealed that the essential oil content of 2.5% of kaffir lime peel contains the main components of 94% limonene, 0.2% geranial, 0.1% citronellal, 11.93% terpinen-4-ol, 0.5% linalol, 0.4% decanal and 0.5% octanal.

Essential oil extracted from lime peel contains a variety of chemical compounds, predominantly monoterpenes and sesquiterpenes, with limonene, geranial, pinene, neral, and citronellal as the main components, and its yield and composition vary depending on the solvent used (ethanol or n-hexane). Other studies state that the essential oil content of lime peel consists of 16% monoterpene compounds, 6.55% sesquiterpene, aromatic and non-aromatic compounds (28). The main components of lime essential oil are 96% limonene, 97% geranial, 96% pinene and 96% neral (29). (30) reported that the use of ethanol and n-hexane solvents obtained essential oil yields of 13.39% and 10.50% with citronellal content of 65.99% and 97.27%.

Citronellal, found in kaffir lime essential oil, has antibacterial and antifungal properties, making it effective as a botanical pesticide and capable of inhibiting the growth of pathogens such as *Streptococcus mutans*, *Staphylococcus aureus*, and *Klebsiella pneumoniae*. (31) revealed that citronellal has antibacterial and antifungal properties so that it can be used as a botanical pesticide. This is in accordance with research by (32), (26), and (20) which stated that kaffir lime essential oil can inhibit the growth of *Streptococus mutans* at a concentration of 25%, *Staphylococus aureus* and against *Klebsiella pneumonia* ATCCThis study was conducted to determine the antibacterial activity against *Staphylococcus aureus* and *Salmonella typhi* bacteria and to characterize the components of the essential oil of Lemo Cuco fruit peel (*Citrus macroptera Mountrous*). The reason for using these two bacteria is because bacteria are generally grouped based on their cell wall structure into 2, namely gram-positive bacteria and gram-negative bacteria (33). The cell wall structure of gram-positive bacteria is layered with low lipid content (1-4%) making it easier for bioactive materials to enter the cell, has a peptidoglycan layer on the outside and plays a less effective role as a permeability defense. While gram-negative bacteria contain fewer peptidoglycan layers with a high lipid content (11-22%) whose cell walls have 3 layers (multilayer) consisting of lipoprotein, phospholipid and lipopolysaccharide layers which make the cell wall difficult to penetrate by antibacterial substances.

MATERIALS AND METHODS

Methods

Materials used in this study were gas chromatography-mass spectrometry (GS-MS) Agilant GC Tipe 7890 A MS Tipe 5975, spectrophotometer UV, rotary evaporator Heidolph Vap-Value, oven kirin and memmert, autoclave Gea Yx-280D, incubator Heraeus Thermo Scientific, Laminar Air Flow Cabinet (LAF) ESCO Isocide, easypure II Barnstead D 3750 Thermo Scientific, fume hood Esco Frontier Tm, analytical balancing Electronic Balance Kern ABJ, , Heating Mantel Stirrer B- One, vortekx advanced mixer IR Wizard Velp Scientifica, UV light 254-336 nm, set of soxhlet Pyrex. Sample used was Lemo Cuco (Citrus *macroptera Mountrous*) peel, it was wild plant obtained from Bijinangka Village, Sinjai Borong, Sinjai, South Sulawesi). Chemical material utilized were alcohol 70% Intraco, ampicilin, cefixime, aquadest (H_2O), concentrated sulfate acid (H_2SO_4), boiling stone, white thread, iron (III) chloride ($FeCl_3$) 5% and 1%, dimethyl sulfoxide (DMSO) p.a Intraco, technical ethanol (C_2H_5OH) Duta Gemini, ethyl acetate ($C_4H_8O_2$) Brataco, cotton, disc paper, Duta Gemini filter paper, TLC (Thin Layer Chromatography) plate Silica gel F_{254}, muller hinton agar (MHA), physiological sodium chloride (NaCl) 0.9% p.a, sodium hydroxide (NaOH) 10% p.a, n-Hexane (C_6H_{14}) Brataco, nutrient agar (NA), dragendorff reagent, Lieberman Burchard reagent, mayer reagent, wagner reagent. While the bacteria used were *Staphylocuccus aureus* and *Salmonella thypi*.

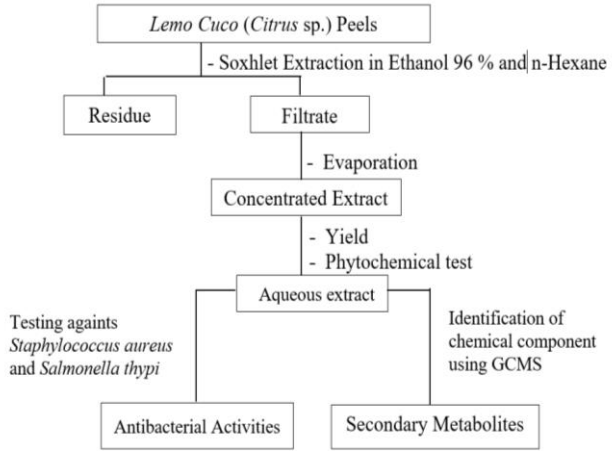

Figure 1. Research Flowchart

Preparation Method of Simplicia

Lemo Cuco fruit samples (*Citrus macroptera Mountrous*) were cleaned then the skin was separated from the flesh. Furthermore, the orange peel was cut into small pieces and dried at room temperature. After that, the Lemo Cuco peel was ground into powder which was then called simplicia.

Extraction

This study was carried out with two variations of solvents, namely n-hexane and ethanol. Weighing 50 grams of simplicia then inserting it into a sleeve made of filter paper. After that, the sleeve was inserted into a soxhlet extractor then extracted with 500 mL of ethanol (C_2H_5OH) 96% at a temperature of 81-96 ºC (heating temperature) until the solvent color returned to its original state. After the extraction process, Lemo Cuco filtrate was obtained. The Lemo Cuco filtrate obtained was then concentrated with a rotary evaporator at a temperature of 50 ºC and 40 rpm until the solvent did not drip, thus producing essential oil. The same treatment was carried out with n-hexane solvent (C_6H_{14}) at a temperature of 72-86 °C. The extract obtained then calculated the yield value and carried out phytochemical tests and characterization of the components of the essential oil of the lemon peel (*Citrus macroptera Mountrous*) with GC-MS.

Phytochemical Test

The content of secondary metabolites in the ethanol and n-hexane extract of lemo cuco peel was tested using various reagents based on standard testing procedures (Harborne, 1996). Phytochemical testing in this study used various reagents to check for the presence of flavonoid, alkaloid, steroid, terpenoid, phenolic and saponin compounds.

Antibacterial Activity Test

Antibacterial activity test using the disc paper diffusion method. The steps in this study include sterilization of equipment, making nutrient agar (na) media, rejuvenating bacteria, diluting essential oils and positive controls, making test suspensions, making test media, soaking disc paper, planting disc paper and measuring inhibition. The disc paper diffusion method was chosen because it is easy and simple to determine the antibacterial activity of the tested extract. Soaking is done for 30 to 60 minutes so that the test solution is completely absorbed into the disc. The absorbed disc paper is aired until no more drips so that when incubated the inhibition zone formed does not spread. The disc paper is planted on a solid medium that has been mixed with the test suspension. The activity test is carried out near the fire so that no other bacteria enter the petri dish which can cause contamination in the media. Furthermore, it is incubated at a temperature of 37 °C for 24 hours. This temperature is used because it is the optimum temperature for bacterial growth (34) (35) (36). Observations are made after incubation to see the clear zone around the disc paper. The measurement of the inhibition zone in this study was carried out for 24 hours to see the response of bacterial growth inhibition by antibacterial compounds in the essential oil of Lemo Cuco fruit skin. The measurement of the inhibition zone used a digital caliper with an accuracy of 0.02 mm per scale. Calculating the diameter of the clear zone formed around the disc paper on 3 sides, namely vertical, horizontal and diagonal, was then averaged as the inhibition zone of the test extract (34) (32).

RESULTS AND DISCUSSION

Results

Soxhlet extraction is a simple and easy extraction to extract volatile compounds from a sample (37). This study used ethanol and n-hexane solvents. Ethanol solvents have a low boiling point so they are easier to evaporate and can dissolve compounds quickly and are affordable (38). Ethanol solvents have hydroxyl groups that can bind polar compounds such as flavonoids and alkaloids (39) (40) (41). While n-hexane solvents are non-polar solvents that can bind non-polar compounds such as steroids and terpenoids (42). The results of the soxhlet extraction were dark green liquid essential oil which was then concentrated with a rotary evaporator to separate the solvent from the essential oil which was indicated by the evaporation of the solvent (43). The yield of essential oil from Lemo Cuco fruit skin obtained consisted of ethanol extract and n-hexane extract were 25.8% and 3.2%. Ethanol is a polar solvent capable of dissolving polar compounds and some non-polar compounds. In contrast, n-hexane is a non-polar solvent that is more limited in dissolving polar compounds. Since many bioactive compounds in plants, such as phenols and flavonoids, are polar, ethanol is more effective in extracting these compounds. Beside that, ethanol can extract various types of compounds, including monoterpenes, sesquiterpenes, and phenolic compounds, which often have therapeutic effects. n-Hexane, which is more suitable for extracting lipids and non-polar compounds, may not be able to isolate all bioactive compounds present in plant materials. Ethanol can also interact more effectively with the cellular matrix of plants, facilitating the release of compounds from cell walls. This process increases the number of compounds extracted into the solvent, thus enhancing the extract yield. The

results obtained in the ethanol extract were greater while the n-hexane extract was smaller than the study by (30) who obtained the yield of ethanol extract and n-hexane extract with the soxhlet extraction method from kaffir lime leaves of 13.39% and 10.50%. The same study was conducted by (44) who conducted a study on the extraction of 20 grams of orange peel with 200 mL of solvent. Meanwhile, (8) obtained an orange peel extract yield of 1.625% using the steam distillation method and (45) also reported that the yield of essential oil from Citrus nobilis orange peel was 19.383% using the maceration method. The amount of yield produced depends on the solubility properties of the bioactive components and the extraction method used.

Based on Table 1, we can see the groups of compounds found in the essential oil of Lemo Cuco fruit peel.

Table 1. Phytochemical Test of Lemo Cuco Fruit Peel Essential Oil (*Citrus macroptera Mountrous*)

Qualitative Test		Test Sample	
Compounds	**Reagents**	**Ethanol Extract**	**n-Heksane Extract**
Flavonoid	NaOH 10%	+	+
	$FeCl_3$ 5%	+	+
	H_2SO_4 pekat	+	+
Alkaloid	Mayer	+	+
	Wagner	+	+
	Dragendorff	+	+
Steroid	Lieberman Burchard	+	+
Terpenoid	Lieberman Burchard	+	+
Phenolic	$FeCl_3$ 1%	+	+
Saponin	Aquades	-	+

Note: (+) to positive result and (–) to negative result

The identification results obtained are in accordance with several studies such as research by Javed, et al., (2014) which explained the test results on 5 types of oranges containing several groups of secondary metabolite compounds quite high. (5) also reported that the components of lime peel are tannins, flavonoids, polyphenols, steroids and alkaloids. However, it is different from (46) which revealed that orange peel extract contains flavonoids, phenols, seroids, triterpenoids. (47) explained that oranges contain tannins, flavonoids and alkaloids, and (48) reported that kalamodin oranges and kaffir limes contain flavonoids and limonoids. General factors that can affect compound identification tests include differences in growing places and climates that cause different metabolic processes. Antibacterial activity test as a test of a compound used to control the growth of harmful bacteria so that it can prevent the spread of disease and infection and prevent decay or destruction of materials caused by bacteria.

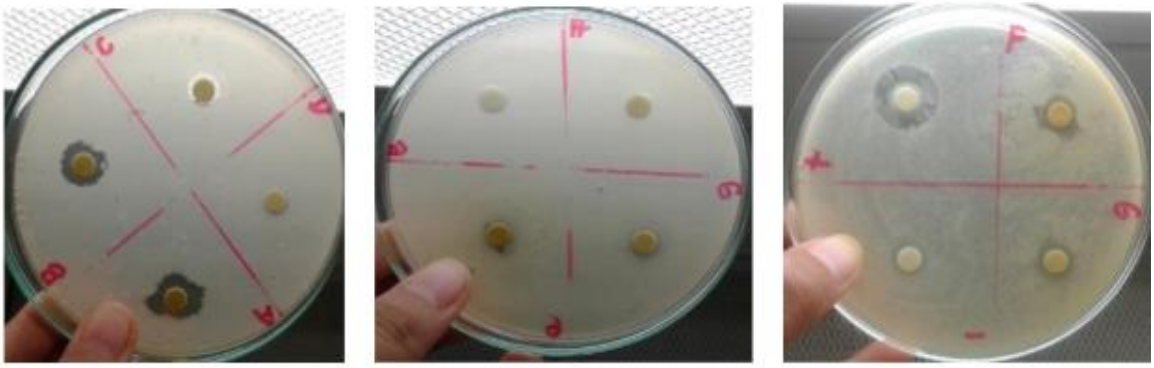

Figure 2. Test against *Staphylococcus aureus* bacteria. A-E (n-Hexane Extract 20%; 10%; 5%; 2.5% and 1.25%), F-J (Ethanol Extract 20%; 10%; 5%; 2.5% and 1.25%), Negative Control (DMSO) and Positive Control (Antibiotic Ampicillin 2%)

Table 2. Antibacterial Activity Test of Essential Oils against *Staphylococcus aureus* Bacteria

Test Sample	Concentration	Diameter of	Inhibition	Category

	(%)	Inhibiton Zone (mm)	Zone (%)	
Extract of n-Heksan	20	16.55	69.79	Strong
	10	12.39	59.65	Strong
	5	9.73	48.62	Medium
	2.5	7.47	33.10	Medium
	1.25	7.17	30.27	Medium
Extract of Ethanol	20	8.39	40.41	Medium
	10	7.68	34.40	Medium
	5	6.62	24.47	Weak
	2.5	5.73	12.74	Weak
	1.25	5.38	7.10	Weak
Control of Negative	0	5.00	0.00	Not Active
Control of Positive	2	14.78	66.17	Strong

Based on the results obtained, it can be said that the essential oil from the n-hexane extract of Lemo Cuco fruit skin from Sinjai Regency has an inhibitory effect on the growth of *Staphylococcus aureus* bacteria. These results are in accordance with the results of research conducted by (49) and (32) that ethanol extract can inhibit the growth of *Staphylococcus aureus* bacteria. The same research was also conducted by (50) who reported that the antibacterial activity of ethanol extract of kaffir lime skin has an inhibition zone of 9 mm against *Staphylococcus aureus* bacteria. This is in accordance with research by (51) reported the best inhibition results at the highest concentration of 25% against *Staphylococcus aureus* bacteria.

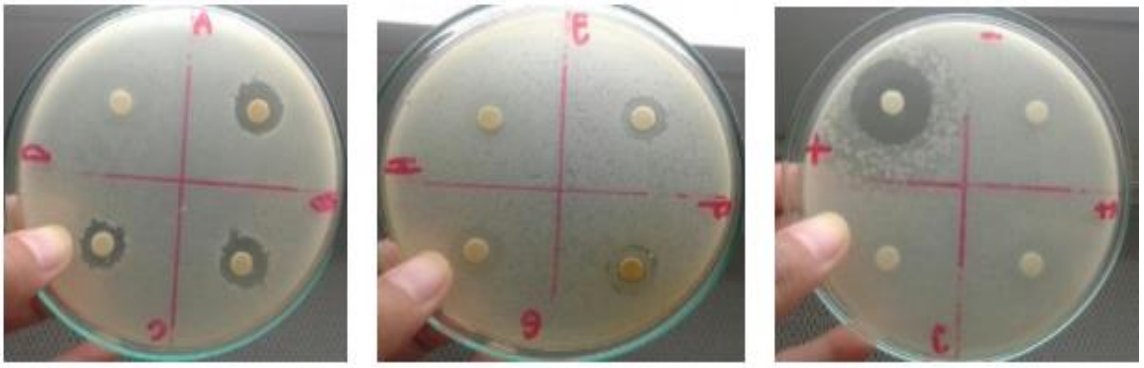

Figure 3. Test against *Salmonella typhi* bacteria A-E (n-hexane extract 20%; 10%; 5%; 2.5% and 1.25%), F-J (ethanol extract 20%; 10%; 5%; 2.5% and 1.25%), negative control (dmso) and positive control (cefixime 2%)

Table 3. Antibacterial Activity Test of Essential Oils against *Salmonella typhi* Bacteria

Test Sample	Concentration (%)	Diameter of Inhibition Zone (mm)	Inhibition Zone (%)	Category
Ekstrak n-Heksan	20	13.08	61.77	Strong
	10	10.72	53.36	Strong
	5	8.61	41.93	Medium
	2.5	6.62	24.47	Weak
	1.25	5.69	12.13	Weak
Ekstrak Etanol	20	9.31	46.29	Medium
	10	5.51	9.26	Weak

	5	5.27	5.12	Weak
	2.5	5.00	0.00	Not Active
	1.25	5.00	0.00	Not Active
Kontrol Negatif	0	5.00	0.00	Not Active
Kontrol Positif	2	14.31	65.10	Strong

Based on Figure 3 and Table 3, the results of the antibacterial activity test of Lemo Cuco fruit peel essential oil obtained against *Salmonella thypi* bacteria in n-hexane and ethanol extracts were able to inhibit bacterial growth. Based on the results obtained, it can be said that the essential oil from the n-hexane extract of Lemo Cuco fruit peel from Sinjai Regency has inhibitory power against the growth of *Salmonella typhi* bacteria. Meanwhile, the results of the measurement of the inhibition zone of essential oil from ethanol extract against *Salmonella typhi* bacteria are in accordance with the results of research conducted by Amin (2012) and Nanasomat (2005) that ethanol extract is able to inhibit the growth of *Salmonella typhi* bacteria.

Research by (17) reported that the juice of purut orange peel (*Citrus hystrix* Dc.) has inhibitory activity at a concentration of 25% to 100% with an average inhibition zone of 3.4 mm. In addition, research of (52) revealed that the decrease in the number of *Salmonella typhi* bacterial colonies was quite sharp after being given a concentration of lime peel extract (*Citrus aurantifolium*) 6.25% which is in line with (53) (54)that lime peel extract can affect the growth of *Staphylococcus* sp, *Salmonella* sp, *Escherichia coli*, *Klebsiella*, *Proteus* sp and *Pseudomonas* sp bacteria.

There is a difference of inhibition zones of the test extract against *Staphylococcus aureus* and *Salmonella typhi* bacteria. This is in accordance with research of (55) which reported that the results of star fruit ethanol extract inhibited gram-positive bacteria more than gram-negative bacteria. This difference is due to the difference in high sensitivity which is indicated by the high level of inhibition produced by a particular antibacterial compound. In addition, it is also influenced by several factors such as the toxicity of the test material, the diffusion ability of the test material in the media, the interaction between the components of the medium and the in vitro microenvironmental conditions. According to (56), the concentration of a test sample as an antibacterial is a determinant of the size of the ability to inhibit the growth of test microbes. The difference is due to differences in cell wall structure between the two bacteria that affect the work of the extract (57). The test results on the positive control using ampicillin antibiotics against *Staphylococcusa aureus* bacteria gave an inhibition zone diameter of 14.78 mm with an inhibition power of 66.17%, in *Salmonella thypi* bacteria using the antibiotic cefixin which gave an inhibition zone diameter of 14.31 mm with an inhibition power of 65.10%.

The results obtained are in line with the research of (58) and (59) who used ampicillin against the activity test of *Staphylococcus aureus* bacteria with an inhibition diameter of 8 mm and 10 mm. Ampicillin as a commercial antibiotic and is one type of penicillin antibiotic that works by inhibiting cell wall synthesis. Research by (60) also reported several antibiotics that are often used against Salmonella thypi bacteria, one of which is the antibiotic cefixime. Cefixime is a third-generation oral cephalosporin antibiotic that has antimicrobial activity including *Enterobacterisceae.* This shows that the antibiotics used are sensitive to both test bacteria. While the test results on the negative control against the two test bacteria did not provide an inhibition zone. This shows that the use of DMSO solvent does not affect the antibacterial test results of Lemo Cuco fruit peel essential oil. the orange peel infusion test had a very strong inhibitory activity level (inhibitory power> 75%), strong (50 <inhibitory power ≤ 75%), medium (25 <inhibitory power ≤ 50%), weak (0 <inhibitory power ≤ 25%) and inactive (0). Based on tables 2 and 3, it can be seen that the inhibitory effect of essential oils from ethanol extract and n-hexane extract of Lemo Cuco fruit skin against *Staphylococcus aureus* bacteria is included in the strong category (concentrations of 20% and 10%) and moderate (5%, 2.5% and 1.25%). While the essential oil from ethanol extract is included in the moderate category (20% and 10%) and weak (5%, 2.5% and 1.25%). The inhibitory power of essential oils from n-hexane extract against *Salmonella typhi* is included in the weak category (1.25% and 2.5%), medium (5%) and strong (10% and 20%). While the essential oil from ethanol extract is included in the moderate category (20%), weak (10% and 5%) and inactive (concentrations of 5%, 2.5% and 1.25%). The difference in the diameter of the inhibition zone produced is due to differences in the components of each part of the plant (20)

Table 4. Characterization of Essential Oil Components from Ethanol Extract of Lemo Cuco Fruit Peel (*Citrus macroptera Mountrous*) with GC-MS

No	Retension	%	Molecule	Molecule	Component of

	Time	Area	Formula	Weight	Compound
1	14.301	0.94	$C_{15}H_{24}$	204	β-Elemen
2	15.558	4.69	$C_{15}H_{24}$	204	Germakren
3	15.708	6.49	$C_{15}H_{24}$	204	α-Farnesen
4	16.027	1.94	$C_{15}H_{24}$	204	δ-Kadinen

Table 5. Characteristics of Essential Oil Components from n-Hexane Extract of Lemo Cuco Fruit Peel (*Citrus macroptera Mountrous*) with GC-MS

No	Retension Time	% Area	Molecule Formula	Molecule Weight	Component of Compound
1	13.506	0.51	$C_{10}H_{16}$	136	α-Terpinen
2	14.107	1.28	$C_{15}H_{24}$	204	Kopaein
3	14.747	1.73	$C_{15}H_{24}$	204	Kariofilen
4	15.214	1.27	$C_{15}H_{24}$	204	α-Humulen
5	15.564	6.43	$C_{15}H_{24}$	204	Germakren D
6	16.027	2.86	$C_{15}H_{24}$	204	δ-Kadinen
7	16.778	4.61	$C_{15}H_{22}$	204	Aromandendren
8	17.334	3.33	$C_{15}H_{24}O$	220	Isospathulenol
9	18.585	1.09	$C_{15}H_{26}O$	238	Oplopanon
10	19.355	0.87	$C_{15}H_{22}O$	218	Nutkaton
11	20.262	1.21	$C_{17}H_{34}O_2$	255	Metil Palmitat
12	21.951	0.75	$C_{19}H_{32}O_2$	294	Metil Linoleat

The results obtained based on Table 4 of the compound components contained in the essential oil from the n-hexane extract of Lemo Cuco fruit skin consist of monoterpenoid, sesquiterpenoid and fatty acid compounds. Based on the results of GC-MS characterization of essential oil from ethanol extract and essential oil from n-hexane extract of Lemo Cuco fruit skin, the dominant compounds are monoterpenoid and sesquiterpenoid compounds. The greater variety of mono- and sesquiterpenes identified from n-hexane extracts is primarily due to its non-polar nature, strong affinity for lipophilic compounds, and focused extraction of volatile hydrocarbons. Ethanol, being polar, is more suitable for extracting polar and semi-polar compounds, which limits its ability to capture the diversity of terpenes. Thus, the choice of solvent significantly affects the range of compounds extracted and detected.The results obtained are in accordance with several studies such as the study of (30) which revealed that the components of essential oil are citronellal 65.99%, nerolidol 19.68%, trans caryopilen 8.61%, sabinen 2.07% and decane 0.66%. Other studies such as research by (27) reported that the largest components of essential oil compounds in kaffir lime are citronellal 66.85%, β-spinen 32.967%. The same study was conducted by Javed, et al., (2014) revealing that the largest components of 5 types of citrus are limonene 87.84%, carvone 16.97% and α-terpineol 12.16%. Research by (61) also reported that the components of essential oil compounds in orange peel are limonene, β-pinene, citronellal, α-terpineol, copaene, cadinene, caryophilene and geranyl acetate. The differences in compound components are caused by the place of growth and climate which cause different metabolic processes (Sari, 2013 and Muntaha 2013).

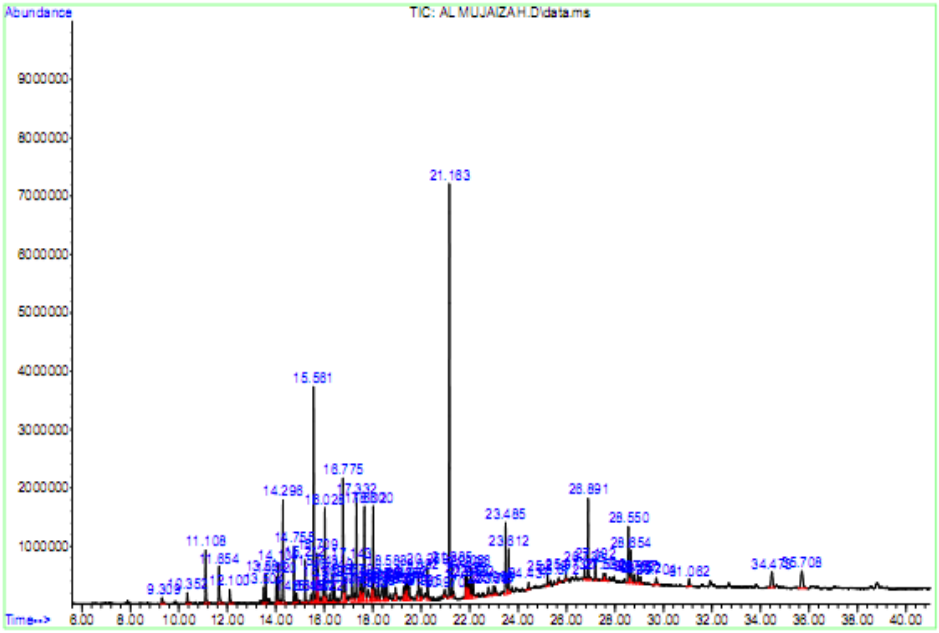

Figure 4. Spectrum of Essential Oils from n-Hexane Extract of Lemo Cuco Fruit Peel (*Citrus macroptera Mountrous*)

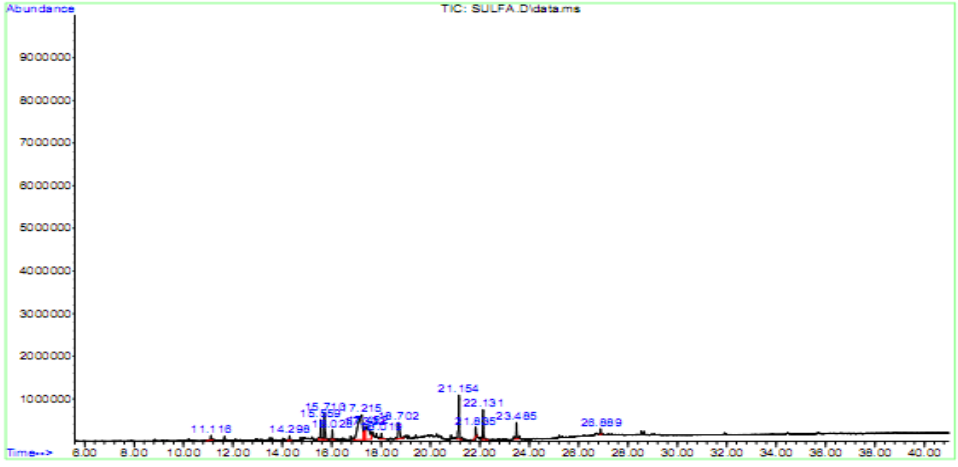

Figure 5. Spectrum of Essential Oils from Ethanol Extract of Lemo Cuco Fruit Peel (*Citrus macroptera Mountrous*)

Based on the characterization of the components of the essential oil of Lemo Cuco fruit skin with GC-MS from ethanol extract and essential oil from n-hexane extract, the dominant compounds are monoterpenoid and sesquiterpenoid compounds. The results obtained indicate that it can be used as an antibacterial.. Research by (62) and (63) also reported that the active antibacterial compounds in lime leaf essential oil are terpene compounds. Terpene compounds work as antibacterials by damaging porins, which are proteins in bacteria so that bacterial growth is inhibited). Research by (64) also explained that essential oils have antibacterial activity because essential oils contain compounds that can inhibit or kill bacterial growth. The difference in the magnitude of the inhibition produced depends on the solubility properties of the bioactive components and the method used (65).

CONCLUSIONS

The antibacterial activity of essential oils from ethanol extract and n-hexane extract of Lemo Cuco fruit peel (*Citrus macroptera Mountrous*) against *Staphylococcus aureus* bacteria is categorized as strong and medium. While from ethanol extract

it is categorized as moderate and weak. The inhibitory power of essential oils from n-hexane extract against *Salmonella typhi* is categorized as weak, moderate and strong. While from ethanol extract it is categorized as moderate, weak and inactive. Bacteria that are more sensitive to lemon coco are *Staphylococcus aureus* which is a Gram-positive bacteria. Ethanol extract is a solvent extract that shows the highest yield percentage and more complex secondary metabolites.

The components of essential oils from Lemo Cuco fruit peel (*Citrus macroptera Mountrous*) from ethanol extract show the presence of α-farnesen, germacrene, δ-cadinene and β-element compounds, while n-hexane extract shows δ-cadinene, germacrene D, aromadendrene, isospathulenol, caryophyllene, copaine, α-humulene, α-terpinene, oplopanone and nutkaton, methyl palmiate and methyl linoleate.

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CONFLICT OF INTEREST

There is no conflict of interest

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