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Exploring Side Effects of Warfarin in Outpatients at Tertiary Hospital in Indonesia

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Abstract

Background*: Patients with atrial fibrillation, venous thromboembolism, valvular disease, cardioembolic stroke, and acute myocardial infarction are prescribed the oral anticoagulant warfarin to treat thrombi. To guarantee its efficacy and safety, anticoagulants must be closely monitored. Furthermore, warfarin has a narrow therapeutic index, which increases the risk of side effects, particularly in the early stages of treatment.* ***Objective****: This study aimed to analyze the incidence of warfarin side effects in outpatients at Dr. Soetomo General Hospital.* ***Methods****: A retrospective cross-sectional design was adopted for outpatients at the Cardiology Department of Dr. Soetomo General Hospital from March to May 2023. Data were collected on the incidence of side effects in outpatients who received warfarin for a minimum of one month through an interview process. Other data, including age, sex, duration of warfarin therapy, comorbidities, and INR at the last scan, were extracted from medical records. The Chi-Square test was used to examine the data.* ***Results****: The results showed that 88 patients (42.7%) had side effects of bleeding (126 events). These included gum bleeding (22.3%), hematoma (20.4%), melena (7.7%), menometrorrhagia (2.4%), epistaxis (1.9%), hematuria (1.5%), hematemesis (1.0%), hemoptysis (1.0%), spontaneous venous bleeding (1.0%), hematochezia (0.5%), hemostasis during blood sampling (0.5%), tongue bleeding (0.5%), and subconjunctival bleeding (0.5%).* ***Conclusion****: The incidence of side effects during warfarin treatment was high, accounting for approximately 42.7% of cases. Furthermore, one patient experienced more than one side effect.*

Keywords: *outpatients, side effects, warfarin*

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INTRODUCTION

The prevalence of mortality due to cardiovascular disease was quite high, around 17.9 million people in 2016, accounting for 31% of the world's mortality (WHO, 2019). According to data from the Indonesian Ministry of Health, 1.5% of people in Indonesia and the province of East Java had cardiovascular disease in 2018 (based on doctors' diagnoses) (Kemkes RI, 2018). Therefore, oral anticoagulants are essential. In 1941, the FDA approved the clinical use of warfarin for the prevention or treatment of thromboembolism (How, 2015). Warfarin, a vitamin K antagonist anticoagulant, is used to prevent and treat thromboembolic problems linked to atrial fibrillation. In patients with atrial fibrillation, medication has been proven to reduce the risk of stroke by 62% (Touchette et al., 2007). The use of anticoagulants raises concerns because of the associated risk of side effects. Warfarin has a high risk of drug interactions with a narrow therapeutic index, necessitating the determination of a therapeutic dose for each patient. This results in side effects, particularly in the early stages of therapy (Morgan et al., 2009; Hanigan et al., 2021).

The side effects of warfarin are generally mild and do not require intervention. However, some patients with complications are at a higher risk of side effects, such as excessive bleeding (Snipelisky & Kusumoto, 2013). The use of anticoagulants raises concerns because they are associated with adverse effects. A hemoglobin drop of at least 2 g/dL or bleeding that necessitates hospitalization or transfusion is a typical definition of this adverse event. The side effects of warfarin include major bleeding (hematuria), lower gastrointestinal bleeding, upper gastrointestinal bleeding, upper gastrointestinal bleeding, intracranial bleeding, minor bleeding (ecchymosis), intramuscular or intra-abdominal hematoma, epistaxis, hemoptysis, and vaginal and retinal hemorrhage (Ozturk et al., 2019). Other side effects include thromboembolic conditions, such as decreased breath sounds, cough, chest pain, irregular heartbeat, redness or skin rash on the hands, deep vein thrombosis, and edema in the feet or hands (Waheed et al., 2021; Vyas et al., 2022). Necrosis can also occur at the start of warfarin therapy and in cases of systemic embolism or cholesterol microembolism (Ranieri et al., 2015).

Previous studies have shown that several factors influence the risk of bleeding in patients taking warfarin, including sex, age, comorbidities, genetic polymorphisms, and drug interactions (Ozturk et al., 2019; Ababneh et al., 2021; Yabeyu et al., 2022; Aulia et al., 2022). In addition, warfarin has nearly 100% oral bioavailability and reaches peak concentrations within 4 h of administration. This drug has a distribution volume of 0.14 L/kg and 99% protein binding, particularly to albumin. Warfarin binding can be influenced by factors that can increase free drug levels, thereby increasing the risk of side effects (Ranieri, 2015). Apart from these variables, habits such as alcohol consumption, smoking, and a diet of foods containing vitamin K can influence the occurrence of side effects during the use of warfarin (Yabeyu et al., 2022). There are few reports on the side effects of warfarin; therefore, existing data are limited. Furthermore, a larger sample size with bleeding type classification was used in this study than in previous studies. Therefore, this study aimed to analyze the incidence of warfarin side effects in outpatients at Dr. Soetomo General Hospital.

MATERIALS AND METHODS

Study area and period

This study was conducted at the Department of Cardiology at Dr. Soetomo General Hospital, Surabaya, between March and May 2023. These departments are primarily responsible for most hospitalized patients prescribed warfarin. Only outpatient clinic patients who had been taking warfarin for at least one month were included.

Study design

This retrospective cross-sectional study included outpatients who received warfarin at the Department of Cardiology of Dr. Soetomo General Hospital, Surabaya, Indonesia, between March and May 2023.

Sampling and selection of patients

The samples were obtained from total sampling, which included inclusions, from March to May 2023. The inclusion criteria were outpatients at the Cardiology Department of Dr. Soetomo General Hospital who had received warfarin for at least a month and were > 18 years old. The exclusion criterion was cognitive impairment.

Data collection method

Data were collected on the incidence of side effects in outpatients who received warfarin minimum of a month through an interview process. Patient information, such as age, sex, length of warfarin treatment, comorbidities, and INR at the latest scan, was obtained from medical records.

Data analysis

Warfarin side effects were grouped using the Bleeding Academic Research Consortium (BARC) classification. In the absence of bleeding, the BARC

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group was classified as type 0. As type 1 bleeding is "non-actionable," the patient did not seek medical assistance. Similar to type 1, type 2 bleeding is characterized by clinically noticeable symptoms that are "actionable" and necessitate medical diagnosis, hospitalization, or treatment. Type 3, which includes clinical, laboratory, and/or imaging evidence of bleeding with a particular healthcare provider response, is separated into three categories, numbered a to c. Type 3a occurs when hemoglobin levels drop from ≥3 to <5 g/dL, and there is open bleeding that necessitates transfusion. Type 3b is the presence of open bleeding and a decrease in hemoglobin level of ≥5 g/dL, cardiac tamponade, surgical intervention requirement (excluding teeth/nose/skin/hemorrhoids), and intravenous vasoactive drugs. Intracerebral and intraocular hemorrhages that affect vision are classified as type 3c. Type 4 bleeding is linked to coronary artery bypass grafting (CABG) (within 48 h) and type 5 bleeding is lethal. Lethal bleeding is classified as genitourinary, pericardial, pulmonary, retroperitoneal, gastrointestinal, cerebral, or other (Hicks et al., 2011). The Chi-Square tests were conducted to investigate differences between patient demographic characteristics and the incidence of warfarin side effects. A P-value <0.05 indicated a statistical significance of patient demographic characteristics affecting the incidence of warfarin side effects. Ethical approval was obtained from the Health Research Ethics Committee of Dr. Soetomo General Academic Hospital (Surabaya No. 0600/KEPK/II/2023).

RESULTS AND DISCUSSION

From March to May 2023, data were obtained from 206 outpatients taking warfarin at the Department of Cardiology at Dr. RSUD. Soetomo General Hospital. The data extracted from the medical records are presented in Table 1.

Measuring the particle size is essential for determining the dimensions of the particles and ensuring that they meet the desired size range. Typically, the diameter of Nanostructured Lipid Carriers (NLC) ranges from 10 to 1000 nm, with a preferred size of 50–300 nm for targeted drug delivery. For topical applications, such as hair and skin, NLCs are generally the most effective when their maximum particle size is approximately 300-500 nm. Although the human pore diameter ranges from 40–80 μm to 0.05–0.2 mm, achieving an NLC particle size of approximately 300 nm is crucial for optimal product stability and effectiveness.

Table 1. Demographic profile characteristics of warfarin medication in outpatients at the Dr. Soetomo general hospital

	Side Effect		P value
	Absent	Present	
	Subject (n=118)	Subject (n=88)	
Sex			
Male	59 (62.8)	35 (37.2)	0.145
Female	59 (52.7)	53 (47.3)	
Age			
<50 year	59 (60.2)	39 (39.8)	0.419
≥ 50 year	59 (54.6)	49 (45.4)	
Duration of Warfarin Medication			
<1 year	41 (64.1)	23 (35.9)	0.187
≥1 year	77 (54.2)	65 (45.8)	
INR*			
< 2,0	77 (69.4)	34 (30.6)	
2,0-3,0	33 (45.2)	40 (54.8)	-
> 3,0	8 (36.4)	14 (63.6)	
Comorbidity			
Absent	32 (60.4)	21 (39.6)	0.597
Present	86 (56.2)	67 (43.8)	

*recent INR

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The chi-square test yielded a p-value > 0.05, as shown in Table 1, indicating that sex did not significantly influence the incidence of side effects. Other studies have shown no discernible variation in the risk of bleeding between males and females corroborating these findings (Barcellona et al., 2022). According to previous studies, women taking warfarin are at higher risk of bleeding (Gieling et al., 2017). According to another study, women typically have a low TTR, which is associated with a higher risk of bleeding and decreased efficacy (Costa et al., 2021). Several studies have found that men taking warfarin have a greater risk of bleeding (Penttilä et al., 2019; Rydberg et al., 2020). Females have a lower tendency to receive anticoagulant prescriptions than males after considering age, comorbidities, and thromboembolic risk factors (Lee et al., 2023).

According to the other data (Table 1), there was no significant difference in the side effects observed between patients aged ≤ 50 and > 50 years (p > 0.05). The incidence of side events, including bleeding is significantly influenced by the age of warfarin users (Shendre et al., 2018). This study demonstrated that the occurrence of side effects was significantly affected by age. These results contradict those of several studies that have shown a correlation between age and the presence of bleeding side effects when using warfarin. However, determining a set age criterion for evaluating bleeding risk is challenging. Previous studies have found that patients aged 60 years have worse anticoagulant control than older adults. Poor compliance, social factors including employment and alcohol consumption, and clinical considerations that are considered while delivering warfarin safely and effectively are the causes of this adverse event (Abohelaika et al., 2016). In contrast, other studies have stated that bleeding as a complication is prone to occur in patients aged ≥ 65 years owing to the risk of thrombosis when using anticoagulants (Gross & Chan, 2021). In addition to age, the duration of warfarin use should be considered.

Table 1 shows that there were no differences between patients who experienced side effects and patient without have experienced side effects and those who did not in the duration of warfarin medication < 1 year or ≥ 1 year (p > 0.05). According to this study, the risk of side effects was not significantly influenced by the duration of warfarin therapy. However, Al Saikhan (2020) stated that 57.4% of patients in Saudi Arabia had a warfarin medication duration ≥1 year. Another study reported that the use of warfarin for ≥1 year causes major bleeding (Ozturk et al., 2019). Patients taking warfarin for > 12 months require close coagulation monitoring to minimize the risk of bleeding. Monitoring was performed using INR values because thrombus formation still occurs 6-12 months after onset (Ardissino et al., 2003).

Table 1 shows the INR grouping into three groups: INR < 2.0, INR 2.0-3.0, and INR > 3.0 in the groups with and without side effects. The INR value in individuals with long-term warfarin consumption can change even with maintenance doses (Ozturk et al., 2019). To reduce the risk of adverse effects, patients on warfarin therapy must have their INR modified (Shikdar et al., 2018). One parameter that varies with warfarin and other treatments is the INR (Hindricks et al., 2021). INR can be influenced by a wide range of external circumstances, including the use of herbal remedies, alcohol, foods high in vitamin K, diet, and compliance (Al-Momany et al., 2019). A study conducted in 2022 revealed that 14 patients with thromboembolic events had an INR< 3.0, and 23 of the 74 patients who experienced bleeding had an INR < 3.0 (Putriana et al., 2022). Furthermore, Ozturk et al (2019) showed that only major and minor bleeding occurred in patients with an INR of 5-8.9, while major bleeding was quite large in INR ≥ 9 (Ozturk et al., 2019). When the last INR scan was <2, monitoring was performed every day until the individual INR target was reached. However, an INR > 3 requires monitoring every 2-3 d. INR was within the target range; therefore, monitoring was performed for 4-6 months. This is primarily performed in patients with comorbidities (Health, 2024).

Tabel 2. Side effect incidence of warfarin medication in outpatients at the Dr.Soetomo general hospital

BARC Classification	n (%)
Type 0 (absent)	118 (57.3)
Type 1 (gums bleeding, hematoma, melena, menometorrhagia, epistaxis, hematuria, hematemesis, hemoptysis, spontaneous venous bleeding, hematochezia, hemostasis during blood sampling, tongue bleeding, and subconjunctival bleeding.	88* (42.7)

*One patient experienced more than one type of side effect

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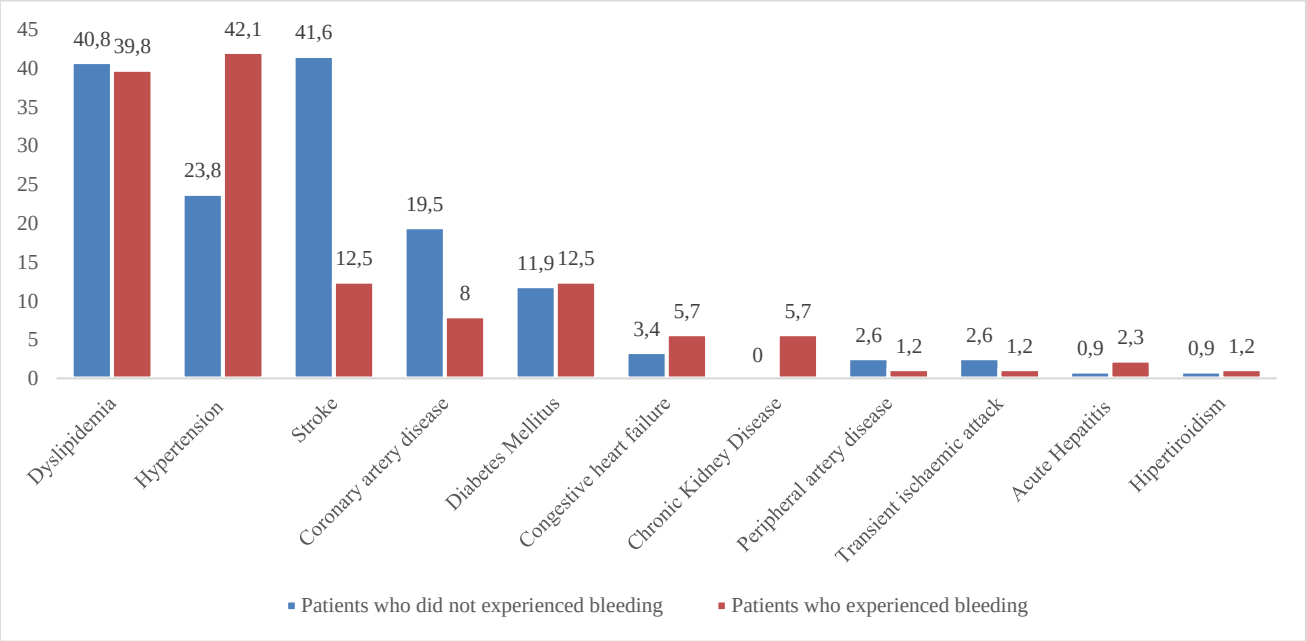

Figure 1. Types of comorbidities in patients receiving warfarin

Table 3. Clinical manifestations of side effects of warfarin medication in outpatients at the Dr.Soetomo general hospital

Side Effect Reaction	n (%)
Gum bleeding	46 (22.3)
Hematoma	42 (20.4)
Melena	16 (7.7)
Menometorrhagia	5 (2.4)
Epistaxis	4 (1.9)
Hematuria	3 (1.5)
Hematemesis	2 (1.0)
Hemoptysis	2 (1.0)
Spontaneous venous bleeding	2 (1.0)
Hematochezia	1 (0.5)
hemostasis during blood sampling	1 (0.5)
tongue bleeding	1 (0.5)
Subconjunctival bleeding	1 (0.5)

The p-value from the *chi-square* test for the presence of comorbidities had no significant influence on the occurrence of side effects ($p > 0.05$) (Table 1). The three most common comorbidities among patients who experienced bleeding were hypertension, dyslipidemia, and stroke (Figure 1). In this study, the most prevalent comorbidity among individuals with bleeding as a side effect was hypertension (Lackland, 2014). Hypertension was the most prevalent comorbid condition in all the black samples (Ciurus et al., 2015). It is possible that the illness may make oral anticoagulants less effective. Individuals without AF who have this comorbidity have been linked to poor anticoagulation control and an elevated risk of stroke (Bertomeu-González et al., 2015). Tapaskar et al (2022) reported that patients using warfarin had several comorbid conditions such as HIV, diabetes mellitus, heart failure, and a previous history of stroke or transient ischemic attack. Warfarin can protect against thrombus formation for 180 days in conditions such as transient ischemic attack, venous thromboembolism, and ischemic stroke (Tapaskar et al., 2022). These conditions are risk factors in increased risk of bleeding in patients with a history of non-cardioembolic stroke, chronic kidney or liver disease, acute, or chronic disease (Ageno et al., 2012).

Patients with comorbidities receive polypharmacy medications, such as antiplatelet agents, nonsteroidal anti-inflammatory drugs, and several other drugs, and many other medications can interact with warfarin or elevate the risk of bleeding (Ikeda et al., 2018). For patients with mechanical prosthetic heart valves, combination antithrombotic therapy comprising oral anticoagulants (OAC) and low-dose aspirin (≤100

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mg/day) is recommended (Salem et al., 2004). Currently, this combination is commonly administered to patients with vascular disease, coronary heart disease, and myocardial infarction (MI). This combination increases the risk of bleeding (So & Eckman, 2017). According to previous studies, both non-selective NSAIDs and selective COX-2 inhibitors carry a significant risk of severe hemorrhage. This condition is not easily controlled by INR monitoring and can be avoided by appropriate management. Previous research has shown that the side effects resulting from the use of warfarin vary (Battistella, 2005; Hauta-Aho et al., 2009).

According to the BARC classification, 88 patients (42.7%) experienced side effects in the form of bleeding (Table 2). The various types of bleeding in Table 3 show that a total of 126 events such as gum bleeding (22.3%), hematoma (20.4%), melena (7.7%), *menometorrhagia* (2.4%), epistaxis (1.9%), hematuria (1.5%), hematemesis (1.0%), hemoptysis (1.0%), **s**pontaneous venous bleeding (1.0%), hematochezia (0.5%), hemostasis during blood sampling (0.5%), tongue bleeding (0.5%), and subconjunctival bleeding (0.5%). In this study, the results showed that the most common bleeding reactions were in the form of blood gums, hematoma, and melena. This was supported by the findings of Ozturk et al (2019) which found that the incidence of bleeding in the form of subcutaneous hematoma (10.4%), hematuria (10.4%), and epistaxis (4.2%) was quite large compared to hemoptysis, intracranial bleeding, lower and upper gastrointestinal bleeding (Ozturk et al., 2019). According to Mascolo et al. (2019), the incidence of side effects included epistaxis (40.4%), hematuria (9.8%), melena (7.3%), gum bleeding (4.7%), hematemesis (3.6%), hematoma (2.1%), and gastric bleeding (0.5%) (Mascolo et al., 2019). In geriatric patients, less than 5% of the side effects occur in the form of hematuria (Ningrum et al., 2020). Another study reported that 44% of gastrointestinal bleeding cases occurred during the use of warfarin. In this context, the use of warfarin as monotherapy is associated with the probability of recurrent gastrointestinal bleeding, thus requiring close monitoring (Tapaskar et al., 2022).

According to Kimmel (2008), genetic variables are substantial. Warfarin is influenced by genetic polymorphisms in vitamin K epoxide reductase complex 1 (VKORC1) and cytochrome P450 2C9 (CYP2C9) genes. Warfarin reactivity is also influenced by clinical variables such height, weight, and age (Kimmel et al., 2008). The polymorphisms contribute to the dose variability among ethnic groups (Takahashi et al., 2006). A racemic mixture of R and S enantiomers forms the warfarin. The more potent S-warfarin is metabolized in the liver by cytochrome P450 2C9. Warfarin is metabolized more slowly by the wild-type allele, CYP2C9*1, and the other two alleles, CYP2C9*2 and CYP2C9*3. This allele may increase the risk of bleeding in patients carrying it (Shurin & Nabel).

CONCLUSION

In conclusion, the incidence of warfarin-induced side effects was significant, accounting for approximately 42.7% of the cases. To reduce the risk of warfarin side effects, such as excessive bleeding, drug administrators must ensure close monitoring, including providing education to patients before initial warfarin administration.

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AUTHOR CONTRIBUTIONS

Conceptualization, B.S., W.N., BZ.; Methodology, B.S., W.N., M.Y., F.Z.; Software, F.Z., D.M.; Validation, B.S., W.N., D.M.; Formal Analysis, F.Z., D.W.; Investigation, F.Z., D.M., B.Z.; Resources, M.Y., D.M.; Data Curation, D.W., D.S.; Writing - Original Draft, F.Z., D.W.; Writing - Review & Editing, B.S., W.M.; Visualization, D.W., F.Z.; Supervision, B.S., M.Y., D.M.; Project Administration, F.Z., B.S.; Funding Acquisition, FZ., D.W., B.S.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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Phytochemical Screening and Antibacterial Test of *Streptococcus mutans* Using Extracts of Five Piperaceae Species

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Abstract

Background*: The Piper genus has 700 species distributed around the world, which have many benefits as traditional medicines. Some of them are red betel, Javanese chili, Chinese betel, green betel, and forest betel, which are included in the Piperaceae family. One of the biggest threats to public health and global development is antimicrobial resistance.* ***Objective****: Therefore, the aim of this study was to conduct phytochemical screening of extracts from five piperaceae species as natural antibiotics for the antibacterial test of Sterptococcus mutans.* ***Methods****: The extraction method used was UAE (ultrasound-assisted extraction) with three solvents of different polarity, namely n-hexane, ethyl acetate and ethanol. MIC (minimum inhibitory concentration) and DDH (diameter of inhibition) tests were conducted by solid agar dilution method, and paper disc diffusion method.* ***Results****: The results of MIC testing of the five piperaceae species show that the best extracts, which have the smallest MIC value, are forest betel leaf extract, namely 5% n-Hexane and red betel leaf extract, namely n-Hexane and 5% ethyl acetate. The results of DDH testing of the five piperaceae species show that the best extract that has the greatest DDH value is green betel leaf extract with ethyl acetate solvent, DDH value of 23.26 mm ± 0.21 included in the Susceptible category.* ***Conclusion****: The results can be concluded that the best extract is green betel leaf extract with ethyl acetate solvent, DDH value of 23.26 mm ± 0.21 against Streptococcus mutans included in the Susceptible category.*

Keywords: *antibacterial, inhibitory diameter, minimum inhibitory concentration, ultrasound-assisted extraction*

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INTRODUCTION

One of the biggest threats to public health and global development is antimicrobial resistance. Resistant bacteria are estimated to be responsible for 1.27 million deaths worldwide in 2019. Overuse of antimicrobials is a major factor driving the development of drug-resistant pathogens. This makes treatment of infections more difficult (WHO, 2023). *Streptococcus mutans* is a Gram-positive facultative anaerobic bacterium that can cause an acidic atmosphere in the mouth and lead to tooth decay. This acidic atmosphere can occur due to the metabolic process of S. mutans feeding on carbohydrate-containing foods and interacting with them, which causes mineral loss from the teeth and ultimately damages the teeth. In the treatment of dental caries infection at RSUD Haji Makassar from January to March 2016, some antibiotics showed resistance to S. mutans bacteria. For example, amoxicillin showed 100% resistance and cefetriasone showed 85% resistance from 10 patients (Handayany & Fany, 2018). New innovations are needed as alternative antimicrobial treatments to stop the development of infection-causing microorganisms in the event of resistance. The Piper genus, which consists of 700 species grown worldwide, is the largest genus that has many benefits as traditional medicine. The Piperaceae family consists of the genus Macropiper, Zippelia, Piper, Peperomia, and Manekia (Nascimento et al., 2012).

Of the many types of Piperaceae, Javanese chili, Chinese betel, green betel, forest betel and red betel were chosen because the five betels have secondary metabolite compounds that function as antimicrobials such as alkaloids, flavonoids, saponins, and others. This study was conducted to compare the extracts of the five betels, which is the best as a natural antibiotic against *Streptococcus mutans* by using three solvents with different levels of solubility, namely n-hexane, ethyl acetate and ethanol. The use of three solvents with different levels of solubility (n-hexane, ethyl acetate and ethanol) in the extraction process aims to separate the compounds in the sample based on their polarity properties.This allows a more in-depth analysis of the components contained in the sample. By using these three solvents, researchers can separate and identify different compounds in the sample. For example, an n-hexane extract may contain chlorophyll and lipid pigments, while an ethyl acetate extract may contain flavonoids and polyphenolic compounds. Ethanol will separate polar compounds, which may include sugars and organic acids (Setyaningrum, 2017; He et al., 2015).

Java chili (*Piper retrofractum* Vahl.) leaves are traditionally used by people to treat fever, flu, diarrhea, beriberi, anemia, and cholera. It is one of the natural medicinal materials that has antimicrobial potential with a very low level of bacterial contamination (Musthapa & Gumilar, 2016). People usually call the betel nut plant (*Peperomia pellucida* L.) a weed or wild plant. However, there are some communities that consume this Chinese betel as an additional food or fresh vegetables. *Piper betle* L, or green betel leaf, has antibacterial properties. In addition, there are active compounds in green betel leaves that can stop the development of Gram-positive and Gram-negative bacteria (Nouri et al., 2014). The forest betel plant (*Piper aduncum* L.) is one of the species that is still very rarely used. Phytochemical testing in the research of Safriana et al. (2019) showed that forest betel leaves contain flavonoids, tannins, saponins, and alkaloids, which showed significant antibacterial activity. Red betel (*Piper cf. fragile* BENTH) is usually cultivated in the yard as an ornamental plant and as an herbal medicine (Anugrahwati et al., 2016). Red betel leaf decoction is traditionally used as a mouthwash, prevention of bad breath, and treatment of diseases such as toothache and mouth ulcers (Suri et al., 2021).

MATERIALS AND METHODS

Materials

The materials used were Javanese chili leaves, Chinese betel leaves, forest betel leaves, green betel leaves and red betel leaves taken and determined at the National Research and Innovation Agency (BRIN) Bogor, *Streptococcus mutans* bacteria from IPB *Culture Collection*, aluminum foil, filter paper, spirtus, discs (Oxioid), Nutrient agar media (Merck), Potato Dextrose Agar media (Merck), 96% Ethanol, 70% Ethanol, Ethyl acetate, n-Hexane, Amoxicillin tablets (Mersi), Nystatin suspension 100.00 IU/mL (Novell), Tween 1%, Bouchardat, Dragendorff, Mayer reagents, hydrochloric acid (Merck), chloroform (Merck), concentrated sulfuric acid (Merck), anhydrous acetic acid (Merck), iron (III) solution (Merck), barium chloride (Merck), $H_2SO_4$1% (Merck), physiological NaCl, sterile distilled water, $FeCl_3$, DPPH, aluminum chloride, quinine and other materials.

Tools

The tools used are autoclave (Hirayama), incubator (Nuve®), digital scale (Labpro), blender (Zppelin®), dropper pipette, measuring cup (pyrex), petri dish, test tube (pyrex), bunsen, ose needle, filter paper no.1 (whatman), ultrasonic (Branson m5800h), hot plate,

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vacuum rotary evaporator, tweezers, vernier, spatula, muffle furnace, oven (Memment®), desiccator (Iwaki®), laminar air flow, crucibles, Sonication ultrasonicator (Sonica®), rotary evaporator (IKA®), UVVis Spectrophotometry (Jasco V730®), dark bottle (brown bottle), digital balance (AND G-120®), furnace (Ney®) and other glassware.

Method

Extraction

Betel leaves as much as 5 Kg were washed thoroughly with running water, dried in an oven at 40˚C until dry. Then mashed and sieved using a 40 mesh sieve until it becomes powder. Next, 300g of powder was weighed and n-Hexan solvent was added in the ratio of material:solvent 1:10. The mixture of powder and solvent was then extracted using ultrasound-assisted extraction at a frequency of 40 KHz for 15 minutes at a temperature of 400˚C. The filtrate obtained was filtered with Whatman no.1 filter paper and the powder dregs were air dried and re-extracted with ethyl acetate solvent with the same treatment, then the dregs were dried again and re-extracted with 96% ethanol solvent. Each filtrate was evaporated with a rotary evaporator at 40˚C until a thick extract was obtained.

Extract characteristics

The three thick extracts produced, namely n-hexan extract, ethyl acetate extract, and ethanol extract, were then characterized which included organoleptic tests (color, aroma and taste), water content test and ash content test. The water content test was carried out using the gravimetric method, namely by weighing a sample of 1g placed in a cup that has been tared. Then dried in an oven at 105ºC for 5 hours, cooled in a desiccator and weighed. The ash content test was carried out by weighing a sample of 1g and placing it in a previously weighed krus, then incinerated in a furnace at a temperature of 600˚C

Phytochemical screening of betel leaves

Dried leaves and betel leaf extracts obtained were tested for phytochemical screening including testing secondary metabolite compounds such as alkaloids, flavonoids, tannins, saponins, and triterpenoids or steroids according to the color change reaction method (Hanani, 2016).

Antimicrobial test preparation

Pure cultures of microbes were propagated in NA (Nutrient Agar) medium for S.mutans bacteria. Then incubated at 37˚C for 24 hours for bacteria. Then a suspension of S.mutans culture was prepared, by mixing with NaCl 0.9%, then equalizing the turbidity with the Mc farland 0.5 standard. Making a standard solution of Mc Farland 0.5 is as much as 0.05 mL of 1% Barium Clorida in distilled water added 9.95 mL of H_2SO_4 1% (Aviany et al., 2020).

Minimum inhibitory concentration testing

MIC testing of S. mutans using agar dilution method. MIC testing was carried out in a petri dish filled with 15 mL of NA medium mixed with each extract concentration (2.5%, 5%, 7.5%, and 10%) and a bacterial suspension of 0.2 mL and then streaked onto the media and incubated for 24 hours at 37˚C. Furthermore, observations were made of the test medium which remained clear and there were no fungal or bacterial colonies indicating the inhibition of microbial growth (Fitriana et al., 2020).

Resistance diameter testing

DDH testing used the Kirby-Bauer plate method with the aim of determining antibacterial activity by measuring the clear zone around the paper disk. Paper disks were soaked for 15 minutes in each betel leaf extract according to the concentration tested with reference to the MIC results. As a negative control for S.mutans, 1% tween was used. Furthermore, the culture was incubated in an incubator at 37ºC for 24 hours for S.mutans bacteria. The parameter measured was the DDH value in the form of a clear zone formed around the disk using a vernier caliper. The clear zone formed indicates the presence of antimicrobial power in the test solution against S.mutans bacteria (Azizah & Antarti, 2019).

Data analysis

The data obtained were analyzed using the SPSS 26 application with a completely randomized design (RAL). Data in this study in the form of numerical variables that have two factors were tested using Two Way ANOVA (Analysis Of Variance) with a confidence level of 95% ($\alpha < 0.05$). If the treatment given shows a significant difference in the results, it is continued with Duncan's further test to determine which treatment is most effective in having similarities with the control.

RESULTS AND DISCUSSION

Results of moisture content and ash content

Based on the table below, the percent moisture content of the dried leaves powder meets the quality requirements of ≤ 10 and the requirement for the total ash content of the extract is ≤ 10% (Rasydy et al., 2019). Ash content should have a small value because this parameter indicates the presence of heavy metal contamination that is resistant to high temperatures (Rasydy et al, 2019). Ash content testing relates to the internal and external mineral content derived from the

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initial process to become dried leaves or extract (Ndumuye et al., 2021). Can be seen in Table 1.

Based on this table of the five species of the family Piperaceae for *Piper retrofractum* Vahl, the water content that meets the requirements is only in the dried leaves powder, namely 4.51 ± 0.046 and for the ash content of the ethyl acetate extract has the lowest value, namely 8.3495 ± 0.069). 96% g that polar compounds make it harder to remove water during drying, leading to higher measured water content., *Peperomia pellucida* L.Kunth best water content is dried leaves with a value of 4.56 ± 0.130 and n-hexane ash content with a value of 5.19 ± 0.910, *Piper betle* L best water content of n-hexane solvent with a value of 5.832 ± 0.197 and ash content of dried leaves with a value of 4.7264 ± 0.122, *Piper aduncum* L best water content of dried leaves with a value of 5.32 ± 0.08 and 96% ethanol ash content with a value of 7.99 ± 0.08 and *Piper cf.* fragile Benth the best water content is dried leaves with a value of 2.83 ± 0.29 and ash content of dried leaves with a value of 4.00 ± 0.41. The ash content of the extract is stated to be standardized for the quality standard of viscous extract which is no more than 10.2%. The higher the percentage of ash content, the higher the ash content in the sample that is not needed. Of the five species of the family Piperaceae that have the best moisture and ash content are *Piper cf.* fragile Benth.

Phytochemical screening

Phytochemical screening was carried out qualitatively to determine the presence of secondary compounds of alkaloid, flavonoid, saponin, steroid/terpenoid, and tannin groups in dried leaves and extracts from the leaves of five Piperaceae family species. The results of compound identification in dried leaves and thick extracts of leaves of five species of the Piperaceae family can be seen in Table 2.

Table 1. Results of moisture content and ash content of leaves of five species of the family Piperaceae

Sample Average	Water Content (%) ± SD	Average Ash Content (%) ± SD
Piper retrofractum Vahl.		
SimpleciaPowder	4.51 ± 0.046	13.5638 ± 0.137
n-Hexane Extract	22.7931 ± 0.139	9.7617 ± 0.112
Ethyl Acetate Extract	23.6804 ± 0.177	8.3495 ± 0.069
96% ethanol extract	24.7554 ± 0.170	9.2445 ± 0.035
Peperomia pellucida L.Kunth		
SimpleciaPowder	4.56 ± 0.130	8.73 ± 0.355
n-Hexane Extract	6.71± 0.195	5.19 ± 0.910
Ethyl Acetate Extract	6.07 ± 0.120	8.09 ± 0.510
96% ethanol extract	6.46 ± 0.045	5.56 ± 0.445
Piper betle L.		
SimpleciaPowder	7.0922 ± 0.138	4.7264 ± 0.122
n-Hexane Extract	5.8325 ± 0.197	8.0348 ± 0.022
Ethyl Acetate Extract	6.2304 ± 0.073	8.0806 ± 0.081
96% ethanol extract	6.7207 ± 0.113	7.8335 ± 0.151
Piper aduncum L.		
SimpleciaPowder	5.32 ± 0.08	9.15 ± 0.12
n-Hexane Extract	7.31 ± 0.20	9.10 ± 0.07
Ethyl Acetate Extract	6.48 ± 0.11	9.02 ± 0.09
96% ethanol extract	6.08 ± 0.07	7.99 ± 0.08
Piper cf. fragile Benth		
SimpleciaPowder	2.83 ± 0.29	4.00 ± 0.41
n-Hexane Extract	3.91 ± 0.06	6.43 ± 0.42
Ethyl Acetate Extract	3.53 ± 0.01	7.31 ± 0.29
96% ethanol extract	5.00 ± 0.36	8.27 ± 0.49

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Table 2. Phytochemical screening results of dried leaves and leaf extracts of five species of the family Piperaceae

Phytochemical Test	Simplecia	N-Hexane Extract	Ethyl Acetate Extract	96% Ethanol Extract
Piper retrofractum Vahl.				
Alkaloids	+	-	-	+
Flavonoids	+	-	+	+
Terpenoids	+	+	-	-
Saponins	+	-	-	+
Tannin	+	-	+	+
Peperomia pellucida L.Kunth				
Alkaloids	+	-	+	+
Flavonoids	+	-	-	+
Terpenoids	-	+	-	-
Saponins	+	+	+	+
Tannin	+	-	+	+
Piper betle L.				
Alkaloids	+	-	-	+
Flavonoids	+	-	-	+
Terpenoids	+	-	-	+
Saponins	+	+	-	+
Tannin	+	-	+	+
Piper aduncum L.				
Alkaloids	+	+	+	+
Flavonoids	+	-	-	+
Terpenoids	+	+	+	-
Saponins	+	+	+	+
Tannin	+	-	+	+
Piper cf. fragile Benth				
Alkaloids	+	+	+	+
Flavonoids	+	-	-	+
Terpenoids	+	+	-	+
Saponins	+	-	+	+
Tannin	+	-	+	+

Information: + (Contains the chemical compounds tested), - (Does not contain the chemical compounds tested)

Table 3. Minimum inhibitory concentration test results (%)

Species	Extract		
	N-Hexane	Ethyl Acetate	Ethanol 96%
Piper retrofractum Vahl.	10	10	20
Peperomia pellucida L.Kunth	25	20	20
Piper betle L.	15	15	15
Piper aduncum L.	5	10	10
Piper cf. fragile Benth	5	5	10

The difference in secondary metabolites in the screening results of the three leaf extracts of five species of the Piperaceae family is due to differences in the polarity of each solvent that can attract these compounds. The withdrawal of metabolite compounds is based on the principle of like dissolve like, namely polar solvents will dissolve polar compounds, and vice versa for non-polar solvents (Damayanti & Ervilita, 2019). Alkaloid compounds flavonoids, saponins, and tannins can generally be attracted by polar solvents such as ethanol and methanol and terpenoid compounds, steroids are non-polar compounds that can generally be attracted to non-polar solvents such as n-hexane and chloroform, while ethyl acetate is a semi-polar compound that may attract polar or non-polar compounds (Dewatisari, 2020).

Minimum inhibitory concentration test results (%) of leaves of five species of the Piperaceae family

MIC testing aims to see the susceptibility or resistance of a particular microbe to antimicrobial agents carried out in vitro (Kowalska-Krochmal & Dudek-Wicher, 2021). The solvent used for extract dilution is 1% tween 80 due to its properties as a surfactant and can reduce surface tension so that it can dissolve the three extracts. The results of MIC testing can be seen in Table 3.

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Based on the results above, MIC testing is carried out with different ranges and produces the best MIC value, namely in forest betel and red betel. Forest betel with a concentration of 5% n-hexane solvent has shown inhibition characterized by no growth of bacterial colonies and the media looks clear and red betel with a concentration of 5% in n-hexane and ethyl acetate solvents. There is no literature testing the MIC of red betel leaf extract against *S.mutans* bacteria, but red betel ethanol extract obtained from maceration with liquid dilution method has a MIC of 25% against *Staphylococcus aureus* bacteria (Gram positive), and 6.25% against *Escherichia coli* (Gram negative) (Rachmawaty et al., 2009).

Based on the test results, it can be seen that this is also associated with the nature of the solubility of compounds that can be extracted in each extract, the difference in solvent polarity can affect the concentration of soluble active compounds. This minimum inhibitory concentration test is used as a reference for the initial concentration in the inhibition width test. In Fahdi (2018) Gram positive bacteria are more sensitive to antibacterials than Gram negative bacteria. This is due to the cell wall of Gram-positive bacteria which is only composed of several layers of peptodoglycan where the cell is easily denatured by compounds contained in green betel leaf extract, one of which is bethel phenol. While Gram-negative bacteria have three wrapping polymers that are found outside the peptodoglycan layer such as outer membranes, lipoproteins and lipopolysaccharides.

Diameter resistance testing (DDH)

Inhibition diameter testing antimicrobial activity was carried out using the inhibition diameter test method or disc diffusion method with NA media for *S. mutans* bacteria. From the test results, the diameter of the inhibitory power indicated by the clear zone around the disk was measured using a vertical and horizontal push rod with mm units. The results of antimicrobial activity against *S. mutans* can be seen in Table 4.

Table 4. Antibacterial activity of leaf extracts of five species of family Piperaceae with different solvent types against *Streptococcus mutans* bacteria

Species	Extract Sample	Concentration (%)	Average DDH (mm)	Category
Piper retrofractum Vahl.	N-Hexane	2.5	$6.67^{b} \pm 0.09$	*Resistant*
		5	$8.19^{c} \pm 0.13$	*Resistant*
		10	$11.23^{d} \pm 0.03$	*Resistant*
		20	$17.37^{f} \pm 0.43$	*Intermediate*
		K+	$25.42^{g} \pm 0.95$	*Susceptible*
		K-	$0.00^{a} \pm 0.00$	*Doesn't hinder*
	Ethyl Acetate	2.5	$7.93^{c} \pm 0.40$	*Resistant*
		5	$8.33^{c} \pm 0.08$	*Resistant*
		10	$11.57^{d} \pm 0.20$	*Resistant*
		20	$15.37^{e} \pm 0.77$	*Intermediate*
		K+	$25.08^{g} \pm 0.52$	*Susceptible*
		K-	$0.00^{a} \pm 0.00$	*Doesn't hinder*
	Ethanol 96%	2.5	$6.75^{b} \pm 0.12$	*Resistant*
		5	$7.58^{c} \pm 0.24$	*Resistant*
		10	$11.10^{d} \pm 0.26$	*Resistant*
		20	$14.77^{e} \pm 0.45$	*Resistants*
		K+	$25.42^{g} \pm 0.79$	*Susceptible*
		K-	$0.00^{a} \pm 0.00$	*Doesn't hinder*
Peperomia pellucida L.Kunth	N-Hexane	25	$9.67^{c} \pm 0.17$	*Resistant*
		35	$12.75^{e} \pm 0.76$	*Resistant*
		45	$15.23^{g} \pm 0.19$	*Intermediate*
		K+	$34.33^{i} \pm 0.62$	*Susceptible*
		K-	$0^{a} \pm 0.000$	*Doesn't hinder*
	Ethyl Acetate	20	$14.23^{f} \pm 0.28$	*Resistant*
		30	$15.55^{g} \pm 0.18$	*Intermediate*
		40	$17.93^{h} \pm 0.23$	*Intermediate*
		K+	$35.33^{j} \pm 0.23$	*Susceptible*
		K-	$0^{a} \pm 0.000$	*Doesn't hinder*
		20	$7.80^{b} \pm 0.20$	*Resistant*
		30	$9.43^{c} \pm 0.12$	*Resistant*
		40	$11.06^{d} \pm 0.41$	*Resistant*

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	Ethanol 96%	K+	$34.67^{j} \pm 0.84$	*Susceptible*
		K-	$0^{a} \pm 0.000$	*Doesn't hinder*
Piper betle L.	N-Hexane	15	$18.13^{c} \pm 0.16$	*Intermediate*
		20	$19.30^{e} \pm 0.02$	*Susceptible*
		25	$23.03^{g} \pm 0.21$	*Susceptible*
		K+	$25.16^{i} \pm 0.31$	*Susceptible*
		K-	$0.00^{a} \pm 0.00$	*Doesn't hinder*
	Ethyl Acetate	15	$18.28^{d} \pm 0.14$	*Intermediate*
		20	$19.85^{f} \pm 0.12$	*Susceptible*
		25	$23.26^{h} \pm 0.21$	*Susceptible*
		K+	$25.42^{i} \pm 0.23$	*Susceptible*
		K-	0.00 ± 0.00	*Doesn't hinder*
	Ethanol 96%	15	$17.08^{b} \pm 0.38$	*Intermediate*
		20	$18.58^{d} \pm 0.10$	*Susceptible*
		25	$22.70^{g} \pm 0.35$	*Susceptible*
		K+	$25.08^{i} \pm 0.07$	*Susceptible*
		K-	$0.00^{a} \pm 0.00$	*Doesn't hinder*
Piper aduncum L.	N-Hexane	5	$3.08^{b} \pm 0.24$	*Resistant*
		15	$4.77^{d} \pm 0.21$	*Resistant*
		25	$7.69^{h} \pm 0.47$	*Resistant*
		K (+)	$11.01^{i} \pm 0.15$	*Resistant*
		K (-)	$0^{a} \pm 0.000$	*Doesn't hinder*
	Ethyl Acetate	10	$3.73^{c} \pm 0.06$	*Resistant*
		20	$5.04^{e} \pm 0.10$	*Resistant*
		30	$6.78^{g} \pm 0.17$	*Resistant*
		K (+)	$11.45^{i} \pm 0.05$	*Resistant*
		K (-)	$0^{a} \pm 0.000$	*Doesn't hinder*
	Ethanol 96%	10	$4.44^{d} \pm 0.08$	*Resistant*
		20	$5.17^{f} \pm 0.07$	*Resistant*
		30	$7.78^{h} \pm 0.05$	*Resistant*
		K (+)	$11.20^{i} \pm 0.05$	*Resistant*
		K (-)	$0^{a} \pm 0.000$	*Doesn't hinder*
Piper cf. fragile Benth	N-Hexane	5	$12.62^{c} \pm 0.13$	*Resistant*
		15	$14.57^{f} \pm 0.23$	*Resistant*
		25	$17.43^{h} \pm 0.20$	*Intermediate*
		K+	$19.19^{i} \pm 0.05$	*Intermediate*
		K- tween 1%	$0.00^{a} \pm 0.00$	-
	Ethyl Acetate	5	$12.10^{b} \pm 0.23$	*Resistant*
		15	$13.99^{e} \pm 0.40$	*Resistant*
		25	$16.09^{g} \pm 0.29$	*Intermediate*
		K+	$19.22^{i} \pm 0.18$	*Intermediate*
		K-	$0.00^{a} \pm 0.00$	-
	Ethanol 96%	10	$12.03^{b} \pm 0.09$	*Resistant*
		15	$12.99^{d} \pm 0.09$	*Resistant*
		25	$15.83^{g} \pm 0.05$	*Intermediate*
		K+	$19.07^{i} \pm 0.10$	*Intermediate*
		K-	$0.00^{a} \pm 0.00$	-

Note: Numbers followed by the same superscript letter in the same column or row indicate that they are not significantly different. Antimicrobial activity according to CLSI, (2013): inhibitory area >20 mm Susceptible, 15-19 mm Intermediate, < 14mm Resistant

Family Piperaceae especially 5 species that have been tested phytochemical screening and antibacterial S.mutans provide diverse results, namely for Java chili the results of DDH against S. mutans including the category of near Susceptible (Intermedate) is a concentration of 20% in each solvent with an average DDH of 15.84 mm, Chinese betel the results of DDH testing, Chinese betel ethyl acetate extract at a concentration of 40% with an average DDH of 17.93 mm is most effective as an antibacterial S.mutans, green betel the most effective solvent of the three solvents used to inhibit the growth of Streptococcus mutans is

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Ethyl acetate 25% with DDH 23.26 mm included in the Susceptible category. Forest betel shows that from n-hexane, ethyl acetate and 96% ethanol extracts, the most effective as an antibacterial in inhibiting S. mutans bacteria has an inhibition of 7.78 mm at a concentration of 30%, namely 96% ethanol extract and red betel.

The results of the study can be concluded that red betel leaf extract has the best antibacterial activity of S. mutans is n-hexane extract at a concentration of 25% with DDH 17.43 mm. Of the five betels, it can be seen that the one with the best antimicrobial potential is green betel with 25% ethyl acetate solvent with a DDH of 23.26 mm against Streptococcus mutans, including the Susceptible category. The results obtained state that the higher the concentration given, the wider the clear zone produced to inhibit the growth of bacteria Sterptococcus mutans can also be said that the levels of secondary metabolites that function as antimicrobials are getting bigger. The inhibition formed in the three types of extracts with each concentration is due to the presence of active compounds in the form of secondary metabolite compounds contained in green betel leaf extract such as alkaloids, flavonoids, tannins, saponins and terpenoids that can inhibit bacterial growth which makes it a natural antibacterial and antifungal agent. The mechanism of action of flavonoids as antimicrobials can be divided into 3, namely first inhibiting nucleic acid synthesis, second inhibiting cell membrane function by damaging the permeability of the bacterial cell wall followed by the release of intracellular compounds and third inhibiting energy metabolism (Hendra et.al, 2011).

This is in accordance with the opinion of Rahmawati (2014) that the greater the concentration of the extract, the greater the diameter of the inhibition formed. And that the higher the concentration of the extract, the better the content of antibacterial ingredients, so that it can inhibit the growth of microbes. (Suciati et al., 2012). The difference in results in the study is caused by several factors, in addition to the extraction method factor, several things can affect the results of this study such as the quality of red betel leaf dried leaves, the storage process and several other factors that can affect the quality of the quality of the extract, especially the red betel leaves used in the study are different places of growth which affect the quality and content of a plant's secondary metabolite compounds due to different environmental factors.

The results of testing the antimicrobial effectiveness of leaf extracts of five species of the Piperaceae family against the growth of S. mutans bacteria are indicated by the presence of a clear area surrounding the disk called the inhibition zone. The results of the inhibition diameter test can be seen in Figure 1.

Figure 1. Antibacterial activity of Java chili leaf extract with different solvent types against *Streptococcus mutans* bacteria

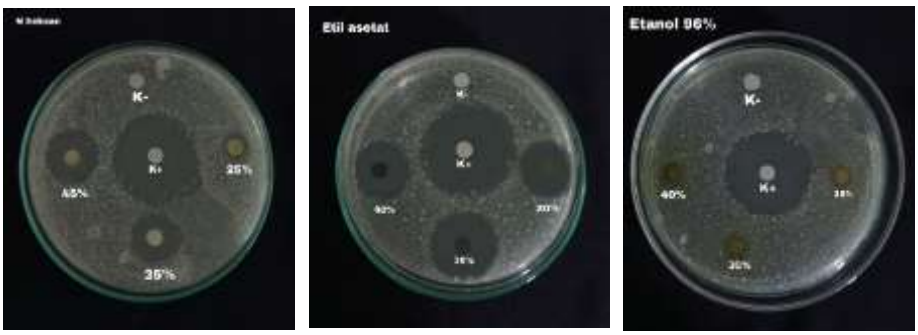

Figure 2. Antibacterial activity of Chinese betel herb extract with different types of solvents against *Streptococcus mutans* bacteria

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Figure 3. Antibacterial activity of green betel leaf extract with different types of solvents against *Streptococcus mutans* bacteria

Figure 4. Antibacterial activity of forest betel leaf extract with different types of solvents against *Streptococcus mutans* bacteria

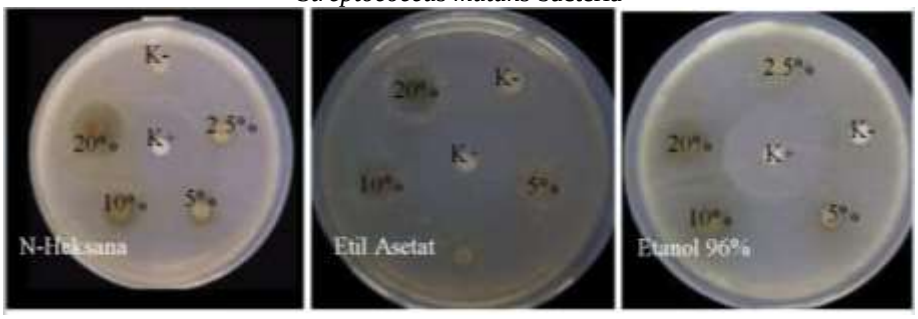
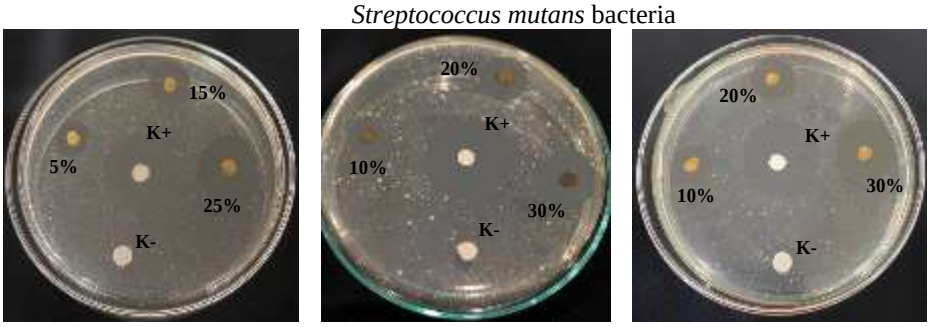


Figure 5. Antibacterial activity of red betel leaf extract with different types of solvents against *Streptococcus mutans* bacteria



a). n-Heksana b). Etil Asetat c). Etanol 96%

CONCLUSION

Of the five Betel species selected from the family piperaceae which has the best antimicrobial potential of *S.mutans* is green betel with 25% ethyl acetate solvent with DDH 23.26 mm ± 0.21 against *Streptococcus mutans* included in the Susceptible category.

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AUTHOR CONTRIBUTIONS

Conceptualization, P.D., S.M., F.D.S., I.K., Y.; Methodology, P.D., S.M., F.D.S., I.K., Y.; Software, A.A.P., J.N.A., N.S., I.M., Z., M.H.; Validation, P.D., S.M., F.D.S., I.K., Y.; Formal Analysis, A.A.P., J.N.A., N.S., I.M., Z., M.H.; Investigation, A.A.P., J.N.A., N.S., I.M., Z., M.H.; Resources, P.D., S.M., F.D.S., I.K., Y.; Data Curation, A.A.P., J.N.A., N.S., I.M., Z., M.H.; Writing - Original Draft, A.A.P.; Writing - Review & Editing, P.D.; Visualization, A.A.P., J.N.A., N.S., I.M., Z., M.H.; Supervision, P.D., S.M., F.D.S., I.K., Y.; Project Administration, P.D., S.M., F.D.S., I.K., Y.; Funding Acquisition, P.D.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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Optimization and Stability Assessment of Clindamycin HCl Transethosome: Exploring the Effects of Ethanol and Tween 80 Concentrations

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Abstract

Background*: Clindamycin HCl is drug commonly used as an anti-acne in conventional topical formulations. However, effectiveness of clindamycin HCl in conventional topical formulations is limited due to poor skin penetration, whereas Propionibacterium acnes colonies in the deeper sebaceous follicle area. To overcome this limitation, transethosome emerged as an innovative drug delivery system capable of enhancing drug permeation through the skin.* ***Objective:*** *This study aimed to optimise clindamycin HCl transethosome formula using a 2^2-factorial design.* ***Methods:*** *The optimisation was carried out with two factors and two levels, ethanol (20% and 40%) and Tween 80 (15% and 25%), on the responses of particle size, polydispersity index, and entrapment efficiency. Transethosomes were prepared using the thin-layer hydration method. Furthermore, the optimum transethosomes were tested for stability using the ICH Q1A(R2) method.* ***Results:*** *The optimum formula contains 20% ethanol and 15% Tween 80. The optimum transethosome shows a particle size of 240.933 ± 1.488 nm, a polydispersity index (PDI) of 0.177 ± 0.013, and an entrapment efficiency (EE) of 89.401 ± 0.118%. The release model follows zero-order kinetics with an activation energy of 2.978758 cal/mol. The shelf life at 25°C ± 2°C / RH 60% ± 5% is 22.536 days, and at 5°C ± 3°C is 24.572 days.* ***Conclusion:*** *The optimum transethosomal formula of clindamycin HCl exhibited good initial physical characteristics, with particle size below 250 nm, polydispersity index (PDI) of less than 0.3, and high entrapment efficiency (EE). However, the low shelf life indicated a need for further optimisation to achieve long-term stability.*

Keywords: *clindamycin HCl, optimization, stability, transethosomes*

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INTRODUCTION

Clindamycin is a topical antibiotic with bacteriostatic and anti-inflammatory properties, commonly used to treat acne (Dawson & Dellavalle, 2013). However, clindamycin in conventional topical formulations faces challenges in achieving adequate skin penetration, with studies reporting only about 5-8% of the active ingredient being absorbed into the deeper skin layers (Abdellatif & Tawfeek, 2016). Additionally, clindamycin faces challenges in penetrating the skin's stratum corneum. In contrast, *Propionibacterium acnes*, the bacterium responsible for acne, resides in the sebaceous glands located in the dermis, necessitating deeper skin penetration to reach the target cells (Dréno, 2017; Mollerup et al., 2016). Therefore, developing an effective drug delivery system, such as transethosomes, is essential.

Transethosomes have been shown to exhibit enhanced vesicle elasticity, which contributes to their superior deformability and, consequently, improved skin permeation, as demonstrated by Garg et al. (2017), in comparison to other nanovesicles. This improvement is attributed to the combination of ethanol and surfactants, which cause structural rearrangements in the lipid bilayer of the vesicles (Ascenso et al., 2015; Kumar et al., 2019). Moreover, transethosomes offer better stability than ethosomes, as evidenced by Esposito et al. (2022), who found that the higher lipid and surfactant content in transethosome formulations contributes to increase steric stability.

The selection of appropriate surfactants and ethanol concentrations is critical in formulating transethosomes. Factorial design is a valuable approach for optimizing all influencing parameters simultaneously, allowing for assessing each factor's effect and interactions. This design reduces the number of trials, time, and costs while providing reliable results. Compared to one-factor-at-a-time experiments, factorial design is more efficient and can detect interactions, making it ideal for this study (Elhalil et al., 2016; Watkins & Newbold, 2020).

The surfactant used in this study was Tween 80. According to Zeb et al. (2016), Tween 80 demonstrates superior deformability and skin permeation compared to sodium cholate. Additionally, El-Laithy et al. (2011) found that Tween 80 offers better entrapment of active ingredients than Span 80, likely due to its longer carbon chain. Ethanol is a crucial excipient optimised in transethosome formulations due to its primary role as a skin penetration enhancer. It works by fluidising the intercellular lipids of the stratum corneum, the outermost layer of the skin, thereby creating temporary pathways that facilitate drug penetration. However, optimising ethanol concentration is critically essential; an inappropriate concentration can compromise the integrity of the transethosome vesicles, leading to drug leakage and reduced entrapment efficiency (Abdulbaqi et al., 2016; Li et al., 2012; Rakesh & Anoop, 2012). Therefore, while Apriani et al. (2023) successfully optimised the ethanol concentration to 40% for clindamycin HCl ethosome systems, achieving high entrapment efficiency, a particle size of less than 200 nm, and a polydispersity index of less than 0.4, specific research on the optimal ethanol concentration for clindamycin HCl transethosomes is yet to be established.

Stability testing is crucial for confirming the quality of pharmaceutical products over time, as environmental conditions like humidity, temperature, and light affect their stability. This testing helps determine the retest period, shelf life of drugs, and storage recommendations (ICH, 2023). The ICH Q1A(R2) guidelines, developed by the FDA, align storage conditions with climatic zones, categorizing regions into four zones. Indonesia falls into Zone IVB, characterized by hot and humid conditions, necessitating accelerated testing. The scientific basis for modelling accelerated test data is the Arrhenius kinetic equation, which predicts the degradation of active ingredients influenced by temperature and humidity. This model analyzes the impact of humidity, time, and temperature on degradation rates, yielding more precise predictions than traditional accelerated testing methods, which assume a linear correlation between temperature and degradation. The reaction kinetics offer a more precise estimation of degradation rates, and the stability of a product can be predicted using kinetic constants, where lower constants correlate with longer stability (Clancy et al., 2017).

Based on the description above, transethosome system was optimized using Tween 80 as the surfactant and 96% ethanol as the penetration enhancer. The concentration of Tween 80, initially set at 20% in previous studies, was modified to 15% and 25% of the total lipid content. Ethanol concentrations of 20% and 40% were selected based on prior research. This combination results in four transethosome formulations using the 2^2 factorial design model. The formulations were evaluated based on particle size, polydispersity index, and entrapment efficiency with the data processed using Design Expert 12® software to identify the optimum formula. The optimum formula was then

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subjected to accelerated stability testing over two months, with the results analyzed using SPSS software.

MATERIALS AND METHODS

Materials

Clindamycin HCl (gift from PT. Dexa Medica, Palembang, Indonesia), Phospholipon 90G (Lipoid®, Ludwigshafen, Germany), methanol (Merck®, Indonesia), dichloromethane (Merck®, Indonesia), Tween 80 (Merck®, Indonesia), KH2PO4 (Merck®, Indonesia), NaOH (Merck®, Indonesia), distilled water (Bratachem, Indonesia), 96% ethanol (Merck®, Indonesia), and propylene glycol (DOW®, Australia) were the materials used for this study.

Method

Tools

The tools used in this study included an analytical balance (NewTech Electronic Balance®), Rotary Evaporator (Dragon Lab®), Magnetic Stirrer (IKA® C-MAG HS 4), Oven (Memmert®), Refrigerator (Sanyo®), micropipette 100-1000 µl (Dragon Lab®), UV-Vis Spectrophotometer (Biobase® BK-UV1900PC), Particle Size Analyzer (Malvern® Zetasizer), pH meter (Lutron® pH Electrode PE-03), Sonicator Bath (GT SONIC®), Ultra-Turrax (Ika® T-25 Digital Ultra Turrax), Centrifuge (DLAB©: D2012 PLUS), Climatic Chamber 25°C/60% RH (Thermolab® Scientific Equipment), Walk-In Climatic Chamber 30°C/75% RH (Thermolab® Scientific Equipment), Climatic Chamber 40°C/75% RH (Maximus®), and glassware (Pyrex®).

Optimization of the clindamycin HCl transethosomal formula

This study made a transethosomes formulation containing clindamycin HCl using a 2^2-factorial design with variations concentrations in ethanol and tween 80 (Apriani et al., 2023). The formulation design of clindamycin HCl transethosomes can be seen in Table 1.

Production of clindamycin HCl transethosomes

Clindamycin HCl transethosomes were made following the research of Apriani et al. (2019) with slight modifications. The rotary evaporator was operated in a vacuum at 54°C with an initial rotation speed of 50 rpm and a maximum rotational speed of 125 rpm to produce thin films from a mixture of Phospholipon 90G, tween 80, and organic solvents dichloromethane and methanol. The round bottom flask was sealed with aluminum foil and cooled for 24 hours after the thin layer was formed. The hydration solution was prepared from a mixture of clindamycin HCl, propylene glycol, ethanol, and phosphate buffer pH 7.4. A clindamycin HCl transethosomes suspension was generated by hydrating the samples in a rotary evaporator at 37°C without a vacuum. The obtained clindamycin HCl transethosomes suspension was then reduced in particle size using ultra turrax for 15 minutes at a speed of 7600 rpm. Subsequently, the suspension was subjected to sonication for three cycles, each running for 10 minutes.

Transetosome characterization

Percentage of entrapment efficiency

The entrapment efficiency test (%EE) was conducted at 15000 rpm, 4°C for 30 mimutes using the indirect method. The suspension of transethosomes will be divided into two phases: entrapped and non-entrapped. The absorbance value was subsequently determined through using a UV-Vis spectrophotometer at 204 nm to measure the non-entrapped phase (Apriani et al., 2023). Free clindamycin HCl levels were calculated based on the previously obtained regression equation. Equation 1 is employed to determine the entrapment efficiency (%EE).

$$\%EE = \left[\frac{(Qt-Qs)}{Qt}\right] x100\% \quad \text{..... (Equation 1)}$$

Note:

%EE : Entrappment Efficiency (%)

Qt : Total clindamycin HCl concentration in transethosomes suspension (µg/mL)

Qs : Clindamycin HCl concentrations that are not entrapped in the transethosomes suspension (µg/mL).

Table 1. Clindamycin HCl transethosomes formulations

Ingredient	Concentration (%)			
	F1	F2	F3	F4
Clindamycin HCl	1	1	1	1
Phospolipon 90G	2	2	2	2
Propylene Glycol	5	5	5	5
Ethanol	20	40	40	20
Tween 80	25	25	15	15
Phosphate Buffer pH 7,4	up to 100 mL	up to 100 mL	up to 100 mL	up to 100 mL

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Particle size and polydispersity index

The Dynamic Light Scattering (DLS) method was employed to determine the polydispersity index and particle size using a Particle Size Analyzer (PSA). The sample was dissolved in 10 ml of phosphate buffer pH 7.4 and then was transferred to a cuvette for measurement (Apriani et al., 2022; Mardiyanto et al., 2023).

Optimum formula determination

The results of the characterization are used to determine the optimum transethosome formula. The Design-Expert 12® software was employed to identify the most optimized formula, which had a desirability value near one. The optimum formula was determined by the following criteria: a polydispersity index value of less than 0.3, a particle size within the 80-250 nm range, and the maximum percentage of drug entrapment efficiency within the formula (Ferrara et al., 2022; Raj et al., 2023).

Stability study

Stability testing followed the guidelines written by ICH (2003) with the accelerated test method. Physical stability testing was conducted using a climatic chamber with three storage conditions. The storage conditions used were 40ºC±2ºC/75% RH±5%, 30ºC±2ºC/75% RH±5% and 25ºC±2ºC/60% RH±5%. Organoleptic (discolouration and phase separation), entrapment efficiency, and pH were conducted on the tested samples on days 0, 22, and 44. Then, the shelf life is calculated using the formula according to the release kinetics model.

Data on the decrease in entrapment efficiency obtained from the ICH Q1A(R2) stability test are used to calculate the kinetic constant by examining the coefficient of determination (R2) in close proximity to one. The summary of the release kinetics model formula can be seen in Table 2.

Table 2. Release kinetics equation

Release Kinetic	Equation
Zero-Order	$C = K_o.t$
First-Order	$\ln C = K_1.t$
Higuchi	$C = K_h.\ t^{1/2}$
Korsmeyer's Peppas	Ln C = n. ln t + ln K

Note:
C = The concentration of drug released at time t
K_0, K_1, K_h, K = Drug release constant
N = Drug diffusion exponent

The Arrhenius equation is acquired by plotting the Arrhenius 1/T vs Log K graph in accordance with the release kinetics that were obtained. The K and Ea values, which indicate the decomposition rate of the vesicles and the minimum energy required for the reaction to occur, are determined using these equations. The K value is obtained by substituting the Log K value from the equation Log K = Log A - (Ea/(2.303 R))x 1/T according to the temperature of the storage conditions. The K value is subsequently employed to determine the shelf life (Dlamini et al., 2019; Lu & Ten Hagen, 2020).

Data analysis

Design-Expert 12® program was employed to conduct data analysis in order to determine the impact of ethanol concentration and Tween 80, as well as their interaction, on the characteristics of the transethosome suspension which resulted. The parameters used to determine the optimum formula are R2, predicted R2, adjusted R2, adequate precision, and p-value. SPSS 25 software is used to process transethosomes stability data that has been tested.

RESULTS AND DISCUSSION

The thin-film hydration method was used to produce clindamycin HCl transethosomes. The obtained transethosome suspension, shown in Figure 1, was milky white with a distinct ethanolic odor consistent across all formulations. This suspension was stored it in the cold temperature (2-8ºC), before further characterization.

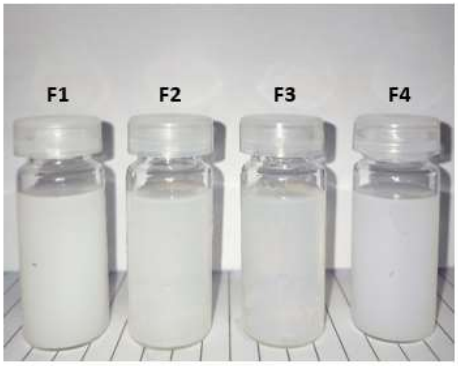

Figure 1. Clindamycin HCl transethosomal suspension at Day-0

Characterization of the Clindamycin HCl transethosomes involved parameters such as entrapment efficiency, particle size, and polydispersity index (PDIs), which are critical for identifying the optimum formula. The transethosomes are considered optimal if they exhibit high entrapment efficiency, particle sizes between 80-250 nm, and a PDIs below 0.3 (Ferrara et al., 2022). Table 3 summarizes the characterization results, which indicate that all formulations met the criteria for satisfactory particle sizes (203-265 nm), PDIs (0.15-0.23), entrapment efficiencies (81-89%).

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Table 3. Characterization results of clindamycin HCl transetosomes

Formula	Entrapment Efficiency (%)	Particle Size (nm)	Polydispersity Index
F1	87.736±0.140	265.467±2.088	0.233±0.003
F2	81.675±0.438	203.067±1.312	0.152±0.010
F3	82.871±0.139	209.800±1.157	0.160±0.039
F4	89.401±0.118	240.933±1.488	0.177±0.013

Note: Data are given as mean±SD, n=3

Table 4. Model analysis result

Parameter	*p-value*	Adjusted R^2	Predicted R^2	The difference between Adjusted R2 and Predicted R2	Adequate Precision
Entrapment Efficiency	<0.0001*	0.9920	0.9869	0.0051	44.1205
Particle Size	<0.0001*	0.9947	0.9914	0.0033	56.8448
Polydispersity Index	0.0232*	0.5551	0.2719	0.2832	5.2560

Note: * showed significant results (p<0.05)

Table 5. Responses analysis result

Parameter		Intercept	A (Tween 80)	B (Ethanol)	AB (Interaction)
Entrapment Efficiency	Coefficient	85.42	-0.7152	-3.15	0.1172
	P-Value		<0.05*	<0.05*	0.217
	%Contribution		4.87408	94.4107	0.130841
Particle Size	Coefficient	229.82	4.45	-23.38	-7.82
	P-Value		<0.0001*	<0.0001*	<0.0001*
	%Contribution		3.14279	86.7777	9.69702

Note: * showed significant results (p<0.05)

To determine the optimum formulation, Design-Expert 12® software was used, considering model and response analysis. The model is considered satisfactory if it fulfills the following criteria: a p-value of less than 0.05, an adjusted R^2 in excess of 0.7, a difference between the adjusted and predicted R^2 of less than 0.2, and a precision of at least 4 (Miksusanti et al., 2023). The model analysis results of the three parameters can be seen in Table 4.

The entrapment efficiency and particle size parameters satisfy the criteria for a satisfactory model, as indicated in Table 4. Nevertheless, the polydispersity index fails to satisfy these criteria, as its adjusted R^2 value and the difference between the adjusted R^2 and predicted R^2. The adjusted R^2 is used because the model includes multiple independent variables, namely ethanol and Tween 80. This value reflects the proportion of variability in the response variable explained by the model, representing the sample population. The model explains 99.20% to 99.47% of the population variance for entrapment efficiency and particle size, respectively, as indicated by the adjusted R^2 values of 0.9920 and 0.9947. This data suggests a robust linear relationship between the dependent variables and the independent variables.

The predicted R^2 parameter reflects the ideal model fit and indicates how closely the regression obtained from the experimental responses matches the regression predicted by the system. The predicted R^2 values for entrapment efficiency and particle size are 0.9869 and 0.9914, respectively, meaning that the observed regression closely aligns with the expected regression by 98.69% and 99.14%. The modeling error is represented by the difference between the adjusted R^2 and predicted R^2. The differences between entrapment efficiency and particle size are 0.0051 and 0.0033, respectively, suggesting a small modeling error. Additionally, the model's robustness and resistance to disturbance are suggested by a precision value that exceeds 4. The dependent variables—entrapment efficiency and particle size—are substantially influenced by the independent variables, as indicated by these results. Consequently, these two parameters are eligible for further optimization process analysis.

Response analysis was conducted to evaluate the effects of the ethanol factor, Tween 80, and their interaction on the entrapment efficiency and particle size parameters. The coefficient, p-value, and percentage contribution of each factor can be used to evaluate the response analysis results. The detailed response analysis results are presented in Table 5 and Figure 2.

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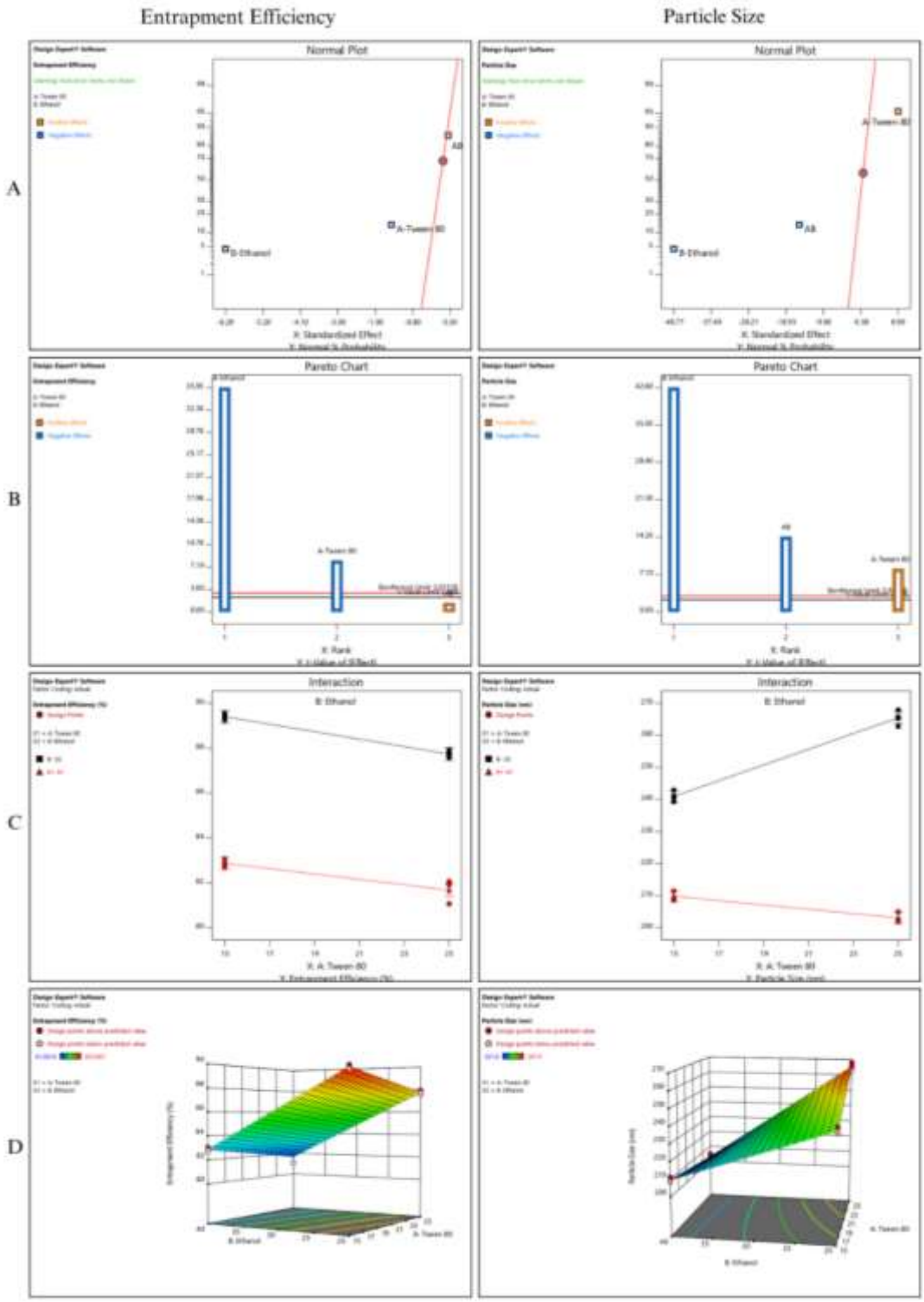

Figure 2. Normal plot (A), pareto chart (B), interaction (C) and 3d surface (D) graphs of entrapment efficiency and particle size parameters

The response analysis, detailed in Table 5 and Figure 2, showed that both ethanol and Tween 80 significantly affected entrapment efficiency and particle size, with their interaction notably influencing particle size ($p<0.05$). Figures 2A and 2B support these findings, showing that the interaction between Tween 80 and ethanol did not significantly affect entrapment efficiency but significantly impacted particle size. The Pareto chart further emphasized that ethanol had the highest contribution, with 94.41% and 86.78% for entrapment efficiency and particle size, respectively.

The interaction between Tween 80 and ethanol is shown in Figure 2C. For entrapment efficiency, the interaction curves do not intersect, indicating no

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interaction between the factors, which is supported by the p-value for interaction between Tween 80 and ethanol on entrapment efficiency, showing no significant effect. In contrast, for particle size, the curves intersect, indicating an interaction between Tween 80 and ethanol, as confirmed by the significant p-value for interaction between Tween 80 and ethanol on particle size. The black line represents low ethanol concentration, while the red line represents high ethanol concentration. At low ethanol concentrations, increasing Tween 80 concentration decreases entrapment efficiency and increases particle size. These results are additionally confirmed by the 3D surface graph in Figure 2D, which shows that the red area represents the highest entrapment efficiency and the blue area represents the smallest particle size.

The influence of factors on entrapment efficiency and particle size can be both positive and negative, as indicated by the coefficients in Table 5. The entrapment efficiency was negatively impacted by ethanol and Tween 80, as indicated by a negative coefficient (-). Specifically, increasing the concentration of ethanol led to a decrease in entrapment efficiency. The highest entrapment efficiency was observed in F4, with concentrations of 96% ethanol and Tween 80 at 20% and 15%, respectively. This result aligns with studies by Bendas & Tadros (2007), and Zhou et al. (2010), which reported that while ethanol concentration generally increases entrapment efficiency up to a certain point, excessive ethanol can cause vesicle instability, leading to decreased entrapment due to leakage of the bilayer coating.

Additionally, lower surfactant concentrations in F4 resulted in higher entrapment efficiency, which corroborates findings by Varia et al. (2022), and Wu et al. (2019). High surfactant concentrations tend to cause vesicle leakage, decreasing drug entrapment efficiency. This is because surfactants enhance vesicle flexibility, increase leakage, and can also lead to micelle and pore formation when their concentration exceeds the critical lamellar transition temperature (Bnyan et al., 2018). In terms of particle size, ethanol and its interaction with other factors had a negative effect, while Tween 80 showed a positive influence. As supported by Somwanshi (2019), Monisha et al. (2019), and Pathan et al. (2016), increasing ethanol concentration reduced vesicle size, likely due to ethanol's ability to penetrate the vesicular bilayer, reducing its thickness. In contrast, higher concentrations of Tween 80 led to smaller particle sizes. This finding is consistent with Varia et al. (2022) and Iskandarsyah et al. (2020), who noted that Tween 80's smaller HLB value and ability to form hydrogen bonds with the phospholipid bilayer facilitate a reduction in particle size. Table 3 summarizes the optimization process, which suggested an optimum formula consisting of 15% of tween 80 and 20% of 96% ethanol. This formula achieved a particle size of 240.93 ± 1.49 nm, a PDI of 0.18 ± 0.01, and entrapment efficiency of 89.40 ± 0.12%.

The stability of this formula was then tested according to ICH Q1A (R2) guidelines at various storage conditions. The results, as shown in Table 6 and Figure 3, indicate that storage at higher temperatures led to color changes (from white to yellowish) and the formation of precipitates, particularly at 40°C. This suggests lipid vesicle breakdown and oxidation.

Table 6. Organoleptic results of stability test in optimum formula

Day	Storage Condition	Result
0	25ºC±2ºC/60% RH ± 5% RH	Clear white colour, ethanol aroma, no precipitate
	30ºC±2ºC/75% RH ± 5% RH	Clear white colour, ethanol aroma, no precipitate
	40ºC±2ºC/75% RH ± 5% RH	Clear white colour, ethanol aroma, no precipitate
22	25ºC±2ºC/60% RH ± 5% RH	Clear white colour, odorless, precipitate
	30ºC±2ºC/75% RH ± 5% RH	Clear white colour, odorless, precipitate
	40ºC±2ºC/75% RH ± 5% RH	Clear white colour, odorless, precipitate
44	25ºC±2ºC/60% RH ± 5% RH	Slightly yellowish in colour, odorless, precipitate
	30ºC±2ºC/75% RH ± 5% RH	Slightly yellowish in colour, odorless, precipitate
	40ºC±2ºC/75% RH ± 5% RH	Yellow in colour, odorless, precipitate

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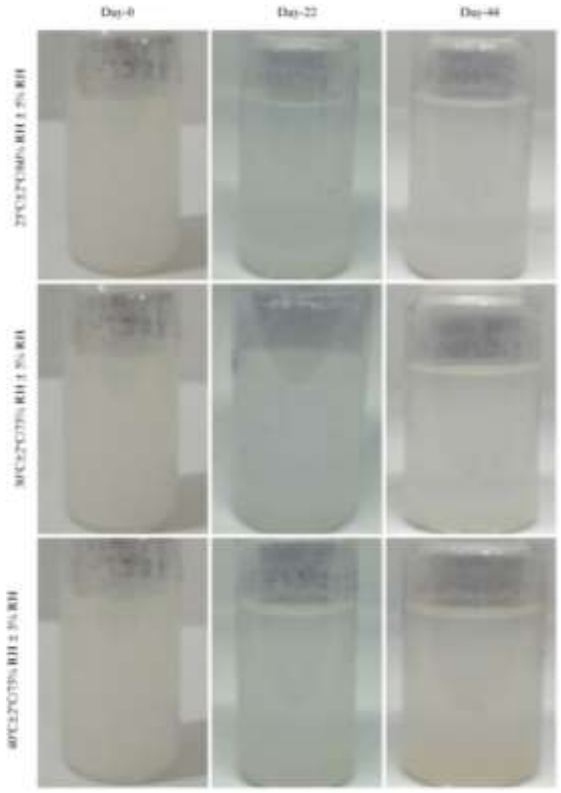
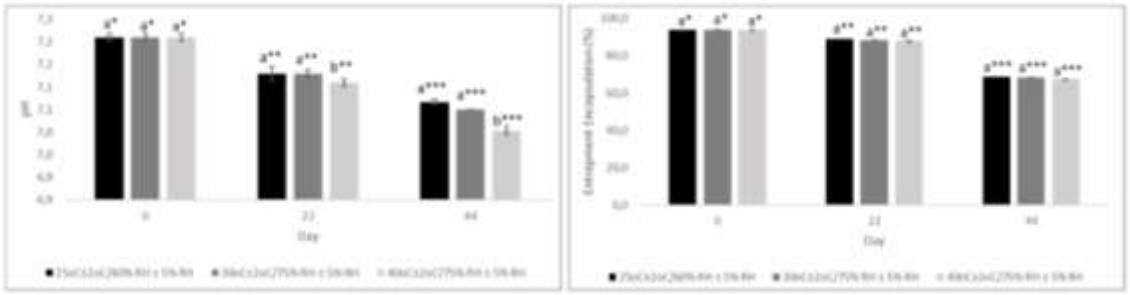


Figure 3. Results of organoleptic of the optimum formula stability test



Note:
The notations "a" and "b" at each time point indicate a significant difference between the three storage conditions based on the Post Hoc-Duncan analysis ($p<0.05$).
The symbols *, **, and *** indicate significant differences between the storage conditions at each time point based on the Post Hoc-Duncan analysis ($p<0.05$).

Figure 4. Results of the pH and entrapment efficiency of the optimum formula stability test

The pH and entrapment efficiency of the optimum formula decreased over time, as depicted in Figure 4, correlating with storage temperature and humidity. A decrease in pH was observed in the optimized formula, which was attributed to the disruption of transethosome vesicles, which released free clindamycin. Clindamycin undergoes degradation upon liberation, which results in the release of H+ ions and a decrease in pH (Apriani et al., 2023). Additionally, the pH of the clindamycin HCl transethosome preparation was influenced by the phospholipon 90G used, which contains phosphatidylcholine. Hydrolysis of phosphatidylcholine produces phosphatidic acid, further lowering the pH (Hong et al., 2023). The optimum formula also showed a decrease in entrapment efficiency. High temperatures increase particle kinetic energy, leading to more collisions between vesicles and a faster reaction rate, which reduces entrapment efficiency (Salawu et al., 2023). Moreover, moisture accelerates the degradation of active ingredients through hydrolysis or oxidation, which can enhance drug release kinetics (Kamaly et al., 2016).

The shelf life of clindamycin HCl transethosomes is based on the release rate kinetics, and the observed release rate follows a zero-order kinetic model. According to Table 7 and Figure 5, the coefficient of

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determination for this model was closest to 1, indicating a strong fit. The zero-order release kinetics imply that the drug's release rate remains constant over time, which is characteristic of systems where the active substance is uniformly dispersed and released at a steady rate, such as in suspension dispersion systems. This suggests that the formulation of clindamycin HCl transethosomes can maintain its release rate for a certain period without significant fluctuations.

The ICH Q1A(R2) guideline offers suggestions for estimating the shelf life of a product by considering the kinetics and storage conditions. The expiration life on the product label should be determined from real-time data if substantial changes are observed during the accelerated stability tests. This approach eliminates the need for six-month testing under accelerated conditions. In this study, extrapolation based on the Arrhenius equation was used to estimate the shelf life by correlating the kinetic constants with temperature, as shown in Table 8. The activation energy (Ea) of 2.978758 cal/mol indicates the minimum energy needed for the release reaction to occur. A lower Ea, which can result from extreme temperature and humidity, may accelerate the release rate due to increased molecular collisions.

Table 9 indicates that under typical storage conditions, the shelf life of clindamycin HCl transethosomes is estimated to be between 21-22 days. As expected, higher temperatures lead to a shorter shelf life, and at a lower temperature of 5°C, the extrapolated shelf life extends to 24.572 days. The study further highlights that preparations in suspension dispersion systems are less stable than those in solution forms, as undissolved particles in suspension can precipitate over time, leading to changes in the formulation's performance.

In conclusion, the shelf life of clindamycin HCl transethosomes is influenced by the temperature and storage conditions, with the Arrhenius equation providing a useful tool for extrapolating stability data. However, the nature of the formulation as a suspension system limits its stability compared to solution-based preparations.

Table 7. Optimum formula release rate kinetics

Release Rate Kinetics	25/60		30/75		40/75	
	R^2	k	R^2	k	R^2	k
Zero-Order	0.886	5.677	0.901	5.777	0.906	6.011
First-Order	0.8718	13.07476	0.8852	13.30506	0.8894	13.84417
Higuchi	0.5167	103.6596	0.6088	102.9963	0.4738	101.4435
Korsmeyer's Peppas	0.5683	0.00000119	0.5885	0.00000103	0.595	0.00000665
		n = 2.9813		n = 2.9812		n = 2.9816

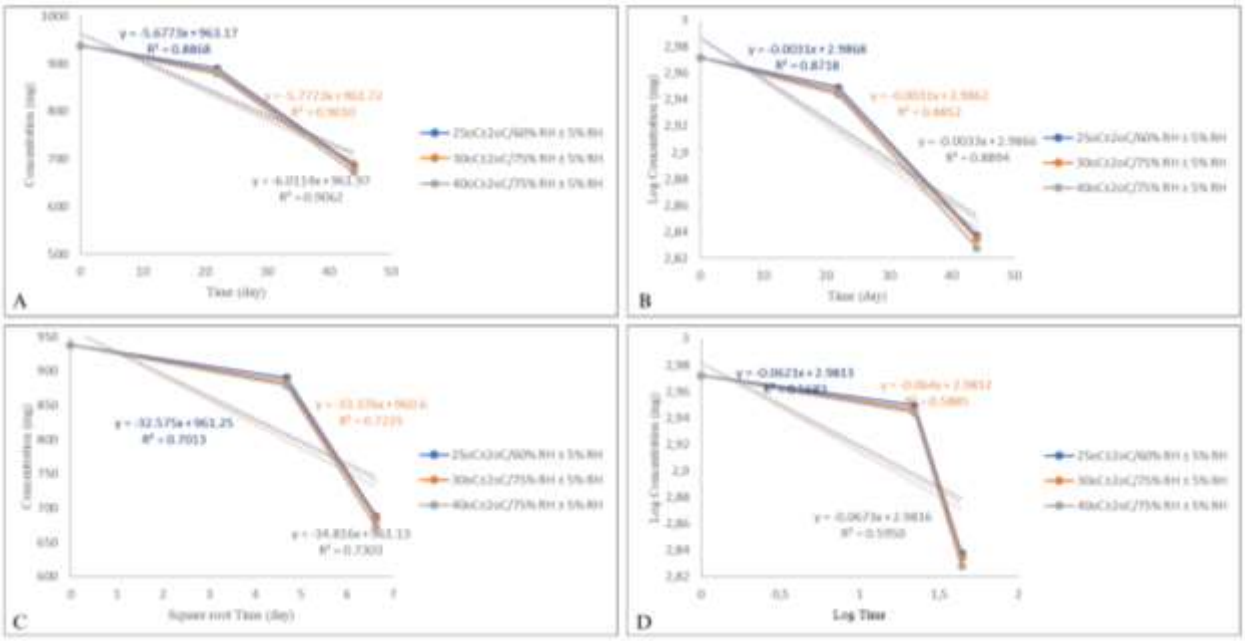

Figure 5. Release profile of optimum formula: A. zero-order, B. first-order, C. higuchi, D. korsmeyer-peppas

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Table 8. Kinetic constants and parameters of the arrhenius in optimum formula

Storage Conditions (°C/%RH)	K	Log K	1/T(K^{-1})
25/60	6.011	0.7789	0.003356
30/75	5.777	0.7617	0.0033
40/75	5.677	0.7541	0.003195
Parameter of Arrhenius			
Ideal Gas Constant (R) (J $K^{-1}.mol^{-1}$)		$8.3134x10^{-3}$	
Arrhenius Factor (A)		13.84522	
Activation Energy (Ea) (cal/mol)		2.978758	

Table 9. Optimum formula shelf life

Storage Conditions (°C/%RH)	Shelf Life (t_{90}) (day)
25/60	22.536
30/75	22.093
40/75	21.274
5°C±3°C	24.572

CONCLUSION

The entrapment efficiency and particle size of clindamycin HCl transethosomes are substantially influenced by the concentrations of ethanol and Tween 80. By increasing the concentration of ethanol, the entrapment efficiency was reduced, and the particle size was larger. Conversely, the entrapment efficiency was reduced and the particle size was increased when the concentration of Tween 80 was increased. The optimum concentrations were determined to be 20% for ethanol and 15% for Tween 80. The clindamycin HCl transethosome formulation exhibited zero-order release kinetics, with an activation energy of 2.98 kcal/mol. The shelf life of the optimum formula was found to be 22.54 days at 25°C/60% RH and 24.57 days at 5°C, indicating the stability of the formulation under these conditions.

AUTHOR CONTRIBUTIONS

Conceptualization, E.F.A., A.A.; Methodology, E.F.A., A.A.; Software, E.F.A., A.A.; Validation, E.F.A., A.A., A.A.; Formal Analysis, E.F.A., A.A., A.A.; Investigation, M.A.M. F.A.; Resources, E.F.A., A.A.; Data Curation, M.A.M. F.A.; Writing - Original Draft, A.A.; Writing - Review & Editing, E.F.A., A.A.; Visualization, A.A.; Supervision, E.F.A., A.A.; Project Administration, E.F.A., A.A.; Funding Acquisition, E.F.A., A.A.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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Efficacy of Ticagrelor Monotherapy in Patients at High Bleeding Risk Undergoing Percutaneous Coronary Intervention: a Systematic Review

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Abstract

Background*: Dual antiplatelet therapy (DAPT) after percutaneous coronary intervention (PCI) prevents ischemic events. However, prolonged therapy increases the risk of bleeding. In this context, an antithrombotic strategy is applied to post-PCI patients by discontinuing aspirin and maintaining P2Y12 receptor monotherapy. Currently, patients with ACS treated with the single antiplatelet agent ticagrelor prefer to apply DAPT for 1 to a few months to open blocked arteries.* ***Objectives:*** *This systematic review aimed to evaluate the clinical efficacy of transitioning high-bleeding-risk patients to ticagrelor monotherapy following a three-month course of DAPT.* ***Methods:*** *A systematic literature review based on the PRISMA statement was conducted to review articles on DAPT, PCI, ticagrelor monotherapy, and high bleeding risk (HBR). The article search was conducted using Internet search databases, including PubMed and ScienceDirect, published between January 2014 and December 2024.* ***Results:*** *Six studies met the inclusion criteria and were included in the analysis. Clinical outcomes were assessed over a follow-up period of up to one year, including endpoints such as all-cause mortality, myocardial infarction, stent thrombosis, stroke, and target vessel revascularization. The secondary endpoints included major adverse cardiovascular and cerebrovascular events (MACCE), significant bleeding defined by Bleeding Academic Research Consortium (BARC) types 2, 3, or 5, and net adverse clinical events (NACE).* ***Conclusion:*** *the use of ticagrelor monotherapy after 3 months of dual antiplatelet therapy is expected to assist healthcare professionals in considering the risk-benefit of single therapy for patients after percutaneous coronary intervention.*

Keywords: *high bleeding risk, monotherapy, percutaneous coronary intervention, ticagrelor*

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INTRODUCTION

Aspirin is commonly administered with P2Y12 inhibitors, such as clopidogrel, prasugrel, or ticagrelor, to patients with acute coronary syndrome (ACS) who undergo percutaneous coronary intervention (PCI). Thrombotic events and platelet inhibition are reduced because patients with ACS need to preserve dual antiplatelet therapy (DAPT) for a complete 12 months after PCI. DAPT reduces stent thrombosis and different ischemic events (Levine et al., 2016; Valgimigli et al., 2018) to improve platelet blockading (Mourikis & Polzin, 2023).

Prolonged DAPT increases bleeding, which may jeopardize the patient's existence, fitness, and economic responsibilities (Valgimigli et al., 2017). Previous research has suggested that the use of aspirin after DAPT could reduce the risk of bleeding without increasing the risk of thromboembolic events (Navarese et al., 2015). Even though aspirin is commonly applied to decrease the chance of cardiovascular troubles, the drug may increase the chance of myocardial infarction in patients at expanded threat stricken by acute coronary syndrome (ACS) (Costa et al., 2019). Different studies have also investigated the role of the P2Y12 receptor in clotting and bleeding events caused by aspirin (Li et al., 2017). Mehran et al. (2019) encouraged treatment with a P2Y12 receptor inhibitor and prohibited the use of aspirin (Gargiulo et al., 2016).

Recent research using subsequent-era drug-eluting stents (DES) has reported a decreased threat of stent thrombosis. In this context, DAPT treatment may be reduced to six months without increasing the risk of thrombosis (Windecker et al., 2020; Valgimigli et al., 2021). This threshold has been decreased through step-by-step methods in research on the use of randomized trials. Currently, patients with ACS treated with the single antiplatelet agent ticagrelor prefer to apply DAPT for 1 to a few months to open blocked arteries (B. K. Kim et al., 2020). Based on the description above, this systematic overview aimed to report the potency level of ticagrelor after three months of DAPT in patients at a high risk of bleeding to open blocked coronary arteries.

MATERIALS AND METHODS

Search strategy

This systematic review was reported according to the PRISMA 2020 standard (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) (Page et al., 2021). PubMed and ScienceDirect were used to retrieve articles, and keyword selection was guided by the PICOT framework, which includes population/problem, intervention, comparison, outcome, and time. Additionally, relevant medical subject terms were identified using the Medical Subject Headings (MeSH) database. Keywords appropriate for the systematic review were used to identify journals, making the search more targeted and comprehensive. For example, literature searches used Boolean operators (AND/OR) to combine terms into search strings such as “dual antiplatelet” OR “DAPT” AND “percutaneous coronary intervention” OR “ticagrelor” OR “monotherapy” AND “HBR,” ensuring that all relevant articles were captured.

Criteria for selection

The selected articles were expected to meet the necessities for the primary research query, which was approximately the potency level of ticagrelor in lowering the hazard of bleeding in patients with ACS undergoing PCI. Articles published from January 2015 to 2025 were used in the systematic overview. This research must meet the following requirements: (1) randomized controlled trials (RCTs); (2) inclusion of ACS patients with high bleeding risk (HBR) who had PCI and published in English; (4) the whole textual content should be available and the effectiveness of ticagrelor monotherapy must be examined; (6) 10-year period guaranteed; and (7) the outcomes of negative cardiovascular activities, all-reason dying, stent thrombosis, or bleeding occasions must be reported. However, the research was limited by a lack of access to full-text content and the inclusion of patients with ACS subjected to PCI without HBR.

For a full search, the method did not use observation groups or clinical effect standards within the first database searches (PubMed and ScienceDirect). In this context, the titles and descriptions were checked before conducting full-text content searches to locate related gadgets. The reference lists of the applicable articles were searched manually to discover more research. Subsequently, resources with greater facts were selected over those without substantial statistics. The selection was checked for every sponsored search to prevent bias. Screening and qualification checks were based on the set parameters. Obtaining the proper articles led to discarding the copies and deciding on the right key phrases and outlines. A content evaluation of important information answered the question concerning the proper use of ticagrelor in patients with ACS subjected to PCI and an excessive chance of bleeding (Table 1).

The quality of the articles was assessed using the JBI Critical Appraisal Checklist for Randomized Controlled Trials to evaluate the validity of the research. The key elements included allocation concealment

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(blinding), group comparison, identical treatment, follow-up, analysis, outcome measurement, statistical analysis, research design, research quality assessment, result evaluation, and decision guidance (Table 2).

Data extraction

The selected articles were obtained from two sources: after removing 14 comparable entries, the titles and summaries of 156 articles were analyzed. Therefore, 126 articles were removed, leaving 30 full-text articles for analysis. A total of 24 were removed because the requirements for the admission standards were not met. In this context, only six articles were left for the closing thorough assessment, as indicated by the PRISMA waft chart in Figure 1.

RESULTS AND DISCUSSION

A search of databases such as PubMed and ScienceDirect yielded 170 articles. However, only six were selected based on their appearance, as shown in Table 1. The primary goal of this study was to determine the effects of ticagrelor on patients with a high risk of bleeding after 3 months of DAPT.

Characteristics of the research

The six reviewed articles evaluated the efficacy of ticagrelor monotherapy in patients with a high chance of bleeding after 3 months of DAPT. Over the past decade, PCI has been performed in a broader and more complex patient population with comorbidities that increase bleeding during the procedure (Neumann et al., 2019). There may be a connection between the duration and severity of antithrombotic therapy and bleeding events after PCI. Therefore, shorter periods and unmarried antiplatelet regimens are becoming readily available for patients with a high risk of bleeding (Levine et al., 2016; Valgimigli et al., 2018). Patients in DAPT clinical research with a high threat of bleeding also have a higher risk of cardiac events, which increases the difficulty of determining unmarried remedies (Cao et al., 2020; Ueki et al., 2020; Singh et al., 2024).

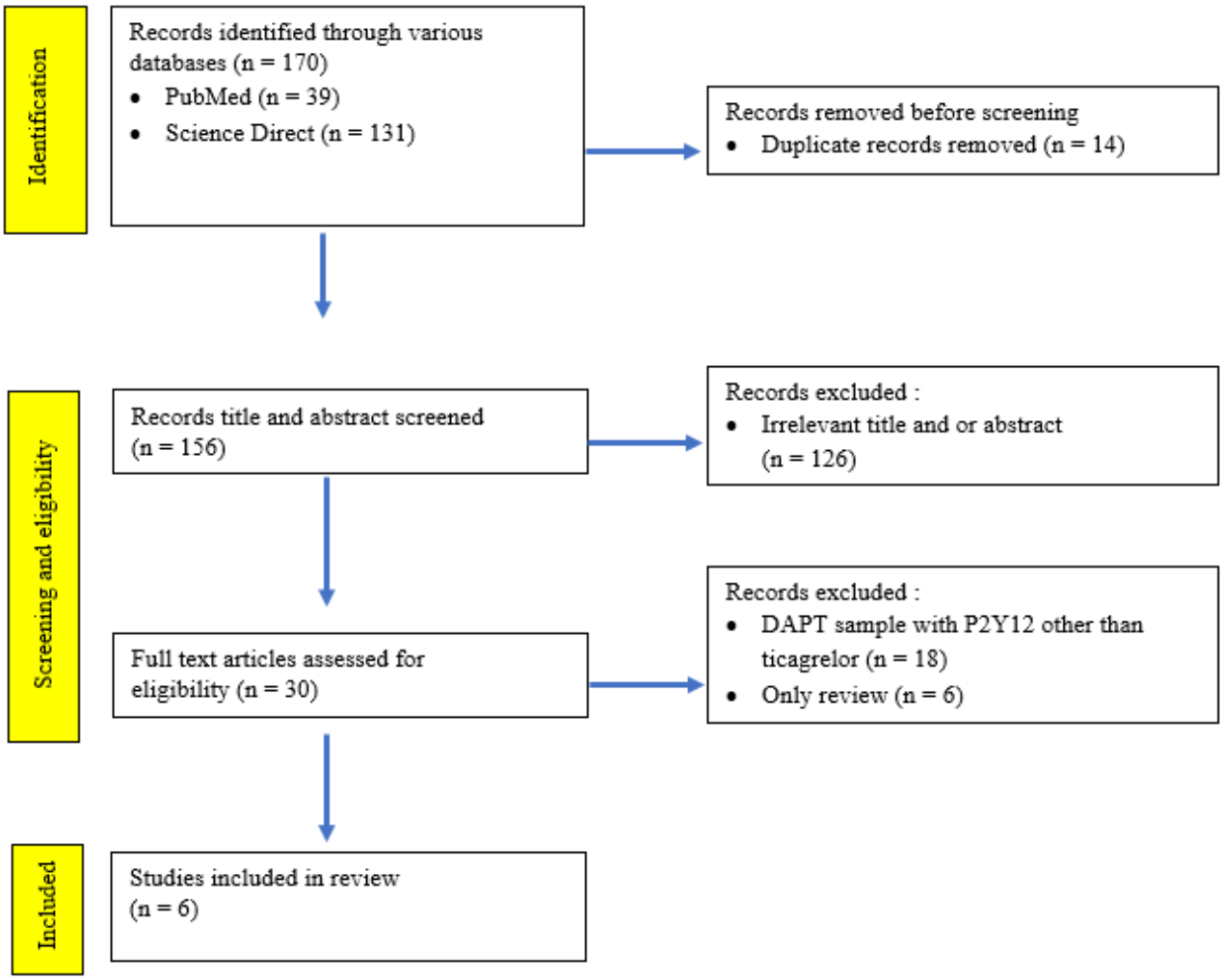

Figure 1. PRISMA

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Table 1. Studies on ticagrelor monotherapy after 3 months of DAPT with ticagrelor 12 months of DAPT

Study Year	Country	Number of patients	Design	Population	Intervention	Control	Main findings (intervention vs control)	Outcome
TWILIGHT HBR (Escaned et al., 2021)	Canada, USA, Italy, UK, Poland, Spain, Austria, Germany, India, Israel.	7,119	Double blind	Elderly HBR patients (≥65 years, age) undergoing PCI	Ticagrelor monotherapy after 3 months of DAPT	DAPT	**BARC 2, 3 or 5 bleeding at 12 months:** HBR: 6.3% vs. 11.4%; HR 0.53, 95% CI 0.35–0.82, Non-HBR: 3.5% vs. 5.9%; HR 0.59, 95% CI 0.46–0.77 with no evidence of heterogeneity (P interaction = 0.673). **BARC 3 or 5 bleeding at 12 months:** HBR: 1.6% vs. 5.0%; HR 0.31, 95% CI 0.14–0.67, Non-HBR: 0.8% vs. 1.3%; HR 0.62, 95% CI 0.36–1.09, P = 0.098, P interaction = 0.48 **Ischemic events:** HBR: 6.5% vs. 5.6%; HR 1.16, 95% CI 0.71–1.90, P = 0.554, Non-HBR: 3.6 % vs. 3.6 %; HR 1.01, 95 % CI 0.75–1.35, P = 0.949, P interaction = 0.637	Patients with excessive bleeding risk (HBR) after PCI have strong records of the efficacy and protection of ticagrelor monotherapy from the TWILIGHT-HBR trial and sub-analyses. These patients also experienced hemorrhages and ischemia. The risk was increased by the satisfaction of extra ARC-HBR standards. After three months of DAPT, the frequency of clinically significant bleeding episodes of type 2, 3, or 5 was considerably reduced when ticagrelor was used compared with aspirin. This was genuine, irrespective of the presence or absence of HBR. Less severe bleeding occurred in patients with HBR than in those with NON-HBR. All ARC-HBR chance classes were favored by ticagrelor monotherapy in reducing bleeding and stroke occurrence. Escaned et al. (2021) discovered that the use of ticagrelor was supported by a selected population of high-risk patients, lending credence to its use in a selection of high-risk patients.
TWILIGHT sub study (Angiolillo et al., 2021)	Canada, USA, Italy, UK, Poland, Spain, Austria, India, Israel.	6,532	Double blind	Elderly HBR patients (≥65 years, age) undergoing DAPT PCI	Ticagrelor monotherapy after 3 months of DAPT	DAPT	**BARC 2, 3 or 5 bleeding at 12 months:** 4.5% vs. 8.2%; HR 0.53, 95% CI 0.40–0.71, P interaction = 0.62 **Death, MI or stroke at 12 months:** 4.2% vs. 4.4%; HR 0.96, 95% CI 0.68–1.35, P interaction = 0.77	The results showed that (i) the frequency of BARC 2, 3, or 5 hemorrhages increased appreciably after the age of 65 years. Meanwhile, the frequency of mortality, myocardial attack, or stroke improved after 70 years of age. In patients aged ≥ 65 years, the risk of BARC bleeding was approximately 50% lower when ticagrelor was used as monotherapy than when it was combined with aspirin. Loss of life, myocardial infarction, and stroke were not significantly associated. Regardless of age,

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								ticagrelor was safe and effective in lowering bleeding without increasing the risk of ischemic events. Reducing the threat of bleeding while preserving ischemia protection in older patients presents a challenge in PCI. In this context, high-risk bleeding patients can transition from DAPT to ticagrelor monotherapy after a short duration.
TWILIGHT Sub study (Chiarito et al., 2022)	Canada, USA, Italy.	7,119	Double blind	Elderly HBR patients (≥65 years, age) undergoing PCI with prior MI	Ticagrelor monotherapy after 3 months of DAPT	DAPT	**BARC 2, 3 or 5 bleeding at 12 months:** Prior MI: 3.4% vs. 6.7%; HR 0.50, 95% CI 0.33–0.76. No prior MI: 4.2% vs. 7.0%; HR 0.58, 95% CI 0.45–0.76. P interaction = 0.54 **Death, MI, or stroke at 12 months:** Prior MI: 6.0% vs. 5.5%; HR 1.09, 95 % CI 0.75–1.58. No prior MI: 3.1% vs. 3.3%; HR 0.92, 95 % CI 0.67–1.28. P interaction = 0.52	Patients at high risk of myocardial infarction (MI) were analyzed in the TWILIGHT study. According to Chiarito et al. (2022), patients with a history of myocardial infarction and PCI have a higher risk of ischemic events. Regardless of the event, ticagrelor monotherapy reduced the risk of clinically critical and dangerous bleeding. Patients receiving ticagrelor as monotherapy or in combination with aspirin did not show significantly higher rates of mortality, myocardial infarction, or stroke. After meeting the PEGASUS-TIMI 54 criteria, treatment with ticagrelor monotherapy showed a lower risk of clinically significant and severe bleeding. Furthermore, there were no substantial differences in the ischemic outcomes between the treatment groups.
TWILIGHT sub study (Mendieta et al., 2023)	Canada, USA, Italy, UK, Poland, Spain, Austria.	7,119	Double blind	Elderly HBR patients (≥65 years, age) undergoing successful DES implantation	Ticagrelor monotherapy after 3 months of DAPT	DAPT	**BARC 2, 3 or 5 bleeding at 12 months:** LBR: 3.1% vs. 5.7%; RR 1.85, 95% CI 1.40–2.46 HBR: 6.0% vs. 9.7%; RR 1.61, 95% CI 1.21–2.14 P interaction = 0.54 LIR: 3.5% vs. 7.0%; RR 2.01, 95% CI 1.55–2.60 HIR: 5.1% vs. 7.3%; RR 1.43, 95% CI 1.04–1.96 P interaction = 0.11 **MACCE at 12 months:** LBR: 3.4% vs. 3.2% HBR: 4.0% vs. 4.7% LIR: 1.9% vs. 2.2% HIR: 7.0% vs. 6.8%	The TWILIGHT sub-study aimed to assess bleeding events (BARC types 2, 3, or 5) and major adverse cardiac and cerebrovascular events (MACCE) at 12 months after a three-month ticagrelor-based DAPT. Patients with high and low bleeding risks experienced significantly fewer events when aspirin was discontinued, with ticagrelor continued as monotherapy. The reduction in bleeding was not associated with an increase in

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								MACCE or ischemic events across various risk profiles. Therefore, this study provides personalized risk estimates that balance the bleeding reduction benefits of ticagrelor monotherapy against potential ischemic risks.
TICO – sub study (S. J. Lee et al., 2021)	South Korea	3,056	Open label	Patients undergoing PCI for ACS (STEMI, NSTEMI and unstable angina)	Ticagrelor monotherapy after 3 months of DAPT	DAPT	**NACE at 12 months:** STEMI: 3.7% vs. 5.0%; HR 0.73, 95% CI 0.41–1.29 NSTEMI: 4.8% vs. 7.4%; HR 0.66, 95% CI 0.40–1.09, Unstable angina: 2.9% vs. 5.2%; HR 0.57, 95% CI 0.29–1.12 P interaction = 0.64 **TIMI major bleeding at 12 months:** STEMI: 0.9% vs. 2.9%; HR 0.32, 95% CI 0.12–0.87 NSTEMI: 2.4% vs. 3.5%; HR 0.69, 95% CI 0.34–1.43 Unstable angina: 1.6% vs. 2.5%; HR 0.64, 95% CI 0.25–1.63 Pinteraction = 0.36 **TIMI major or minor bleeding at 12 months** STEMI: 3.1% vs. 5.0%; HR 0.62, 95% CI 0.34–1.13 NSTEMI: 3.3% vs. 7.4%; HR 0.45, 95% CI 0.25–0.79 Unstable angina: 4.1% vs. 3.9%; HR 1.05, 95% CI 0.55–2.00 P interaction = 0.29 **Ischemic outcomes:** **MACCE at 12 months:** STEMI: 2.7% vs. 2.5%; HR 1.10, 95% CI 0.53–2.27 NSTEMI: 2.6% vs. 4.5%; HR 0.58, 95% CI 0.30–1.13 Unstable angina: 4.1% vs. 3.1%; HR 0.44, 95% CI 0.17–1.13 P interaction = 0.14	Ticagrelor monotherapy following a short course of DAPT was safe and feasible. The key results were as follows: (i) the rates of MACCE in patients with STEMI were comparable to those in patients with non-STEMI or unstable angina; (ii) there was no significant interaction between treatment strategy and clinical presentation, as ticagrelor monotherapy after three months of DAPT was consistently effective; and (iii) ticagrelor monotherapy reduced major bleeding across all clinical presentations without increasing the risk of MACCE. These results support the discontinuation of aspirin after three months of DAPT, with continued ticagrelor monotherapy, as a strategy for reducing bleeding risk without ischemic events across a broad range of patients.
TICO – sub analysis from the TICO Trial (Y. J. Lee et al., 2022)	Korea	3,056	Open label	Patients undergoing PCI for ACS (unstable angina, non-ST-elevation	Ticagrelor monotherapy after 3 months of DAPT	DAPT	**ARC-HBR** **NACE**, HBR vs. non-HBR : (5.4% vs. 1.9%; HR, 2.87; 95% CI, 1.76–4.69; p<0.001).	The research examined the impact of ticagrelor monotherapy initiated after three months of DAPT in patients with ACS treated with a new-generation sirolimus-eluting stent. The primary outcome was the incidence of adverse clinical events, while

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				myocardial infarction [MI], or ST-elevation MI)			**Major bleeding**, HBR vs. non-HBR (2.7% vs. 0.6%; HR, 4.91; 95% CI, 2.27–10.61; p<0.001) **MACCE**, HBR vs. non-HBR (3.2% vs. 1.4%; HR, 2.34; 95% CI, 1.26–4.36, p=0.006) **PRECISE-DAPT** **NACE**, HBR vs. non-HBR (5.5% vs. 1.9%; HR, 3.09; 95% CI, 1.92–4.98; p<0.001). **Major Bleeding**, HBR vs. non-HBR (2.9% vs. 0.5%; HR, 5.96; 95% CI, 2.76– 12.88; p<0.001) **MACCE,** HBR vs. non- HBR (3.1% vs. 1.4%; HR, 2.31; 95% CI, 1.25–4.25; p=0.006).	the secondary outcomes included all-cause mortality, myocardial infarction, stent thrombosis, stroke, major bleeding, target vessel revascularization, and MACCE. The results reported the following: (i) patients with ACS treated with DES and identified as HBR had a higher incidence of primary outcomes, including bleeding and ischemic events. (ii) Regardless of HBR status, switching to ticagrelor monotherapy after three months of DAPT significantly reduced major bleeding and primary outcomes compared to continuing ticagrelor-based DAPT for 12 months. No significant interaction was observed between the treatment strategy and bleeding risk. (iii) These results were consistent whether HBR status was defined using the PRECISE-DAPT score or the ARC-HBR criteria**.**
TWILIGHT HBR (Escaned et al., 2021)	Canada, the USA, Italy, the UK, Poland, Spain, Austria, Germany, India, Israel.	7,119	Double blind	Elderly HBR patients (≥65 years) undergoing PCI	Ticagrelor monotherapy after 3 months of DAPT	DAPT	**BARC 2, 3 or 5 bleeding at 12 months:** HBR: 6.3% vs. 11.4%; HR 0.53, 95% CI 0.35–0.82; P=0,004, Non-HBR: 3.5% vs. 5.9%; HR 0.59, 95% CI 0.46–0.77; P<0,001 with no evidence of heterogeneity (P interaction = 0.673). **BARC 3 or 5 bleeding at 12 months:** HBR: 1.6% vs. 5.0%; HR 0.31, 95% CI 0.14–0.67; P= 0,003, Non-HBR: 0.8% vs. 1.3%; HR 0.62, 95% CI 0.36–1.09, P=0.098, P interaction = 0.148. **Ischemic events:** HBR: 6.5% vs. 5.6%; HR 1.16, 95% CI 0.71–1.90, P=0.554, Non-HBR: 3.6 % vs. 3.6 %; HR 1.01, 95% CI 0.75–1.35, P=0.949, P interaction = 0.637.	Patients with excessive bleeding risk (HBR) after PCI have strong records of the efficacy and protection of ticagrelor monotherapy from the TWILIGHT-HBR trial and sub-analyses. These patients also experienced hemorrhages and ischemia. The risk was increased by the satisfaction of extra ARC-HBR standards. After three months of DAPT, the frequency of clinically significant bleeding episodes of type 2, 3, or 5 was considerably reduced when ticagrelor was used compared with aspirin. This was genuine, irrespective of the presence or absence of HBR. Less severe bleeding occurred in patients with HBR than in those with NON-HBR. All ARC-HBR chance classes were favored by ticagrelor monotherapy in reducing bleeding and stroke occurrence. Escaned et al. (2021) discovered that the use of

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								ticagrelor was supported by a selected population of high-risk patients, lending credence to its use in a selection of high-risk patients.
TWILIGHT sub study (Angiolillo et al., 2021)	Canada, the USA, Italy, the UK, Poland, Spain, Austria, India, Israel.	6,532	Double blind	Elderly HBR patients (≥65 years) undergoing DAPT PCI	Ticagrelor monotherapy after 3 months of DAPT	DAPT	**BARC 2, 3 or 5 bleeding at 12 months:** 4.5% vs. 8.2%; HR 0.53, 95% CI 0.40–0.71, P interaction = 0.62 **Death, MI or stroke at 12 months:** 4.2% vs. 4.4%; HR 0.96, 95% CI 0.68–1.35, P interaction = 0.77	The results showed that (i) the frequency of BARC 2, 3, or 5 hemorrhages increased appreciably after the age of 65 years. Meanwhile, the frequency of mortality, myocardial attack, or stroke improved after 70 years of age. In patients aged ≥ 65 years, the risk of BARC bleeding was approximately 50% lower when ticagrelor was used as monotherapy than when it was combined with aspirin. Loss of life, myocardial infarction, and stroke were not significantly associated. Regardless of age, ticagrelor was safe and effective in lowering bleeding without increasing the risk of ischemic events. Reducing the threat of bleeding while preserving ischemia protection in older patients presents a challenge in PCI. In this context, high-risk bleeding patients can transition from DAPT to ticagrelor monotherapy after a short duration.
TWILIGHT Sub study (Chiarito et al., 2022)	Canada, the USA, Italy.	7,119	Double blind	Elderly HBR patients (≥65 years) undergoing PCI with prior MI	Ticagrelor monotherapy after 3 months of DAPT	DAPT	**BARC 2, 3 or 5 bleeding at 12 months:** Prior MI: 3.4% vs. 6.7%; HR 0.50, 95% CI 0.33–0.76. No prior MI: 4.2% vs. 7.0%; HR 0.58, 95% CI 0.45–0.76. P interaction = 0.54 **Death, MI, or stroke at 12 months:** Prior MI: 6.0% vs. 5.5%; HR 1.09, 95 % CI 0.75–1.58. No prior MI: 3.1% vs. 3.3%; HR 0.92, 95 % CI 0.67–1.28. P interaction = 0.52	Patients at high risk of myocardial infarction (MI) were analyzed in the TWILIGHT study. According to Chiarito et al. (2022), patients with a history of myocardial infarction and PCI have a higher risk of ischemic events. Regardless of the event, ticagrelor monotherapy reduced the risk of clinically critical and dangerous bleeding. Patients receiving ticagrelor as monotherapy or in combination with aspirin did not show significantly higher rates of mortality, myocardial infarction, or stroke. After meeting the PEGASUS-TIMI 54 criteria, treatment with ticagrelor monotherapy

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								showed a lower risk of clinically significant and severe bleeding. Furthermore, there were no substantial differences in the ischemic outcomes between the treatment groups.
TWILIGHT sub study (Mendieta et al., 2023)	Canada, the USA, Italy, the UK, Poland, Spain, Austria.	7,119	Double blind	Elderly HBR patients (≥65 years) undergoing successful DES implantation	Ticagrelor monotherapy after 3 months of DAPT	DAPT	**BARC 2, 3 or 5 bleeding at 12 months:** LBR: 3.1% vs. 5.7%; RR 1.85, 95% CI 1.40–2.46 HBR: 6.0% vs. 9.7%; RR 1.61, 95% CI 1.21–2.14 P interaction = 0.54 LIR: 3.5% vs. 7.0%; RR 2.01, 95% CI 1.55–2.60 HIR: 5.1% vs. 7.3%; RR 1.43, 95% CI 1.04–1.96 P interaction = 0.11 **MACCE at 12 months:** LBR: 3.4% vs. 3.2% HBR: 4.0% vs. 4.7% LIR: 1.9% vs. 2.2% HIR: 7.0% vs. 6.8%	The TWILIGHT sub-study aimed to assess bleeding events (BARC types 2, 3, or 5) and major adverse cardiac and cerebrovascular events (MACCE) at 12 months after a three-month ticagrelor-based DAPT. Patients with high and low bleeding risks experienced significantly fewer events when aspirin was discontinued, with ticagrelor continued as monotherapy. The reduction in bleeding was not associated with an increase in MACCE or ischemic events across various risk profiles. Therefore, this study provides personalized risk estimates that balance the bleeding reduction benefits of ticagrelor monotherapy against potential ischemic risks.
TICO – sub study (S. J. Lee et al., 2021)	South Korea	3,056	Open label	Patients undergoing PCI for ACS (STEMI, NSTEMI and unstable angina)	Ticagrelor monotherapy after 3 months of DAPT	DAPT	**NACE at 12 months:** STEMI: 3.7% vs. 5.0%; HR 0.73, 95% CI 0.41–1.29 NSTEMI: 4.8% vs. 7.4%; HR 0.66, 95% CI 0.40–1.09, Unstable angina: 2.9% vs. 5.2%; HR 0.57, 95% CI 0.29–1.12 P interaction = 0.64 **TIMI major bleeding at 12 months:** STEMI: 0.9% vs. 2.9%; HR 0.32, 95% CI 0.12–0.87 NSTEMI: 2.4% vs. 3.5%; HR 0.69, 95% CI 0.34–1.43 Unstable angina: 1.6% vs. 2.5%; HR 0.64, 95% CI 0.25–1.63 Pinteraction = 0.36 **TIMI major or minor bleeding at 12 months** STEMI: 3.1% vs. 5.0%; HR 0.62, 95% CI 0.34–1.13 NSTEMI: 3.3% vs. 7.4%; HR 0.45, 95% CI 0.25–0.79 Unstable angina:	Ticagrelor monotherapy following a short course of DAPT was safe and feasible. The key results were as follows: (i) the rates of MACCE in patients with STEMI were comparable to those in patients with non-STEMI or unstable angina; (ii) there was no significant interaction between treatment strategy and clinical presentation, as ticagrelor monotherapy after three months of DAPT was consistently effective; and (iii) ticagrelor monotherapy reduced bleeding events across all clinical presentations without increasing the risk of MACCE. These results support the discontinuation of aspirin after three months of DAPT, with continued ticagrelor monotherapy, as a strategy for reducing

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							4.1% vs. 3.9%; HR 1.05, 95% CI 0.55–2.00 P interaction = 0.29 **Ischemic outcomes:** **MACCE at 12 months:** STEMI: 2.7% vs. 2.5%; HR 1.10, 95% CI 0.53–2.27 NSTEMI: 2.6% vs. 4.5%; HR 0.58, 95% CI 0.30–1.13 Unstable angina: 1.4% vs. 3.1%; HR 0.44, 95% CI 0.17–1.13 P interaction = 0.14	bleeding risk without ischemic events across a broad range of patients.
TICO – sub analysis from the TICO Trial (Y. J. Lee et al., 2022)	Korea	3,056	Open label	Patients undergoing PCI for ACS (STEMI, NSTEMI and unstable angina)	Ticagrelor monotherapy after 3 months of DAPT	DAPT	**ARC-HBR** **NACE**, HBR vs. non-HBR : (5.4% vs. 1.9%; HR, 2.87; 95% CI, 1.76–4.69; p<0.001). **Bleeding events**, HBR vs. non-HBR (2.7% vs. 0.6%; HR, 4.91; 95% CI, 2.27–10.61; p<0.001) **MACCE**, HBR vs. non-HBR (3.2% vs. 1.4%; HR, 2.34; 95% CI, 1.26–4.36, p=0.006) **PRECISE-DAPT** **NACE**, HBR vs. non-HBR (5.5% vs. 1.9%; HR, 3.09; 95% CI, 1.92–4.98; p<0.001). **Bleeding events**, HBR vs. non-HBR (2.9% vs. 0.5%; HR, 5.96; 95% CI, 2.76– 12.88; p<0.001) **MACCE,** HBR vs. non- HBR (3.1% vs. 1.4%; HR, 2.31; 95% CI, 1.25–4.25; p=0.006).	The research examined the impact of ticagrelor monotherapy initiated after three months of DAPT in patients with ACS treated with a new-generation sirolimus-eluting stent. The primary outcome was the incidence of adverse clinical events, while the secondary outcomes included all-cause mortality, myocardial infarction, stent thrombosis, stroke, bleeding events, target vessel revascularization, and MACCE. The results reported the following: (i) patients with ACS treated with DES and identified as HBR had a higher incidence of primary outcomes, including bleeding and ischemic events. (ii) Regardless of HBR status, switching to ticagrelor monotherapy after three months of DAPT significantly reduced bleeding events and primary outcomes compared to continuing ticagrelor-based DAPT for 12 months. No significant interaction was observed between the treatment strategy and bleeding risk. (iii) These results were consistent whether HBR status was defined using the PRECISE-DAPT score or the ARC-HBR criteria**.**

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Table 2. JBI critical appraisal

No.	JBI Critical Appraisal	TWILIGHT HBR (Escaned et al., 2021)	TWILIGHT sub study (Angiolillo et al., 2021)	TWILIGHT Sub study (Chiarito et al., 2022)	TWILIGHT sub study (Mendieta et al., 2023)	TICO – sub study (S. J. Lee et al., 2021)	TICO – sub analysis from the TICO Trial (Y. J. Lee et al., 2022)
1.	Was true randomization used for assignment of participants to treatment groups?	√	√	√	√	√	√
2.	Was allocation to treatment groups concealed?	√	√	√	√	X	X
3.	Were treatment groups similar at the baseline?	√	√	√	√	X	X
4.	Were participants blind to treatment assignment?	√	√	√	√	X	X
5.	Were those delivering treatment blind to treatment assignment?	√	√	√	√	X	X
6.	Were outcomes assessors blind to treatment assignment?	X	X	X	X	X	X
7.	Were treatment groups treated identically other than the intervention of interest?	√	√	√	√	X	X
8.	Was follow up complete and if not, were differences between groups in terms of their follow up adequately described and analyzed?	√	√	√	√	√	X
9.	Were participants analyzed in the groups to which they were randomized?	√	√	√	√	√	√
10.	Were outcomes measured in the same way for treatment groups?	√	√	√	√	√	√
11.	Were outcomes measured in a reliable way?	√	√	√	√	√	√
12.	Was appropriate statistical analysis used?	√	√	√	√	√	√
13.	Was the trial design appropriate, and any deviations from the standard RCT design (individual randomization, parallel groups) accounted for in the conduct and analysis of the trial?	√	√	√	√	√	√

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The RCTs listed in Table 1 analyzed the effects of discontinuing aspirin and administering ticagrelor monotherapy (a P2Y12 inhibitor) instead of DAPT. In the TWILIGHT-HBR study conducted by Escaned et al. (2021), primary and secondary targets were identified. The final result examined was the occurrence of bleeding of types 2, 3, or 5 in one year, as determined with the aid of the Bleeding Academic Research Consortium (BARC). Secondary bleeding results were reported by (Costa et al. (2017) and Ueki et al. (2019), Urban et al. (2019) and Corpataux et al. (2020). Subsequent secondary ischemic events, such as death from any cause, myocardial infarction, stroke, cardiovascular death, non-fatal myocardial infarction, ischemic stroke, and stent thrombosis, were protected. Patients with excessive bleeding risk (HBR) after PCI have strong records of the efficacy and protection of ticagrelor monotherapy from the TWILIGHT-HBR trial and sub-analyses. The TWILIGHT HBR study in 2021 included 7.119 patients from Canada, the USA, Italy, the UK, Poland, Spain, Austria, Germany, India, and Israel. These patients also experienced hemorrhages and ischemia. The risk was increased by the satisfaction of extra ARC-HBR standards. After three months of DAPT, the frequency of clinically significant bleeding episodes of type 2, 3, or 5 was considerably reduced when ticagrelor was used compared with aspirin. This was genuine, irrespective of the presence or absence of HBR. Less severe bleeding occurred in patients with HBR than in those with NON-HBR. All ARC-HBR chance classes were favored by ticagrelor monotherapy in reducing bleeding and stroke occurrence. Escaned et al. (2021) discovered that the use of ticagrelor was supported by a selected population of high-risk patients, lending credence to its use in a selection of high-risk patients.

The analysis of the TWILIGHT subtitle in 2021 included 6.532 patients from Canada, the USA, Italy, the UK, Poland, Spain, Austria, India, and Israel. Angiolillo et al. (2021) examined the connection between age and detrimental activities. The results showed that (i) the frequency of BARC 2, 3, or 5 hemorrhages increased appreciably after the age of 65 years. Meanwhile, the frequency of mortality, myocardial attack, or stroke improved after 70 years of age. In patients aged $\geq$ 65 years, the risk of BARC bleeding was approximately 50% lower when ticagrelor was used as monotherapy than when it was combined with aspirin. Loss of life, myocardial infarction, and stroke were not significantly associated. Regardless of age, ticagrelor was safe and effective in lowering bleeding without increasing the risk of ischemic events. Reducing the threat of bleeding while preserving ischemia protection in older patients presents a challenge in PCI. In this context, high-risk bleeding patients can transition from DAPT to ticagrelor monotherapy after a short duration.

Meanwhile, the TWILIGHT sub-study in 2022 included 7.119 patients from Canada, the USA, and Italy. Patients at high risk of myocardial infarction (MI) were analyzed in the TWILIGHT study. According to Chiarito et al. (2022), patients with a history of myocardial infarction and PCI have a higher risk of ischemic events. Regardless of the event, ticagrelor monotherapy reduced the risk of clinically critical and dangerous bleeding. Patients receiving ticagrelor as monotherapy or in combination with aspirin did not show significantly higher rates of mortality, myocardial infarction, or stroke. After meeting the PEGASUS-TIMI 54 criteria, treatment with ticagrelor monotherapy showed a lower risk of clinically significant and severe bleeding. Furthermore, there were no substantial differences in the ischemic outcomes between the treatment groups.

Additionally, the TWILIGHT sub-study in 2023 included 7.119 in Canada, the USA, Italy, the UK, Poland, Spain, and Austria. This information shows that ticagrelor significantly lowers the threat of bleeding without increasing the risk of ischemic activities. This is particularly appropriate for patients at high risk of myocardial infarction. Extraordinary predictive models have been developed through interventions (Mendieta et al., 2023). The TWILIGHT sub-study aimed to assess bleeding events (BARC types 2, 3, or 5) and major adverse cardiac and cerebrovascular events (MACCE) at 12 months after a three-month ticagrelor-based DAPT. Patients with high and low bleeding risks experienced significantly fewer events when aspirin was discontinued, with ticagrelor continued as monotherapy. The reduction in bleeding was not associated with an increase in MACCE or ischemic events across various risk profiles. Therefore, this study provides personalized risk estimates that balance the bleeding reduction benefits of ticagrelor monotherapy against potential ischemic risks.

In another trial, the TICO sub-study in 2021 included 3.056 in South Korea. S. J. Lee et al., 2021 investigated patients with ST-segment elevation myocardial infarction (STEMI) treated with DES. In this context, ticagrelor monotherapy following a short course of DAPT was safe and feasible. The key results were as follows: (i) the rates of MACCE in patients with STEMI were comparable to those in patients with non-

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STEMI or unstable angina; (ii) there was no significant interaction between treatment strategy and clinical presentation, as ticagrelor monotherapy after three months of DAPT was consistently effective; and (iii) ticagrelor monotherapy reduced bleeding events across all clinical presentations without increasing the risk of MACCE. These results support the discontinuation of aspirin after three months of DAPT, with continued ticagrelor monotherapy, as a strategy for reducing bleeding risk without ischemic events across a broad range of patients.

The TICO trial in 2022 included 3.056 patients in Korea. Y. J. Lee et al., 2022 reported the primary and secondary clinical outcomes of post-hoc analysis of the TICO-HBR trial. The research examined the impact of ticagrelor monotherapy initiated after three months of DAPT in patients with ACS treated with a new-generation sirolimus-eluting stent. The primary outcome was the incidence of adverse clinical events, and the secondary outcomes included all-cause mortality, myocardial infarction, stent thrombosis, stroke, bleeding events, target vessel revascularization, and MACCE. The results reported the following: (i) patients with ACS treated with DES and identified as HBR had a higher incidence of primary outcomes, including bleeding and ischemic events. (ii) Regardless of HBR status, switching to ticagrelor monotherapy after three months of DAPT significantly reduced bleeding events and primary outcomes compared to continuing ticagrelor-based DAPT for 12 months. No significant interaction was observed between the treatment strategy and bleeding risk. (iii) These results were consistent whether HBR status was defined using the PRECISE-DAPT score or the ARC-HBR criteria**.**

HBR stratification assessment

Patients were classified as having HBR according to the standardized definition developed by the ARC, which requires the presence of a major criterion or two minor criteria. HBR is defined by the ARC as a risk of 4% for BARC type 3 or 5 bleeding or 1% for intracranial hemorrhage (ICH) within one year (Urban et al., 2019).

ARC-HBR criteria were used for HBR categorization in the TWILIGHT-HBR experiment performed by Escaned et al., 2021. Several criteria for HBR include advanced or end-stage chronic kidney disease (CKD) with an estimated glomerular filtration rate (eGFR) of less than 30 mL/min/1.73 m² or patients subjected to dialysis, hemoglobin levels below 11 g/dL, moderate to severe thrombocytopenia with a platelet count below 100×10^9cells/L, a history of significant bleeding, and liver disease. The minor criteria include age ≥ 75 years, moderate CKD (eGFR 30–59 mL/min/1.73 m²), hemoglobin levels between 11 and 13 g/dL and 11–12 g/dL in men and women, respectively, and the use of non-steroidal anti-inflammatory drugs (NSAIDs). The eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (Corpataux et al., 2020).

Patients were categorized into HBR and non-HBR in the TICO-HBR trial by Y. J. Lee et al., 2022 throughuseduse of precise DAPT rating and ARC-HBR criteria (Urban et al., 2019; Costa et al., 2017). The correct DAPT score was based on five characteristics taken from massive randomized clinical research. The ARC-HBR was composed of 20 evidence-based medical criteria labeled as essential and minor categories. According to PCI registries, both tools are highly predictive of patients at risk for bleeding or ischemic complications following PCI (Costa et al., 2017; Natsuaki et al., 2019; Ueki et al., 2019; Urban et al., 2019; Ueki et al., 2020). In TICO-HBR, patients with HBR had an increased hazard of net adverse clinical events (NACE), including extreme bleeding and MACCE, compared to those without HBR.

Previous trials

The duration of the twin antiplatelet medicinal drugs and their association with the expanded bleeding threat have been the subject of several investigations. Costa et al. stated that patients with HBR with a prolonged DAPT period (12-24 months) experienced greater bleeding (Costa et al., 2017). Longer DAPT duration may also decrease the risk of ischemia in 20.8% of patients undergoing hard PCI. However, extended durations of DAPT appear to reduce ischemic events in patients without HBR subjected to complex or standard PCI. The decisions regarding the duration of DAPT following PCI should be primarily guided by bleeding risk, since the benefit has not been reported in HBR patients (Costa et al., 2019). An opportunity for ordinary DAPT has been proposed, which includes the prevention of aspirin use and the use of P2Y12 inhibitor monotherapy (Capodanno et al., 2018).

The GLOBAL LEADERS trial was the first large-scale randomized study to evaluate antiplatelet therapy strategies. In the general population, ticagrelor monotherapy for 23 months following only one month of DAPT did not lead to superior outcomes compared with the standard 12-month DAPT. Generally, 16% of the participants were classified as having HBR, defined by a PRECISE-DAPT score of 25 or higher (Vranckx et al., 2018 ; Gragnano et al., 2022). In contrast, the TWILIGHT trial focused on patients at an elevated risk

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of bleeding or ischemic events. Transitioning from DAPT to ticagrelor monotherapy after three months significantly reduced bleeding events without compromising antithrombotic efficacy compared to the continued use of ticagrelor in combination with aspirin (Baber et al., 2016; Mehran et al., 2019). The TICO trial showed that ticagrelor monotherapy following three months of DAPT led to fewer NACE events than ticagrelor-based DAPT continued for the full 12 months (B. K. Kim et al., 2020).

Net adverse clinical events (NACE)

NACE is a composite outcome of bleeding events and MACCE, comprising all-cause mortality, myocardial infarction, stent thrombosis, cerebrovascular accident, and target vessel revascularization (C. Kim et al., 2019). Ticagrelor monotherapy after three months of DAPT was associated with a lower incidence of bleeding. Furthermore, the PRECISE-DAPT score showed no significant interaction between the treatment strategy and bleeding risk. B. K. Kim et al., (2020) reported that switching to ticagrelor monotherapy after three months of DAPT reduced NACE compared to the standard 12-month ticagrelor-based DAPT. Among patients with ACS treated with DES, ticagrelor monotherapy was associated with a lower risk of NACE and bleeding events, regardless of bleeding risk status. The treatment showed consistent efficacy across the HBR and non-HBR groups.

Major adverse cardiac or cerebrovascular events (MACCE)

MACCE describes deaths, myocardial infarctions, and crashes resulting from the brain and coronary heart. This measure is used to assess the mortality risk and severity of ischemic outcomes. In the TWILIGHT-HBR subanalysis, patients were selected based on a broad spectrum of clinical and angiographic factors not related to HBR or ischemic risk classification criteria. Patients who were on long-term oral anticoagulants, had a history of stroke, were scheduled for surgery within 90 days, and had conditions associated with an increased risk of bleeding events were excluded from the trial. Although patients with thrombocytopenia (platelet count $<100\times10^9$/L), liver disease, or those on dialysis were excluded, some were included in the population. According to the ARC criteria, only 17.2% of patients were classified as HBR, which is lower than the proportions reported in previous large-scale community registries. This reflects the study design, which limited enrollment to patients suitable for long-term ticagrelor-based DAPT. In the TWILIGHT trial, two-thirds of the patients were subjected to PCI for non-ST-elevation acute coronary syndrome (NSTE-ACS), one-third had complex PCI procedures, and 37% had diabetes (Angiolillo et al., 2020; Baber et al., 2020; Dangas et al., 2020).

The use of ticagrelor after 3 months of DAPT lowers the risk of bleeding in patients with HBR and a higher risk of ischemia. This is mainly true when doctors recall a robust P2Y12 inhibitor. Additionally, the widespread distribution of critical and small threat factors is related to ARC-HBR validation (Cao et al., 2020; Ueki et al., 2020). Aspirin and strong P2Y12 inhibitors effectively increase antiplatelet motion, supporting the treatment benefits of ticagrelor (Baber et al., 2020; Armstrong et al., 2011).

Death/myocardial infarction and stent thrombosis

According to (Levine et al. (2016) and Valgimigli et al. (2018), patients who receive DES for ACS are typically prescribed 12 months of DAPT, including newer and more effective antiplatelet agents. In the TWILIGHT-HBR study, patients who received ticagrelor plus placebo showed similar rates of all-cause mortality, myocardial infarction, and stroke compared to those receiving the drug in combination with aspirin.

The TICO trial exclusively used an ultrathin bioresorbable polymer sirolimus-eluting stent (Orsiro; Biotronik AG, Bülach, Switzerland). This stent has been reported to have superior clinical outcomes owing to its ability to reduce thrombus formation, inflammation, and neointimal proliferation (Kandzari et al., 2017; Roguin et al., 2018; C. Kim et al., 2019; B. K. Kim et al., 2020). Similarly, DAPT with P2Y12 receptor inhibitors (ticagrelor) is better at reducing ischemic events than clopidogrel alone. In this context, a longer duration of DAPT is needed to forestall ischemic events with other DES and P2Y12 receptor blockers.

Y. J. Lee et al., 2022 reported that the most significant reduction in bleeding occurred among HBR patients who were not receiving anticoagulation therapy. This reduction was not accompanied by an increase in MACE in patients expected to receive anticoagulation therapy. Evidence from research, including HBR patients treated with DAPT for 1 to 3 months, shows that shorter DAPT durations do not significantly increase MACE or all-cause mortality. Stent thrombosis rates remained below 0.5% in the short- and standard-duration DAPT groups with extended follow-up. Previous research has reported that the risk increases within the first 30 days post-implantation and declines sharply thereafter. Therefore, short-term DAPT was sufficient to provide protection during the critical period. Consistently low rates of stent

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thrombosis were largely attributed to the use of modern DES, such as biodegradable polymer and second-generation DES, used in the TICO and TWILIGHT trials. Additional research supports the safety of shorter DAPT durations in patients subjected to complex PCI (Valgimigli et al., 2022).

Bleeding

European guidelines for the management of NSTE-ACS recommend ticagrelor monotherapy following three months of DAPT in selected low-risk patients. This recommendation is based on the TWILIGHT trial, which reported a relatively low incidence of adverse events within the first year of follow-up (Collet et al., 2021). The safety and efficacy of discontinuing aspirin and continuing ticagrelor monotherapy were evaluated during the initial phase of dual antiplatelet therapy (DAPT). Patients with HBR experienced more than three times the incidence of severe BARC type 3 or 5 compared to without those HBR. These individuals had approximately twice the rate of bleeding events (BARC types 2, 3, and 5). The research design contributed to an underestimation of the overall bleeding rates, but the risk of BARC type 3 or 5 bleeding in high-risk patients receiving ticagrelor monotherapy was significantly reduced from 5.0% to 1.6% (Koo et al., 2021).

Direct comparisons of clopidogrel and aspirin monotherapies for secondary prevention show that early discontinuation of aspirin may reduce the risk of bleeding beyond the first year after percutaneous coronary intervention (PCI). In patients with HBR, shortening the duration of DAPT to 1–3 months led to a statistically significant and clinically meaningful reduction in bleeding events. However, this benefit is less pronounced in non-HBR patients (Yin et al., 2019; Khan et al., 2020; Benenati et al., 2021; Benenati et al., 2022; Bainey et al., 2023).

Heterogeneity in patient populations

In the TWILIGHT HBR study, there was no evidence of heterogeneity (Escaned et al., 2021). In the TICO trials short (3-6 months) and long (12-24 months) duration DAPT was heterogeneous, with aspirin continuation after DAPT in previous studies; the definition was strictly limited to 3 months of DAPT with P2Y12 receptor inhibitor continuation versus 12 months of DAPT (Y. J. Lee et al., 2022). However, the TICO pre-specified subgroup analysis did not reveal heterogeneity in the effect of ticagrelor monotherapy after 3 months of DAPT, compared with 12 months of DAPT, on the primary outcomes, bleeding events, and MACCE across clinical presentations, including STEMI (S. J. Lee et al., 2021)

Intervention protocols

The risk of ischemic events is highest within the first two weeks and gradually declines. In contrast, the bleeding risk associated with prolonged DAPT is consistently elevated. This divergence supports the rationale for de-escalation strategies that tailor both the duration and selection of antiplatelet agents based on individual patient risk profiles to minimize bleeding without compromising ischemic protection in patients with ACS. Current clinical guidelines recommend the use of potent P2Y12 inhibitors, such as prasugrel or ticagrelor, in combination with aspirin. This is followed by a reduced DAPT duration (1–6 months) or a transition to clopidogrel in patients with HBR. Evidence from large-scale clinical trials, including TALOS-AMI and STOPDAPT-2 ACS, showed that short-term DAPT followed by monotherapy provided a favorable balance between ischemic protection and bleeding risk, reinforcing the value of individualized antiplatelet therapy (Singh et al., 2024).

Switching to ticagrelor monotherapy after a brief course of DAPT

Transitioning to ticagrelor monotherapy after a brief period of DAPT significantly reduced the bleeding risk without increasing the risk of ischemic events in post-PCI patients. Studies such as ULTIMATE-DAPT and TWILIGHT have shown that ticagrelor monotherapy leads to a marked reduction in bleeding complications compared to continued DAPT, while maintaining comparable rates of ischemic events. These benefits are consistent across diverse patient subgroups experiencing reduced bleeding risk without an increase in ischemic complications. Similar outcomes have been observed across various types of DES, as well as in high- and low-bleeding-risk populations.

Ticagrelor monotherapy effectively lowers bleeding rates without adversely affecting the risks of death, myocardial infarction, or stroke in patients with NSTE-ACS and stable coronary artery disease (CAD). The TICO trial reported that switching to ticagrelor monotherapy after 3 months of DAPT in patients with ACS treated with new-generation sirolimus-eluting stents reduced adverse and bleeding events compared with 12-month DAPT, with no significant difference in ischemic risk. Furthermore, comparable results have been reported in patients with STEMI and other subgroups (Angiolillo et al., 2021; Escaned et al., 2021; S. J. Lee et al., 2021; Y. J. Lee et al., 2022; Chiarito et al., 2022; Mendieta et al., 2023)

According to T-PASS research, patients with ACS who received DES and transitioned to ticagrelor

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monotherapy after a short course of DAPT experienced fewer adverse and bleeding events. Collectively, these results suggest that ticagrelor monotherapy following short-term DAPT is a safer and more effective strategy than prolonged DAPT across a broad spectrum of post-PCI patient populations (Singh et al., 2024).

Follow-up durations

In the TWILIGHT-HBR study, follow-up occurred 1 month after randomization via telephone and in-person at 6 and 12 months after randomization (Escaned et al., 2021). Meanwhile, for the TICO trial, patient follow-up was 365 days for the primary outcome (net adverse clinical events) (S. J. Lee et al., 2021).

Clinical implications and research limitations

The TWILIGHT study suggests that ticagrelor monotherapy following a short course of DAPT provides greater benefits to patients with HBR than to those at a lower risk. Patients with HBR experienced a high reduction in bleeding events, leading to an overall lower net clinical risk. Therefore, this systematic review evaluated the safety of bleeding-reduction strategies and the potential for further shortening of the duration of DAPT, as well as optimizing outcomes through antiplatelet regimens (Voudris & Feldman, 2023). However, this study had several limitations, as the TICO-HBR subgroup analysis was not prespecified in the original trial design. The TWILIGHT-HBR study was restricted to data available within the trial. The ARC-HBR and PRECISE-DAPT criteria used to identify HBR were not available at the time of the trials, leading to retrospective application for hypothesis generation and post-hoc analysis. This highlights the need for future prospective and randomized studies. Most of the included evidence stemmed from subgroup analyses of HBR patients in larger randomized controlled trials. Additionally, not all ARC-HBR criteria could be assessed because of the relatively small number of HBR patients compared to non-HBR patients. This study lacked sufficient statistical power to detect clinically meaningful differences in ischemic events, and the results were limited to ticagrelor, with no applicability to other P2Y12 inhibitors.

Different studies have been conducted in South Korea, India, the United States, and certain parts of Europe. This highlights the importance of gathering data from diverse populations in countries such as Indonesia. Therefore, further investigation is needed to evaluate ticagrelor monotherapy following three months of DAPT in HBR patients. The HBR subgroup sample reported the need for more comprehensive research, including larger patient cohorts. Despite these limitations, clinicians consider ticagrelor monotherapy after a short DAPT course a valuable method for enabling more personalized treatment strategies and potentially offering better therapeutic options.

CONCLUSION

The prevention of aspirin and administration of ticagrelor monotherapy after 3 months of DAPT lowered the risk of the most important or clinically significant bleeding in high-risk patients undergoing PCI and DES. This could increase the risk of ischemic events, such as myocardial infarction and stroke. Treatment lasting for 3 months was more effective than the usual 12-month ticagrelor-based DAPT in lowering the incidence of NACE and bleeding events in HBR and non-HBR patients. The results showed that ticagrelor was used to lower bleeding after PCI in patients experiencing bleeding.

AUTHOR CONTRIBUTIONS

Conceptualization, B.S.Z.; Methodology, E.N.A.; Software, B.S.Z.; Validation, B.S.Z.; Formal Analysis, E.N.A.; Investigation, B.S.Z.; Resources, B.S.Z.; Data Curation, B.S.Z., E.N.A.; Writing - Original Draft, B.S.Z., E.N.A.; Writing - Review & Editing, B.S.Z., E.N.A.; Visualization, B.S.Z., E.N.A.; Supervision, B.S.Z.; Project Administration, B.S.Z., E.N.A.; Funding Acquisition, E.N.A.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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Medication Adherence, Quality of Life, and Rehospitalization in Post-Acute Coronary Syndrome Patients

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Abstract

Background: *Post-ACS patients often face an increased likelihood of mortality, rehospitalization, and diminished quality of life as a consequence of poor medication adherence.* ***Objective:*** *This study aimed to evaluate the level of adherence to medication in post-ACS patients and its relationship with quality of life and rehospitalization rates.* ***Methods:*** *A cross-sectional, observational, single-center, prospective study conducted at Universitas Airlangga Teaching Hospital, Surabaya. Compliance with medication was evaluated using the Adherence Refill Medication Scale-7 (ARMS-7) questionnaire, while quality of life was measured using the Short Form-36 (SF-36) Quality of Life questionnaire. Rehospitalization rates were obtained through direct interviews and medical record observations within 45 days of hospital discharge.* ***Results:*** *In total, 39 patients participated in this study, with overall adherence rates of 35.89% for all prescribed medications, 53.85% for antiplatelets, 38.46% for statins, 55.56% for beta-blockers, and 58.06% for ACEIs/ARBs. Among the quality-of-life dimensions, social functioning had the highest score (93.01 ± 15.89), whereas physical role functioning had the lowest score (40.39 ± 35.18). Within 45 days of hospital discharge, 26% of the patients experienced rehospitalization. Statistical analysis indicated a positive correlation between adherence to all prescribed medications and physical role functioning in relation to QoL ($p = 0.038$). In addition, overall medication adherence was negatively correlated with the risk of rehospitalization ($p = 0.019$).* ***Conclusion:*** *Total medication adherence was associated with improved physical function and rehospitalization events. Providing education can lead to better therapeutic outcomes, improved quality of life, and reduced rehospitalization in patients.*

Keywords: *acute coronary syndrome, medication adherence, quality of life, rehospitalization*

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INTRODUCTION

Acute Coronary Syndrome (ACS), such as Unstable Angina (UA) and Myocardial Infarction (MI), is a manifestation of ischemic cardiovascular conditions, which are among the primary contributors to fatal cardiovascular outcomes. According to data reported by the Global Burden of Disease (GBD), this condition accounts for approximately 49.2% of deaths. The 2019 GBD data also reported that 197 million patients worldwide were diagnosed with ischemic cardiovascular conditions, with approximately 5% of these cases leading to death. Other reports show that one American experiences an MI every 40 s, with 109,199 deaths recorded annually (Tsao et al., 2023). Data from the Jakarta Acute Coronary Syndrome (JAC) Registry showed that non-ST-elevation acute coronary syndrome was detected in 1223 patients in 2013, 1915 patients in 2007, and 1925 patients in 2010 (Yusniawati et al., 2018).

Individuals diagnosed with ACS often face an elevated likelihood of mortality, rehospitalization, and a decline in quality of life (Oliveira et al., 2019; Dou et al., 2022). According to research on ACS patient rehospitalization in Indonesia, the rehospitalization rate for ACS patients was approximately 36.7%. Medical issues and patient education impacted this incident. (Romalina et al., 2018). A study at Hasan Sadikin Central Hospital in Bandung indicated that 48% of patients with acute coronary syndrome experienced a diminished quality of life significantly associated with the physical domain (Nurhamsyah et al., 2021). These adverse outcomes can be prevented by ensuring proper implementation of post-ACS therapeutic management. Pharmacological and non-pharmacological treatments are typically used to manage post-ACS patients. As outlined in the 2020 European Society of Cardiology (ESC) recommendations, the primary classes of medications recommended for pharmacological therapy include antiplatelets, statins, and beta-blockers (Collet et al., 2021). In addition, angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin II receptor blockers (ARBs) are also suggested for individuals diagnosed with ACS and heart failure (O'Gara et al., 2013). Since post-ACS treatment is a long-term therapy, medication adherence is a major factor influencing the effectiveness of cardiovascular treatment (Bansilal et al., 2016; Shang et al., 2019).

Several studies have concluded that poor compliance with prescribed medications is a key factor affecting the success of efforts to prevent rehospitalization and the decline in quality of life (Zhang et al., 2014; Suhail et al., 2021; Thomson et al., 2020). In Indonesia, studies on post-ACS patients are very limited, particularly regarding the association between adherence to medication and treatment results, such as rehospitalization rates and quality of life. Therefore, this study aimed to investigate the correlation between adherence to prescribed medications and QoL in post-ACS patients receiving treatment at the Cardiology Clinic of Universitas Airlangga Hospital (RSUA) in Surabaya.

MATERIALS AND METHODS

Materials

The inclusion criteria were individuals aged ≥ 18 years who had received a diagnosis of Non-ST-Elevation Acute Coronary Syndrome (NSTEACS) or ST-Elevation Myocardial Infarction (STEMI), were discharged from the hospital, and attended follow-up visits at the Cardiology Clinic of Universitas Airlangga Hospital. Patients who consented to participate and whose caregivers were literate and had no communication difficulties were included in the study. The exclusion criteria included patients with unknown history of rehospitalization.

Tools

Complications with medication were assessed using the Adherence to Refill Medication Scale-7 (ARMS-7) questionnaire. The ARMS-7 scale followed a 4-point Likert-type rating, consisting of four response options: "Never" (1 point), "Sometimes" (2 points), "Often" (3 points), and "Always" (4 points). The total score ranged from 7 to 28, with a lower score indicating better adherence. In this study, adherence was categorized into adherent and non-adherent groups; those who scored exactly 7 were classified as adherent, while those who scored greater than 7 were classified as non-adherent. The ARMS-7 questionnaire has undergone validity and reliability testing with r=0.906 and was considered good with Cronbach's alpha >0.72. Overall quality of life was evaluated using the Short Form-36 (SF-36) Quality of Life Questionnaire, which consists of eight dimensions covering both physical and mental aspects. The questionnaire included 36 items evaluating the following dimensions: physical capability (10 questions), role limitations due to physical conditions (4 questions), bodily discomfort (2 questions), perceptions of general health status (6 questions), social interactions (4 questions), role limitations due to emotional challenges (3 questions), vitality (2 questions), and mental health (5 questions). All dimensions were scored on a scale ranging from 0 to 100, with higher values

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reflecting improved overall medication adherence. The internal consistency of the Indonesian version of the SF-36 questionnaire, measured using Cronbach's alpha, was rated as good, with a value of >0.70.

Method

Study design and data collection

This non-interventional, single-center, prospective, cross-sectional study was conducted at Universitas Airlangga Hospital (RSUA), Surabaya, from June to August 2023. The independent variable in this study was patient medication adherence. The dependent variables were patients' quality of life and the incidence of rehospitalization. The sample was collected prospectively using a quantitative approach that required numerical data. In addition, the cross-sectional data collection method was used, suggesting that data were obtained at a specific time to analyze patient compliance with prescribed medications and overall quality of life. Rehospitalization was assessed through direct interviews and a review of medical records 37–45 days post-hospitalization.

RESULTS AND DISCUSSION

Sociodemographic characteristics data

A total of 39 patients fulfilled the eligibility requirements and consented to participate in this study. The sociodemographic attributes and clinical data of the study participants are displayed in Table 1.

Table 1. Sociodemographic characteristics and clinical data of patients with ACS

Variables	Total (n=39)	Adherent* (n=14)	Non-adherent* (n=25)	*p-value*
Age, years (mean ± SD)	56,43±12,90	54,29±13,19	57,64±12,85	
Age range n (%)				0,443[a]
- ≤50 years	11 (28,21)	5 (35,71)	6 (24)	
- >50 years	28 (71,79)	9 (64,29)	19 (76)	
Gender n (%)				
- Male	22 (56,41)	9 (64,29)	13 (52)	0,458[c]
- Female	17 (43,59)	5 (35,71)	12 (38)	
Marriage status n (%)				
- Married	29 (74,36)	12 (85,71)	17 (70,83)	0,279[d]
- Widow/Widower	10 (25,64)	2 (14,29)	8 (29,17)	
Occupation n (%)				
- None/retired	25 (64,10)	10 (71,43)	15 (60)	
- Private worker	9 (23,08)	3 (21,43)	9 (36)	0,807[c]
- Entrepreneur	4 (10,26)	1 (7,14)	3 (12)	
- Civil servant/police	1 (2,56)	0	1 (4)	
Education level n (%)				
- Primary	10 (25,64)	2 (14,38)	8 (32)	
- Middle high	6 (15,38)	4 (28,57)	2 (8)	0,308[c]
- High school	12 (30,77)	4 (28,57)	8 (32)	
- University	11 (28,21)	4 (28,57)	7 (28)	
BMI (*mean*±SD)	25,02±3,94	25,28±3,84	24,87±4,06	
BMI Status n (%)				0,756[a]
- Underweight	3 (7,7)	1 (7,14)	2 (8)	
- Normal weight	18 (46,2)	6 (42,86)	12 (38)	
- Overweight	13 (33,3)	5 (35,71)	8 (32)	
- Obesity	5 (12,8)	2 (14,29)	3 (12)	
Comorbidity n (%)				
- Hypertension	29 (74,36)	10 (71,43)	19 (76)	1,000[d]
- Diabetes Mellitus	12 (30,77)	4 (28,57)	8 (32)	1,000[d]
- Dyslipidemia	9 (23,08)	3 (21,43)	6 (24)	1,000[d]
- Heart failure	21 (53,85)	7 (50)	14 (56)	0,718[c]
Diagnosis on discharged n (%)				
- Unstable angina	16 (41,03)	5 (35,71)	11 (44)	0,854[c]
- NSTEMI	16 (41,03)	6 (42,86)	10 (40)	0,854[c]
- STEMI	7 (17,94)	3 (21,43)	4 (16)	0,854[c]
History of Stent Insertion n (%)				
- Yes	19 (48,72)	10 (71,43)	9 (36)	0,034[c]
- No	20 (51,28)	4 (28,57)	16 (64)	0,034[c]

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Variables	Total (n=39)	Adherent* (n=14)	Non-adherent* (n=25)	*p-value*
Disease duration n (%)				
- ≤ 1 year	27 (69,23)	12 (85,71)	15 (60)	0,151[d]
- > 1 year	12 (30,77)	2 (14,49)	10 (40)	0,151[d]
Smoking history				
- Yes	22 (56,41)	9 (64,29)	13 (52)	0,458[c]
- No	17 (43,59)	5 (35,71)	12 (48)	0,458[c]

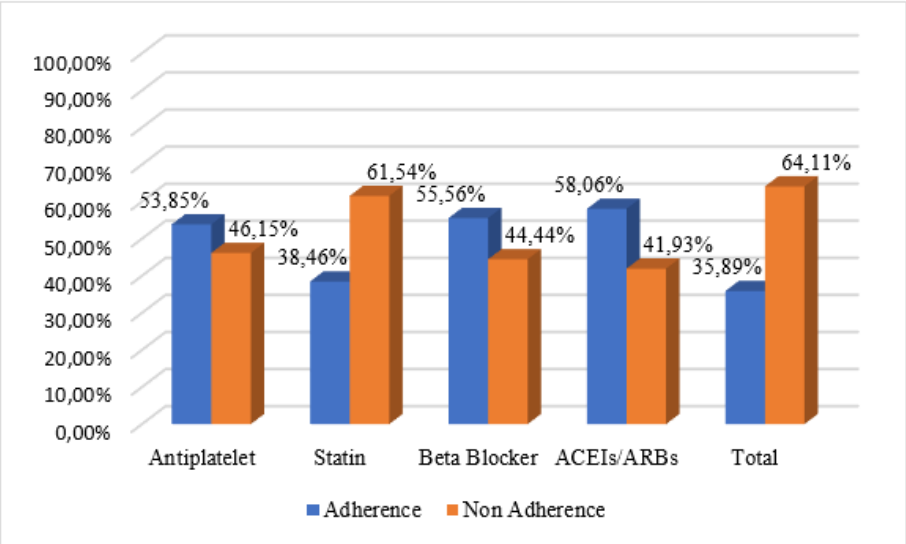

Figure 1. Medication adherence data of post-acute coronary syndrome patients based on the arms questionnaire

Among the patients who agreed to participate, 56.41% were male and 43.59% were female. The average patient age was 56.43 ± 12.90 years, with the oldest being 87 years and the youngest 31 years. A total of 29 participants (74.36%) were married, while 10 (25.64%) were widowed or divorced. The highest level of education attained was high school (30.77%), followed by higher education (28.21%), while the remaining patients had completed only elementary or middle school education. In addition, the majority of patients (64.1%) were retired, while the rest were employed.

The most common diagnosis at hospital discharge was NSTEMI or Unstable Angina (UA), affecting 16 patients (41.03%), while only a small proportion were diagnosed with STEMI (17.94%). Approximately 90% had comorbidities, with hypertension being the most prevalent (74.36%). In addition, 12 participants (30.77%) had a history of diabetes mellitus, and 9 (23.08%) had a history of dyslipidemia. A total of 21 patients (53.85%) also had other heart diseases, particularly heart failure. Regarding BMI classification, the majority had an ideal BMI (46.2%), while 33.3% were overweight, 12.8% were obese, and 7.7% were underweight. The duration of heart disease was ≤1 year in most patients (69.23%, 27 patients), while 30.77% (12 patients) had heart disease for more than 1 year. More than 50% of the participants were former smokers (56.4%), 19 (48.72%) had undergone stent placement, and 20 had not undergone or were not scheduled for stent placement.

Medication adherence data based on the adherence to refills and medications scale (arms) questionnaire

This study showed that the overall patient adherence rate was 35.89%. The adherence rates for specific medication classes were as follows: 53.85% for antiplatelet therapy, 38.46% for statin therapy, 55.56% for beta-blockers, and 58.06% for ACEIs/ARBs (Figure 1). Non-adherence across all medication categories is displayed in Table 2. The primary reason for non-adherence, based on the adherence to refill indicator, was running out of medication stock (25.6%). The most common cause of non-adherence, based on the adherence to medication indicator, was forgetting to take the medication (25.6%).

Quality of life assessment data based on the short-form 36 (sf-36) questionnaire

The overall well-being of the patients in this study was obtained from structured interviews using the SF-36 questionnaire. Table 3 outlines the distribution of

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patients' QoL data, showing that patients had the highest level of medication adherence in the social interaction dimension, with an average score of 93.01±15.89. The lowest-scoring dimension was physical role limitation, with an average score of 40.39±35.18.

Rehospitalization incidence in post-acute coronary syndrome patients

After a 45-day observation period following hospital discharge, data showed that 10 patients (26%) were readmitted due to a cardiovascular disease event, whereas the remaining 29 (74%) did not experience rehospitalization (Figure 2). The primary diagnoses associated with rehospitalization included Acute Coronary Syndrome (ACS), Acute Decompensated Heart Failure (ADHF), and a combination of ADHF and ACS.

Table 2. Frequency distribution of arms questionnaire total adherence items

n = 39 Patients						
Question Number	**Question**	**Never**	**Sometimes**	**Often**	**Always**	**Average score**
Indicator: Adherence to Refill (Compliance in Refilling Prescriptions)						
3	How often do you forget to refill your heart medication prescription? (Unintentional)	33 (94,6%)	4 (10,3%)	1 (2,6%)	1 (2,6%)	1,23±0,63
4	How often do you run out of your heart medication? (Intentional)	21 (53,8%)	10 (25,6%)	7 (17,9%)	1 (2,6%)	1,69±0,86
7	How often do you plan and refill your heart medication prescription before it runs out? (Intentional)	1 (2,6%)	3 (7,7%)	3 (7,7%)	32 (82,1%)	1,31±0,73
Indicator: Adherence to Medication (Compliance in Taking Medication						
1	How often do you forget to take your heart medication? (Unintentional or conscious)	22 (56,4%)	10 (25,6%)	6 (15,4%)	1 (2,6%)	1,64±0,84
2	How often do you decide not to take your heart medication? (Intentional)	34 (87,2%)	0 (0%)	4 (10,3%)	1 (2,6%)	1,28±0,76
5	How often do you skip taking your heart medication when you feel better? (Intentional)	32 (82,1%)	4 (10,3%)	2 (5,1%)	1 (2,6%)	1,28±0,69
6	How often do you skip taking your heart medication when you still feel unwell? (Intentional)	36 (92,3%)	1 (2,6%)	1 (2,6%)	1 (2,6%)	1,69±0,86

Table 3. Quality of life data based on the sf-36 questionnaire

Dimension of SF-36 (Scores = 0-100)	Mean N = 39 patients	Standard Deviation
Physical Function	65,13	26,94
Physical Role	40,39	35,18
Pain	72,69	37,01
General Health	62,82	18,54
Social Function	93,01	15,89
Emotional Well-being	56,84	31,47
Vitality	77,86	18,21
Mental Health	79,59	17,79

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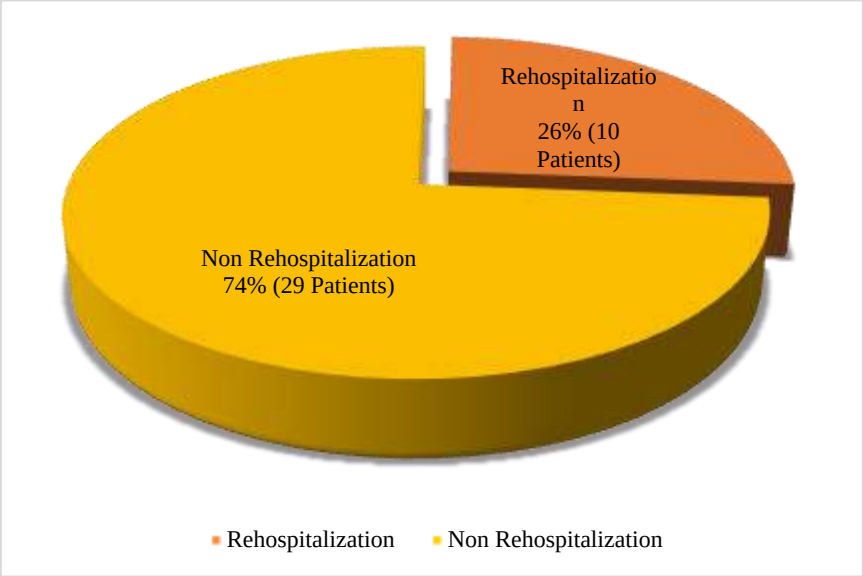

Figure 2. Rehospitalization incidence in post-acute coronary syndrome patients

Table 4. Analysis of the association between medication adherence and quality of life in patients post-acute coronary syndrome at the cardiology clinic of UNAIR Hospital

Quality of life Dimension	Adherent (Mean ± SD)	Non-Adherent (Mean ± SD)	p-value
Total of Adherence (n=39)			
Physical Function	68,93±18,73	63±30,75	0,758[b]
Physical Role	55,36±32,75	32±34,25	0,046[b]
Pain	71,96±24,24	73,1±28,92	0,590[b]
General Health	63,89±18,54	62,15±18,91	0,628[b]
Social Function	93,33±17,59	92,81±15,31	0,451[b]
Emotional Well-being	59,52±32,49	55,33±31,45	0,737[b]
Vitality	81,79±15,39	75,67±19,56	0,375[b]
Mental Health	79,42±17,17	79,68±18,47	0,917[b]

Table 5. Analysis of the association between medication adherence and rehospitalization in patients post-acute coronary syndrome at the cardiology clinic of UNAIR Hospital

Adherence Level	No Rehospitalization	Rehospitalization	Total	p-value (CI 95%)
Total Adherence (n=39)				
Non-Adherent (%)	22 (88%)	3 (12%)	24 (100%)	0,019[d]
Adherent (%)	7 (50%)	7 (50%)	15 (100%)	(1,484-36,239)
Total (%)	29 (74,4%)	10 (25,6%)	39 (100%)	

Analysis of the association between medication adherence and quality of life in patients post-acute coronary syndrome

Bivariate analysis revealed a significant relationship between total medication adherence and the physical role limitation dimension of well-being (p = 0.038). The average score in the physical role limitation dimension was greater in patients who adhered to their medication regimen than in those who did not (Table 4).

Analysis of the association between medication adherence and rehospitalization in patients post-acute coronary syndrome

The relationship between total adherence and rehospitalization was analyzed using the bivariate Fisher's Exact Test. In addition, the bivariate analysis results in Table 5 indicate a significant association between total treatment adherence and rehospitalization (p = 0.019).

Analysis of the association between drug category and rehospitalization in patients post-acute coronary syndrome

The relationship between each medication category and rehospitalization was analyzed using the bivariate Fisher's Exact Test. The results of the bivariate analysis in Table 6 show no significant relationship between each medication and rehospitalization (p > 0.05). The statin group could not be tested because all patients were using statins; therefore, there was no comparison group.

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Table 6. Analysis of the association between drug category and rehospitalization in patients post-acute coronary syndrome at the cardiology clinic of UNAIR Hospital

Drug Category (n (%))	Rehospitalization	No Rehospitalization	Total	*p-value*
Beta Blocker				
No (n (%))	0	2 (100%)	2 (100%)	
Yes (n (%))	10 (27 %)	27 (73%)	37 (100%)	1.000[d]
Total (n (%))	10 (25.6%)	29 (74.4%)	39 (100%)	
ACEi/ARB				
No (n (%))	0	8(100%)	8 (100%)	
Yes (n (%))	10 (32.3%)	21 (67.7%)	31 (100%)	0.086[d]
Total (n (%))	10 (25.6%)	29 (74.4%)	39 (100%)	
Statin				
No (n (%))	0	0	0	
Yes (n (%))	10 (25.6%)	29 (74.4%)	39 (100%)	.
Total (n (%))	10 (25.6%)	29 (74.4%)	39 (100%)	
CYP Inhibitor				
No (n (%))	2 (14.3%)	12 (85.7%)	14 (100%)	
Yes (n (%))	8 (32.0%)	17 (68.0%)	25 (100%)	0.279[d]
Total (n (%))	10 (25.6%	29 (74.4%)	39 (100%)	
ASA				
No (n (%))	1 (50%)	1 (50%	2 (100%)	
Yes (n (%))	9 (24.3%)	28 (75.7%)	37 (100)	0.452[d]
Total (n %))	10 (25.6%)	29 (74.4%)	39 (100%)	

Notes:
[a] Statistical test conducted using the independent t-test
[b] Statistical test conducted using the Mann-Whitney test
[c] Statistical test conducted using the Chi-square test
[d] Statistical test conducted using Fisher's Exact Test

Discussion

An analysis of medication adherence concerning QoL and rehospitalization was conducted on post-ACS participants at the Cardiology Outpatient Clinic of RSUA. Rehospitalization events were observed 37–45 days after hospital discharge. In addition, medication adherence and quality of life assessments were conducted through structured interviews using the ARMS-7 and SF-36 questionnaires, which were validated for their reliability. In total, 39 patients participated in this study.

The average age of the patients was 56.43±12.9 years, ranging from 31 to 87 years, with 28.21% being under 50 years old. These findings were consistent with studies in Indonesia reporting an average ACS patient age of 56.9±11.7 years, as well as European studies showing that 26.3% of ACS patients were < 50 years old (Adam et al., 2021; Zanchin et al., 2022). Most patients were men (56.4%), married (74.4%), and unemployed or retired. In addition, the majority of patients had a high school education (30.8%), followed by university graduates (28.2%), and 25.64% had only completed primary education. Several studies conducted in Indonesian hospitals also reported that ACS was more prevalent in men than in women (Adam et al., 2021; Qothi et al., 2021; Pramudyo et al., 2022). European reports have shown that married patients exhibit better physical and social QoL (Lisiak et al., 2016). Other studies have indicated that patients with low incomes experienced more frequent rehospitalization and that employment status influenced ACS patients' overall quality of life, especially regarding physical function, emotional well-being, and general health. Most patients had an ideal BMI (46,2%). A multicenter study by Pocock et al. (2021) reported that obese patients had lower well-being scores than individuals with ideal body weight (Pocock et al., 2021).

As many as 89.7% of the patients (35 participants) had comorbidities, with hypertension being the most prevalent (74.36%), followed by heart failure and diabetes. Hypertensive patients have twice the risk of ACS compared to those without hypertension, due to high blood pressure causing vascular damage through oxidative and mechanical stress (Rathore, 2018). These comorbidities have been linked to 30-day rehospitalization events and a decline in quality of life (Tsoulou et al., 2023; Hess et al., 2016). At hospital discharge, 41.03% of the patients were diagnosed with NSTEMI and UA, with 16 and 19 participants (48.7%) having undergone percutaneous coronary intervention

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(PCI). Studies have indicated that patients with UA experience reduced well-being and an increased likelihood of rehospitalization (Aljabery et al., 2022; Rathore, 2018). Tripathi et al. and Tsoulou et al. suggested that patients with ACS who underwent PCI exhibited improved overall quality of life and lower rehospitalization rates within 30 days post-discharge (Tripathi et al., 2019; Tsoulou et al., 2023). In addition, 56.4% of patients had a history of smoking, which exacerbated heart disease severity by increasing the risk of MI and early atherosclerosis, influencing rehospitalization rates (Oliveira et al., 2019; Rathore, 2018).

This study aimed to analyze post-ACS patients' medication adherence to antiplatelets, statins, beta-blockers, and ACEIs/ARBs, as illustrated in Figure 1. The overall medication adherence rate among patients with ACS was 35.89%. This percentage was lower than that in studies conducted in developed countries such as the United States (U.S.) and Germany, where ACS patients' adherence to all cardiovascular medications exceeded 70% (Goss et al., 2017; Mathews et al., 2018). Several factors contributed to the discrepancy in adherence rates between this study and previous studies, such as (1) differences in healthcare systems, where hospitals in developed countries provided higher-quality services, leading to better patient outcomes; (2) limited healthcare infrastructure in Indonesia, where logistical challenges and insurance coverage barriers hindered adherence; and (3) education and health literacy levels, as 25.64% of patients had only completed primary education, and lower health literacy was associated with poorer medication adherence. A study in the U.S. reported that hospitals with high-quality services correlated with better patient adherence to follow-ups and treatments, thereby improving clinical outcomes (Mathews et al., 2018). Higher health literacy enables patients to better understand and follow medical instructions, leading to improved adherence (Luu et al., 2019).

The ARMS questionnaire identified whether non-adherence was due to patient behavior in taking medications or filling prescriptions. Questions 1, 2, 5, and 6 assessed medication-taking behavior, and questions 3, 4, and 7 evaluated prescription-filling behavior. The primary reason for non-adherence to medication was that patients forgot to take their medications. In terms of prescription filling, the major reasons for non-adherence were running out of medication and failing to attend follow-up appointments. These findings were consistent with previous studies identifying forgetfulness and missed follow-ups as major adherence barriers for patients with ACS. Other reasons for non-adherence included forgetting to take medication due to a lack of reminders from family, forgetting midday doses due to work, stopping medication due to the absence of symptoms, and discontinuing medication due to boredom without consulting healthcare providers. Stopping medication due to unpleasant side effects, receiving atorvastatin for only 7 days, and follow-up appointments being delayed beyond 30 days due to hospital holiday schedules were also factors of non-adherence.

The next variable examined was rehospitalization due to cardiovascular events. The 37 to 45-day rehospitalization rate for post-ACS patients at RSUA's Cardiology Clinic was 26% (Figure 2), consistent with Asian studies reporting rehospitalization rates exceeding 25% (Karim et al., 2018). However, this percentage was higher than the ACS rehospitalization rate in the U.S., which was recorded at less than 20% over a 30-day observation period (Rymer et al., 2019). In this study, a correlation was found between total medication adherence and rehospitalization during the 45-day observation period ($p = 0.019$). This finding contrasts with those of previous studies, which concluded that high medication adherence reduces rehospitalization in patients with ACS (Murad et al., 2022). Bivariate analysis was conducted to evaluate the relationship between medication categories and the incidence of rehospitalization in patients with post-acute coronary syndrome. Based on the test results, no statistically significant relationship was found between medication use and rehospitalization ($p > 0.05$). These findings suggest that drug category did not represent a confounding factor for the incidence of rehospitalization in this study.

In addition to adherence and rehospitalization events, another variable observed in this study was the patients' overall QoL. The quality of life of post-ACS patients was measured using the Short-Form 36 (SF-36) questionnaire, which has been widely applied to evaluate quality of life among patients with chronic diseases, including ACS, across various dimensions, such as physical capability, physical role, bodily discomfort, general health, social interactions, emotional role, vitality, and mental health. In this study, the score for each dimension in the SF-36 varied between 0 and 100, with higher scores reflecting greater medication adherence.

The physical function dimension scored 65.13 ± 26.94, reflecting the patients' ability to perform daily

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activities. This finding is consistent with a study conducted in Greece involving patients with ACS undergoing PCI (Tsoulou et al., 2023). The physical role dimension, with a score of 40.39 ± 35.18, indicated limitations in the patients' activities due to a decline in physical health. Previous studies also reported that the physical role dimension score during the 30-day post-ACS period was 40.3 (de Carvalho Costa et al., 2022). The pain dimension scored 72.69 ± 37.01, which was consistent with prior studies on patients with STEMI 6 months after undergoing PCI (Tsoulou et al., 2023). In addition, the general health dimension scored 62.82 ± 18.54, which was higher than the findings of a study in Brazil that reported a lower general health score in ACS patients. This dimension evaluates patients' overall perception of their health and their belief in whether their health has improved or worsened (de Carvalho Costa et al., 2022). The emotional role dimension in this study scored 56.84 ± 31.47, consistent with previous studies. This dimension reflects the emotional impact on the patients' work or other activities (Tsoulou et al., 2023; de Carvalho Costa et al., 2022). The vitality dimension scored 77.86 ± 18.21, which is better than that reported in previous studies. This dimension illustrates the extent to which patients feel energetic in their activities or how frequently they feel fatigued while working (Tsoulou et al., 2023; de Carvalho Costa et al., 2022; Serrano-Rosa et al., 2021). The social interaction and mental health dimensions achieved the highest scores (93.01 ± 15.89 and 79.59 ± 17.79, respectively). Previous studies have also noted that patients experiencing their first hospitalization had the highest overall QoL scores in the social interactions and mental health dimensions (Anchah et al., 2017; de Carvalho Costa et al., 2022).

The statistical test results in Table 4 indicate a variation in overall QoL scores between adherent and non-adherent patients. Patients who adhered to their treatment generally exhibited a better overall quality of life than those who were less adherent. Total medication adherence was significantly correlated with overall QoL scores in post-ACS patients (p = 0.046). This finding is aligned with research on ACS patients at a teaching hospital in Bandung, which concluded that medication adherence in post-ACS patients was related to overall QoL (p-value = 0.009) (Ramadhan et al., 2022). Similarly, a study in Korea stated that overall patient adherence to medication enhanced the overall quality of life. This is because patients who complied with treatment also tended to follow lifestyle changes, including adhering to a low-fat, low-sodium diet, participating in regular physical exercise, and managing stress, which ultimately affected their quality of life (Lee et al., 2018).

In this study, those who were more adherent to their overall treatment had higher scores in the physical role dimension than non-adherent patients (55.36 ± 32.75 vs. 32 ± 34.25; p = 0.046). Adherence to prescribed medications was linked to the benefits received by patients. Regular medication intake reduces the risk factors for ACS and prevents major adverse cardiovascular events (MACE), including rehospitalization due to recurrent ACS episodes or other cardiovascular events, thereby improving the patients' quality of life (Suhail et al., 2021; Murad et al., 2022; Lee et al., 2018). Furthermore, no significant relationship was found between medication adherence and the bodily discomfort dimension of patients' overall QoL. The pain dimension reflects the extent to which patients experience pain and whether any activities are restricted. Patients with ACS who experienced MACE, including recurrent attacks or relapses. The intensity and recurrence of pain in patients are influenced not only by medication adherence but also by various factors, including diabetes, disease severity, left ventricular dysfunction, and patients who have undergone PCI (Sadrnia et al., 2013; Vafaie et al., 2020).

In this study, most patients reported that their pain intensity significantly decreased after receiving treatment, both in the adherent and non-adherent groups. Consequently, there was no significant difference in pain dimension scores between the two groups. Another possible explanation for this result is that some patients had already undergone PCI. Patients who had undergone PCI tended to experience significantly less pain than before, even when they were less adherent to their medication (Pujowaskito et al., 2021). The SF-36 quality-of-life questionnaire was used in this study to assess general pain rather than the frequency of heart attacks experienced by patients, unlike the Seattle Angina Questionnaire (SAQ).

This study presents several novelties compared to other studies in Indonesia. First, it analyzed both total medication adherence and adherence by drug category in post-ACS patients, allowing for the identification of adherence levels for each drug class and providing a foundation for developing educational strategies focused on improving medication adherence in post-ACS patients. Second, in addition to identifying patient issues through questionnaires, this study presented data on patient-reported barriers to taking or refilling medications. These findings could serve as a reference

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for pharmacists to address medication adherence barriers. Finally, this study provided insights into the association between adherence to medication, rehospitalization, and QoL among post-ACS patients, which has not been widely studied in Indonesia.

The main limitation of this study was the use of a generic quality-of-life questionnaire. While this questionnaire has been extensively applied to assess overall medication adherence in various chronic diseases, it was not specifically developed to assess the overall quality of life in coronary artery disease patients with CAD. For instance, it did not capture the frequency of relapses in patients with post-ACS. In addition, the sample size was smaller compared to similar studies. Owing to the short duration of this study, a long-term cohort study is recommended to assess adherence over multiple observation points over time.

CONCLUSION

The overall rate of medication adherence was 35.89%. Medication adherence was associated with improved physical function and rehospitalization events. Providing education to patients and families about acute coronary syndrome and its treatment can lead to better therapeutic outcomes, improve patients' quality of life, and reduce rea.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y., W.P.N., H.S., D.M.N.R.; Methodology, Y., W.P.N., H.S., D.M.N.R.; Software, Y., W.P.N., H.S., D.M.N.R.; Validation, Y., W.P.N., H.S., D.M.N.R.; Formal Analysis, Y., W.P.N., H.S., D.M.N.R.; Investigation, Y., W.P.N.; Resources, Y., W.P.N.; Data Curation, Y., W.P.N., H.S., D.M.N.R.; Writing - Original Draft, Y., W.P.N., F.M.B.; Writing - Review & Editing, Y., W.P.N., F.M.B.; Visualization, Y., W.P.N., F.M.B.; Supervision, W.P.N., H.S.; Project Administration, Y., W.P.N., H.S.; Funding Acquisition, W.P.N.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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Implementing Electronic Forms for Prescription Screening during Pregnancy in Outpatient Obstetric Clinic

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Abstract

Background: *Prescription screening is an assessment of the suitability of a prescription performed by a pharmacist to minimize medication errors. Numerous drugs can cross the placenta; therefore, caution is required when using medications during pregnancy.* ***Objective:*** *This study aimed to determine the problems of prescribing based on administrative, pharmaceutical, and clinical requirements using electronic forms and to analyze drug safety during pregnancy using electronic prescription archives from the outpatient obstetric clinic.* ***Methods:*** *This research employed an observational study utilized quantitative methods and retrospective data collection. The analysis focused on descriptive statistics to summarize and interpret the data according to the Pharmaceutical Care Standard in hospitals.* ***Results:*** *Administrative problems included the absence of a digital signature from the prescriber, patient weight, and allergy history in 100 electronic prescriptions (100%). Pharmaceutical requirement problems included the absence of dosage strength (8.00%) and unclear usage rules (9.00%). Clinical considerations included indications for drug selection (8%), potential drug interactions (3.00%), dose appropriateness (3.00%), duplication (2.00%), and contraindications (6.00%). Based on drug safety in pregnant women, 40 types of drugs were identified as category A drugs (7.50%), category B (32.50%), category C (50.00%), off-label (2.50%), and unknown (7.50%).* ***Conclusion:*** *Electronic prescription screening, which uses digital forms to review prescriptions, is a tool developed to improve patient safety by efficiently identifying potential drug therapy problems. The system accommodates structured screening based on specific criteria, such as pregnancy category and potential drug interactions, which helps prevent errors and ensures appropriate medication use.*

Keywords: *electronic forms, pregnancy, prescription screening*

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INTRODUCTION

Pregnancy is a specific physiological process that requires preparation and safe passage. The mother and fetus are inseparable functional units during pregnancy. The health of the pregnant mother must be maintained to ensure the optimal function and development of both units. During pregnancy and breastfeeding, mothers may experience various ailments or health problems that require medication. The use of drugs during pregnancy requires careful monitoring. Numerous drugs can cross the placenta; therefore, the use of medicines in pregnant women needs to be precise. Drugs can undergo biotransformation in the placenta, which is a protective effort as well as the potential to form reactive intermediate compounds that are teratogenic/dysmorphogenic. Drugs can cause unintended effects on the fetus that are often unnoticed. During the first trimester, drugs can cause birth defects (teratogenesis), and the most prone risk is at 3-8 weeks of gestation. During the second and third trimesters, drugs may functionally affect the growth and development of the fetus or poison the placenta. If possible, medications should be avoided during the first trimester. Changes in physiology during pregnancy and breastfeeding can affect drug kinetics, potentially leading to changes in a pregnant woman's response to medications (Kepley et al., 2023).

Obstetric healthcare providers often inquire about the safety of medications recommended or prescribed to pregnant patients. Most women use at least one medication during their pregnancies. However, information on the safety and appropriate dosing of many drugs during pregnancy is limited. Some drugs are also used "off-label" during pregnancy, which means they are used in ways not mentioned in the FDA-approved drug label. Important off-label uses in pregnancy include antenatal glucocorticoids (betamethasone and dexamethasone) to improve fetal lung maturity and non-steroidal anti-inflammatory drugs (indomethacin) to prevent preterm labor. Prescription drug use during pregnancy includes drugs approved for pregnancy-related conditions, approved medical indications (on-label use), or unapproved uses (off-label use) (Wesley et al., 2021).

Pharmaceutical care is a patient-oriented service in which pharmacists are responsible for optimizing therapeutic outcomes. Pharmaceutical service activities initially focused on managing drugs as commodities and have become comprehensive services to improve patients' quality of life. One of the clinical pharmacy services performed by pharmacists is prescription assessment. Pharmacists must understand and be aware of the possibility of errors in this process. Notably, hospital pharmacists are required to expand the paradigm of pharmaceutical services that focus on patients. For this reason, the competence of pharmacists needs to be improved continuously so that this paradigm shift can be implemented (Kementrian Kesehatan RI, 2016).

Research by Yani & Fardin (2021) evaluated the administrative and pharmaceutical requirements for outpatient BPJS prescriptions in Bantaeng Regency and found that the completeness of administrative requirements was 0% and 43.43% in pharmaceutics. Administrative requirements that are 100% unmet are related to gender and physicians' SIP. Administrative requirements related to treatment effectiveness and safety that were not met were related to the patient's age (41%) and weight (39%). Fortinguerra et al. (2021) related to monitoring drug prescriptions before, during, and after pregnancy in Italy showed that approximately 73.1% of pregnant women received at least one drug prescription during pregnancy, 57.1% before pregnancy, and 59.3% postpartum. The prevalence of prescription drug use increased with maternal age, particularly during the first trimester of pregnancy. The most prescribed drug was folic acid (34.6%), followed by progesterone (19%), both of which were concentrated in the first trimester of pregnancy (29.2% and 14.8%, respectively). Eight of the 30 most prescribed drugs were antibiotics, the prevalence of which was higher during the second trimester of pregnancy in women >40 years (21.6%).

Pharmacists are pharmaceutical personnel who ensure the effectiveness and safety of drugs. The management of pharmaceutical services for pregnant and lactating women includes screening and assessment of prescriptions, monitoring drug use, and providing information and education. This study used an electronic form to conduct the prescription screening process by reviewing the factors associated with drug prescriptions during pregnancy (Kementrian Kesehatan RI, 2016).

MATERIALS AND METHODS

Materials

This study used one of the elements of pharmaceutical service management under the Guidelines for Pharmaceutical Services for Pregnant and Breastfeeding Women (Kementrian Kesehatan RI, 2016), namely screening/assessment of prescriptions for patients with specific conditions, namely pregnant women at outpatient clinics. Prescription

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screening/assessment was performed based on administrative, pharmaceutical, and clinical requirements using electronic prescription archives at the obstetrics and gynecology clinic.

Tools

The tool used for the screening/prescribing assessment was an electronic form (Google form) to determine whether new problems were detected after the previous manual screening. The electronic form was designed specifically for pregnancy conditions. Clinical judgment assessment criteria were added from the Pharmaceutical Care Network Europe (PCNE) to facilitate the analysis of drug-related problems (MTOs).

Method

Study design

This study was based on observational research (non-experimental) using quantitative methods by screening/assessing prescriptions based on Permenkes No. 72/2016. The data obtained were analyzed descriptively using Microsoft Excel and presented in tables and percentages of findings.

Selection of the study population and sources of data

Retrospective data were collected using electronic prescription archive data from the obstetrics and gynecology outpatient clinic between January and December 2022. Electronic prescription archives were selected based on the following criteria

1. diagnosis: pregnant
2. patient data: same medical record number only taken 1 (one) time
3. visit history: the last visit at the time the data was taken

Electronic prescription records were printed according to the subject-selection criteria. The study sample size was the total number of prescription records of pregnant women who met the standards for data completeness for analysis.

Research variable

The variables used in this study included:

1. Drug prescription: paper or electronic prescription from the obstetrics and gynecology clinic.
2. Drug characteristics: Oral drugs prescribed to pregnant women.
3. Patient characteristics: based on age, comorbidities, and pregnancy conditions
4. Administrative requirements include the patient's name, age, sex, and weight; doctor's name, license to practice number, address, telephone number, doctor's initials, and date of prescription writing.
5. Pharmaceutical requirements include dosage form, dosage strength, stability, and incompatibility.
6. Clinical considerations include the appropriateness of the indication and dose of the drug, rules, mode, and duration of drug use, duplication and/or polypharmacy, adverse drug reactions, contraindications, and drug interactions.

Ethical approval

This research was approved by the Ethics Committee of the University of Surabaya, Surabaya, Indonesia (number 40/KE/I/2023) for researcher Lisa Aditama with a study entitled "Prescription Review for Specific Conditions Geriatric and Pregnancy Using Electronic Forms at outpatient clinic" for December 26, 2022-January 14, 2023.

RESULTS AND DISCUSSION

Data collection began in December 2022, namely, electronic prescription archives originating from obstetrics and gynecology clinics. The sample obtained consisted of 101 prescription sheets according to the research criteria. The research instrument for prescription screening used an electronic form (Google Form), consisting of patient characteristics, prescription information, drug names in the prescription, screening administrative requirements, screening pharmaceutical requirements, and screening clinical requirements.

Patient characteristics and treatment

The characteristics of the participants are presented in Table 1. The majority were pregnant women in the 26–30 year age range (43.00%). There was 1 patient aged < 20 years (1.00%) and based on the table, the number of patients over the age of 35 exceeded 21.

Tabel 1. Age characteristics of research subjects

Age (Years)	Number of Patients	Percentage (%)
< 20	1	1.00
20 - 25	14	14.00
26 - 30	43	43.00
31 - 35	21	21.00
36 - 40	20	20.00
> 40	1	1.00
	100	100.00

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Table 4. Prescription profile of symptomatic medication

Pharmacology Group	Number of Prescriptions	Frequency (%)
Symptom relief : antihistamines		9.30
Cetirizine	2	
Desloratadine, cetirizine	1	
Mebhydrolin	1	
Symptomatic relief : anti-inflammatory and mucolytic		2.33
Dexamethasone, ambroxol	1	
Symptom relief : anti-inflammatory		9.30
Mefenamic acid	1	
Meloxicam	2	
Na Diclofenac	1	
Symptomatic relief : anti-inflammatory and hemostatic		4.65
Paracetamol, Na diclofenac, tranexamic acid	1	
Dexketoprofen, tranexamic acid	1	
Symptom relief : hemostatic		9.30
Tranexamic acid	4	
Symptom relief : antiemetic		46.51
Metoclopramide	1	
Ondansetron	17	
Pyridoxine, pyrathiazine	2	
Symptomatic relief : gastric acid lowering agents		18.60
Lansoprazole	3	
Ranitidine	5	
	43	100.00

Pregnant women <20 years of age experience many health risks that can trigger miscarriage, anemia, prematurity, low birth weight, and other pregnancy complications (Ratnaningtyas & Indrawati, 2023). Pregnancy in women aged >35 years has the potential to cause loss of pelvic elasticity, and complications are prone to occur both during pregnancy and childbirth, such as pre-eclampsia, diabetes mellitus, hypertension, and anemia, which cause premature birth or LBW (low birth weight) (Susanti et al., 2020).

The characteristics of drug prescriptions in pregnant women were mostly supplements, with 78 patients (78.00%). The majority of pregnant women in this study received supplements containing folic acid (64 patients, 69.57% of supplement prescriptions) and iron (42 patients, 45.65% of supplement prescriptions).

There were prescriptions for astaxanthin antioxidants in eight patients (8.70% of supplement prescriptions). Patients who received astaxanthin also received other drugs, including 2 patients who received furosemide, 2 patients who received misoprostol, and 1 patient who received oral albumin. Astaxanthin has antioxidant effects on the vascular endothelium and improves symptoms of pre-eclampsia; however, its safety during pregnancy requires further investigation (Xuan et al., 2016).

Table 2. Supplement prescription profile for pregnant women

Supplement Composition	Number of Prescriptions	Frequency (%)
Astaxanthin	8	8.70
Vitamin E	1	1.09
B1 B6 B12	1	1.09
Folate, B12, DHA	22	23.91
Folate and Fe	7	7.61
Multivitamins, folate, Fe	35	38.04
Calcium lactate	3	3.26
Calcium D3	6	6.52
Calcium D3 and mineral	9	9.78
	92	100.00

The prescribing profiles of gynecologic enhancers are presented in Table 3. The most common age groups receiving gynecological boosters were 31-35 years (47.62%) and >40 years (100%). The most common antenatal boosters in pregnant women were micronized progesterone (33 patients, 91.67%), dydrogesterone (1 patient, 2.78%), and isoxsuprine (2 patients, 5.56%).

One patient (out of 35) received a combination of micronized progesterone and isoxsuprine. Dydrogesterone is a synthetic form of progesterone with better bioavailability and tolerability than micronized progesterone. Micronized progesterone is more effective in preventing preterm birth and LBW than is

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dydrogesterone (Ansari et al., 2023). Isoxsuprine is a class of β_2 adrenoreceptor agonists that directly induces uterine relaxation and improves blood circulation by selectively vasodilating the blood vessels supplying the uterine smooth muscle (Coelho & Gupta, 2024).

Table 3. Prescription profile of progesterone supplement

Progesterone Supplement	Number of Prescriptions	Frequency (%)
Micronized progesterone	33	91.67
Dydrogesterone	1	2.78
Isoxsuprine	2	5.56
	36	100.00

The prescription profile of symptomatic relief drugs in pregnant women is presented in Table 4, consisting of antiemetics (46.51%), gastric acid-lowering agents (18.60%), anti-inflammatory agents (9.30%), antihistamines (9.30%), hemostatic agents (9.30%), anti-inflammatory and hemostatic agents (4.65%), and anti-inflammatory and mucolytic agents (2.33%). In the antiemetic group, ondansetron was the most prescribed drug. The most prescribed gastric acid-lowering agent was ranitidine.

Pyridoxine is the first-line medication for nausea and vomiting during pregnancy. Metoclopramide is the second choice for nausea and vomiting and should be used for no more than 5 days. Another factor to consider when using metoclopramide is the occurrence of extrapyramidal symptoms (EPS). Ondansetron is the most widely prescribed nausea and vomiting reliever but should not be used in the first trimester. In pregnancy-related nausea and vomiting and hyperemesis gravidarum, single pyridoxine is often less effective, and its combination with H1 antihistamines, such as doxylamine or pyrathiazine, is recommended (Nelson-Piercy et al., 2024).

The prescribing profile of condition-specific drugs in pregnant women is presented in Table 5, including medications for cervical ripening (6.00%), pre-eclampsia prevention (4.00%), antihypertensives (3.00%), and gestational diabetes (1.00%). The most common age groups that received misoprostol were < 20 years (100.00%), 31-35 years (9.52%), and 36-40 years (5.00%). The use of misoprostol in obstetrics and gynecology has expanded with the ability to diagnose fetal abnormalities in the prenatal period for indications of pregnancy termination in the second trimester and cervical ripening in preparation for parturition at a dose of 400 mg intravaginally every 3-6 hours.

The largest age groups who received acetylsalicylic acid (ASA) were 31-35 years (14.29%) and 26-30 years (2.33%). In this study, some patients received antihypertensive and antidiabetic medications, namely furosemide (two patients), methyldopa (one patient), and metformin (one patient). Prevention of pre-eclampsia has been considered to improve pregnancy safety for both the mother and child, given at a dose of 80 mg ASA during weeks 12-28 (optimally before week 16). The mechanism of pre-eclampsia prevention with ASA is based mainly on in vitro studies and may work by improving placentation. The functional systems modulated by ASA involve cytokine release through antiapoptotic and vasoprotective mechanisms. The thromboxane/prostacyclin ratio, which is altered in pre-eclampsia, can be normalized by ASA-induced thromboxane synthesis inhibition. Dysregulation of angiogenic growth factors also plays a role in the pathogenesis of preeclampsia and can be positively affected by ASA (Stubert et al., 2023).

Table 5. Prescription profile for specific-condition drugs

Pharmacology Group	Number of Prescriptions	Proportion (%) (n=100)
Cervical ripening		6.00
Misoprostol	2	
Misoprostol and tranexamic acid	4	
Pre-eclampsia prevention		4.00
Acetylsalicylic 100 mg	4	
Antihypertensive		3.00
Furosemide 40 mg	2	
Methyldopa 250 mg	1	
Antidiabetic		1.00
Metformin 500 mg	1	

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Table 6. Antibiotic prescription profile in pregnancy conditions

Pharmacology Group	AB Combination or Specific Drugs	Number of Prescriptions	Frequency (%)
Cephalosporins		11	91.67
Cefadroxil 500 mg		1	
Cefadroxil 500 mg	Na diclofenac, paracetamol, tranexamic acid,	1	
Cefadroxil 500 mg	Misoprostol, tranexamic acid	4	
Cefixime 100 mg		3	
Cefixime 200 mg	Metronidazole 500 mg	2	
Azole			8.33
Metronidazole 500 mg		1	
		12	100.00

Table 7. Profile of drug category for pregnancy

Drug Categories in Pregnancy	Type of Medicine	Percentage
A	3	7.50
B	13	32.50
C	20	50.00
D	0	0.00
Off-label	1	2.50
NA	3	7.50
	40	100000

Description
Category A: Adequate research studies using control groups that failed to demonstrate any risk to the fetus in the first trimester of pregnancy (and there is no evidence of risk in later trimesters).
Category B: Animal reproduction studies failed to demonstrate any risk to the fetus, and there are no adequate studies using control groups in pregnant women.
Category C: Animal reproduction studies suggest adverse effects on the fetus, and there have been no adequate studies using control groups in humans, but the potential benefits warrant the use of the drug in pregnant women despite the potential risks.
Category D: There is positive evidence of risk to the human fetus based on adverse reaction data from investigational or marketing experience or human studies, but the potential benefit may warrant the use of the drug in pregnant women despite the potential risk.
Off-label: the prescription or use of a drug for a purpose, dose, or patient population not specifically approved or labeled by the FDA.

Based on the review of drug data input in electronic forms, antibiotic prescriptions were obtained, as shown in Table 6. Antibiotic prescriptions were received by 12 patients (12.00%), the majority of which were from the cephalosporin group (11 prescriptions, 91.67%), and one from the azole group as many as 1 prescription (8.33%).

During pregnancy, there is a potential risk of infection of the genitourinary system, and if untreated, it increases morbidity, including low birth weight, premature birth, and spontaneous abortion. However, it is estimated that only 10% of drugs have sufficient data related to their safe and effective use during pregnancy. Antibiotics such as beta-lactams, vancomycin, nitrofurantoin, metronidazole, clindamycin, and fosfomycin are considered safe and effective in pregnancy.

Physiological changes during pregnancy lead to an increased glomerular filtration rate, total body volume, and cardiac output. These changes can lead to pharmacokinetic changes in antibiotics that require dose adjustments or careful monitoring and assessment (Bookstaver et al., 2015).

In this study, data on drug prescriptions during pregnancy were obtained for 40 types of drugs. The profile of drug categories during pregnancy is presented in Table 7, with category C accounting for 50.00% of the cases. Category C drugs can be administered if the benefits of the drug outweigh the risks to pregnant women and fetuses. There was one type of drug with six prescriptions (6.00%), including the off-label category, namely misoprostol. In this study, three types of drugs were found that were not yet known for their safety category in pregnancy: astaxanthin, coral calcium, and mebhydroline napadisylate.

Administrative requirements screening

The prescription review in this study used electronic forms with the selection of administrative

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requirements based on the Technical Guidelines for Pharmaceutical Service Standards in Hospitals (PMK No 72 of 2016) and Permenkes No 24 of 2022 concerning Medical Records. Health Service Facilities (Fasyankes) are required to process electronic medical record information by coding, reporting, and reviewing; therefore, administrative requirements are needed, especially in the review of electronic prescriptions. Administrative screening using electronic forms can identify gaps in electronic prescribing, based on regulatory requirements. Table 8 presents issues related to administrative requirements.

Three administrative requirements were not met: the doctor's signature, patient's weight, and allergy history. This needs to be considered in terms of legal aspects, professional responsibilities, and the safety of drug selection for patients. Utami et al. (2024) evaluated the implementation of electronic prescribing and identified the level of conformity of electronic prescribing features at Roemani Muhammadiyah Semarang hospital at 87.50%, where there was still no data on body surface area in pediatric patients, drug ID based on the national formulary, and prescription progress status.

Pharmaceutics requirements screening

The screening of pharmaceutical requirements in this study used an electronic form based on the Technical Guidelines for Pharmaceutical Service Standards in Hospitals (PMK No. 72 of 2016). The problems related to pharmaceutical requirements are presented in Table 9.

Two pharmaceutical requirements were not met, namely, writing the dosage strength and the administration rules. There were prescriptions for ondansetron without dosage strength (2 out of 17 prescriptions), paracetamol (1 out of 1 prescription), and dexamethasone (1 out of 1 prescription), which could potentially provide dosage strengths that do not follow the expected effects. Ondansetron is available in 4 and 8 mg doses, dexamethasone in 0.5 and 0.75 mg doses, and paracetamol in 500 and 625 mg doses. The recommended dose of ondansetron for hyperemesis gravidarum is 8 mg at intervals of every 8 hours. Dexamethasone is used in a wide dose range, depending on the expected indication. Paracetamol, a pain reliever and antipyretic, also depends on the level of pain and severity of the condition (American Pharmacists Association, 2022).

Table 8. Administrative requirements screening

Administrative aspects (n=100 prescriptions)	No problem		Problem found	
	Frequency	Percentage (%)	Frequency	Percentage (%)
Completeness of Prescriber Data				
Doctor Name	100	100.00	-	-
SIP	100	100.00	-	-
Prescription Date	100	100.00	-	-
Signature	-	-	100	100.00
Completeness of Patient Data				
Patient Name	100	100.00	-	-
RM Number	100	100.00	-	-
Patient Address	100	100.00	-	-
Patient Age	100	100.00	-	-
Patient Weight	-	-	100	100.00
Allergy History	-	-	100	100.00
Completeness of Drug Data				
Medicine Name	100	100.00	-	-
Drug Instruction Writing	100	100.00	-	-
Quantity Requested	100	100.00	-	-
Usage Instruction	100	100.00	-	-

Table 9. Pharmaceutics requirements screening

Pharmaceutical Aspects (n=100 prescriptions)	No problem		Problem found	
	Prescription Frequency	Percentage (%)	Prescription Frequency	Percentage (%)
Dosage Forms	100	100.00	-	-
Dosage Strength	92	92.00	8	8.00
Stability	100	100.00	-	-
Medication Instruction	91	91.00	9	9.00
Duration of Administration	100	100.00	-	-

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Table 10. Clinical considerations screening

Clinical Aspects (n=100 prescriptions)	No problem		Problem found	
	Prescription Frequency	Percentage (%)	Prescription Frequency	Percentage (%)
Presence of Allergies	NA	NA	NA	NA
Indication	92	92.00	8	8.00
Side Effects	100	100.00	-	-
Interaction	97	97.00	3	3.00
Dosage Accuracy	97	97.00	3	3.00
Duration Accuracy	100	100.00	-	-
Duplication/Polypharmacy	98	98.00	2	2.00
Contraindications	94	94.00	6	6.00

Table 11. Causes of DRPs in drug selection and prescribing

1. Drug Selection	DRP	Drug Name
1.1 Not following guidelines/Fornas		
1.2 No indications	√	Astaxanthin
1.3 Unsuitable combination	√	Ca lactate and Blood Supplement Tablets
1.4 Duplication of therapy class	√	Desloratadine and cetirizine
1.5 Incomplete prescription of medication		
1.6 Overuse of drugs for one indication	√	Isoxsuprine and micronized progesterone
2. Dosage Form Selection		
2.1 Incompatible dosage form		
3. Dose Selection		
3.1 Insufficient dose	√	Metoclopramide 5 mg
3.2 Excessive dose		
3.3 Under-dosing instruction		
3.4 Over-dosing instruction	√	Meloxicam and Na diclofenac
3.5 Insufficient/incorrect instructions	√	Misoprostol
4. Determination of Treatment Duration		
4.1 The treatment duration is too short		
4.2 The treatment duration is too long		

There were unclear rules for misoprostol administration (6 out of 6 prescriptions), that is, the method of administration was not written. This is related to the off-label use of the drug and should be closely monitored by health workers, as it can lead to termination of pregnancy. There was an error in writing the rules of administration of meloxicam and Na diclofenac which were given with the rule of 3 times a day when they should have been 2 times a day. The administration of NSAIDs is often associated with cardiovascular events, which are vital during pregnancy.

Clinical considerations screening

Screening of clinical considerations in this study used an electronic form based on the Technical Guidelines for Pharmaceutical Service Standards in Hospitals (PMK No. 72 of 2016) and PCNE Classification for Drug-Related Problems (DRP) V9.1. Problems related to clinical considerations are presented in Table 10. Five clinical considerations were identified in this study: the presence of a consideration of indications for drug selection, potential drug interactions, dose appropriateness, duplication, and contraindications.

The causes of DRP in drug selection and prescription are presented in Table 11. Drug selection requires clinical consideration, namely, there is insufficient evidence for the indication of astaxanthin antioxidants for pregnant women (8 out of 100 prescriptions). The prescription of a combination of calcium lactate and blood supplement tablets has a potential interaction, as calcium can inhibit the absorption of ferrous fumarate if taken together (3 out of 100 prescriptions).

A prescription request was made for metoclopramide 5 mg (1 out of 100 prescriptions), where the dosage strength of the available tablet preparation was 10 mg. In this case, drug delivery could not be confirmed. In this study, there was a duplication of therapeutic classes, namely desloratadine and cetirizine in one prescription, and one indication treated with two drugs with the same pharmacology as a gynecological booster, namely isoxsuprine and micronized progesterone. There was off-label treatment of misoprostol for cervical ripening, which is contraindicated in pregnancy (6 out of 100 prescriptions).

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CONCLUSION

Prescription screening using electronic forms to conduct prescription reviews in pregnant women can improve patient safety by identifying and addressing potential problems based on specific criteria.

By considering the regulatory provisions in electronic prescription services, standard elements of pharmaceutical services, and clinical review classifications elaborated in electronic forms developed by pharmacists, pharmaceutical practice is expected to be optimized.

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AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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Effect of Glycerin on Stability and Antioxidant Activity of Ethyl Acetate Fraction of Secang Wood Face Mist

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Abstract

Background: *Secang wood (Caesalpinia sappan L.) is a plant that contains brazilin, a natural antioxidant. A potential problem in the brazilin formulation process is that brazilin becomes unstable due to environmental influences. Face mist is one of the formulations that contains glycerin to maintain the stability. Glycerin can stabilize brazilin by forming hydrogen bonds between glycerin and brazilin.* ***Objective:*** *The main objective of this research was to evaluate the effect of glycerin on the physical stability and antioxidant activity in ethyl acetate fraction of secang wood face mist.* ***Methods:*** *Face mist formulation was prepared using different glycerin concentrations, such as F1 (10%), F2 (15%), and F3 (20%). Face mist was formulated using ethyl acetate fraction of secang wood, glycerin, phenoxyethanol, and aquadest. Physical evaluation stages included organoleptic, homogeneity, pH, specific gravity, viscosity, spray spreadability, skin moisture, cycling tests, and hedonic tests. 2,2-Diphenyl-1-picrylhydrazyl* ***(****DPPH) method was used to examine antioxidant activity.* ***Results:*** *The physical evaluation showed that face mist produced good results and complied with the requirements of face mist. The antioxidant activity test showed that face mist with the* IC_{50} *value for F1 was 22.114±0.046 µg/mL, F2 was 21.828±0.033 µg/mL, and F3 was 21.378±0.025 µg/mL.* ***Conclusion:*** *Based on these observations, the best formula was F3 because the antioxidant activity was classified as very strong, and the physical evaluations were considered more stable than others. In addition, F3 had the highest average score on the hedonic test.*

Keywords: *antioxidant, Caesalpinia sappan, face mist, glycerin, secang wood*

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INTRODUCTION

Skin is part of the body that is most frequently exposed to UV radiation and pollution from the environment. This exposure generates free radicals or reactive oxygen species (ROS) which can cause dullness, early aging, and even skin cancer.

Antioxidants are mostly sourced in the natural ingredients. Therefore, skincare with natural ingredients can help reduce the risk of adverse reactions caused by harsh synthetic compounds (Utami et al., 2015). That fact encourages the researchers to develop sources of natural antioxidant compounds from plants containing bioactive compounds (Haerani et al., 2018) such as terpenoids, alkaloids, and flavonoids (Hadi et al., 2023).

Secang wood contains phenolic compound such as brazilin, a natural antioxidant that helps protect the body from free radicals (Aloksan et al., 2022) (Vij et al., 2023). Brazilin generally partitioned with ethyl acetate (Nirmal et al., 2015). The ethyl acetate fraction of secang wood has a strong antioxidant capacity with an IC_{50} value of 9.236 ppm (Hidayat et al., 2021). Brazilin is a flavonoid compound which is able to protect body from the damage caused by free radical exposure. Thus, secang wood is potential to use in the formulation of face mist. Face mist is chosen because of its ease of application, practicality, and efficient skin absorption (Lisyanti et al., 2022). Brazilin is easily oxidized when exposed to light and oxygen, resulting in hydrogen loss and structural transformation from the compound (Luxsika & Sakaman, 2023). Brazilein has indeed widely been used as a natural colorant (Ngamwonglumlert et al., 2020)

Glycerin is a hygroscopic component that attracts water molecules from the environment and resists water's evaporation from the skin and preparations, thereby providing a moisturizing and hydrating effect on the skin. The addition of glycerin in the preparation formulation can affect the formula's effectiveness and stability (Sukmawati & Laeha, 2017). It shows that increasing glycerin concentration will affect the preparation's effectiveness; the higher the glycerin concentration, the greater the moisturizing effectiveness. Glycerin helps maintain the stability of preparations by balancing the humidity. It preserves the rate of water absorption from the air and slows down water evaporation of the preparation (Lin et al., 2019). Therefore, it is necessary to research the effect of glycerin concentration on the stability and effectiveness of face mist preparations containing brazilin. This study examined the impact of varying glycerin concentrations in face mist preparation containing ethyl acetate fraction of secang wood. The research focused on antioxidant activity and physical stability tests which findings are expected to serve as a reference for developing skincare products containing unstable active ingredients such as brazilin. The stability of antioxidant compounds is influenced by pH; at higher pH levels, these compounds tend to become unstable, resulting in structural changes in the compound (Sigi et al., 2015).

MATERIALS AND METHODS

Materials

Secang wood (*Caesalpinia sappan* L.) was obtained from traditional healthcare unit managed by Sardjito Hospital, distilled water was taken from Water One, glycerin and formic acid were taken from Brataco, Tangerang, while DPPH was obtained from TCl, Tokyo, Japan. Meanwhile, Ethyl acetate, n-hexane, ethanol 96%, phenoxyethanol, ascorbic acid, GF254 silica gel plate, HCl, Magnesium powder, boric acid, acetone, oxalic acid, ether, toluene, and methanol were taken from Merck, Darmstadt, Germany.

Tools

The tools utilized for this research include a set of Soxhlet tools (Pyrex), rotary evaporator (IKA RV 10. BASIC), water bath (Memmert), beaker glass (Pyrex), test tube (Pyrex), Erlenmeyer (Pyrex), pH meter (HANNA), Oswald viscometer (Pyrex), UV-Vis spectrophotometer double beam UV 1780 (SHIMADZU), skin moisture meter (U-track), stopwatch, separating funnel (Pyrex), UV lamp, chamber, and glass plate.

Methods

Sample preparation

Three kg of secang wood were chopped and dried in an oven at 55°C. The dried material was then ground into powder and sieved using a 40-mesh screen.

Extraction of secang wood

A 30 g secang wood powder was extracted using the soxhletation method with 300 mL of 96% ethanol. The extraction was carried out for 15 cycles at 60ºC, repeatedly circulating the same solvent through the extractor. The extract was then concentrated using rotary evaporator at the temperature of 60°C, and was further placed in a water bath at 50°C to be thickened (Pattananandecha et al., 2022).

Fractionation of secang wood ethanol extract

Ten grams of extract were dissolved in 10 mL of 30% ethanol, fractionated with 100 mL of n-hexane solvent (1:10), and shaken until the color cleared. The extracts were then left to stand until two distinct layers were formed, namely the water fraction (bottom) and the

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n-hexane fraction (top). The water fraction was separated and further fractionated with 100 mL of ethyl acetate. The mixture was agitated untul the color became clear. Two layers were again formed, the water fraction (bottom) and the ethyl acetate fraction (top). After that, the ethyl acetate fraction was separated from the water fraction and evaporated in a water bath at 60°C (Islamiati, 2022).

Flavonoid phytochemical screening

Willstatter method

The ethyl acetate fraction was homogenized after being dissolved in distilled water, 0.5 mg of magnesium (Mg) powder, and 2 drops of 2 M HCl. The presence of flavonoids was indicated by a color change to green, orange, or red (Pratiwi et al., 2021).

Taubeck method

After 1 mL of the ethyl acetate fraction was dissolved in distilled water and evaporated until completely dry, boric acid, acetone, and oxalic acid were added. Further, 5 mL of ether was added after the mixture was evaporated over a water bath and measured at 366 nm in UV. Intense yellow fluorescence indicated positive flavonoid results (Hidayat et al., 2021).

Thin layer chromatography

Brazilin was identified by dissolving the ethyl acetate fraction of secang wood in 1 mL of methanol. The sample solution was spotted on a GF_{254} silica gel plate and eluted in the mobile phase chloroform: methanol (5:5). The plate was left to stand until the spots moved with the mobile phase and dry, then observed under UV light at 254 nm and 366 nm. The brazilin compound showed an Rf value of 0.84 (Kementerian Kesehatan RI, 2017).

Formulation of face mist ethyl acetate fraction of secang wood

The ethyl acetate fraction of secang wood was dissolved in warm distilled water (±40˚C). Then, glycerin, phenoxyethanol, and more distilled water were homogenized until the total volume reached 100 mL. Three formulations were prepared: F1 = 10% glycerin, F2 = 15% glycerin, and F3 = 20% glycerin.

Evaluation of secang wood ethyl acetate fraction face mist

a. Organoleptic

Organoleptic preparation was observed for its shape, color, and smell (Sakka & Hasma, 2023).

b. Homogeneity

The face mist was observed under a lamp to see whether or not there were undissolved coarse particles. A good face mist requires no coarse particles to completely dissolve in the preparation (Asjur et al., 2019).

c. pH

A calibrated pH meter was used to determine the face mist pH. According to SNI 16-4399-1996, good topical medications should have a pH between 4.5 and 8.0, depending on the skin's pH.

d. Specific gravity

The empty pycnometer (W1), the pycnometer filled with distilled water (W2), and the pycnometer filled with sample (W3) were weighed. The standard specific gravity of face mist preparations is 1.01-1.1 g/mL (Lisyanti et al., 2022).

e. Viscosity

The viscosity of the face mist was measured using an Oswald viscometer, which measures the time required for the liquid to pass through two points of the vertical capillary tube. If the solution follows Newton's rules, the preparation would be easy to spray. The viscosity requirement for spray preparations is 1.27-1.87 Cps (Lisyanti et al., 2022).

f. Dry time

The face mist was sprayed onto the surface of the forearm, and the time required for the preparation to dry was recorded. The standard drying time for a good spray preparation is <5 minutes (Sakka & Hasma, 2023).

g. Spray spread power

The face mist was sprayed once on the mica sheet at a distance of 5 cm, and then the diameter of the spray was observed. A good face mist has a spray diameter of 5-7 cm (Badriyah & Ifandi, 2020).

Table 1. The formula of face mist ethyl acetate fraction of secang wood

Material	Formula (%)			Function
	F1	F2	F3	
Secang wood ethyl acetate fraction	0.1	0.1	0.1	Active substance that contains brazilin compound (Nirmal et al., 2015)
Glycerin	10	15	20	Humectant
Phenoxyethanol	0.5	0.5	0.5	Preservative
Aquadest	ad 100 mL	ad 100 mL	ad 100 mL	Base and solvent

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h. Humidity

Skin moisture on the forearms was measured before and after spraying face mist using a skin analyzer device (Asjur *et al.*, 2019). The skin analyzer handbook states that the usual moisture reference value is < 33% for dry skin, 38–42% for normal skin, and ≥ 47% for moist skin.

i. Stability

The physical stability was evaluated using a cycling test which was conducted in six cycles. The face mist preparation was stored at a temperature of ±4ºC and ±40ºC for 24 hours each. It was calculated as one cycle. After that, the preparation was compared with the previous condition. A formulation is considered unstable if there are significant changes in the organoleptic test, homogeneity, pH, specific gravity, viscosity, dry time, and spreadability (Asjur *et al.*, 2019).

j. Hedonic

The hedonic test evaluated the three face mist formulas of the ethyl acetate fraction of secang wood. Seventeen respondents aged 17 to 25 years participated in the evaluation which was carried out under ethical approval No 70/EC/KEPK/III/2024. The questionnaire assessed the color, aroma, and absorption capacity of the preparation using a 5-point Linkert scale, where (1) indicated "really dislike", (2) "dislike", (3) "neutral", (4) "like", and (5) "really like".

Antioxidant activity test

A 0.04 mM DPPH solution was prepared by weighing ±0.3943 mg DPPH powder. The powder was then dissolved with ethanol p.a. to the specified limit and further homogenized. The maximum wavelength was determined by measuring the absorbance of the DPPH solution in the 400-800 nm range after homogenization. The control absorbance was determined by measuring the DPPH solution at the maximum wavelength obtained. A stock solution of 1000 µg/mL secang wood ethyl acetate fraction was prepared by weighing 100 mg of the fraction, dissolving it in ethanol p.a. to the specified limit, and homogenizing it. The operating time (OT) of the ethyl acetate fraction and face mist samples was determined by mixing the ethyl acetate fraction stock solution or face mist with DPPH solution, then homogenizing, and measuring the absorbance every 5 minutes. The antioxidant activity test of ethyl acetate fraction was carried out by preparing various concentrations of the solution, mixing them with DPPH solution, homogenizing the mixture, incubating it at 37°C, and measuring the absorbance at the maximum wavelength. The antioxidant activity test of the face mist was carried out using a similar procedure, using various concentrations of face mist samples.

Data analysis

The results were analyzed, and the face mist's physical evaluation was statistically carried out using SPSS One-Way ANOVA software. In that case, a non-parametric test, such as the Kruskal-Wallis test, could be carried out. For a stability test, if the data is normal, the Paired T-test is used; if the data is not normal, the Wilcoxon test is used. An equation for linear regression was used to classify the IC_{50} values based on the analysis of the antioxidant activity data.

RESULTS AND DISCUSSION

Extraction and fractionation

The soxhletation method was chosen to increase the diffusion speed so that the solubility of an active substance would increase and the level of the compound obtained would be greater. The soxhletation method produced 244.8 grams of ethanol extract with an extract yield of 13.74%. According to the Indonesian Herbal Pharmacopoeia, the yield value of secang wood ethanol extract should not be less than 8.10% to meet the specified standard (Anonim, 2017).

Multilevel fractionation using the liquid-liquid method is a technique for separating and classifying chemical compounds based on their polarity level (Amaliah et al., 2020) with the principle "like dissolves like", which means that the solvent will dissolve compounds with a similar polarity level (Sogandi et al., 2019). The multistage fractionation process produced 44.9 grams of ethyl acetate fraction, corresponding to a yield of 29.93%. The ethyl acetate fraction was expected to contain semi-polar compounds (Asmah et al., 2020).

Flavonoid phytochemical screening

Flavonoid phytochemical screening was conducted to confirm that the ethyl acetate fraction of secang wood contains flavonoid compounds as antioxidants. The screening which was performed using the Willstatter method showed that the ethyl acetate fraction of secang wood contained flavonoids as characterized by the formation of an orange-to-red color after adding the reagent. The subsequent addition of HCl and magnesium would reduce the benzopyrone structure, which comprises a benzene ring fused with a pyrone. This occurred due to the reduction formation of an orange-to-red complex falavylium salt (Mukhriani *et al*., 2019).

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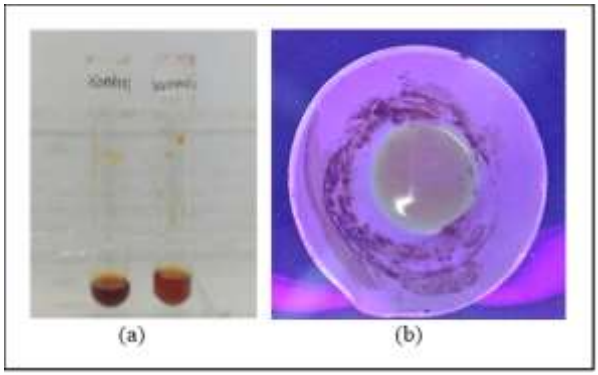

Figure 1. (a) The result of the Wilstater method for flavonoid test produced a red coloration (b) The result of the Taubeck method for flavonoid test indicated the formation of intensive yellow fluorescence

Phytochemical screening using the Taubeck method showed that the ethyl acetate fraction of secang wood contained flavonoids as characterized by the formation of intensive yellow fluorescence at UV 366 nm. Adding reagents could prolong the bathochromic shift, thereby providing intensive yellow fluorescence. Flavonoids have ortho-hydroxy groups, which give fluorescence at UV 366 nm (Pratiwi et al., 2021).

Thin layer chromatography

Thin layer chromatography (TLC) was used to confirm the results of phytochemical screening by separating a compound mixture into pure compounds based on their polarity level. Based on the Indonesian Herbal Pharmacopoeia (Edition II), on a GF_{254} silica gel plate and eluted in the mobile phase chloroform: methanol (5:5), the brazilin compound has an Rf value of 0.84. Brazilin has an absorption capacity within the wavelength range of 328-515 nm (Landuma, 2014), so brazilin spot stains with a blue fluorescent, it can be seen at 366 nm. The TLC test results showed a blue fluorescent stain at UV 366 nm with an average Rf value of 0.83, as shown in Figure 2. These results are similar to those of the literature and confirm that Brazilin is identified in the ethyl acetate fraction of secang.

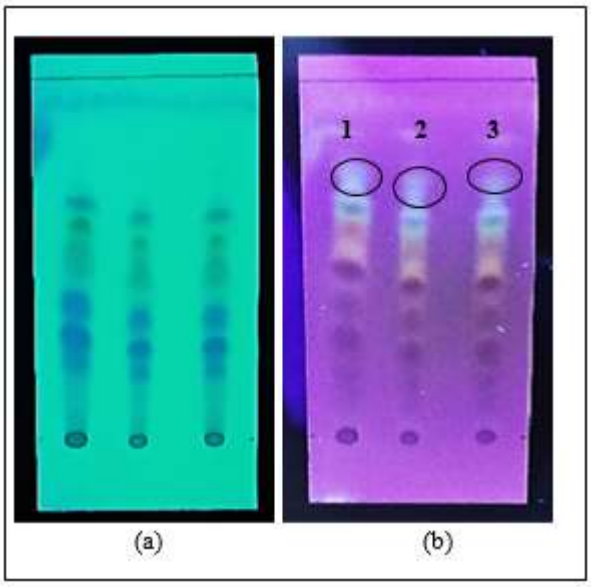

Figure 2. TLC profile of ethyl acetate fraction of secang wood observed under UV light (a) 254 nm and (b) 366 nm with Rf values of 0.83 (spot1), 0.81 (spot 2), and 0.82 (spot 3)

Physical evaluation of ethyl acetate fraction of secang wood face mist

Organoleptic

The organoleptic test aims to observe the formula's color, shape, and odor that will affect the user's comfort. The organoleptic test results in Table 2 show that F1, F2, and F3 had no difference in odor, all being odorless, and shared a consistent orange color. Regarding shape parameters, there were slight differences between the three formulations due to differences in glycerin concentration; F1 was liquid, F2 was slightly more concentrated, and F3 was more concentrated than F1 and F2. These results indicate that the higher the glycerin concentration produced, the thicker the formula because glycerin can increase viscosity by binding more water to increase the molecular size. Thus, the resistance to flow will also increase (Wulandari et al., 2023).

Table 2. Physical evaluation of ethyl acetate fractions of secang wood face mist

Parameter	F1	F2	F3	*p-value*
Organoleptic	Orange Liquid No smell	Orange Liquid No smell	Orange Liquid No smell	-
Homogeneity	Homogeneous	Homogeneous	Homogeneous	-
pH	5.9±0.0	5.9±0.0	5.9±0.0	1,000
Specific gravity (g/mL)	1.029±0.001	1.042±0.000	1.053±0.000	0,000
Viscosity (Cps)	1.067±0.017	1.260±0.035	1.326±0.029	0,000
Dry time (minutes)	3.00±0.37	5.32±0.15	7.13±0.11	0,000
Spreadability (cm)	5.5±0.2	4.5±0.2	4.2±0.1	0.031

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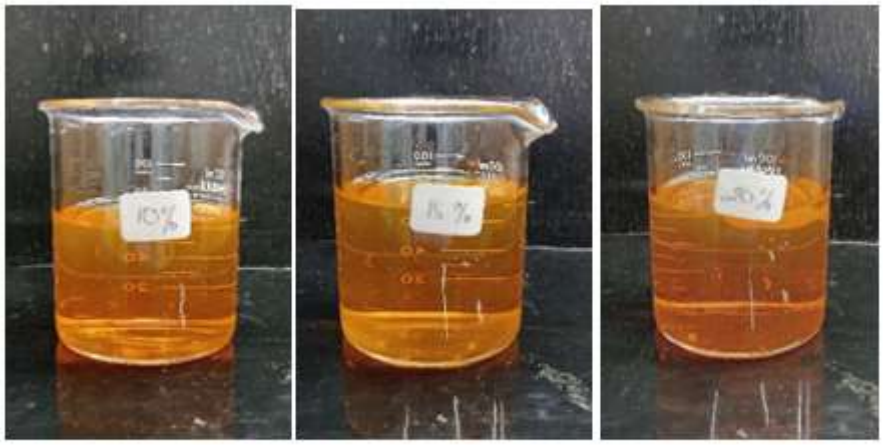

(a) (b) (c)

Figure 3. Face mist ethyl acetate fraction of secang wood (a) = F1; (b) = F2; (c) = F3

Homogeneity

The purpose of the homogeneity test is to determine if the active ingredient and any other ingredients are uniformly mixed, as indicated by the lack of granules or coarse particles in the composition. Based on the homogeneity test results shown in Table 2, F1, F2, and F3 were homogeneous because there were no coarse particles or grains in the face mist, meaning that all the ingredients dissolved and mixed evenly. The homogeneity test results of the three face mist formulas for the ethyl acetate fraction of secang wood met the homogeneity requirements. The Homogeneity is measured by observing under a lamp to see whether or not there are insoluble particles.

pH

The purpose of the pH test is to evaluate the formula; whether it is suitable for the natural pH of human skin and safe for use without causing irritation. Based on the results shown in Table 2, the formulations of F1, F2, and F3 had the same pH value of 5.9, which indicates that the three formulas comply with the normal pH range for human skin, so they are safe to use. In addition, the test results showed that the three secang wood ethyl acetate fraction face mist formulas met the pH value requirements for topical preparations according to SNI 16-4399-1996 because they were in the range of 4.5-8.0.

Specific gravity

The specific gravity test aims to determine the density of the formulation by comparing the mass of a substance to a volume of water at a certain temperature (25^{o}C). Based on Table 2, the specific gravity values of F1, F2, and F3 met the specific gravity requirements because the results were in the range of 1.01-1.1 g/mL, indicating that the resulting face mist was not too thick. The number of components influenced the specific gravity value in the sample, where the more components in the sample, the more the specific gravity value would increase, and vice versa (Kurniawan & Nugraha, 2023). In this study, the higher the glycerin concentration, the higher the specific gravity value, and vice versa.

Viscosity

Based on the viscosity test results presented in Table 2, F2 and F3 met the viscosity requirements for spray preparations because the results were in the range of 1.27-1.87 Cps. Meanwhile, F1 did not meet the requirements because the results were <1.27-1.87 Cps. The viscosity value was influenced by the concentration of glycerin; the higher the concentration of glycerin used, the more concentrated the solution will be, so the higher the viscosity value, and vice versa (Ratriyantari & Santosa, 2017). However, excessively high viscosity can cause the face mist to have difficulty flowing, making it difficult to dispense from the packaging Conversely, very low viscosity will cause the face mist to drip when applied, preventing it from staying completely on the skin (Asjur *et al.*, 2019).

Dry time

The dry time test aims to determine the time required for the formula from the start of application to drying. Based on the results shown in Table 2, F1 met the dry time requirements because it is < 5 minutes, while F2 and F3 did not meet the dry time requirements because it is > 5 minutes. Viscosity is influenced by the concentration of glycerin used. A low glycerin concentration causes a low viscosity value, so the drying time of the preparation becomes faster, and vice versa (Ratriyantari & Santosa, 2017).

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Spray spread power

The purpose of the spray spread power test is to determine the effectiveness of the spray face mist to disperse effectively across the skin surface. The wider the spray spread, the more active substances will contact the skin surface. Based on the results shown in Table 2, F1 met the spray spread power requirements with a spread range of 5-7 cm. However, F2 and F3 did not meet the requirements as their measurements were below 5-7 cm. The glycerin concentration affects the spray weight of the preparation. The spray weight of the formula decreases with increasing glycerin content and vice versa (Asjur et al., 2019). The spray weight is influenced by viscosity (Ramadhani & Listiyanti, 2021). A high viscosity value causes the formula to become more concentrated and the spray output becomes lower. As a result, only a small amount of the formula is dispensed from the applicator, and it fails to spread evenly on the skin surface. A low viscosity value causes the preparation to become more liquid so that the spray weight is too high and causes the preparation to drip when applied (Indalifiany et al., 2023).

Humidity

The humidity test determines skin moisture before and after the formulation based on ethical clearance No 70/EC/KEPK/III/2024. The humidity test results of 17 respondents showed that the highest percentage of humidity was obtained by F3, followed by F2 and F1, which can be seen in Table 3. Skin moisture is affected by glycerin concentration; the higher the glycerin concentration, the more the skin moisture increases (Sukmawati & Laeha, 2017). Glycerin can absorb moisture from the environment by binding water molecules and reducing the evaporation of water from the preparation (Becker et al., 2019), thereby providing a moisturizing effect. Glycerin with a concentration of 10% can increase skin moisture (Sawiji & Utariyani, 2022).

Table 3. Humidity results

Formula	Increased skin moisture (%)	*p-value*
F1	11.76±8.61	0,000
F2	18.05±8.84	0,000
F3	24.64±7.00	0,000

Stability

The stability test was conducted using the cycling test method which is a method for evaluating the stability of cosmetics with variations in storage temperature within a certain time interval. A face mist will be stable if it maintains its physical, microbiological, and chemical qualities during storage. The physical instability of face mist is characterized by changes in color, loss of clarity, the appearance of an unexpected or pungent odor, and change in consistency. Based on the stability test results, it could be seen that the face mist F1, F2, and F3 were unstable, as indicated by physical changes. Face mist F1 and F3 were unstable in organoleptic and dry time, while face mist F2 was unstable in organoleptic, viscosity, and dry time.

Table 4. Results of physical stability evaluation of face mist F1

Parameter	Day-0	Day-12	*p-value*
Organoleptic	Orange Liquid No smell	Orange Liquid No smell	-
Homogeneity	Homogeneous	Homogeneous	-
pH	5.9±0.0	5.6±0.0	0.083
Specific gravity (g/mL)	1.029±0.001	1.027±0.000	0.109
Viscosity (Cps)	1.067±0.017	1.024±0.027	0.083
Dry time (minutes)	3.00±0.37	2.08±0.06	0.036
Spread ability (cm)	5.5±0.2	5.6±0.5	0.593

Table 5. Results of physical stability evaluation of face mist F2

Parameter	Day-0	Day-12	*p-value*
Organoleptic	Orange Liquid No smell	Orange Liquid No smell	-
Homogeneity	Homogeneous	Homogeneous	-
pH	5.9±0.0	5.7±0.0	0.083
Specific gravity (g/mL)	1.042±0.000	1.038±0.000	0.102
Viscosity (Cps)	1.260±0.035	1.192±0.023	0.021
Dry time (minutes)	5.32±0.15	4.18±0.12	0.000
Spread ability (cm)	4.5±0.2	5.1±0.2	0.109

Hedonic

The hedonic test aims to determine differences in the quality of similar products by assessing their level of likeability. The hedonic test data was analyzed using Two-Way ANOVA; respondents gave a color preference value in the average range of 3.88-4.00 (neutral-like) and showed that there was no significant difference in the level of liking for color parameters (p-value 0.462 >0.05). Aroma preference was scored in the average range of 3.88 to 3.94 (neutral) with no

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significant difference (p-value 0.563>0.05). Meanwhile, the absorption capacity preference was scored in the average range of 3.06 to 4.47 (neutral-like) and showed a significant difference in their level of preference for the absorption capacity parameter (p-value 0.00<0.05). Based on the data, F3 got the highest average score.

Antioxidant activity test

The secang wood antioxidant activity test aims to determine its ability to combat free radicals by evaluating the IC_{50} value. The DPPH method was carried out for this test based on natural ingredients' ability to inhibit free radicals. The mechanism by which antioxidant compounds react as antioxidant radical scavengers is by reducing DPPH, which is characterized by a change in the color of DPPH from purple to yellow, which occurs when the odd electron of the DPPH radical pairs with the hydrogen of the free radical scavenging compound, forming reduced DPPH-H, followed by a decrease in absorbance at a wavelength of 516 nm (Musykuroh & Abna, 2022).

The antioxidant activity of ascorbic acid was determined to ensure that this method could be used in this research. The antioxidant activity of the ethyl acetate fraction was determined to identify compounds that could prevent damage from free radicals. These compounds could serve as natural alternatives to the chemical antioxidants currently used in various products. Based on the results in Table 8, the IC_{50} values of the ethyl acetate fraction of secang wood, face mist F1, F2, and F3 were classified as very strong antioxidants. This suggests that these fractions can be further developed as sources for new natural antioxidant compounds. Antioxidant test data from the three statistically tested (F1, F2, and F3) shows that there is a significant difference in the average IC50 value between F1, F2, and F3 (p-value 0.027<0.05), which means the variations in the concentration of glycerin affect the antioxidant activity of the face mist ethyl acetate fraction of secang wood in each formula.

The data shows that IC_{50} value of all face mist formulas was greater than the IC_{50} value of the ethyl acetate fraction of secang wood which indicates that the IC_{50} value showed a decrease in antioxidant activity in the ethyl acetate fraction of secang after it was formulated. This is because the formulation applied pH of 5.9, while the ethyl acetate fraction of secang has a pH of 3.9. The stability of compounds' role as antioxidants is influenced by pH, where these compounds become unstable at higher pH levels, leading to structural changes in the compound (Sigi et al., 2015). Even though there has been a decrease in antioxidant activity, it was still included in the very strong antioxidant category.

The IC_{50} values of F1, F2, and F3 indicate that the higher the concentration of glycerin added, the greater the antioxidant activity provided, although the differences were not too significant. It caused glycerin to protect phenolics in the fraction ethyl acetate of secang wood. Glycerin would make hydrogen bonds with phenolic compounds so that the more concentration of glycerin added; the more phenolic compounds would be bound. This condition could help reduce chemical interactions that cause compound damage and increase antioxidant activity (Nuansa et al., 2017).

Table 6. Results of physical stability evaluation of face mist F3

Parameter	Day-0	Day-12	*p-value*
Organoleptic	Orange Liquid No smell	Orange Liquid No smell	-
Homogeneity	Homogeneous	Homogeneous	-
pH	5.9±0.0	5.7±0.0	0.083
Specific gravity (g/mL)	1.053±0.000	1.050±0.000	0.109
Viscosity (Cps)	1.326±0.029	1.281±0.023	0.280
Dry time (minutes)	7.13±0.11	6.15±0.22	0.026
Spread ability (cm)	4.2±0.1	4.4±0.1	0.180

Table 7. Hedonic test

Parameter	F1	F2	F3
Color	3.88±0.60	4.00±0.61	4.00±0.79
Aroma	3.88±0.69	3.82±0.80	3.94±0.74
Absorption capacity	4.47±0.51	3.65±0.86	3.06±1.14
Average score	4.08±0.60	3.82±0.76	4.67±0.89

Information:
(1) very dislike (2) Dislike (3) neutral (4) Like (5) really like it

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Table 8. IC_{50} value of ascorbic acid, ethyl acetate fraction of secang wood, face mist F1, F2, and F3 secang wood ethyl acetate fraction

Sample	IC_{50} (µg/mL)	Average of IC_{50} (µg/mL)
Ascorbic acid	8.862 8.945 8.983	8.930±0.061
Ethyl acetate fraction of secang wood	9.665 9.637 9.646	9.649±0.014
F1	22.094 22.168 22.082	22.114±0.046
F2	21.866 21.794 21.825	21.828±0.033
F3	21.360 21.408 21.368	21.378±0.025

CONCLUSION

The Face mist containing ethyl acetate fraction of secang wood demonstrated a strong antioxidant ability. However, the addition of glycerin concentrations at some variations (10 %, 15%, and 20%) did not positively affect the stability of the preparations, This is evidenced by significant differences observed in several parameters of the physical properties of the preparations.

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AUTHOR CONTRIBUTIONS

Conceptualization, N.H.R., N.U., D.P.; Methodology, N.H.R., N.U., D.P.; Software, N.H.R.; Validation, N.H.R.; Formal Analysis, N.H.R.; Investigation, N.H.R.; Resources, N.H.R.; Data Curation, N.U., D.P.; Writing - Original Draft, N.H.R.; Writing - Review & Editing, N.U., D.P.; Visualization, N.H.R.; Supervision, N.U., D.P.; Project Administration, N.U.; Funding Acquisition, N.U.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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Preliminary In Vitro Antiplatelet Potential of *Ipomoea pes-caprae* from North Lombok with Adenosine Diphosphate-Induced Platelet Aggregation

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Abstract

***Background**: Cardiovascular diseases, particularly ischemic stroke, remain a global health burden, necessitating potential candidate for further antiplatelet with fewer side effects. **Objectives**: This study aimed to evaluate the antiplatelet potential of ethanolic extracts from the leaves and stems of Ipomoea pes-caprae (Katang-katang) from North Lombok, Indonesia, through ADP-induced platelet aggregation. Phytochemical screening, total tannin quantification, and in vitro antiplatelet assays were conducted. **Methods**: The leaves and stems were macerated with 96% ethanol, followed by qualitative phytochemical tests, Folin-Ciocalteu-based tannin analysis, and platelet aggregation inhibition assays using human platelet-rich plasma. **Results**: The extracts contained alkaloids, flavonoids, and tannins, with higher tannin levels in leaves (4.02 ± 0.02 mgEAT/g) than stems (3.67 ± 0.17 mgEAT/g). Concentration-dependent antiplatelet activity was observed, with leaf extracts showing inhibition (85.9% at 2000 µg/mL) compared to stems (79.5%) and aspirin (77.3%). IC_{50} values were 727.78 µg/mL (leaves) and 349.95 µg/mL (stems). Statistical analysis confirmed significant differences across concentrations ($p < 0.05$). **Conclusion**: These findings demonstrate that Ipomoea pes-caprae exhibits potent antiplatelet activity, attributed to its tannin and phytochemical content, with leaves being more effective. Although these findings suggest preliminary antiplatelet potential, further analysis is required to validate the method using aspirin IC_{50}, and subsequent in vivo and pharmacological investigations are necessary before therapeutic application can be claimed.*

***Keywords:** adenosine diphosphate, antiplatelet, Ipomoea pes-caprae, phytochemical, tannin*

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INTRODUCTION

Platelets, or thrombocyte fragments, are non-nucleated cytoplasmic fragments of megakaryocytes formed in the bone marrow. In their mature form, they measure 2–4 µm and exhibit a biconvex disc shape. Platelets play a critical role when blood vessels are damaged or the skin is injured, leading to leakage that causes blood to exit the vessels, resulting in bleeding (Lobang, 2021). Platelets can contribute to thrombosis through activation, leading to adhesion and aggregation, ultimately forming a clot. These clots form when platelet levels in the blood exceed normal levels. Thrombosis can result in cardiovascular diseases, particularly ischemic stroke, when clots form in the brain's blood vessels (Ashorobi et al., 2024).

Globally, approximately 15 million people suffer from stroke annually. Of these, 5 million die and another 5 million are left permanently disabled, placing a heavy burden on families and communities. Stroke is rare in individuals under 40; when it does occur, hypertension is the primary cause. However, stroke also affects approximately 8% of children with sickle cell disease **(**WHO, 2025). In Indonesia, the prevalence of stroke increased by 3.9 per 1,000 population between 2013 and 2018 (Kemenkes RI, 2018). As the predominant form of stroke, ischemic stroke highlights the urgent need for effective prevention, especially through antiplatelet therapy (Hackam et al., 2019).

Antiplatelet agents commonly used in the treatment of non-cardioembolic ischemic stroke and transient ischemic attacks include aspirin, aspirin-dipyridamole, clopidogrel, and ticagrelor (Hackam et al., 2019). Aspirin inhibits platelet aggregation by blocking thromboxane A2 synthesis in platelets (Arif & Aggarwal, 2023). Dipyridamole inhibits phosphodiesterase and adenosine deaminase, preventing the conversion of cAMP and cGMP to their inactive forms. This increases intracellular cAMP and cGMP levels, thereby reducing platelet aggregation and thrombosis (Kerndt & Nagalli, 2023). While dipyridamole has relatively weak antiplatelet activity alone, its combination with aspirin is effective for secondary stroke prevention (Phillips & Gibson, 2021). Clopidogrel irreversibly inhibits the platelet P2Y12 adenosine diphosphate (ADP) receptor, preventing activation of the glycoprotein IIb/IIIa receptor complex and reducing aggregation (Beavers et al., 2025). Ticagrelor acts by reversibly binding to the P2Y12 ADP receptor to inhibit platelet activation and aggregation (Fuller & Chavez, 2012).

Despite their effectiveness, antiplatelet agents also have adverse effects. Aspirin, clopidogrel, and ticagrelor can cause bleeding, while dipyridamole may lead to dizziness, chest tightness, and abdominal pain (Wei et al., 2024; Whitlock et al.,2016; Lee et al., 2016). Therefore, alternative treatments derived from natural sources are needed. Natural compounds are considered due to their abundance in Indonesia (Indriani & Ardiana, 2023), their generally milder side effects compared to synthetic drugs (Sumayyah & Salsabila, 2017), and their substantial potential for therapeutic development (Novianti, 2017).

One plant with demonstrated antiplatelet properties is katang-katang (*Ipomoea pes-caprae*). According to research by Rogers et al. (2000), *I. pes-caprae* significantly inhibits ADP-induced platelet aggregation. This plant contains secondary metabolites such as alkaloids, flavonoids, tannins, and terpenoids (Akinniyi et al., 2022), all of which contribute to its antiplatelet effects. Alkaloids inhibit platelet aggregation by blocking thromboxane A2 synthesis induced by ADP, arachidonic acid, and collagen (Ain et al., 2016). Flavonoids interfere with arachidonic acid metabolism, thus disrupting platelet function (Zaragoza et al., 2022). Tannins exert antiplatelet effects through their antioxidant properties and inhibition of reactive oxygen species (ROS) production in platelets. They also increase antioxidant enzyme levels and prevent protein modification due to oxidative stress (Marcinczyk et al., 2022). The tannin content in plant extracts has been positively correlated with antiplatelet activity (Wiyono et al., 2018), indicating that higher tannin concentrations result in stronger antiplatelet effects. Therefore, quantification of tannin levels in katang-katang extracts is essential.

Additionally, the unique and relatively unexplored coastal environment of North Lombok presents an opportunity to investigate variations in the bioactive compound content of katang-katang. One method commonly used to determine antiplatelet activity in natural products is the ADP-induced platelet aggregation assay. This study aims to identify the qualitative presence of secondary metabolite compounds, quantify total tannin content, and evaluate the antiplatelet activity of the katang-katang plant using ADP induction methods.

MATERIALS AND METHODS

Materials

The materials used in this study include $AlCl_3$ p.a (Merck), amyl alcohol p.a (Merck), anhydrous acetic

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acid p.a (Merck), aspilet tablet, chloroform p.a (Merck), concentrated H_2SO_4 p.a (Merck), concentrated HCl p.a (Merck), Dragendorff reagent, ethanol 96%, $FeCl_3$ 5%, Folin-Ciocalteu reagent, tannic acid standard, gelatin 5%, HCl 2 Nildu, Katang-katang (*Ipomoea pes-caprae*) leaf and stem dry powder, Mayer Reagent, Mg powder, Na_2CO_3, Platelet Poor Plasma (PPP), Platelet Rich Plasma (PRP) sodium acetate 1 M, sodium citrate 3.2%, syringe 22, Wagner Reagent, Whole Blood sample (Inclusion criteria included healthy adult volunteers aged 18–40 years with normal blood pressure, glucose, and lipid levels, non-smokers, and not currently on any medication. Volunteers were selected regardless of gender, but with moderate and stable physical activity levels (no recent intense exercise or physical stress).

Tools

The tools used in this study include 1000 uL micropipette (Dragonlab), analytical balance (Ohauss), dehydrator, hot plate (Labnet), UV-Vis spectrophotometry, vacuum rotary evaporator (Heidolph).

Method

***Ipomoea pes-caprae* determination**

The katang-katang plant (*Ipomoea pes-caprae*) collected from Pemenang Beach, North Lombok Regency, was taxonomically identified as *Ipomoea pes-caprae* at the Advanced Biology Laboratory, Faculty of Mathematics and Natural Sciences, University of Mataram.

Extraction of *Ipomoea pes-caprae*

The leaves and stems of *Ipomoea pes-caprae* were extracted using the maceration method with a sample-to-solvent ratio of 1:10, utilizing 96% ethanol as the solvent. The maceration process was conducted for 24 hours with two replications, accompanied by occasional stirring. After 24 hours, the extract was filtered using mori cloth and subsequently evaporated using a vacuum rotary evaporator at 45 °C. The remaining liquid macerate was further dried using a hotplate at 40 °C to obtain a thick extract. This extract was then analyzed both qualitatively and quantitatively, and evaluated for secondary metabolite activity.

Phytochemical screening

Alkaloid test

Qualitative test of alkaloids used three reagents consisting of Dragendorff, Mayer, and Wagner reagents. A 0.1 g of thick extract was dissolved into 10 mL of 96% ethanol. The extract solution was heated on a hotplate for 2 minutes. Then, the extract solution was made and put into three different test tubes (Ananta et al., 2024). Each tube was filled with 3 mL of extract solution and added with 1 mL of 2 N HCl. Tube I was added 2-3 drops of Mayer reagent with positive results of white precipitate formation. Tube II added 2-3 drops of Wagner's reagent with positive results of orange-to-brown precipitate formation. Tube III added 2-3 drops of Dragendorff reagent with positive results of orange precipitate formation (Harahap, 2023). Qualitative positive results of alkaloids were indicated by positive results in two of the three experiments (Meigaria et al., 2016).

Flavonoid test

0.1 g of thick extract and 10 mL of 96% ethanol were combined in a beaker. In addition, 1 mL of concentrated HCl, 1 mL of amyl alcohol, and magnesium powder were added. The extract solution was thoroughly shaken (Ananta et al., 2024). The appearance of red, yellow, or orange color in the amyl alcohol layer indicates that this extract containds flavonoids (Zahra et al., 2021).

Tannin test

Qualitative tannin test used 5% FeCl3 reagent and 10% gelatin. A 0.1 g of thick extract was dissolved with 10 mL of 96% ethanol. The extract solution was divided into 2 test tubes. In tube I were given 5 drops of 5% FeCl3 reagent and positive results were marked by a color change to dark blue or greenish black (Puspitasari et al., 2013). Tube II was given drops of 10% gelatin and positive results were characterized by forming a white precipitate (Astarina et al., 2013).

Saponin test

A 0.1 g of concentrated extract was mixed with 10 mL of ethanol and boiled with water at 100°C for five minutes. The filtrate was collected and used as the test solution. In a closed test tube, the filtrate was agitated for ten seconds and left for ten minutes. A 1 mL of 2 M HCl developed the foam after shaking. When a steady foam forms for 30 seconds and has a 1-3 cm height, it indicates that the extract contains saponins (Padmasari et al., 2013; Marami et al., 2021)

Total tannin test

Preparation of saturated sodium carbonate

A total of 37 g of sodium carbonate was dissolved in distilled water. A 100 mL volumetric flask was filled with the solution and then distilled water was added to finish the capacity. Several minutes were spent vortexing the solution.

Preparation of standard solution and concentration series

A 100 mL of distilled water was mixed with 10 milligrams of tannic acid to achieve a 100 µg/mL concentration (Ryanata et al., 2015). The stock solution

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was diluted into 5 series of 20, 40, 60, 80, and 100 µg/mL.

Determination of maximum wavelength

One mL of a standard solution containing 20 µg/mL was combined with 7.5 mL of distilled water and 0.5 mL of Folin-Ciocalteu reagent (Kurniawati et al., 2024). One milliliter of saturated sodium carbonate was added after the solution had stood for three minutes (Pratama et al., 2019) and then incubated for 15 minutes. The absorbance was measured in the 400-800 nm range (Pratama et al., 2019). The maximum lambda was determined at the highest absorbance.

Preparation of standard curve

Each concentration series was taken up to 1 mL and combined with 7.5 mL of distilled water and 0.5 mL of Folin- Ciocalteu reagent. After standing for 3 minutes, 1 mL of saturated sodium carbonate was added and incubated for fifteen minutes. The absorbance was determined using the highest wavelength. The linear regression equation and standard curve were created by plotting the concentration and absorbance data onto a curve.

Determination of sample tannin content

A 0.5 g of the extract was weighed and then dissolved in 10 mL of distilled water. One milliliter of the solution was extracted and combined with 0.5 milliliters of Folin-Ciocalteu reagent and 7.5 milliliters of distilled water. After letting the solution stand for three minutes, one milliliter of saturated sodium carbonate was added. After that, it was incubated once again for fifteen minutes. The highest wavelength attained was used to measure the absorbance. Three replications of the measurement were made. The regression equation was used to compute the levels.

Antiplatelet activity

Preparation of whole blood, platelet rich plasma (PRP), and platelet poor plasma (PPP)

The samples used were Whole Blood, Platelet Rich Plasma (PRP), and Platelet Poor Plasma obtained from volunteers with inclusion criteria are normal blood pressure, glucose levels, and blood cholesterol. PRP was prepared by taking blood samples from the veins of the arms of healthy volunteers. A total of 20 mL of blood was transferred into a centrifuge tube containing 3 mL of 3.2% sodium citrate. The blood was centrifuged for 15 min at 1000 rpm. The upper plasma layer was carefully separated. The plasma layer is PRP (Platelet Rich Plasma). The remaining blood in the centrifuge tube was again centrifuged for 15 minutes at 3500 rpm. The upper plasma layer was carefully separated. The plasma layer is PPP (Platelet Poor Plasma). PPP was used as a blank (Lubis, 2015).

Preparation of aspirin stock solution

A mortar and pestle were used to grind the 80 mg aspilet (aspirin tablet). Up to 25 milligrams of powdered aspirin pill was weighed. After that, a 25 mL volumetric flask was filled with it. After adding distilled water to the maximum, the mixture was homogenized for ten minutes using a sonicator set at 35°C (Wijayanti et al., 2022).

Preparation of ADP (adenosine diphospate) 5 uM

In a 5 mL volumetric flask, 0.125 mL of ADP (Adenosine Diphospate) was dissolved with 5 mL of pure water, and then homogenized using a vortex mixer. Prior to usage, the solution was kept in the refrigerator (Wijayanti et al., 2022).

Preparation of katang-katang (*Ipomoea pes-caprae*) extract solution

Four concentration series—250, 500, 1000, and 2000 µg/mL —were created from the extract solution. 50 mg of the thick extract was weighed and then dissolved with a small amount of distilled water until the limit was reached in a 25 mL volumetric flask. A vortex mixer was used to homogenize the mixture. A 2000 µg/mL of the extract solution was diluted to 1000, 500, and 250 µg/mL. In a 5 mL volumetric flask, each dilution was pipetted in accordance with the calculation and mixed with distilled water until the limit was reached.

Antiplatelet activity test

PPP, as much as 3 mL was used as a blank, aspirin 0.4 mL: PRP 1.8 mL was positive control, distilled water 0.4 mL: PRP 1.8 mL was negative control, while each series contained 0.4 mL of test solution: PRP 1.8 mL were the test solutions. All of them were homogenized using a vortex for 3 minutes and measured using UV-VIS spectrophotometry at 600 nm. This absorbance result was the absorbance before ADP administration. Then positive control, negative control, and concentration series solution were each added 0.08 mL of ADP and incubated for 10 minutes in an incubator at 37°C. All of them were measured absorbance using UV-Vis spectrophotometry at 600 nm. The result of the absorbance was the absorbance after ADP administration (Lubis, 2015).

Data analysis

Data were obtained by measuring absorbance values before and after the addition of ADP to calculate the percentage of platelet aggregation. The percentage of platelet aggregation inhibition was determined by comparing the negative control group with the treatment

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groups, which included the positive control (aspirin) and the Katang-katang stem and leaf extracts. Statistical analysis was conducted using SPSS, which included the Shapiro–Wilk test for normality, Levene's test for homogeneity of variance, one-way ANOVA, and Tukey's HSD post hoc test. The IC_{50} value was determined through linear regression analysis between the logarithm of extract concentrations and the mean percentage of platelet aggregation inhibition. The formulas used to calculate the percentage of platelet aggregation and its inhibition are presented below:

$$\%\ platelet\ aggregation = 1 - \frac{B}{A} x\ 100\% \quad \text{.....1)}$$

Notes:

A = Absorbance before the addition of ADP

B = Absorbance after the addition of ADP

$$\%\ platelet\ aggregation\ inhibition = \frac{A-B}{A} x\ 100\% \text{.....(2)}$$

Notes

A = Persentase agregasi platelet pada kontrol negatif (%)

B = Persentase agregasi platelet pada kelompok perlakuan (%)

IC_{50} calculation

The IC_{50} values were calculated using linear regression analysis between the logarithm of concentration and the percentage of platelet aggregation in the stem and leaf extracts of *Ipomoea pes-caprae*. The log concentrations were obtained by applying a logarithmic transformation to the concentration data. These two variables were then plotted to produce a linear graph. From the linear regression analysis, the R^2 value and the regression equation were obtained. This equation was subsequently used to determine the IC_{50} values by setting y to 50, from which the corresponding x value representing the IC_{50} of each extract was derived.

RESULTS AND DISCUSSION

Results

Extracts yield

Based on the data in Table 1, the 96% ethanol extract of *Ipomoea pes-caprae* leaves yields 20,63%, while the stem extract only reaches 10,09%.This yield is calculated from the ratio of the weight of the extract to the weight of the initial dry plant.The leaf extract is produced from 300 g of dry plant into 61.91 g of extract, while the stem with an initial weight of 213 g of dry plant yields 21.491 g of extract.

Tabel 1. Ethanolic extracts of leaf and stem *Ipomoea pes-caprae* yields

No.	Extract Name	Dry plant weight (g)	Extract Weight (g)	Yield (%)
1	Ethanol extract of katang-katang leaves (*Ipomoea pes-caprae*)	300	61.9	20,63%
2	Ethanol extract of katang-katang stems (*Ipomoea pes-caprae*)	213	21.5	10,09%

Table 2. Phytochemical screening results for the 96% ethanol extract of *Ipomoea pes-caprae*

Testing		Leaf extract			Stem extract		
		Results	Interpretation	Result Image	Results	Interpretation	Result Image
Alkaloid	Dragendorff	Orange-colored sediment	(+)		Orange-colored sediment	(+)	
	Mayer	white precipitate	(+)		white precipitate	(+)	
	Wagner	Brownish-red sediment	(+)		Brownish-red sediment	(+)	
Tannin	FeCl 5%	Dark greenish color	(+)		Dark greenish color	(+)	
	Gelatin 10%	white precipitate	(+)		white precipitate	(+)	

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Flavonoid	Brick red color	(+)		Brick red color	(+)	
Saponin	A stable foam not form for 15-20 minutes.	(-)		A stable foam not form for 15-20 minutes	(-)	

Information:
(+) = positive contains compounds
(-) = negative contains compounds

Phytochemical screening

The results of the phytochemical screening of 96% ethanol extracts of the leaves and stems of *Ipomoea pes-caprae* in Table 2 show that both types of extracts contain various bioactive compounds.The alkaloid test using Dragendorff, Mayer, and Wagner reagents showed positive results, indicated by the formation of orange precipitates, white precipitates, and reddish-brown precipitates, both in the leaf and stem extracts.Tannin content was also detected in both extracts, indicated by a color change to dark green when tested with 5% $FeCl_3$ solution and the formation of a white precipitate with 10% gelatin. The flavonoid test showed positive results with the formation of a brick-red color, while the saponin test not produced stable foam for 15–20 minutes, which was also confirmed negative in both leaf and stem extracts.

Tannic acid standard curve

The standard curve data of tannic acid from 20 to 100 µg/mL shows a positive relationship between concentration and absorbance value, in accordance with the Beer-Lambert Law principle. At a concentration of 20 µg/mL, the absorbance recorded was 0.177, then gradually increased to 0.443 at 100 µg/mL. This increase indicates that the higher the concentration of tannic acid, the more light is absorbed by the solution.

Table 3. Tannic acid standard curve 20 to 100 µg/mL

Concentration (µg/mL)	Absorbance
20	0.177
40	0.250
60	0.318
80	0.407
100	0.443

The results of the linear regression from the standard curve of tannic acid show a very strong relationship between the concentration of tannic acid (20–100 µg/mL) and the absorbance value.The obtained regression equation is y = 0.0034x + 0.1128, with a coefficient of determination (R^2 = 0.9881).The R^2 value close to 1 (98.81%) indicates that 98.81% of the variation in absorbance can be explained by changes in tannic acid concentration, while the remaining 1.19% may be due to experimental factors such as measurement errors or minor variations in sample preparation.

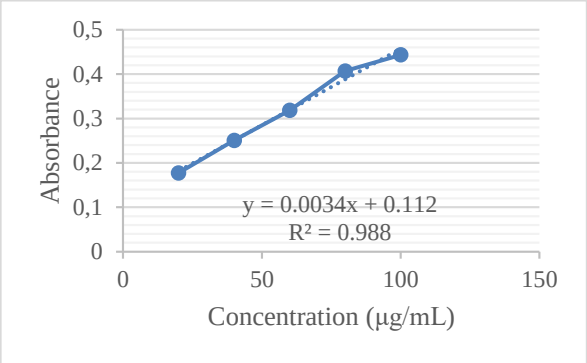

Figure 1. Tannic acid standard curve graph

Determination of total tannin content

The data on tannin content in the leaf and stem extracts of *Ipomoea pes-caprae* show a significant difference between the two parts of the plant.Based on three replications, the leaf extract has an average tannin content of 4.02 ± 0.02 mgEAT/g, while the stem extract has an average tannin content of 3.673 ± 0.168 mgEAT/g.The absorbance values of the leaf samples in the three replicates were very consistent (0.799; 0.797; 0.794), resulting in stable tannin levels (4.04; 4.02; 4.00 mgEAT/g).The low standard deviation (±0.02) indicates high precision in the measurements.There is greater variation in the absorbance of the stem samples across the three replicates (0.755; 0.754; 0.705), especially in the third replicate which is lower.This causes the tannin content to vary (3.78; 3.76; 3.48 mgEAT/g) with a relatively high standard deviation (±0.17), indicating instability or external factors affecting the results.The tannin content in the leaves (4.02 mgEAT/g) is 9.4% higher than in the stems (3.67 mgEAT/g).

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Platelet aggregation data

The results of the platelet aggregation test in Table 5 show that the positive control (aspirin 1000 µg/mL) had the lowest aggregation value, at 2.15% ± 1.15, while the negative control (aquadest) showed a high aggregation value of 9.34% ± 2.03. In the stem extract of *Ipomoea pes-caprae*, aggregation decreased with increasing concentration, from 5.08% ± 1.80 at 250 µg/mL to 1.98% ± 0.91 at 2000 µg/mL. A similar pattern was observed in the leaf extract, where aggregation decreased from 6.17% ± 0.73 at 250 µg/mL to 1.21% ± 0.50 at 2000 µg/mL. Overall, both leaf and stem extracts exhibited concentration-dependent inhibition of platelet aggregation, with leaf extracts showing greater inhibition than stem extracts at the same concentration.

Platelet aggregation inhibiton data

The results of the platelet aggregation inhibition test in Table 6 showed that the positive control (aspirin 1000 µg/mL) produced an inhibition of 77.26% ± 12, while the negative control (aquadest) showed no inhibition activity (0%). In the stem extract of *Ipomoea pes-caprae*, the percentage of inhibition increased with the concentration, from 46.07% ± 15.34 at 250 µg/mL to 79.51% ± 5.50 at 2000 µg/mL. Similarly, in the leaf extract, the inhibition of aggregation increased from 32.80% ± 8.90 at 250 µg/mL to 85.90% ± 7.78 at 2000 µg/mL. Overall, the leaf extract showed a higher percentage of inhibition compared to the stem extract at concentrations of 1000 µg/mL and 2000 µg/mL, indicating a stronger potential for antiplatelet activity in the leaf extract.

Table 4. Tannic content in leaves and stems extract of *Ipomoea pes-caprae*

Sample	Replication	Absorbance	Tannin Concentration (µg/mL)	Tannin Content (mgEAT/g)	Mean Tannin Content (mgEAT/g) ± SD
Leaves	1	0.799	202.03	4.04	4.02 ± 0.02
	2	0.797	201.5	4.02	
	3	0.794	200.44	4	
Stems	1	0.755	189.09	3.78	3.67 ± 0.17
	2	0.754	188.88	3.76	
	3	0.705	174.29	3.48	

Table 5. Platelet aggregation data for the 96% ethanol leaf and stem extract of *Ipomoea pes-caprae*

Groups	Concentration (µg/mL)	Mean Platelet Agregation (%) ± SD
Positive Control	1000	2.15 ± 1.15
Negative Control	-	9.34 ± 2.03
Stem	250	5.08 ± 1.78
	500	4.28 ± 1.24
	1000	3.16 ± 1.28
	2000	1.98 ± 0.91
Leaf	250	6.17 ± 0.73
	500	3.13 ± 0.36
	1000	1.83 ± 0.63
	2000	1.21 ± 0.50

Table 6. Platelet aggregation inhibition data for the 96% ethanol leaf and stem extract of *Ipomoea pes-caprae*

Groups	Concentration (µg/mL)	Mean Platelet Agregation Inhibition (%) ± SD
Positive Control	1000	77.26 ± 12
Negative Control	-	0
Stem	250	46.07 ± 15.34
	500	53.66 ± 13.51
	1000	66.79 ± 10.21
	2000	79.51 ± 5.5
Leaf	250	32.8 ± 8.9
	500	65.69 ± 6.23
	1000	79.31 ± 8.94
	2000	85.9 ± 7.78

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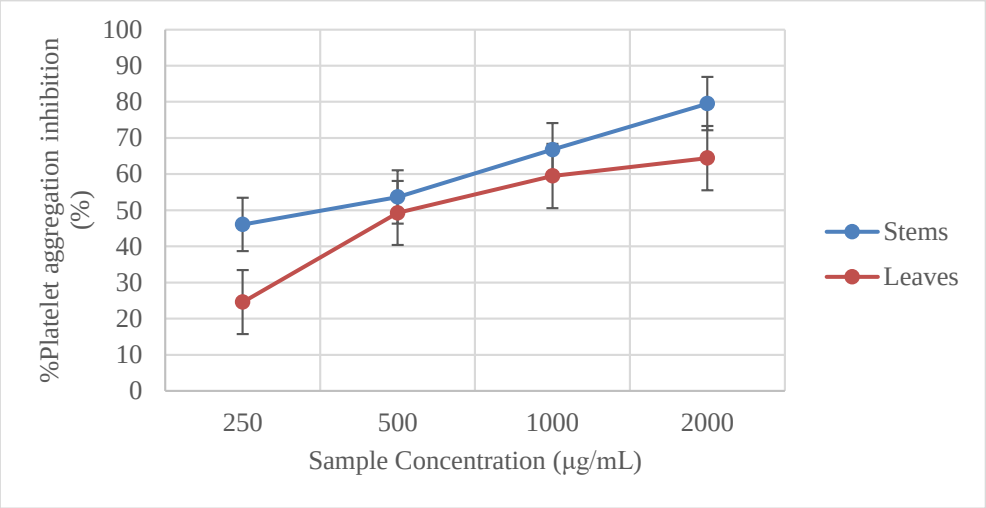

Figure 2. Graph of sample concentration vs % platelet aggregation inhibition from leaf and stem extracts of katang-katang (*Ipomoea pes-caprae*)

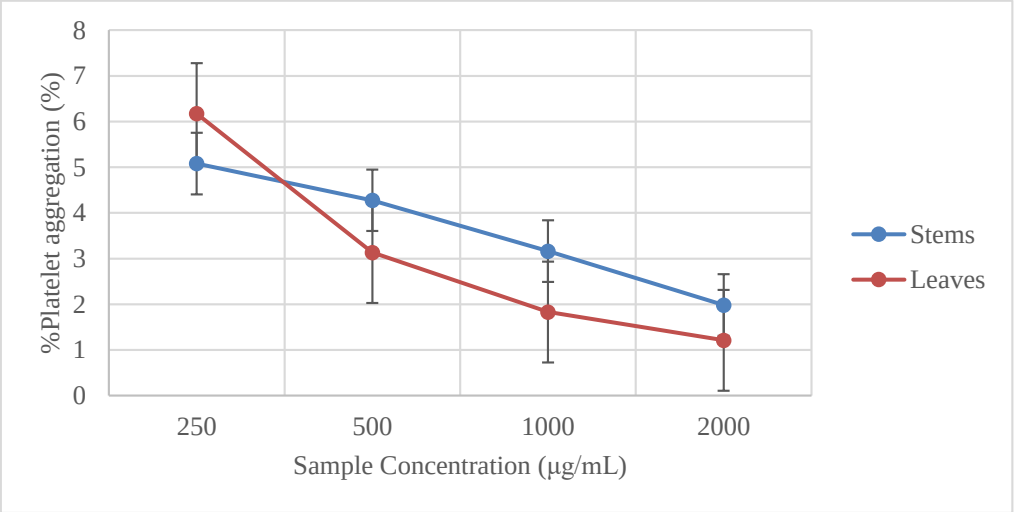

Figure 3. Graph of sample concentration vs % platelet aggregation from leaf and stem extracts of katang-katang (*Ipomoea pes-caprae*)

Graph of concentration vs response

The graph of the relationship between the concentration of leaf and stem extracts of *Ipomoea pes-caprae* and the percentage of platelet aggregation inhibition shows an interesting pattern. At a concentration of 250 µg/mL, the stem extract provided an inhibition of 46.07%, while the leaf extract only showed 24.60%, indicating that the stem is more effective at lower doses. However, this trend changes with increasing concentration. At 500 µg/mL, the inhibition of the stem extract increased to 53.66%, while the leaf extract reached 49.26%, indicating a narrowing difference. A significant increase was observed at a concentration of 1000 µg/mL, where the leaf extract showed the highest inhibition at 79.51%, far surpassing the stem extract which was only 66.79%. This confirms that the leaf extract has greater potential as an anti-platelet aggregation agent at the optimal dose. However, at the highest concentration (2000 µg/mL), the inhibition of the leaf extract decreased to 64.43%, while the stem extract remained stable at 64.43%. This decrease in the leaf extract may be due to the toxic effects of active compounds or receptor saturation at excessively high concentrations.

The graph of the relationship between the concentration of leaf and stem extract samples of *Ipomoea pes-caprae* and the percentage of platelet aggregation shows a trend of decreasing aggregation with increasing concentration, indicating a stronger inhibitory effect. At a concentration of 250 µg/mL, the stem extract resulted in platelet aggregation of 6.17%, while the leaf extract was more effective with an

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aggregation of 5.08%. Increasing the concentration to 500 µg/mL enhances this effect: stem aggregation decreases to 4.28%, and leaf aggregation reaches 3.16%. At a concentration of 1000 µg/mL, both extracts showed a significant decrease in aggregation. The stem extract reached 3.13%, while the leaf extract was superior with an aggregation of 1.98%. This trend continues at the highest concentration (2000 µg/mL), where the stem extract recorded an aggregation of 1.83%, while the leaf extract showed the lowest value, which is 1.21%.

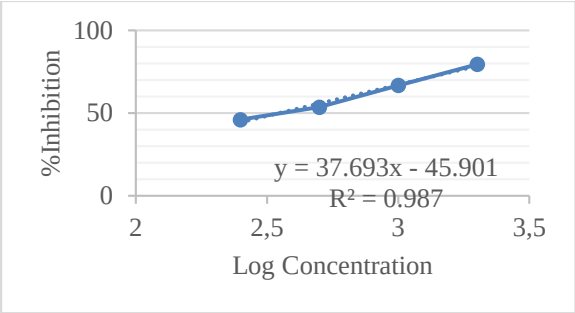

Figure 3. Linear regression curve of log concentration vs %inhibition of platelet aggregation of the stem sample of katang-katang (*Ipomoea pes-caprae*)

The results of the linear regression in Figure 3, which analyzes the relationship between the log concentration of *Ipomoea pes-caprae* stem extract and the percentage of platelet aggregation inhibition, show a very strong relationship. The obtained regression equation is $y = 37.693x - 45.901$, with a coefficient of determination ($R^2 = 0.987$). The R^2 value approaching 1 (98.7%) indicates that 98.7% of the variation in % inhibition can be explained by changes in log concentration, while the remaining 1.3% may be influenced by other factors or experimental error. The slope (37.693) indicates that for every increase of 1 unit in log concentration, the percentage of platelet aggregation inhibition increases by 37.693%. Meanwhile, the intercept (–45.901) represents the theoretical inhibition value when the log concentration is 0 (concentration of 1 µg/mL), although this value is not practically relevant because it falls outside the range of the tested data.

The linear regression analysis in Figure 4, which examines the relationship between the log concentration of *Ipomoea pes-caprae* leaf extract and the percentage of platelet aggregation inhibition, yields the equation $y = 43.087x - 73.333$ with a coefficient of determination ($R^2 = 0.8923$). This R^2 value indicates that 89.23% of the variation in % inhibition can be explained by changes in log concentration, while the remaining 10.77% may be influenced by other factors such as biological variation or measurement error. The positive slope (43.087) shows that for every 1-unit increase in log concentration, platelet aggregation inhibition increases by 43.087%. However, the negative intercept (–73.333) has no practical significance because it falls outside the tested concentration range (250–2000 µg/mL).

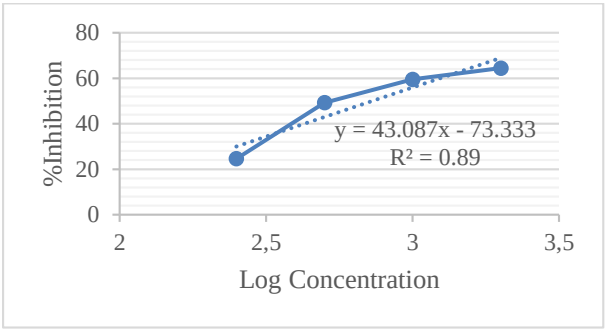

Figure 4. Linear regression curve of log concentration vs %inhibition of platelet aggregation of the leaf sample of katang-katang (*Ipomoea pes-caprae*)

Statistical analysis

The results of the Shapiro-Wilk normality test on the stem and leaf samples of *Ipomoea pes-caprae* indicate that all data groups do not significantly deviate from a normal distribution, with p-value (Sig.) values above 0.05. In the stem samples, the p-value ranged from 0.173 (500 µg/mL) to 0.772 (250 µg/mL), while in the leaf samples, the p-value was more homogeneous, ranging from 0.385 (250 µg/mL) to 0.556 (2000 µg/mL). The control group (+) also meets the normality assumption with a p-value of 0.689.

The results of the Levene's homogeneity test show that the data on the percentage of platelet aggregation inhibition in the stem extract (Sig. = 0.56) and leaves (Sig. = 0.78) have homogeneous variances (p-value > 0.05). This meets the basic assumption for One-Way ANOVA analysis, so the ANOVA results can be considered valid. In the stem extract, the One-Way ANOVA results yielded Sig. = 0,23 (< 0.05), indicating a significant difference in the effects of platelet aggregation inhibition among the concentrations (250, 500, 1000, 2000 µg/mL). In other words, the increase in the concentration of the stem extract statistically significantly affects the percentage of inhibition. On the other hand, in the leaf extract, the One-Way ANOVA results showed Sig. = 0.00 (< 0,05), which means there is a significant difference in the inhibition effect of platelet aggregation between concentrations. Statistically, the leaf samples can affect platelet antiaggregation activity.

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Table 11. Tukey HSD post hoc test for the stem extract sample of katang-katang (*Ipomoea pes-caprae*)

Test Groups	p-value	Interpretation
C (+) vs B1	0.054	Not significantly different
C (+) vs B2	0.179	
C (+) vs B3	0.809	
C (+) vs B4	0.999	
B1 vs B2	0.928	
B1 vs B3	0.272	
B1 vs B4	0.038	Significantly different
B2 vs B3	0.662	Not significantly different
B2 vs B4	0.127	
B3 vs B4	0.686	

Table 12. Tukey HSD post hoc test for the leaf extract sample of katang-katang (*Ipomoea pes-caprae*)

Test Groups	p-value	Interpretation
C (+) vs D1	0.001	Significantly different
C (+) vs D2	0.540	Not significantly different
C (+) vs D3	0.998	
C (+) vs D4	0.763	
D1 vs D2	0.008	Significantly different
D1 vs D3	0.001	
D1 vs D4	<0.001	
D2 vs D3	0.395	Not significantly different
D2 vs D4	0.113	
D3 vs D4	0.891	

Discussion

Phytochemical screening

This study used dry powder derived from the leaves and stems of Katang-katang (*Ipomoea pes-caprae*), obtained from fresh samples collected at Pemenang Beach, North Lombok Regency. After the extraction process, the yield of the leaf and stem extracts was determined by comparing the weight of the extract obtained to the weight of the dry plant used. Extract yield refers to the ratio of the dry weight of the extract to the amount of raw material utilized. A higher yield indicates a greater concentration of compounds extracted from the raw material (Senduk et al., 2020). As shown in Table 1, the extract yields from the leaves and stems of Katang-katang were 20.63% and 10.09%, respectively. According to Farmakope Herbal Indonesia (Kemenkes RI, 2017), a good extract should have a yield greater than 10%. Thus, both extracts meet the quality standard for yield.

However, the difference in yield between the two extracts may be attributed to anatomical differences between the samples. Leaves, as the main photosynthetic organ, are rich in secondary metabolites, comprising approximately 83–88% of their content (Destailleur et al., 2021). The parenchyma tissue in the leaves stores these metabolites and facilitates their extraction by ethanol, which can penetrate and disrupt cell walls, thereby enhancing the release of secondary compounds (Yulianti et al., 2020). Moreover, ethanol's polarity allows it to extract both polar and non-polar compounds (Karepu et al., 2020). In contrast, the stem contains more structural tissue dominated by lignin and cellulose. Although most lignin can dissolve in pure ethanol, the presence of water in the solvent—such as in 96% ethanol, which contains 4% water—reduces the amount of lignin that can be dissolved (Tindall et al., 2020).

Phytochemical screening was performed on both extracts to detect the presence of alkaloids, tannins, steroids/triterpenoids, flavonoids, and saponins, as summarized in Table 2. The results showed that both extracts contain secondary metabolites such as alkaloids, tannins, and flavonoids, while saponins were not detected.

Alkaloids in both extracts were identified using Dragendorff's, Mayer's, and Wagner's reagents. Dragendorff's reagent forms a precipitate through the coordination of potassium ions (K^+) with alkaloids, resulting in potassium-alkaloid complexes (Sangkal et al., 2020). Mayer's reagent similarly produces a

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precipitate by forming a potassium-alkaloid complex via the interaction between K^+ ions and nitrogen atoms within the alkaloid structure (Wardhani & Supartono, 2015). Wagner's reagent also forms a potassium-alkaloid complex through coordination bonds between K^+ ions and alkaloid nitrogen atoms (Adhariani et al., 2018).

Tannins were detected through the addition of 5% $FeCl_3$ and 5% gelatin. The phenolic groups in tannins react with $FeCl_3$ to form a dark-colored triscianoferric (III) complex (Desinta, 2015; Ananta et al., 2024). Meanwhile, gelatin reacts with tannins to form stable, water-insoluble copolymers, leading to the crystallization of proteins and the formation of a white precipitate (Lestari et al., 2024).

Flavonoids were identified using the Shinoda test, which involves the addition of magnesium powder and concentrated HCl. Concentrated HCl hydrolyzes O-glycosides by replacing the glycosidic group with protons due to its electrophilic nature, while magnesium reduces the flavonoid carbonyl group, allowing it to form complexes. The addition of amyl alcohol facilitates the development of a colored complex or precipitate, confirming the presence of flavonoids (Ananta et al., 2024).

Total tanin test

The determination of total tannin content in the leaf and stem extracts of *Ipomoea pes-caprae* (katang-katang) was conducted using an in vitro method. Tannic acid was used as the standard, as it represents a hydrolyzable form of tannin and is commonly found in most aerial parts of plants (Safitri et al., 2023; Maharani et al., 2022). Tannic acid is a natural polyphenolic compound that contains phenolic hydroxyl and carboxyl groups, and shares structural similarities with tannins found in plants (Basri et al., 2023).

The standard tannic acid solution and the sample extracts were reacted with the Folin–Ciocalteu reagent, which forms a blue-colored complex due to the interaction between phenolic compounds and the molybdenum-tungsten components of the reagent. Since the formation of this blue complex requires an alkaline environment, sodium carbonate was added to the reaction mixture (Riyanti et al., 2023). Once the reaction was complete, the absorbance of the colored solution was measured using a UV-Vis spectrophotometer.

To determine the maximum wavelength, the absorbance of the sample was scanned in the range of 400–800 nm. The maximum absorbance for tannic acid was found at 650 nm. A standard curve was generated using five concentration series (20, 40, 60, 80, and 100 µg/mL) prepared by diluting a stock solution of 1000 µg/mL. Each concentration was reacted with the reagents and its absorbance was measured at 650 nm. From the absorbance versus concentration data, the linear regression equation obtained was $y = 0.0034x + 0.1128$, with a correlation coefficient (r) of 0.994, indicating strong linearity (Karim et al., 2021). To determine whether the obtained correlation coefficient (R) from the regression is statistically significant, the degrees of freedom (df) were calculated as $n - 2$, where n represents the number of data points. In this study, five concentration levels of tannic acid were used (20, 40, 60, 80, and 100 µg/mL), resulting in $n = 5$, and thus $df = 3$. Referring to the Pearson correlation significance table, the critical value of r at df = 3 for a two-tailed test at the 0.05 significance level is 0.8783. The calculated correlation coefficient $r = 0.994$ exceeds the critical value, indicating a statistically significant linear relationship between tannic acid concentration and absorbance. This confirms that the standard curve exhibits a valid and reliable linearity for further quantification analysis.

For the sample measurements, 500 mg of each extract (leaf and stem) was dissolved in 10 mL of distilled water. The resulting solution was reacted with the Folin–Ciocalteu reagent and incubated for 15 minutes to allow the reaction to stabilize (Riyanti et al., 2023). Each sample was analyzed in triplicate. The absorbance values obtained from the samples were then plotted into the standard regression equation to calculate the total tannin content in the extracts.

In Table 3, five concentration levels of tannic acid (20, 40, 60, 80, and 100 µg/mL) were used to construct the standard curve. The selection of these concentrations was intended to minimize potential errors that could affect the accuracy of the regression line. As the concentration of tannic acid increases, the absorbance also increases, due to the compound's ability to absorb ultraviolet light measured by the spectrophotometer (Kriechbaum & Bergstrom, 2020).

As shown in Figure 1, the standard curve of tannic acid exhibits a linear relationship with an R^2 value of 0.9881, and a linear regression equation of $y = 0.0034x + 0.1128$. The R^2 value indicates a strong linear correlation, as a good correlation coefficient generally ranges from 0.7 to 0.99 (Gupta et al., 2024). The closer the R^2 value is to 1, the more accurate the linearity of the regression model becomes. This linear regression equation was then used to determine the total tannin content in the sample extracts.

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The calculation of total tannin content is based on the tannin concentration derived from the regression equation, the volume of the solution, the dilution factor, and the weight of the extract. The results are expressed in mg EAT/g (milligrams of tannic acid equivalent per gram), indicating the amount of tannin in the sample equivalent to a certain amount of pure tannic acid.

Based on Table 4, the average tannin content in the leaf extract was 4.02 mg EAT/g, while the stem extract contained 3.673 mg EAT/g. This difference reflects the variation in tannin compound distribution between the plant organs. Although tannins are generally present in various plant parts, including leaves, fruits, bark, and stems (Suhaenah et al., 2024), the higher lignin content in stems compared to leaves can inhibit the extraction efficiency. Since the solvent used is less effective at dissolving lignin, less tannin is extracted from the stems than from the leaves.

Antiplatelet activity in vitro

The antiplatelet activity of the leaf and stem extracts of Katang-katang (*Ipomoea pes-caprae*) was evaluated using the in vitro ADP-induced platelet aggregation method with UV-Vis spectrophotometry (Wijayanti et al., 2022). ADP (adenosine diphosphate) is a platelet agonist that induces changes in platelet shape and promotes aggregation, playing a key role in the formation of thromboxane A2 (Jin et al., 2002). ADP contributes to primary platelet aggregation by interacting with specific surface receptors, leading to platelet activation, morphological changes, aggregation, thromboxane A2 formation, and granule release (Puri & Colman, 1997; Daniel et al., 1998). The in vitro antiplatelet testing method with ADP induction serves as an initial approach to determine the antiplatelet activity of katang-katang extract (*Ipomoea pes-caprae*), thus providing a foundation for future katang-katang antiplatelet research.

Fresh blood samples (20 mL) were collected from human volunteers who met the inclusion criteria. The positive control used was aspilet (1 mg/mL), containing aspirin, a COX-1 inhibitor that suppresses thromboxane A2 synthesis, thereby inhibiting platelet aggregation. Distilled water served as the negative control.

Sodium citrate was added to the blood samples as an anticoagulant. It functions by chelating calcium ions, thereby preventing the formation of thrombin, which is necessary for converting fibrinogen to fibrin during the coagulation process. Sodium citrate also stabilizes erythrocytes and maintains blood integrity for up to 4 hours post-collection. From the blood samples, PRP (Platelet-Rich Plasma) and PPP (Platelet-Poor Plasma) were prepared. PRP contains a high concentration of platelets, while PPP contains relatively few. The platelet aggregation response was measured using a UV-Vis spectrophotometer. ADP serves to induce platelet activation and aggregation (Wijeyeratne & Heptinstall, 2011). The leaf and stem extracts of *Ipomoea pes-caprae* are expected to exhibit inhibitory effects on platelet aggregation in response to ADP stimulation.

The parameters assessed in this study were the percentage of platelet aggregation and the percentage of platelet aggregation inhibition. Platelet aggregation refers to the adhesion and clumping of platelets, which play a crucial role in hemostasis during vascular injury. Aggregation is typically induced by agonists such as adenosine diphosphate (ADP) and collagen, which are present at the injury site (Rumbaut et al., 2010).

The percentage of platelet aggregation was calculated using Formula 1, based on the change in turbidity of the blood sample during the aggregation process. Prior to aggregation, platelets are uniformLy suspended in the plasma, causing the sample to appear turbid and resulting in high absorbance due to limited light transmission. As aggregation occurs, the platelets clump together, reducing turbidity and increasing light transmission, which leads to lower absorbance readings. ADP functions as an aggregation inducer by promoting platelet adhesion and aggregate formation.

The percentage of platelet aggregation inhibition was calculated using Formula 2, which quantifies the reduction in aggregation in treated samples compared to the negative control. As shown in Table 4, platelet aggregation increased with increasing extract concentrations, suggesting that the leaf and stem extracts exert inhibitory effects on platelet aggregation in a concentration-dependent manner.

The positive control, aspirin at a concentration of 1 mg/mL, exhibited a significantly lower platelet aggregation value compared to the negative control. This outcome is consistent with the known antiplatelet properties of aspirin, which functions by inhibiting the cyclooxygenase-1 (COX-1) enzyme, thereby reducing the synthesis of thromboxane A_2, a key mediator of platelet aggregation. Aspirin exerts its antiplatelet effect primarily through the irreversible inhibition of cyclooxygenase-1 (COX-1) by acetylating the serine residue at its active site. This modification leads to a substantial reduction in prostaglandin biosynthesis. In platelets, COX-1 is not rapidly regenerated due to the lack of a nucleus, and thus, its enzymatic activity can only be restored through the formation of new platelets. As a result, aspirin significantly impairs the synthesis of

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thromboxane A_2, prostaglandin E_2, and prostacyclin (PGI_2), all of which play critical roles in platelet aggregation and vasoconstriction. This inhibition ultimately disrupts normal hemostatic function by diminishing the capacity for platelet-driven coagulation (Ornelas et al., 2017). The platelet aggregation value obtained for the positive control was 2.1506%, substantially lower than that of the negative control. Correspondingly, the percentage inhibition of platelet aggregation reached 77.261%. This high inhibition value reflects the potent mechanism of aspirin in suppressing platelet activity, validating its role as an effective antiplatelet agent in this in vitro model.

The negative control group exhibited the highest platelet aggregation among all groups, as it only received distilled water, which lacks antiplatelet properties. Conversely, Table 5 demonstrates that the percentage of platelet aggregation inhibition increased with concentration in both the leaf and stem extract groups. Notably, the 2000 µg/mL concentrations of both extracts exhibited higher inhibition percentages than the positive control (aspirin), suggesting that *Ipomoea pes-caprae* leaf and stem extracts may have potential as potential candidate for further antiplatelet.

Based on Figure 2, the inhibition of platelet aggregation by the stem extract was greater than that of the leaf extract. This may be due to the higher baseline aggregation observed in the stem extract group, resulting in greater inhibition values, as illustrated in Figure 3.

The half-maximal inhibitory concentration (IC_{50}) is a widely used and informative measure of a drug's efficacy, representing the concentration required to inhibit a biological process by 50%. It serves as an important indicator of the potency of an antagonist drug in pharmacological research (Aykul & Martinez-Hackert, 2016). In this study, IC_{50} values were determined using the logarithm of extract concentrations and their corresponding percentages of platelet aggregation inhibition. Transforming concentration data into logarithmic form facilitates comparison of dose-response curves and provides a more manageable scale at low concentrations, where responses change rapidly (Yartsev, 2015).

The regression equation for the stem extract was $y = 37.693x + 45.901$ with an R^2 value of 0.982, while the leaf extract yielded $y = 43.087x – 73.333$ with an R^2 of 0.8923. Based on these models, the IC_{50} values calculated from Tables 8 and 10 were 349.945 µg/mL for the stem extract and 772.779 µg/mL for the leaf extract. According to these results, the stem extract exhibits moderate inhibitory potency, whereas the leaf extract shows lower potency.

The observed differences in antiplatelet activity between the stem and leaf extracts are likely influenced by the varying composition of secondary metabolite compounds present in each plant part. Although the total tannin content was found to be higher in the leaves (4.02 ± 0.02 mgEAT/g) compared to the stems (3.673 ± 0.16773 mgEAT/g), this does not necessarily correlate directly with greater biological activity. It is possible that the stems contain other bioactive compounds with more potent antiplatelet effects than tannins. This suggests that while tannins are present in higher amounts in the leaves, the primary contributors to platelet aggregation inhibition may be other secondary metabolites that are more abundant or more active in the stems. Compounds such as alkaloids and flavonoids have been previously reported to exhibit antiplatelet activity and may play a significant role in this effect (Ain et al., 2016; Zaragoza et al., 2022). When compared to aspirin, which has an IC_{50} of 4.45 µg/mL (24.7 µM), both katang-katang extracts demonstrate significantly lower antiplatelet activity, indicating lower efficacy relative to this standard drug.

Statistical analysis

Statistical analysis was conducted using SPSS software to evaluate the platelet aggregation inhibition activity of *Ipomoea pes-caprae* leaf and stem extracts, alongside a positive control (aspirin). Normality and homogeneity tests confirmed that all data sets were normally distributed and had equal variances ($p \geq 0.05$; Tables 11 and 12).

One-Way ANOVA revealed a significant difference in platelet aggregation inhibition between the stem and leaf extracts ($p \leq 0.05$; Table 12). Subsequent Post Hoc Tukey HSD tests for the stem extract (Table 13) indicated no significant differences between most concentration groups ($p > 0.05$), except for a significant increase in inhibition at 2000 µg/mL (B4) compared to 250 µg/mL (B1) ($p = 0.038$). The mean difference of -33.45% demonstrates a statistically higher inhibition of platelet aggregation at 2000 µg/mL relative to the lowest tested concentration.

In contrast, the leaf extract showed more distinct differences among concentration groups (Table 12). The positive control (C (+)) significantly inhibited platelet aggregation more than the 250 µg/mL group (D1) ($p = 0.001$), with a mean difference of 44.47%. No statistically significant difference was observed between the positive control and the higher concentrations of 500

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μg/mL (D2), 1000 μg/mL (D3), and 2000 μg/mL (D4) ($p > 0.05$).

Within the leaf extract concentrations, 250 μg/mL (D1) was significantly less effective than 500 μg/mL (D2), 1000 μg/mL (D3), and 2000 μg/mL (D4) ($p < 0.05$). The largest inhibition increase was observed between 250 μg/mL and 2000 μg/mL (-53.10%), indicating a dose-dependent enhancement of platelet aggregation inhibition. However, no significant differences were detected among the higher concentration groups (D2 vs D3, D2 vs D4, D3 vs D4), suggesting a saturation effect where inhibition plateaus beyond 500 μg/mL.

These findings indicate that both stem and leaf extracts of *Ipomoea pes-caprae* exhibit significant antiplatelet activity in a concentration-dependent manner, with maximal inhibitory effects reached at higher concentrations. The stem extract showed a more gradual dose-response, while the leaf extract achieved near-maximal inhibition at concentrations above 500 μg/mL.

Study limitations

The antiplatelet activity test was conducted as a preliminary in vitro screening using ADP-induced platelet aggregation, observed through turbidity changes via UV-Vis spectrophotometry. This method is adapted from Lubis (2015) and Wijayanti et al. (2022), and while it offers a simplified model, it does not replace the precision of aggregometry-based assessments. Further validation using standard aggregometry is required to confirm the findings. This study provides early-stage insights into the potential antiplatelet activity of *Ipomoea pes-caprae* extracts. However, future studies using aggregometry, in vivo models, and different agonists are necessary for more accurate and conclusive results.

CONCLUSION

The study demonstrated that ethanol extracts from the leaf and stems of *Ipomoea pes-caprae* (Katang-katang) from North Lombok contain bioactive compounds, including alkaloids, flavonoids, and tannins. The leaf exhibited higher tannin content (4.02 mgEAT/g) than the stems (3.67 mgEAT/g). Both extracts showed antiplatelet activity with IC_{50} value 349.95 μg/mL for stem extract and 727.78 μg/mL for leaf extract of katang-katang (*Ipomoea pes-caprae*), indicating moderate inhibitory potency. These results suggest preliminary potential of *I. pes-caprae* as a natural antiplatelet agent; however, comparison with aspirin IC_{50} and further pharmacological evaluations are needed to validate its therapeutic relevance.

Further studies are required to determine the IC_{50} value of the positive control (aspirin) to validate the method, followed by in vivo antiplatelet activity tests using animal models. Comprehensive safety assessments including acute and subchronic toxicity, as well as pharmacokinetic and pharmacodynamic profiling in appropriate dosage forms, are essential prior to therapeutic exploration.

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AUTHOR CONTRIBUTIONS

Conceptualization, I.H.; Methodology, M.N.F.A., L.H.H., M.I.F.; Software, I.H.; Validation, L.H.H.; Formal Analysis, M.N.F.A., I.H.; Investigation, M.I.F.; Resources, L.H.H.; Data Curation, M.N.F.A.; Writing - Original Draft, M.N.F.A.; Writing - Review & Editing, N.I.H., R.H., D.S.; Visualization, M.I.F.; Supervision, I.H.; Project Administration, I.H.; Funding Acquisition, I.H.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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Formulations and Antibacterial Activity of Shallot (*Allium cepa* L.) Peel Extract Patch against *Streptococcus pyogenes*

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Abstract

Background: *Bacterial pharyngitis is an inflammatory condition in the back of the throat caused by Streptococcus pyogenes. Patients are often prescribed antibiotics and antiinflammatories to alleviate pain and discomfort while reducing bacterial growth in the throat. However improper and prolonged use of antibacterial and antiinflammatory agents increases the risk of bacterial resistance and side effects. An often discarded Shallot (Allium cepa L.) peel rich in flavonoids with great antibacterial and anti-inflammatory properties is potentially used as an alternative treatment for bacterial pharyngitis.* ***Objective:*** *This study aimed to develop shallot peel extract as an antibacterial against Streptococcus pyogenes.* ***Methods:*** *Patch was formulated with variations in extract concentration of 5% (F1), 10% (F2), and 15% (F3) to observe their influence on weight uniformity, thickness, folding endurance, surface pH, moisture content, and antibacterial activity using disc diffusion.* ***Results:*** *All formulations produce slightly heavy and thicker but uniform patches (CV<5%), surface pH suitable for application in the skin (4.6-4.9), flexible and durable patches with high folding endurance (> 300 folds), good moisture content (<10%) and moderate to strong antibacterial activity (inhibition zone diameter ranging from 9 to 13.67 mm). Variations in extract concentration in the formula significantly influenced the thickness, weight, folding endurance, and also the antibacterial activity of the patches. Higher concentrations of extract produce thicker and heavier patches but stronger antibacterial activity against pharyngitis pathogens.* ***Conclusion:*** *Therefore, antibacterial patches containing up to 15% shallot peel extract are potentially used as an alternative treatment for pharyngitis.*

Keywords: *antibacterial, patch, pharyngitis, shallot peels, streptococcus pyogenes*

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INTRODUCTION

Bacterial pharyngitis is an inflammatory condition caused by a bacterial infection in the pharynx, nasopharynx, and tonsils. *Streptococcus pyogenes* or group A beta-haemolytic streptococcus is the predominant cause of bacterial pharyngitis in children and adults accounting for up to 30% of all cases (Tadesse et al., 2023). In Yogyakarta, pharyngitis remains among the top 10 infectious diseases with the highest incidence affecting 10,269 patients in 2023 (Badan Pusat Statistik Yogyakarta, 2023). Pharyngitis features persistent symptoms such as burning sensation, pain, itching, dryness, recurrent cough, and irritation that worsen during swallowing which affect the patient's daily life (Ding et al., 2020). Bacterial pharyngitis treatment management aims to alleviate pain and discomfort while reducing bacterial growth in the throat. Therefore, patients are often prescribed antibiotics and antiinflammatory/analgesic agents to shorten the course of the disease and improve symptoms. However, misidentification of the pathogenic bacteria and irrational use of antibiotics can cause bacterial resistance, leading to severe complications, especially in children (Cots et al., 2015). Moreover, long-term usage of oral anti-inflammatory and analgesic drugs potentially leads to cellular toxicity, allergic reactions, and side effects. This clarifies the need to find safer alternatives for pharyngitis therapy.

Recently, plant-derived herbal medication has gained substantial attention from researchers for its lower toxicity. In Indonesia, shallot *(Allium cepa* L.*)* plants have been used orally or topically as folk remedies to treat various diseases such as colds, fever, ulcers, stomachaches, and asthma (Henri & Hakim, 2020). Shallot is the most produced horticultural crop in Indonesia accounting for nearly 2 million tons annually (Badan Pusat Statistik, 2024). However, the bulbs and leaves are the most utilized part of shallot plants in the medicinal field. Whereas, shallot peels which are often discarded as waste in shallot cultivation are also proven as an abundant source of phytochemicals such as phenolics and flavonoids. Metabolomic profiles of shallot peels extracted by ultrasound-assisted extraction display rich bioactive such as flavonoids, stilbenes, terpenoids, phenolic acids, and sulphur compounds. These bioactives are responsible for their strong antioxidant activity and inhibition of several enzymes involved in metabolism, memory, and pigmentation (Moldovan et al., 2024). According to Fredotović et al. (2021), 100 g of shallot peel extract contains ± 600 mg of quercetin glucosides, ± 70 mg of quercetin aglycone, and ± 4 mg of anthocyanins. Shallot peel extract has proven to show a strong antibacterial activity against pharyngitis major pathogen, *Streptococcus pyogenes* with MIC of 500 µg/mL (Fredotović et al., 2021). A similar study also reported concentrations of 5%, 10%, and 15% of shallot peel extract had an average inhibition zone diameter of 0.683 cm, 0.830 cm, and 1.137 cm respectively (Wirdia et al. 2017). Shallot peel extract also has high antioxidant activity with an IC_{50} value of 86 μg/mL which contributes to its antiinflammatory activity. Juliadi & Agustini (2019), developed a cream formulation containing 0.32% shallot peel extract that can reduce up to 50% swelling in carrageenan-induced paw edema which shows that the extract has great anti-inflammatory activity. A compress made from shallot is also able to reduce children's body temperature during fever, highlighting the antipyretic ability of the plants via promoting vasoconstriction in response to the cold sensation of shallot exposure to the skin (Mardhiah, 2022). Therefore, shallot peel extract is potentially used as an alternative medicine for pharyngitis treatment.

Despite its medicinal properties, shallot peel extract has an extremely unpalatable bitter taste and strong sulphuric odor, which may decrease patient adherence, especially in pediatrics. A study reported that medicines with poor palatability lower the patient's adherence, whereas those with good palatability have a positive influence (Alkilani et al., 2023). Therefore in this study, shallot peel extract was formulated in the patch and intended to be applied on the skin preferably around the throat region. Skin application via transdermal routes has several advantages including bypassing first-pass metabolism, preventing therapeutic discomfort, and increasing patient compliance while ensuring precise dosing (Wong et al., 2023). In this study, three patch formulations containing different concentrations of shallot peel extract were developed and evaluated for their influence on the physical characteristics and antibacterial activity of the patchs.

MATERIALS AND METHODS

Materials

Shallot peels were collected from the local market (Bantul, Yogyakarta), 70% ethanol (Brataco), excipients used for patch formulation were distilled water, hydroxypropyl methylcellulose 4000 (HPMC) (Sigma), propylene glycol (Bratachem), methylparaben (Bratachem), menthol (Bratachem), and phosphate buffer (Merck). Reagents and bacteria used for antibacterial activity assay were H_2SO_4 (Merck), *Streptococcus pyogenes* ATCC 19615 (Yogyakarta

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Health and Calibration Laboratory Center), Mueller Hinton Agar (MHA) (Merck), Nutrient Broth (Merck), $BaCl_2$ 1% (Merck), and amoxicillin Disc Oxoid CT0061B (Oxoid).

Equipments

Rotary evaporator (Heidolph Hei-Vap Platinum 1), oven (Binder), pH meter (Ohaous Aquasearcher AB33M1-F), digital caliper (Mitutoyo 500-196-30) and biological safety cabinet (Monmouth Guardian Class II).

Methods

Preparation of shallot peels extract

The freshly collected shallot peels were sorted and washed with water to remove impurities. Then, the peels were dried at a temperature of 60^{o}C. Subsequently, dry sorting was carried out and dried shallot peels were pulverized with a blender (Setiani et al., 2017). A total of 100 g powdered shallot peels was macerated using 1 L of 70% ethanol with gentle stirring every 1 hour for 10 hours, and the mixture was left at room temperature away from the light for one day. The mixture was then strained and the solid residue was remacerated twice using 1 L of 70% ethanol. All the filtrates were combined and concentrated using a rotary evaporator at 60°C and 80 rpm (Setiani et al., 2017). The concentrated extract was then further tested to determine the presence of flavonoids qualitatively. A shallot peel extract sample (2 mL) was taken in test tubes and was added 5 drops of concentrated H_2SO_4. A red color formation in the test tube indicates the presence of flavonoid in the extract (Atika, 2021).

Preparation of shallot peels extract containing patch

Three patches with varied concentrations of shallot peel extract were prepared using the solvent evaporation method with a formula modified from Wardani & Saryanti (2021) as listed in Table 1. Initially, HPMC 4000 was dispersed in distilled water and let swell properly until a clear and viscous solution was formed. The shallot peel extract was solubilized in 15 mL of water: ethanol 70% mixture (1:2). The dissolved extract was then mixed with HPMC 4000 and stirred until homogeneous. Futhermore, methylparaben was dissolved in a separate container with propylene glycol and added to the HPMC 4000 and extract mixture. Lastly, menthol and the remaining water and ethanol 70% were added to the mixture and stirred until homogeneous. The resulting mixture was allowed to stand for 20 hours to remove bubbles and then poured into square mold of 3.5x3.5 cm per patch. The mixture was then dried in the oven at 50°C until a dried patch was formed. The patches were placed in the desiccator for 20 hours and peeled from the mold. Patches were stored in a closed container (Wardani & Saryanti, 2021).

Physical characterization of shallot peels extract patches

Physical appearance evaluation

Physical appearance of patches were observed for its color, transparency, roughness of the surface texture, and odor (Mariadi & Bernardi, 2023).

Weight uniformity test

Three patches from each formula were weighed in an analytical balance. Then the average weight, standard deviation (SD), and % coefficient of variation (CV) of each formula were calculated. A patch was considered to have a uniform weight if the CV value ≤ 5% (Setiawan & Setiawan, 2024).

Patch thickness measurements

Patch's thickness for each formula was measured using a digital caliper having an accuracy of 0.01 mm at three different points. The thickness of each patch was determined as the average of measurements on three points (Surpiadi & Sherlyke, 2023).

Folding endurance

The patch was folded repeatedly in the same position until it broke. A patch was considered to have good folding endurance if it resists>200 folds without breaking (Setiawan & Setiawan, 2024).

Table 1. Formula of shallot peel extract patches

Composition	Function of Material	Concentration (%)		
		F1	F2	F3
Shallot peels extract	Active ingredient	5	10	15
HPMC 4000	Polimer	7	7	7
Propylene glycol	Plasticizer	10	10	10
Methylparaben	Preservative	0,3	0,3	0,3
Menthol	Perfume	3	3	3
Ethanol 70%	Solvent	40	40	40
Aquadest	Solvent	to 100 mL	to 100 mL	to 100 mL

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pH measurements

To measure the surface pH of the patch, the patch needs to be soaked in 10 mL phosphate buffer pH 6.8. After 2 hours, a pH meter was used to measure the surface pH. Reading on the pH meter was determined as surface pH. The measurement was replicated three times for each formula (Maddeppungeng et al., 2023).

Moisture content analysis

The initial weight (w1) of each patch was independently measured using an analytical balance. Then patch was transferred into an oven at 40ºC for 24 hours and dried until constant final weight (w2) was achieved. Moisture content was calculated as a percentage of [w1-w2]/w2. Three readings were recorded and the average was calculated as a percentage of moisture content (Hikma et al., 2024).

Antibacterial activity of shallot peel extract patch

A disk diffusion method was chosen to determine the antibacterial activity of the three patches. Bacterial suspensions were adjusted to a bacterial cell density of 1.0×10^8 CFU/mL (or 0.5 McFarland turbidity units). A sterile swab soaked in the bacterial suspension was used to inoculate the entire surface of blood agar medium. Patches from each formula were cut into 6 mm in diameter using a paper cutter and placed with slight pressed onto the agar. A 6 mm in diameter placebo patch was used as the negative control and amoxicillin Disc Oxoid 25 µg as a positive control. The petri dish was placed in incubator at 37°C for 24 hours, then the inhibition zone was observed by measuring the clear zone diameter using a caliper (Sfeir et al., 2013). The assay was done in triplicates.

Data analysis

The results of the physical evaluation and antibacterial activity of the patch were analyzed using Statistical Products and Services Solutions (SPSS) software. The testing process started with determining normality using Kolmogorov-Smirnov method and assessing homogeneity through the Levene test. The analysis continued using One-way Analysis of Variance (ANOVA) method to observe differences between formulas since the data were normal and uniform ($p>0.05$). The data were reported to be significant when p-value <0.05.

Yield and flavonoid identification of shallot peels extraction

The maceration of 100-gram shallot peels with 70% ethanol as a solvent yielded 10% w/w concentrated extract. The maceration method used in this study was more effective compared to previous studies with the same solvent which obtained a lower yield of 5.92% w/w due to the short maceration time (1 day). In a study conducted by (Handoyo, 2020) it was stated that the length of extraction time used was one of the factors that influenced the yield value obtained. In addition, the type of solvent polarity, the ratio or concentration of the solvent used, and the particle size of the simplicia could also be factors that influenced the yield value. As seen in Figure 1, shallot peel extract obtained in this study tested positive for flavonoid as signified by a red color change after the sample was treated with concentrated H_2SO_4. The red color. Indicates a complexation between sulfuric acid and flavonoids presence in the extract via an oxidation-reduction reaction (Atika, 2021).

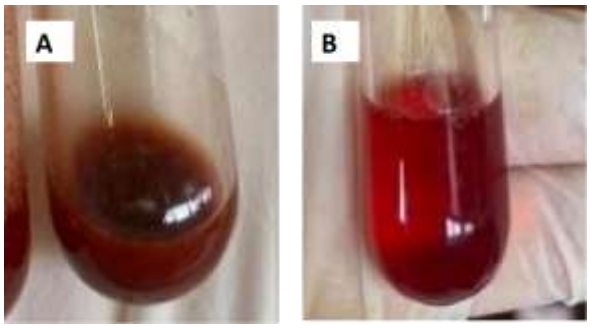

Figure 1. Flavonoid identification in shallot peels extract. (A) shallot peels extract and (B) shallot peels extract and concentrated H_2SO_4 which resulted in a bright red color solution

RESULTS AND DISCUSSION

Results

Extracts yield

Based on the data in Table 1, the 96% ethanol extract of *Ipomoea pes-caprae* leaves yields 20,63%, while the stem extract only reaches 10,09%.This yield is calculated from the ratio of the weight of the extract to the weight of the initial dry plant.The leaf extract is produced from 300 g of dry plant into 61.91 g of extract, while the stem with an initial weight of 213 g of dry plant yields 21.491 g of extract.

Physical characteristics evaluation of shallot peels extract patch

Physical appearance of all patches

The variation of extract concentration (5-15%) in the patch formulation using HPMC as matrix polymer resulted in patches with different colors and transparency. Higher extract concentration intensifies the reddish-brown color and lowers the transparency of the patch. As seen in Figure 2 and Table 2, all patches have a smooth surface, and the addition of menthol to the formulation successfully masks the sulphuric smell

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of shallot peel extract. Thus increasing patient acceptability.

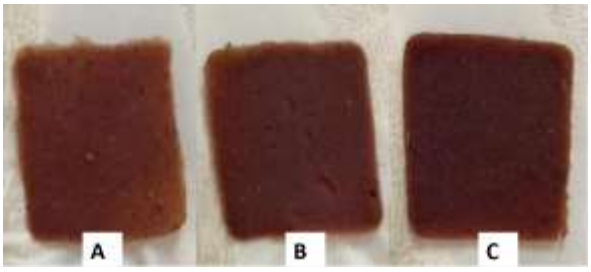

Figure 2. Physical appearance of shallot peel extract patches (A) F1 with 5% extract, (B) F2 with 10% extract, (C) F3 with 15% extract

Weight uniformity and thickness of shallot peel extract patch

A weight uniformity test was done to confirm the consistency of the patch preparation. A low % coefficient of variation (CV) value indicates that each patch produced with the same formula and production process has uniform weight (Maddeppungeng et al., 2023; Andriani et al., 2024). The data presented in Table 3 showed that the CV value of all formulations ranging from 2.29-2.8% meet the weight uniformity requirements. According to Wardani & Saryanti (2021), patch weight with a CV value of ≤ 5% is considered uniform. The weight uniformity of the patch correlates with the drug content uniformity, consistent weight ensures that each patch delivers the intended dose, preventing under or over-dosing, which could compromise treatment efficacy or safety (Lall & Rathore, 2024). As observed in Table 3, each formula resulted in a patch with a different weight, and heavier patches were obtained in the formula using higher concentrations of the extract. Statistical analysis was used to determine the effect of extract concentrations on the weight of patches. The result found that all three formulations have significantly different weights ($p < 0.05$), which indicates that variation in extract concentration in the formula affects the weight of the resulting patch. A similar pattern was observed in the thickness measurements of the patch. Formula made of higher extract concentration of extract resulted in thicker patches, with the formula with the highest extract concentration (F3) showing maximum thickness. The average thickness of all formulations was significantly different ($p < 0.05$), which indicated that extract concentration influenced patch thickness. However, all formulas form thick patches with a thickness larger than 1 mm which is slightly larger than the requirements (≤ 1 mm in thickness) (Fuziyanti et al. 2022). Thin and light patches were preferred by the patient for their wearability and comfort leading to patient compliance. Thicker patches tend to detach more easily from the skin. However, this problem can be resolved by adding an adhesive backing membrane to the matrix patch. The thickness of the patches also affects the drug release rate from the patch matrix, which in this case will influence the patch performance (Gunarti et al., 2024). Thinner patches offer a faster drug release rate due to shorter diffusion pathways for drug molecules to penetrate through the skin (Lall & Rathore, 2024). The thickness of the patch matrix produced by the solvent casting method is not only influenced by matrix composition but also by the mold dimension and volume of the matrix poured onto each mold. In this study, the use of small mold of 3.5x3.5 cm per patch for 30 mL of matrix mass resulted in thicker patches.

Table 2. The physical appearance of shallot peel extract patches

Test Parameters	F1	F2	F3
Color	Light reddish brown	Reddish brown	Dark reddish brown
Smell	Mint	Mint	Mint
Texture	Smooth	Smooth	Smooth
Transparency	Low	No	No

Table 3. Physical properties test results of shallot peel extract patch

Test Parameters	Formula		
	F1	F2	F3
Weight (gram)	0.784±0.02[a]	0.855±0.02[b]	0.972±0.03[c]
Weight uniformity/CV (%)	2.29	2.80	2.67
Thickness (mm)	1.10±0.03[a]	1.15±0.01[b]	1.20±0.04[c]
Folding endurance (folds)	>750	586.33±6.66[b]	367±19.86[c]
Surface pH	4.80±0.04	4.78±0.06	4.90±0.00
Moisture content (%)	8.65±0.68	7.65±0.95	8.39±1.72

*a, b,c (significant difference $p<0.05$)

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Folding endurance

Folding endurance is a parameter to evaluate the durability and flexibility of the patch. Good quality patches should maintain their integrity with general skin folding when applied or during storage (Mo et al., 2022). The flexibility of the patches is greatly affected by the concentration of plasticizer used in the formula. However, based on the result tabulated in Table 3. extract concentration also influenced the elasticity of the patch, with lower extract concentration generating more tear resistance patches due to its thinness. Higher extract concentrations produce a thicker patch which is difficult to fold and tear easily. The folding resistance of the three patch formulations exceeds the requirements of ≥ 200 times recommended by Nisa et al. (2016). This is influenced by the presence of the appropriate amount of plasticizer (propylene glycol) in the formula to regulate the patch flexibility. The plasticizer will bind to the polymer matrix and increase the volume of the cavity between the polymer chains, which reduces the crystallinity of the polymer chains. Enhanced polymer movement can increase the flexibility and elasticity of the patch. However, if the excess amount of extract was introduced, it disrupted the crosslinking between plasticizer and polymer, creating more cavities between polymer chains causing it to break more easily (Maddeppungeng et al., 2023).

Surface pH measurements

Safety is an important quality in developing a dosage form. Patch that is intended to be used on the skin must not irritate the skin upon usage. A Surface pH of the patch should be inside 4.5-6.5 to be tolerated by the skin (Gunarti et al., 2024). pH of the patch is also essential to maintain the stability of the active ingredients. Quercetin a compound belonging to the flavonoid family is the major bioactive in shallot peel extract. Quercetin has molecular stability in the pH range of 1-6 and undergoes autooxidation in pH 7-10 (Momić et al., 2007). Data tabulated in Table 3 showed that the surface pH of all three patch formulas was around 4.7-4.9 which was acceptable for use while also maintaining the stability of the active ingredients. Although the formulas made from different extract concentrations resulted in a patch with varied surface pH, the effect was observed to be not significant ($p>0.05$).

Moisture content analysis

Moisture content can affect the microbial stability and physical integrity of the patch during storage (Andriani et al., 2024). High moisture content patches are prone to microbial contamination which increases skin irritation and compromises the patient's health. High moisture content can also reduce patch adhesion to the skin, leading to early detachment of the patch from the skin (Lall & Rathore, 2024). However, completely dried patches with low moisture content create brittleness, therefore a good quality patch is required to have moisture content in the range of 1-10% % (Fuziyanti et al., 2022). The results in Table 3 showed that all formulations produced patches with good moisture content (≤10%) which ensured the patch stability and tolerability to the skin during application. Although the extract concentration did not significantly affect the moisture content of the patch, the hygroscopic sugar-derivate bioactive that is present in the shallot peels extract works with propylene glycol as a humectant and plasticizer in the formula to help retain the moisture inside the patch (Nurhamidah & Nurrochman, 2022).

Antibacterial activity assay

In the disc diffusion method, the inhibition zone is often measured to evaluate the antibacterial effect of the material, and a larger zone area means stronger antibacterial activity. Table 4 and Figure 3 showed that shallot peel extract formulated in the patch still had great antibacterial activity against *Streptococcus pyogenes* with inhibition zones ranging from 9-11.67 mm, and that the excipients in the patch did not influence the antibacterial activity of the extract (the placebo patch had zero inhibition zone). Although the inhibition zone were smaller than the standard antibiotic, the result still indicated promosing potential of shallot peel extract as a natural antibacterial agent. The inhibition zone diameter increased as the concentration of the shallot peel extract increased ($p<0.05$). The result in this study was correlated to the study conducted by Wirdia et al. (2017), that the inhibition zone of 5-15% extract was 6.83 mm, 8.30 mm, and 11.37 mm respectively, showing that increasing extract concentration will increase the inhibition zone. According to Safitri et al. (2017), the antibacterial activity of a compound based on the inhibition zone is divided into 4 categories, very strong (>20 mm), strong (10-20 mm), moderate (5-10 mm), and weak (<5 mm). Therefore, from the result presented in Table 3, we can summarize that F1 with the lowest extract concentration has moderate antibacterial activity. Meanwhile, F2 and F3 with higher extract concentrations have strong antibacterial activity though slightly lower than the positive control (amoxicillin disc 25 µg).

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Table 4. Antibacterial activity test results of shallot peel extract patch

Replication	Inhibition Zone Diameter (mm)				
	Concentration of Shallot Skin Extract			Amoxicilin Disc 25 µg	Placebo Patch
	5%	10%	15%		
1	9	12	13	30	0
2	9	11	14	31	0
3	9	12	14	30	0
Average	9.00	11.67	13.67	30.33	0
SD	0	0.58	0.58	0.58	-

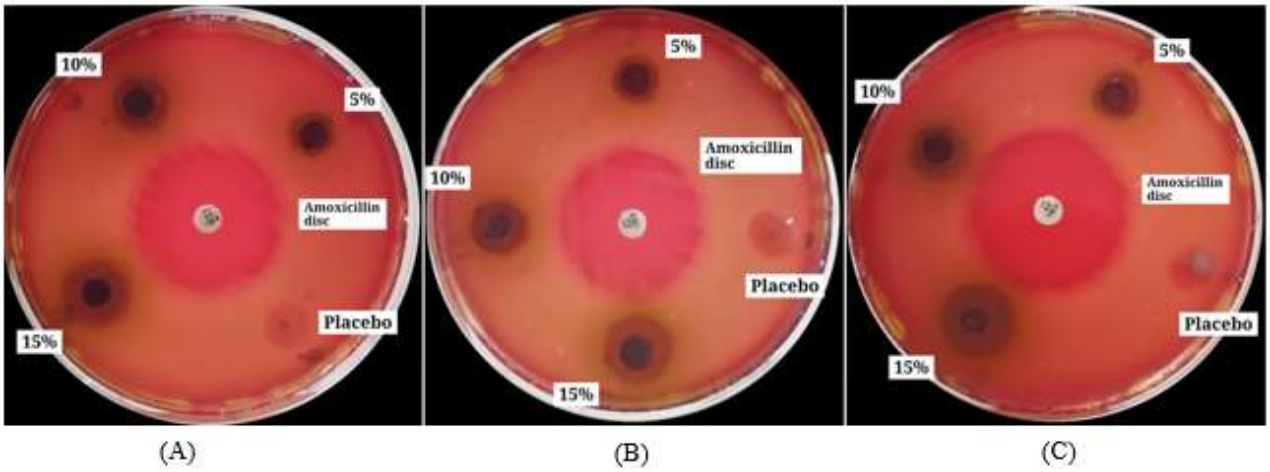

Figure 3. Inhibition zone of shallot peels extract against agar containing *Streptococcus pyogenes* culture , replication 1(A), replication 2(B), replication 3(C)

CONCLUSION

Good physical properties and antibacterial activity of the patch formulation intended to be used in the skin for pharyngitis treatment are important to ensure the safety and effectiveness of the treatment, along with increasing patient acceptability and compliance. In this study, all formulations produced slightly heavy and thicker but uniform patches, surface pH suitable for application in the skin, flexible and durable patches with high folding endurance, and good moisture content which ensure microbial stability and physical integrity during storage. Variations in shallot peel extract concentration in the formula significantly influenced the thickness, weight, folding endurance, and also the antibacterial activity of the patches. Higher concentrations of the extract produced thicker and heavier patches but stronger antibacterial activity (the average inhibition zone is 13.67 mm) against pharyngitis pathogen. Therefore, antibacterial patches containing up to 15% shallot peel extract were potentially used as an alternative treatment for pharyngitis. However, more assays such as in vitro release and permeation studies are needed to confirm that the matrix patch is capable of delivering the active ingredients through the skin. Skin irritation and hedonic tests with panelists are also needed to ensure the safety and comfortability of the antibacterial patch made of shallot peel.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.N.A., L.B.S., C.A.E.; Methodology, L.B.S., C.A.E.; Software, N.A.Z.; Validation, S.N.N., D.A.A., C.A.E.; Formal Analysis, K.S.M., N.A.Z.; Investigation, D.A.A., C.A.E.; Resources, S.N.A., L.B.S., K.S.M., N.A.Z., S.N.N.; Data Curation, K.S.M., N.A.Z., D.A.A., C.A.E.; Writing - Original Draft, S.N.A., L.B.S., K.S.M., N.A.Z., S.N.N., D.A.A., C.A.E.; Writing - Review & Editing,

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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A Systematic Review: Cost-Effectiveness of SGLT2 Inhibitors versus DPP-4 Inhibitors as Add-on to Metformin

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Abstract

Background: *The prevalence of type 2 diabetes mellitus (T2DM) is increasing worldwide. Sodium-glucose cotransporter-2 inhibitors (SGLT2i) and dipeptidyl peptidase-4 inhibitors (DPP-4i) are two second-line therapy alternatives for T2DM patients inadequately controlled with metformin.* ***Objective:*** *This study aimed to systematically review the cost-effectiveness of combining metformin+SGLT2i vs metformin+DPP-4i for T2DM treatment.* ***Methods:*** *A systematic search was conducted in PubMed, Scopus, and ScienceDirect for articles published between 2015-2025, using predefined keywords and following the PRISMA and PICOS frameworks (P: T2DM patients uncontrolled on metformin monotherapy; I: Metformin+SGLT2i therapy; C: Metformin+DPP-4i therapy; O: Cost, clinical outcomes (HbA1c% reduction), Incremental Cost-Effectiveness Ratio (ICER) values, Quality Adjusted Life Years (QALY); S: Study with cost-effectiveness analysis design). Additional studies were identified through reference screening. Eligible articles were independently reviewed and assessed for reporting quality using the CHEERS-2022 standards.* ***Results:*** *Five studies met the inclusion criteria. Considerable heterogeneity was observed with mean patient ages ranging from 55-61 years old and baseline HbA1c levels from 7.9%-9.4%. The studies were conducted in the US, UK, Mexico, and Greece, all funded by the pharmaceutical industry, and used economic models. Despite these differences, all studies consistently demonstrated that combining metformin+SGLT2i was more cost-effective than metformin+DPP-4i. SGLT2i improved the quality of life by 0.032–0.04 QALYs, reduced hypoglycemia, and provided additional benefits for patients with cardiovascular risk, although it was associated with higher initial costs.* ***Conclusion:*** *This review showed that the combination of metformin+SGLT2i was more cost-efficient and effective in managing T2DM than the combination of metformin+DPP-4i.*

Keywords: *cost-effectiveness analysis, diabetes mellitus, DPP-4 inhibitors, metformin, SGLT2 inhibitors*

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INTRODUCTION

Diabetes mellitus is a chronic disease, and the number of patients continues to increase every year. Based on data from the International Diabetes Federation (IDF), there are 589 million adults living with diabetes with an age range of 20-79 years old, of which 20,426 million are from Indonesia. This positioned Indonesia in the 5th position with the most DM patients in the world in 2024 (International Diabetes Federation, 2024). According to the American Diabetes Association (ADA), the annual cost of diabetes in the US will reach $413 billion by 2022, including $307 billion in direct healthcare costs and $106 billion in productivity lost. Diabetes and its associated health complications impose a significant financial burden on individuals and society (American Diabetes Association, 2025).

Metformin is an oral antidiabetic drug (OAD) that is most commonly used to treat type 2 diabetes mellitus (T2DM) (Müller et al., 2018). However, given the progressive nature of T2DM, many patients require additional therapy to maintain adequate glycemic control. The use of a combination of drugs, such as dipeptidyl peptidase-4 inhibitors (DPP-4i) and sodium glucose cotransporter 2 inhibitors (SGLT2i), which have complementary mechanisms of action, can help control blood sugar levels and promote weight loss while reducing the risk of hypoglycemia (Hadjadj et al., 2016; Rosenstock et al., 2016).

Sodium-glucose cotransporter 2 inhibitors (SGLT2i), introduced in 2012 as the newest class of non-insulin antidiabetic agents, provide glycemic control comparable to that of traditional therapies while offering additional advantages, including a lower risk of hypoglycemia and clinically meaningful weight reduction. Moreover, evidence has demonstrated their ability to decrease major adverse cardiovascular events and mortality, as well as to improve clinical outcomes in patients with chronic kidney disease (Yoshida et al., 2020). SGLT2i and DPP-4i have been shown to have a lower risk of hypoglycemia and are beneficial in reducing CVD events and mortality in patients with type 2 diabetes (T2DM) and cardiovascular risk compared to conventional therapies, such as sulfonylureas (SU) and insulin (Zhu et al., 2023).

To ensure that patients receive the best treatment at the available cost, the clinical benefits of each drug must be compared to the cost impact (Charokopou et al., 2015). Many cost-effectiveness analysis (CEA) studies have shown that SGLT2i are more cost-effective than DPP-4i. Due to differences in settings, healthcare costs, and populations, the results of these studies vary, and there is no clear consensus on which of the two combinations is more cost-effective in T2DM therapy (Peng et al., 2022).

A previous systematic review assessed the cost-effectiveness of SGLT2i compared with that of multiple antidiabetic classes, including DPP-4i. However, it did not specifically address SGLT2i versus DPP-4i, despite both being commonly prescribed and frequently considered therapeutic alternatives in routine clinical practice. Therefore, the purpose of this review was to conduct a cost-effectiveness analysis of the combination of metformin + SGLT2i compared to metformin + DPP-4i, especially in T2DM patients.

This study aimed to review and evaluate the data thoroughly to provide a deeper understanding of the economic aspects of the two comparable treatment approaches. The results are expected to serve as a reference for medical personnel and policymakers in making appropriate decisions regarding the management of patients with T2DM. Although these two combinations have been shown to effectively control blood sugar level, cost considerations remain a crucial factor in determining the therapy used. As the burden of healthcare costs increases, economic analysis becomes increasingly important in T2DM management strategies.

METHODS

Search Strategy

The literature search was limited to studies published between 2015 and 2025, considering that SGLT2 inhibitors were first approved by the FDA in 2013 (canagliflozin) and 2014 (dapagliflozin), with relevant publications on clinical effectiveness and cost-effectiveness analyses consistently emerging from 2015 onward. The databases used were from PubMed, ScienceDirect, and Scopus. The search strategy focused on the topic "Cost Effectiveness of metformin+SGLT2i compared to metformin+DPP-4i in patients with type 2 Diabetes Mellitus," using several keywords, namely "Cost Effectiveness," "Diabetes Mellitus," "Dipeptidyl Peptidase-4 Inhibitors," "Metformin," "Sodium Glucose Cotransporter 2 Inhibitors," which were applied to the database. This literature study used a structured search technique that used Boolean operators such as "AND" or "OR."

Inclusion and Exclusion Criteria

The search scheme was adjusted to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Page et al., 2021) and Participants,

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Intervention, Comparator, Outcome, and Study Design (PICOS) method (P: Type 2 diabetes mellitus patients inadequately controlled on metformin monotherapy; I: Metformin + SGLT2i therapy; C: Metformin + DPP-4i therapy; O: Cost and clinical outcomes such as HbA1c% reduction, Incremental Cost-Effectiveness Ratio (ICER) values, and quality adjusted life years (QALY); S: studies with cost-effectiveness analysis design). The excluded articles were articles from journals that could not be accessed, written in languages other than English, research protocols, opinions, notes, letters, editorials, books, conference abstracts, and review articles.

Data extraction and analysis

After reviewing, the information obtained will be included in the table: 1) bibliography, including authors, year, and country of publication; (2) study design, including study perspective, time horizon, interventions, type of modeling, costs included in the study, discount rates, clinical outcomes, and sensitivity analysis; (3) results and conclusions, including the incremental cost-effectiveness ratio (ICER) and QALY. Quality assessment was performed using the Consolidated Health Economic Evaluation Reporting Standards (CHEERS) 2022, which consists of 28 items (Husereau D et al., 2022). Each item with an answer of "yes" was given a score of 1, while the answers of "no" or "not applicable" were given a score of 0. Study quality was classified into three categories: high (scores of 22-28 or more than 75%), moderate (scores of 14-21 or 50% - 75%), and low (scores of <14 or less than 50%).

RESULTS AND DISCUSSION

Search results

From the initial search results, 69 studies were identified. There was one duplicate article, 51 articles had titles that were not in accordance with CEA, two were systematic review articles, and nine articles did not focus on discussing the CEA combination of metformin + SGLT2i compared to metformin + DPP-4i, two articles were not in full text, and one article was not in English. Next, a manual search was conducted, and two articles were obtained. Five articles were included in the final review. The article selection process used PRISMA diagrams (Figure 1).

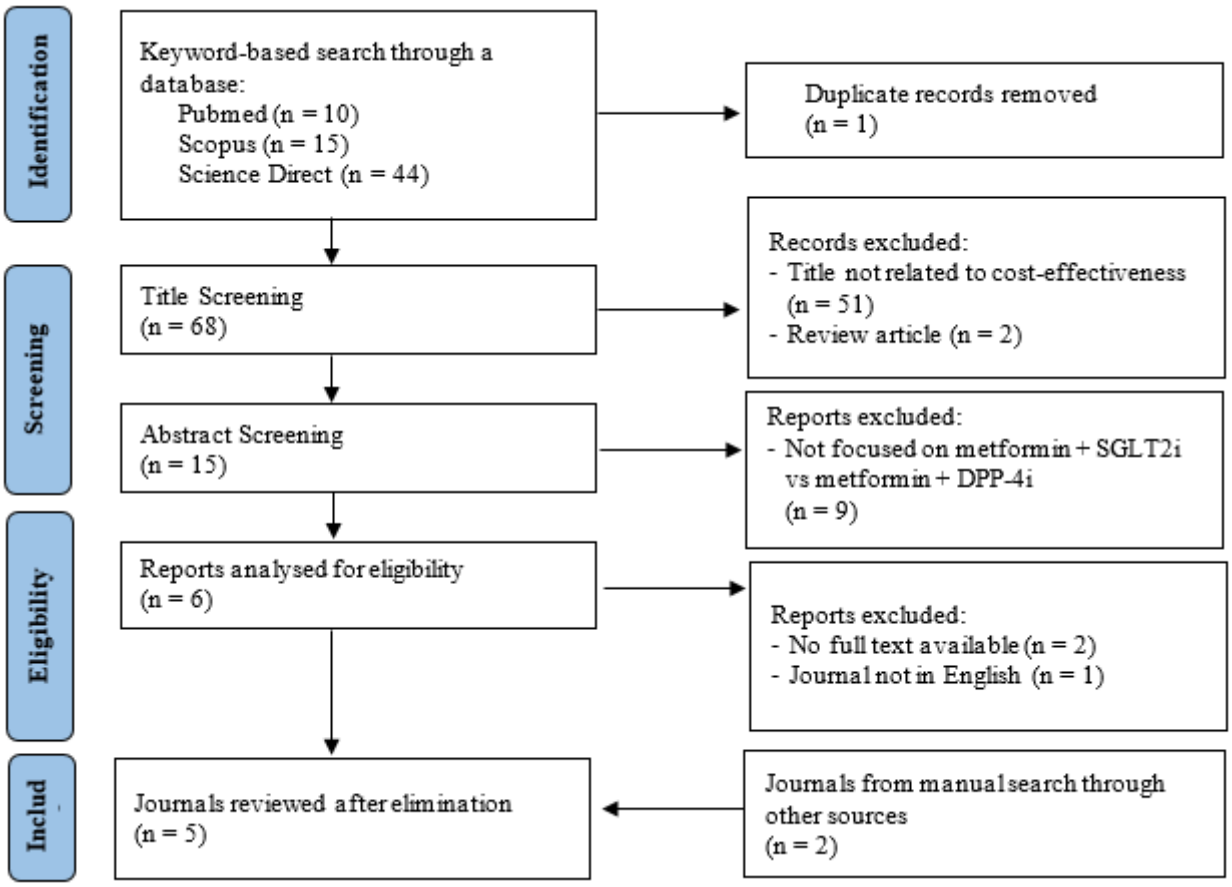

Figure 1. The source search flow using the PRISMA method is cost-effectiveness of Metformin+ SGLT2i compared to Metformin+ DPP-4i

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Table 1. General characteristics of the included studies

Author/ Year	Population	Perspectives	Cost	Interventions & Comparisons	Model	Time Horizon	Discount Rate (%)	Funding Sources
UK, Charokopou et al., 2015	Average age: 58 years old Average HbA1c: 8.05% Patients who failed to achieve adequate control on previous metformin monotherapy and require modifications to their treatment regimen	UK national health services	Direct medical costs: micro- and macrovascular complications (IHD, MI, CHF, stroke, amputation, ESRD, blindness), CV death, non-CV death, hypoglycemia, adverse events	MET+DAPA, MET+DPP4i	Cardiff diabetes model	40 years (Lifetime)	3.5	Bristol-Myers Squibb and AstraZeneca
Mexico, Neslusan et al., 2015	Average age: 55 years old Average HbA1c: 7.9% Patients with T2DM inadequately controlled on metformin monotherapy	Third-party payer in the US healthcare system	Direct costs: drug costs, complications (MI, stroke, nephropathy, neuropathy, retinopathy), adverse events (genital infections, dehydration, etc.), long-term management costs	MET+Canagliflozin 300mg, MET+Sitagliptin	Economic and Health Outcomes Model of T2DM (ECHO-T2 DM)	30 years	5	Janssen Global Services, LLC.
Greece, Tzanetakos et al., 2016	Average age: 58 years old Average HbA1c: 7.98% T2DM patients inadequately controlled with metformin monotherapy	Greece healthcare system	Direct costs: drugs, micro- and macrovascular complications (DM, MI, CHF, stroke, amputation, ESRD, blindness); Costs of hypoglycemia, Adverse events	MET+DAPA, MET+DPP-4i	Cardiff diabetes model	40 years	3.5	AstraZeneca
US, Chakravarty et al., 2018	Average age: 57 years old Average HbA1c: 7.98% Patients with T2DM treated with DAPA vs other glucose-lowering therapy classes added as add-on therapy to metformin.	US third-party payer perspective	Direct medical costs: related to changes in HbA1c, weight, blood pressure, and risk of hypoglycemia; drug costs (wholesale prices); and medical costs (visit, hospitalization, lab)	MET+DAPA, MET+DPP-4i	A short-term economic model (1year)	1 year	-	AstraZeneca
US, Reifsnider et al., 2021	Average age: 61 years old Average HbA1c: 9.4% T2DM patients with or with out CVD on metformin plus empagliflozin or metformin plus sitagliptin	*US healthcare payer perspective*	Direct medical costs: medication, diabetes complications: CVD, kidney failure, stroke, amputation, neuropathy, blindness; Adverse events; Therapy escalation costs.	MET+Empagliflozin, MET+Sitagliptin	Individual patient-level simulation model	10 years	3	Boehringer Ingelheim Pharma-ceuticals Inc. of Ridgefield, CT, USA

Description: DAPA: dapagliflozin, MET: metformin, SITA: sitagliptin

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Table 2. CHEERS assessment result

Author/ Year	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	%
UK, Charokopou et al., 2015	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	-	✓	-	✓	✓	✓	-	✓	✓	✓	89
Mexico, Neslusan et al., 2015	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	-	✓	-	✓	✓	✓	-	✓	✓	✓	89
Greece, Tzanetakos et al., 2016	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	-	✓	-	✓	✓	✓	-	✓	✓	✓	89
US, Chakravarty et al., 2018	✓	✓	✓	-	✓	✓	✓	✓	✓	-	✓	✓	✓	✓	✓	✓	✓	✓	-	✓	-	✓	✓	✓	-	✓	✓	✓	86
US, Reifsnider et al., 2021	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	-	✓	-	✓	✓	✓	-	✓	✓	✓	89

Table 3. The economic outcomes of the literature study analysis of Metformin+ SGLT2i compared with Metformin+ DPP-4i

Country, Author, Year	Sensitivity Analysis	Clinical Outcomes/ QALY	Cost	ICER	WTP Threshold (Cost/ QALY)	Cost Effective
UK, Charokopou et al., 2015	*One way*, PSA	MET+DAPA: 11.86 years MET+DPP4i: 11.83 years	MET+DAPA : £13,809 MET+DPP4i: £13,593	MET+DAPA vs MET+DPP4i: £6,761/ QALY	£20,000/QALY	MET+DAPA
Mexico, Neslusan et al., 2015	*One way,* PSA	MET+Canagliflozin 300mg: 6.35 years MET+Sitagliptin: 6.19 years	MET+Canagliflozin 300mg: MXP 330,087 Sitagliptin MET: MXP 328,290	MET+Canagliflozin 300mg vs MET+Sitagliptin: MXP 11,210/QALY	MXP 141,200/QALY	MET+Canagliflozin 300mg
Greece, Tzanetakos et al., 2016	PSA	MET+DAPA:12.24 years MET+DPP-4i: 12.19 years	MET+DAPA : €25,088 MET+DPP-4i: €24,332	MET+DAPA vs MET+ DPP-4i: €17,695/QALY	€34,000/QALY	MET+DAPA
US, Chakravarty et al., 2018	*One way* and *PSA*	MET+DAPA : +0.0587	MET+DAPA:-$795	MET+DAPA Dominates	$50,000/QALY	MET+DAPA
US, Reifsnider et al., 2021	DSA and PSA	MET+Empagliflozin: 8.85 years MET+Sitagliptin: 8.66 years	MET+Empagliflozin: $89,436 Sitagliptin MET: $88,118	MET+Empagliflozin VS MET+Sitagliptin: $6967/QALY	$50,000/QALY	MET+Empagliflozin

Description: Euro (€), Pound (£), US Dollar ($), Mexican Peso (MXP), DAPA: DAPAGLIFLOZIN, MET: metformin, SITA: sitagliptin, WTP: willingness to pay

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Characteristics of the study

An analysis of the characteristics of the five studies is presented in Table 1. All five studies used economic models to evaluate the cost-effectiveness of SGLT2i in T2DM patients. The most widely used model is the Cardiff Diabetes Model, which has been previously validated and shown to be able to project key clinical endpoints related to the natural course of T2DM, therapeutic effects, and their impact on patient cost and quality of life over the long term (Charokopou et al., 2015). In addition to the Cardiff Diabetes Model, three other models are used: echo-T2DM, a short-term decision-analytic model (1 year), and an individual patient-level simulation model using a discretely integrated condition event (DICE). Each model has a different structure and assumptions, which may affect the results of the cost-Effectiveness evaluation. The pharmacoeconomic perspective is one of the considerations in pharmacoeconomic research. Pharmacoeconomic perspectives are used to consider who pays the costs and who receives the benefits. Of the five studies, most were conducted from the payer's perspective (three studies), and two were conducted from the healthcare perspective.

Most studies used a lifetime horizon to capture the long-term benefits of SGLT2i therapy. However, there is one study that uses short-term models that are considered more suitable for estimating costs and benefits in a short period (Chakravarty et al., 2018). Although a lifetime time horizon is generally used, the time horizon is limited to 20-40 years; this is because the range represents the remaining life expectancy of T2DM patients and is sufficient to capture all relevant benefits and costs (Reifsnider et al., 2021).

The variation in discount rates (3%, 3.5%, and 5%) influences the valuation of future costs and health outcomes in the present (Andayani, 2013). A higher discount rate undervalues long-term benefits, potentially making preventive or chronic disease interventions appear less cost effective. In comparison, a lower rate gives greater weight to the future outcomes. Even small differences in discount rates can meaningfully shift incremental cost-effectiveness ratios (ICERs), particularly when the results are close to the willingness-to-pay thresholds (Kemenkes, 2013). For the study using a one-year horizon, discounting was not applied because it is only relevant for longer timeframes.

The participants in this study were T2DM patients who did not achieve optimal glycemic control with metformin monotherapy, thus requiring second- or third-line additional therapy. All studies involved adults aged > 55 years, in accordance with data from the IDF, which states that in 2024, there will be 589 million adults aged 20-79 years with T2DM (International Diabetes Federation, 2024). The average Body Mass Index of the five studies was above 30 kg/m2. According to the WHO, the ideal BMI classification is in the range of 18.5-24.9 kg/m2; therefore, it is included in class 1 obesity. The HbA1c levels in the five studies ranged from 7.5% to 9.4%.

Quality assessment

All studies in this review showed a high quality of reporting based on the CHEERS checklist, with a score of more than 80%. All studies conducted a sensitivity analysis, including univariate analysis, one-way sensitivity analysis, and Probabilistic Sensitivity Analysis (PSA), and the results obtained were assessed as consistent or stable. The reporting quality of the studies is presented in Table 2.

Document evaluation

The results of the analysis of the cost-effectiveness literature study of metformin + SGLT2i compared with metformin + DPP-4i (Table 3) showed that in the UK, dapagliflozin resulted in an increase in QALY with ICER £ 6.761/ QALY, and a cost-effective probability of 85% at a threshold of £20,000 (Charokopou et al., 2015). In a study in Mexico, canagliflozin (300 mg) showed an ICER well below the national WTP limit and was very cost-effective, mainly due to its impact on blood sugar, body weight, and blood pressure (Neslusan et al., 2015). In a Greek study, dapagliflozin provided additional QALY and remained cost-effective (ICER € 17.695/QALY) compared to the €34,000 threshold (Tzanetakos et al., 2016). In studies conducted in the US (two studies), dapagliflozin and empagliflozin were more cost-effective than DPP-4i and sitagliptin, with lower costs or higher clinical benefits. Empagliflozin is particularly beneficial in patients at risk of CVD, as it reduces CVD mortality and extends CVD-free life (Chakravarty et al., 2018; Reifsnider et al., 2021).

SGLT2 inhibitors are one of the newest classes of antidiabetic drugs that are currently increasingly used as the main choice in T2DM treatment. This is because SGLT2i not only lowers blood glucose levels but also provides protective benefits against cardiovascular and renal complications in T2DM patients (Hsia et al., 2017). However, the prices of drugs in this class are relatively high (Charokopou et al., 2015). Therefore, evaluating the cost-effectiveness of SGLT2i therapy is important. This systematic review shows that SGLT2i is an effective and safe therapeutic option for T2DM patients, especially in patients who have not reached the HbA1c

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target with metformin, because it can reduce blood sugar levels while reducing the risk of cardiovascular complications and mortality.

The results of this systematic review are generally consistent with those of a previous review, which also concluded that SGLT2i is cost-effective. The addition of dapagliflozin to metformin provided a small but significant increase in benefits compared with the addition of DPP-4i, namely an additional life expectancy of 0.01 years and an increase in quality of life of 0.04 QALY during the patient's lifetime in a study conducted in Greece (Tzanetakos et al., 2016). Meanwhile, in the UK, dapagliflozin + metformin increases the quality of life by 0.032 QALY. These differences are mainly due to differences in patient weight, which are known to affect the health-related quality of life (Charokopou et al., 2015). The improvement in QoL was also supported by a decrease in hypoglycemia rates in the dapagliflozin group.

The results of this study align with the Health Technology Assessment (HTA) framework used in various health systems. The economic models used in these studies have generally been validated and used in official HTA assessments, ensuring that their findings have a strong methodological foundation. Furthermore, HTA principles, such as the use of a GDP-based cost-per-QALY threshold and an emphasis on healthcare resource efficiency, were also reflected in the analysis. Consistently, dapagliflozin, compared with DPP-4i, demonstrated more cost-effective results in both short- and long-term models (Chakravarty et al., 2018; Charokopou et al., 2015; Cummins et al., 2012; Neslusan et al., 2015; Tzanetakos et al., 2016).

When interpreting the results of this review, caution is required because of some limitations. First, data on effectiveness are often limited in scope. Efficacy data generally originate from older clinical trials with selected patient populations (Lopez et al., 2015). Second, the wide variation in methodology between studies and in terms of data use, discount rate, modelling, perspective, types of cost, time horizon, and so on, plus differences in population and country contexts, make research results difficult to compare directly and general conclusions are limited (Yoshida et al., 2020). Third, all of the studies analyzed were funded by pharmaceutical companies, which may tend to report more favorable results regarding the cost-effectiveness ratio of recent diabetes therapies such as SGLT2i. The study also did not include non-English-language publications, which could be a source of publication bias. In addition, some paid journals do not provide the full text, which can limit the completeness of the data analyzed.

CONCLUSION

Studies conducted in the UK, Mexico, Greece, and the US found that the combination of metformin with SGLT2i, such as dapagliflozin and empagliflozin, was more cost-effective than metformin and DPP-4i. Despite higher initial costs, SGLT2i improved the quality of life by 0.032–0.04 QALYs, reduced hypoglycemia, and provided additional benefits for patients at cardiovascular risk.

AUTHOR CONTRIBUTIONS

Conceptualization, I.A.H., Y.N..; Methodology, I.A.H., Y.N.; Software, I.A.H.; Validation, I.A.H., A.R.; Formal Analysis, I.A.H., L.; Investigation, I.A.H., L.; Resources, I.A.H.; Data Curation, I.A.H., Y.N., L.; Writing - Original Draft, I.A.H.; Writing - Review & Editing, I.A.H., Y.N., L., A.R.; Visualization, V.; Supervision, I.A.H.; Project Administration, I.A.H.; Funding Acquisition, I.A.H.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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In-Vitro and *In-Silico* Study: The Anti-Inflammatory Activity of Ethanol Extract from Cogon Grass Roots (*Imperata cylindrica* L.)

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Abstract

Background: *Inflammation is a protective reaction triggered by harmful substances, microbes, or physical trauma. Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used to treat inflammation, though they have certain drawbacks, such as the potential for chronic kidney failure and unfavorable gastrointestinal side effects. Therefore, alternative treatments are needed. Cogon grass (Imperata cylindrica L.) roots contain secondary metabolites that may offer potential for inflammation treatment.* ***Objective:*** *This study aims to investigate the potential of secondary metabolites from cogon grass roots as anti-inflammatory agents, both in vitro using protein denaturation inhibition techniques and in silico against the COX-1 and COX-2 enzyme receptors.* ***Methods:*** *Molecular docking of COX-1 (PDB ID 6Y3C) and COX-2 (PDB ID 1PXX) using AutoDock Tool 1.5.6 was used to test the anti-inflammatory activity. In parallel, the in vitro technique involved spectrophotometric denaturation inhibition of the BSA (bovine serum albumin) protein.* ***Results:*** *The in silico results showed that the cyclovalone ligand exhibited the highest interaction and stability, with Gibbs free energies of -9.3 kcal/mol against COX-1 and -9.8 kcal/mol against COX-2, compared to the control ligand diclofenac, which had Gibbs free energies of -6.5 kcal/mol against COX-1 and -8.5 kcal/mol against COX-2. The 30% ethanol extract of cogon grass roots demonstrated anti-inflammatory activity in the in vitro analysis, with an IC50 value of 71.79 µg/mL.* ***Conclusions:*** *These preliminary findings suggest that the ethanol extract of cogon grass roots contains cyclovalone compounds with potential as anti-inflammatory agents.*

Keywords: *anti-inflammatory, cyclooxygenase enzyme, cogon grass, ethanol extract, Imperata cylindrica L.*

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INTRODUCTION

Inflammation is a natural bodily response to combat illnesses, infections, and injuries. It can be triggered by various factors such as immune disorders, cancer, infections, and exposure to harmful chemicals (Mangkulion et al., 2023). Health research conducted by the Indonesian Ministry of Health in 2021 revealed a high incidence of diseases involving inflammation, including diabetes mellitus (2.0%), asthma (2.4%), dermatitis (6.8%), acute respiratory infections (4.4%), pneumonia (2.0%), joint diseases (7.3%), tumors/cancer (1.8%), and hepatitis (0.4%) (Kemenkes, 2021). Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used to manage inflammation and provide pain relief. These medications, including aspirin, ibuprofen, and naproxen, work by inhibiting the enzymes cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), thereby reducing the production of inflammatory molecules like prostaglandins and thromboxanes (Ugwu et al., 2018).

While NSAIDs offer effective relief, their use comes with significant drawbacks. Blocking COX-1, which plays a role in maintaining the protective lining of the gastrointestinal tract, can lead to side effects such as gastrointestinal bleeding, kidney damage, and central nervous system disturbances. On the other hand, selective inhibition of COX-2 offers anti-inflammatory and analgesic benefits without many of these harmful effects. However, long-term use of NSAIDs can still cause complications, including chronic kidney failure, ulcers, and impaired healing of tendons, ligaments, and cartilage (Fischbach, 2019). As a result, there is an urgent need for alternative anti-inflammatory treatments that are both effective and less harmful.

Cogon grass (*Imperata cylindrica* L.), a plant native to tropical and subtropical regions, has emerged as a promising natural anti-inflammatory remedy. Numerous bioactive compounds have been isolated from *Imperata cylindrica*, including saponins, glycosides, coumarins, flavonoids, and phenols. These compounds have been shown to exhibit a wide range of biological activities, such as anti-inflammatory, antibacterial, and anticancer properties (Jung & Shin, 2021). Notably, *Imperata cylindrica* has demonstrated potent anti-inflammatory effects. Studies by Park et al. (2015) revealed that isogeunin, a compound derived from the roots of cogon grass, can reduce inflammation by inhibiting nitric oxide production and downregulating the expression of pro-inflammatory markers such as iNOS, COX-2, and cytokines like TNF-α, IL-6, and IL-1β. Furthermore, research has shown that extracts from the roots of *Imperata cylindrica* can protect against kidney inflammation in animal models (Chen et al., 2015), and its ethanol extract has been found to reduce nitric oxide secretion in fibroblast cells (Mangkulion et al., 2023). Additionally, Putri et al. (2020) found that the ethanol extract from cogon grass roots can effectively reduce sepsis scores, a condition characterized by inflammation.

Despite promising evidence of the anti-inflammatory potential of *Imperata cylindrica*, limited *in vitro* and *in silico* research exists on its ability to modulate COX-1 and COX-2 activity. This study aims to fill this gap by investigating the anti-inflammatory properties of ethanol extracts from the roots of *Imperata cylindrica in vitro*. Through protein denaturation assays, we will explore the anti-inflammatory effects of these extracts and examine their interactions with COX-1 and COX-2 receptors *in silico*.

MATERIALS AND METHODS

Materials

The resources utilized in this investigation included *Cogon Grass Roots* roots, aquadest, ethanol (Smart Lab), glacial acetic acid (Merck), H_2SO_4 (Merck), NaOH (Merck), reagents (Liebermann-Bouchard, Dragendorff, Mayer), BSA standard (Sigma Aldrich), $FeCl_3$ (Merck), Tris buffer (Sigma Aldrich), sodium chloride (Merck), sodium diclofenac, formic acid (Merck), and acetonitrile (Merck).

Tools

The tools used in this research include: Analytical balance (Ohaus), Glassware (Pyrex Iwaki), Water bath (Memmert), Blender (Miyako), Oven (Memmert), Rotary evaporator (Buchi), Mortar, Whatman filter paper No. 1, Vortex (Labnet), UV-Vis spectrophotometer (Shimadzu), LC-MS/MS system (Thermo Scientific). Hardware used includes a Dell laptop with Intel Core i5-1135G7 processor, 12.00 GB RAM, and Windows 11 (64-bit). Software used includes: ChemDraw Professional 15.0, SwissADME: www.swissadme.ch, AutoDock Tool 1.5.6, BIOVIA Discovery Studio Visualizer, and ProTox Online Tool: tox-new.charite.de/protox_II.

Methods

Sample collection

The roots of *Imperata cylindrica* were collected in February 2024 from Ciherang District, Bogor Regency, West Java, Indonesia. The voucher specimen was identified at the National Research and Innovation Agency. The roots were cleaned to remove dirt and contaminants, washed with running water, and drained

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to eliminate excess water. The cleaned roots were dried in an oven set at 40-50°C. After drying, the roots were cleaned again to remove any remaining impurities. The powdered dried roots were sieved using a 40-mesh sieve (Sahidin et al., 2023).

Preparation of extract

A total of 100 grams of *Imperata cylindrica* roots were macerated at a 1:10 (w/v) ratio in a 30% ethanol (E30) solution for three days. The solvent was changed daily, and Whatman filter paper No. 1 was used for filtration. After filtration, the solvent was removed using a rotary evaporator at 40°C under reduced pressure. The same procedure was followed for maceration using 70% ethanol (E70) and 96% ethanol (E96) solutions at the same ratio (1:10, w/v) (Dahlan et al., 2020).

Phytochemical screening

The extracts obtained from E30, E70, and E96 were screened for secondary metabolites using standard colorimetric phytochemical screening methods. Following Harborne's methods, the presence of flavonoids, phenols, tannins, terpenoids, steroids, and alkaloids was tested (Sahidin et al., 2023).

Bovine serum albumin assay

To assess the anti-inflammatory properties of *Imperata cylindrica* extracts, a modified version of the BSA assay (Bailey-Shaw et al., 2017) was used. A 0.2% w/v BSA solution was prepared using Tris Buffered Saline (TBS), where one TBS tablet was dissolved in 15 mL of deionized water to form a 0.15M NaCl and 0.05M tris solution with a pH of 7.6. The pH was adjusted to 6.4 using glacial acetic acid. A negative control was prepared with 50 µL of solvent added to the BSA solution, bringing the total volume to 5 mL. For the positive control, a stock solution of sodium diclofenac (4000 ppm) was prepared by dissolving 100 mg of the drug in ethanol, and serial dilutions were made to achieve concentrations of 130, 250, 500, 1000, 2000, and 4000 ppm. A stock solution of *Imperata cylindrica* root extract (20,000 ppm) was prepared by dissolving 500 mg of extract in ethanol and making serial dilutions for testing concentrations of 100, 500, 1000, and 5000 ppm. The test solutions were incubated at 72°C for 5 minutes, followed by cooling at room temperature. Absorbance was measured at 660 nm using a UV-Vis spectrophotometer.

Identification of secondary metabolite compounds using LC-MS/MS

A total of 1.4 mg of *Imperata cylindrica* root extract was obtained by dissolving it in 100 mL of methanol. The solution was subsequently inserted through the UPLC system following filtration via a 0.2 m GHP filter. LC-MS/MS analysis was conducted using a Xevo-ToF-1 system (Thermo Scientific) with a C-18 column (1.8 µm, 2.1 × 100 mm). The elution system consisted of 0.1% formic acid in distilled water (A) and 0.1% formic acid in acetonitrile (B), with a gradient run for 20 minutes at 100°C (Warnasih et al., 2024).

Data were processed using the MassLynx 4.1 application and subsequently compared with online spectral databases, including ChemSpider (chemspider.com), MassBank (massbank.eu/massbank.jp), the Human Metabolome Database (hmdb.ca), and PubChem (pubchem.org). The acceptance criteria is a mass error of less than 5 ppm.

Molecular docking, lipinski's rule, and toxicity test

Molecular docking is a crucial method in drug discovery to predict the binding affinities and interactions between ligands (small molecules) and receptors (proteins). In this study, molecular docking was used to evaluate the binding efficiency of compounds derived from the root of Cogon Grass Roots. AutoDock Vina (version 4.2) and AutoDock Tools 1.5.6 were employed for the docking simulations (Trott & Olson, 2010). The crystal structures of Cyclooxygenase-1 (COX-1) (PDB ID 6Y3C) and Cyclooxygenase-2 (COX-2) (PDB ID 1PXX) were obtained from the RCSB Protein Data Bank (Muthukrishnan et al., 2024; Ibrahim et al., 2018). To ensure accurate protein representation, polar hydrogen atoms were added, and the heteroatoms were replaced. The proteins were assigned partial atomic charges and solvation parameters, and the data was converted to the PDBQT format.

The structures of the compounds identified by LC-MS/MS analysis were sourced from the PubChem database in three-dimensional format. Hydrogen atoms were added, and the Gasteiger charges for each structure were calculated. The preparation process for each molecule was carried out using AutoDock Tools 1.5.6. Docking simulations were performed with AutoDock v.4.2 using a population size of 100, employing the Lamarckian genetic algorithm to study protein-ligand interactions and affinity. The docking validation was conducted by redocking diclofenac to COX-2, and the RMSD criterion was set at < 2 Å. The compounds were docked using the appropriate grid coordinates (for COX-1: x = -36.654; y = -49.843; z = 0.492, with a spacing of 1 Å; for COX-2: x = 26.655; y = 27.965; z = 10.933, with a spacing of 1 Å). Protein-ligand interactions were visualized using Discovery Studio Visualizer, and the sum of affinity energies was calculated. Bioavailability of the compounds was

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assessed using Lipinski's Rule of Five, and the toxicity of the compounds was predicted using the Protox-II tool.

Statistical analysis

Data were analyzed using IBM SPSS 20.0 for Windows. Results are presented as means ± standard deviation (X ± SD). One-way ANOVA and Duncan's test were employed to analyze group differences. A p-value < 0.05 was considered statistically significant. IC50 values, representing the concentrations required to achieve 50% of the maximum inhibitory effect, were determined using dose-response curves.

RESULTS AND DISCUSSION

Phytochemical screening

The extraction and phytochemical screening results for E30, E70, and E76 of cogon grass roots are displayed in Table 1. The findings revealed that E30, E70, and E76 samples of cogon grass roots included terpenoids, saponins, tannins/polyphenols, flavonoids, and alkaloids. Multiple investigations found that cogon grass roots include polyphenols, flavonoids, saponins, terpenoids, tannins, and alkaloids (Jung & Shin, 2021; Indriyanti et al., 2022; Nayim et al., 2023; Nayim et al., 2021).

***In-vitro* anti-inflammatory activities**

A heat-induced albumin denaturation assay was used to assess the anti-inflammatory properties of cogon grass root extract. The advantages of this method include its simplicity, the use of small sample sizes, and a relatively short duration. This assay measures the ability of anti-inflammatory substances to inhibit protein denaturation induced by high heat, which is the basis for assessing anti-inflammatory activity (Kaur et al., 2018). Sodium diclofenac, a common non-steroidal anti-inflammatory drug (NSAID), was used as a positive control. Diclofenac inhibits protein denaturation by preventing conformational changes in heat-treated proteins at physiological pH (Aidoo et al., 2021). It also competes with arachidonic acid for binding to cyclooxygenase (COX)-1 and COX-2 enzymes, blocking the inflammatory pathway (Rowlinson et al., 2003).

At a concentration of 200 µg/mL, the 30% ethanol extract (E30) of cogon grass roots exhibited the most promising anti-inflammatory activity, with an inhibition percentage of 88.09 ± 0.07%. The percentage of inhibition for each concentration was determined based on absorbance values, and the IC50 value was calculated using a linear regression equation. The IC50 is the concentration required to inhibit 50% of inflammation (Gaffar et al., 2018). According to Ghasemian et al. (2016), strong anti-inflammatory activity is defined by an IC50 value below 50 µg/mL, weak activity by an IC50 between 50 and 100 µg/mL, and very weak activity by an IC50 between 101 and 250 µg/mL.

In this study, the IC50 value for sodium diclofenac (positive control) was 15.56±0.09 µg/mL, indicating high anti-inflammatory activity. Sodium diclofenac, a synthetic drug that has undergone clinical testing and proven effective in treating inflammation (Suciu et al., 2019), exhibits high effectiveness. The 30% ethanol extract (E30) showed the best activity, with an IC50 value of 71.79±0.45 µg/mL. In contrast, the 70% ethanol extract (E70) exhibited lower anti-inflammatory activity, with an IC50 of 96.94±0.92 µg/mL, while the 96% ethanol extract (E96) showed even weaker activity, with an IC50 value of 141.84±10.35 µg/mL. Based on these values, the anti-inflammatory activity of the 30% and 70% ethanol extracts is considered weak, while the 96% ethanol extract is classified as very weak (Table 2, Figure 1).

Table 1. Phytochemical screening of E30, E70, and E96 of *Imperata cylindrica* L. roots

Sample	Yield (%)	Phenolic	Flavonoid	Saponin	Terpenoid/ steroid	Tannin	Alkaloid
E30	22.87	++	+++	++	+	+	++
E70	19.98	++	++	++	+	+	++
E96	19.42	++	++	++	+	+	++

+++ = Strong presence, ++ = Moderate presence, and + = Weak presence

Table 2. Inhibition percentage of cogon grass root extracts

Samples	Concentration (µg/mL)				
	1	5	10	50	200
30% Ethanol (E30)	21.01±0.51	22.92±0.43	32.32±0.79	44.34±0.13	88.20±0.17
70% Ethanol (E70)	30.85±0.26	32.99±0.62	38.52±0.17	51.71±0.23	63.36±0.60
96% Ethanol (E96)	13.83±0.26	25.97±1.01	26.61±0.39	39.57±0.90	59.56±4.20

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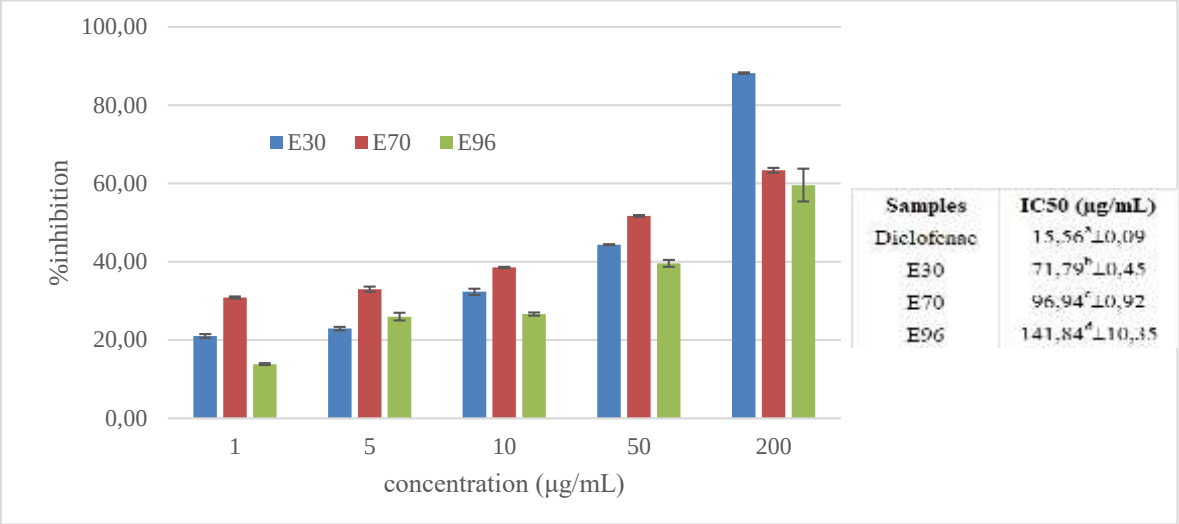

Figure 1. In vitro anti-inflammatory assessment of cogon grass roots extracts via albumin denaturation. Each value is represented as mean ± SD (n = 3). Means with different superscript (a–d) letters in the column are significantly ($p < 0.05$) different from one another

The differences in anti-inflammatory activity among the extracts are likely due to the polarity effects of the solvents used. The 30% ethanol extract (E30), being more polar, may have extracted more active compounds, while the less polar 70% and 96% ethanol extracts possibly extracted fewer bioactive compounds or compounds with lower anti-inflammatory potential. This difference in extract activity can be attributed to the varying solubilities of different phytochemicals in solvents of different polarities, which can influence the biological activity of the extracts (Karta et al., 2024). One compound that acts as an anti-inflammatory is flavonoids. Previous research has shown that the presence of phytochemical compounds such as phenolics, especially flavonoids, plays a role in anti-inflammatory activity (Razafindrakoto et al., 2021). This is also proven based on the results of phytochemical screening (Table 1) in flavonoid testing showing that 30% ethanol extract produces a more concentrated color, which indicates that the flavonoid content in 30% ethanol extract is higher than that in 70% and 96% ethanol extracts, so that the anti-inflammatory activity of 30% ethanol extract is also better than other extracts.

The anti-inflammatory properties of cogon grass roots have been explored in various studies. For example, Razafindrakoto et al. (2021) demonstrated that methanol extracts of the aerial parts of cogon grass roots reduced inflammation significantly through the inhibition of chemical mediator release at doses of 50, 100, and 200 mg/kg. Helma et al. (2024) found that both methanol and ethanol extracts from the leaves of cogon grass roots possess anti-inflammatory compounds, although their activities were relatively weak, with IC50 values of 194.15 µg/mL for methanol extract and 209.36 µg/mL for ethanol extract.

Although this study suggests that the ethanol extract of cogon grass roots demonstrates weak anti-inflammatory activity, further research is needed. It is recommended to conduct fractionation of active compounds and determine the total flavonoid and phenolic content in the extracts, which may provide insights into the active components contributing to the observed anti-inflammatory effects.

Chemical screening using LC-MS/MS

The identification of secondary metabolites in the 30% ethanol extract of cogon grass roots (E30) was carried out using LC–MS/MS. The crude extract chromatogram (Figure 2) displayed multiple peaks, reflecting the presence of various compounds since the sample had not undergone purification. Data were processed using the MassLynx 4.1 application and subsequently compared with online spectral databases, including ChemSpider (chemspider.com), MassBank (massbank.eu/massbank.jp), the Human Metabolome Database (hmdb.ca), and PubChem (pubchem.org). In positive ionization mode (ESI+), most compounds were detected as protonated ions $[M+H]^+$, whereas in negative ionization mode (ESI–), they appeared as deprotonated ions $[M–H]^-$. Therefore, the calculation of the neutral molecular mass was adjusted by considering the protonation or deprotonation process during ionization. Based on data interpretation, a total of 26 metabolites were putatively identified (MSI Level 2), among which six compounds are known to possess anti-inflammatory potential (Table 3).

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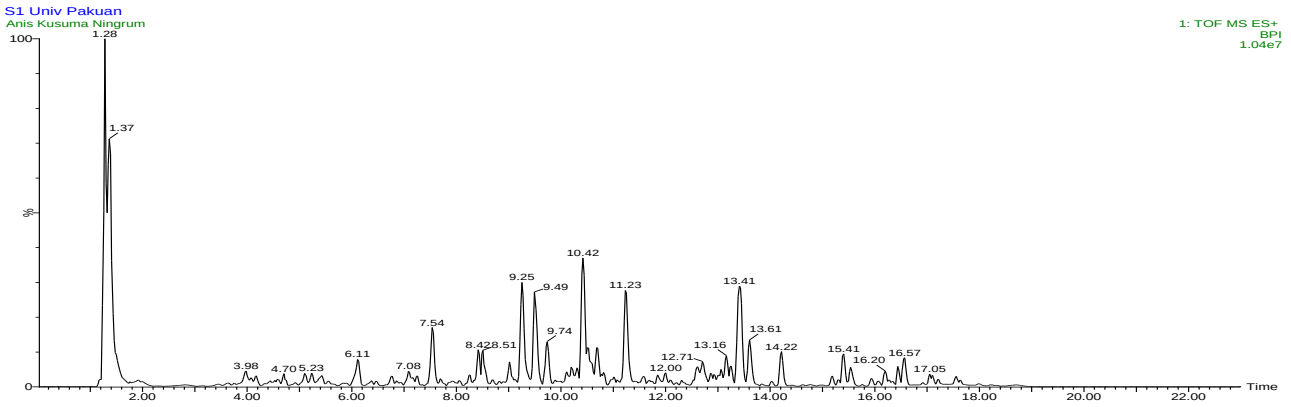


Figure 2. Chromatogram of ethanol extract of cogon grass roots (E30)

The findings indicate that the flavonoid, alkaloid, acetogenin, and phenolic groups are present in the cogon grass root ethanol extract. An investigation by Xiao et al. (2011), the chemical quercetin was discovered to impair COX-2 expression by blocking the transactivator NF-kB and limiting the recruitment of coactivator p300, which functions as a promoter for COX-2. According to Rahmawati et al. (2020), 1.57µM cyclovalone inhibited protein denaturation due to heat by 19.64%. Cyclovalone, a monocarbonyl analog of curcumin, exhibits anti-inflammatory properties. *In vitro* experiments by González et al. (2007) revealed showed the chemical umbelliferone may suppress serotonin and histamine production at the site of inflammation. Additionally, it appears to block the synthesis of prostaglandins from arachidonic acid by inhibiting cyclooxygenase (COX) activity. In vivo, isorhamnetin demonstrates anti-inflammatory properties. A study by Xu et al. (2022) states that isorhamnetin significantly reduces the inflammatory response in COPD-induced rats exposed to cigarette smoke (CS), particularly by affecting the Nrf2/Keap1 pathway. Strychnine and brucine are closely related to alkaloids. The main chemical substances present in the plant's seeds, leaves, roots, and bark are brucine and strychnine. According to pharmacological testing, brucine has analgesic, anti-inflammatory, and antitumor properties (Lu et al., 2020). Annohexocin, an acetogenin discovered in plants of the Annonaceae family, has prospective as an anti-inflammatory compound (Enema et al., 2024).

***In-silico* docking results**

Cogon grass roots contain 6 phytochemical compounds. Based on several studies, these compounds have the potential to be anti-inflammatory. The main mediators of the production of prostaglandins causing inflammation have been identified as cyclooxygenases (COX-1 and COX-2). COX-1, an enzyme that maintains homeostasis, can be found in all tissues, while COX-2 is highly sensitive to cytokines and pro-inflammatory stimuli (Deepika et al., 2023).

Table 3. Secondary metabolite compounds of ethanol extract of cogon grass roots

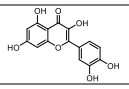
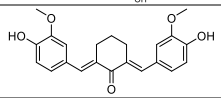
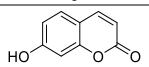
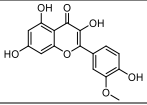
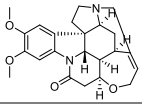
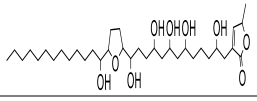
No	RT (Min.)	Observed $[M+H]^+$ m/z	MS^n Fragmentation	Structure	Compound Name	Group
1	2.730	303.0512 $C_{15}H_{10}O_7$	153.0213 97.0295		Quercetin	Flavonoid
2	3.546	367.1508 $C_{22}H_{22}O_5$	277.1200 205.0979 163.0403		Cyclovalone	Phenolic
3	3.981	163.0405 $C_9H_6O_3$	151.0766 89.0392		Umbelliferone	Phenolic
4	7.383	317.2121 $C_{16}H_{12}O_7$	189.0918 105.0705		Isorhamnetin	Flavonoid
5	9.739	395.1857 $C_{23}H_{26}N_2O_4$	377.1754 283.0977 257.1186		Brucine	Alkaloid
6	17.627	617.4416 $C_{35}H_{64}O_9$	469.4251 379.1909 338.3419		Annohexocin	Acetogenin

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Table 4. Docking scores (ΔG Values) for COX-1 and COX-2 binding of ligands

Compound	COX-1 ΔG (kcal/mol)	COX-2 ΔG (kcal/mol)
Diclofenac	-6.5	-8.5
Quercetin	-9.0	-9.0
Umbelliferone	-6.9	-7.6
Brucine	-8.7	-8.2
Annohexocin	-7.0	-7.2
Isorhamnetin	-8.6	-8.9
Cyclovalone	-9.3	-9.8

Table 5. Physicochemical Properties, Lipinski's Rule, and Toxicity Classification of Ligands

Compound	Molecular Weight (Da)	Log P	Hydrogen Bond Donors	Hydrogen Bond Acceptors	Refractivity Molar	Lipinski's Violation	LD50 (mg/kg)	Toxicity Class
Diclofenac	318.13	3.1	1	2	75.61	0	53	3
Quercetin	310.13	1.99	5	6	78.04	0	159	3
Umbelliferone	163.04	1.5	1	2	44.51	0	10,000	6
Brucine	395.18	2.11	0	6	114.04	0	150	3
Annohexocin	617.441*	5	6*	9	175	2	400	4
Isorhamnetin	317.212	0,24	7*	11*	114,63	2	5,000	5
Cyclovalone	367.15	4.34	2	5	105.3	0	5,000	5

Note: *Not by Lipinski's Rule of Five

Docking analyses were performed to identify the binding ability of potential ligands for COX-1 and COX-2 proteins (Table 4). These studies compared the ligand's binding affinity to that of the reference drug, diclofenac. The docking results, including the Gibbs free energy (ΔG) values, were analyzed to assess the strength of the binding interactions. The docking simulations demonstrated that cyclovalone exhibited the strongest binding affinity toward both COX-1 and COX-2, with docking scores of –9.3 and –9.8 kcal/mol, respectively. These values were lower than those of the reference drug diclofenac (–6.5 and –8.5 kcal/mol), indicating that cyclovalone may form more stable interactions with both enzymes. Quercetin and isorhamnetin also showed high affinities, though not as strong as cyclovalone. Importantly, cyclovalone's stronger binding to COX-2 suggests potential selectivity, which is associated with reduced gastrointestinal adverse effects compared to non-selective NSAIDs such as diclofenac (El-Malah et al., 2022).

Based on the molecular docking results, the conformation with the lowest Gibbs free energy was identified. The ability of the drug to bind to the receptor is quantified by its Gibbs free energy value; a lower value indicates a stronger potential for compound interaction with the test protein (Trott & Olson, 2010). This shows that secondary metabolites from cogon grass roots have good stability and ability to bind to receptors, as some compounds have lower binding energy to receptors than diclofenac controls. Referring to the ΔG value, the most stable compounds are cyclovalone, quercetin, and isorhamnetin. In this test, diclofenac was used as a comparison because this compound is commonly used in anti-inflammatory drugs (Savitri et al., 2023).

Lipinski's rule and toxicity

A key criterion for assessing a compound's suitability as a drug candidate and determining its capacity to cross biological membranes during bodily reactions is Lipinski's Rule of Five. According to this rule, the compound must have a molar refractivity value between 40 and 130, a molecular weight of no more than 500 Daltons, a log (P) (lipophilicity) of no more than 5, no more than 5 hydrogen bond donors, and no more than 10 hydrogen bond acceptors. Based on the physicochemical analysis of the ligands (Table 5), annohexocin and isorhamnetin fail to meet Lipinski's Rule due to violations.

Toxicity prediction was carried out using ProTox-II, a program that classifies toxicity into six stages. Substances classified as class 1 have a lethal dose 50 (LD50) of 5 mg/kg or less, making them extremely dangerous to swallow. Class 2 drugs have LD50s ranging from 5 mg/kg to 50 mg/kg, making them lethal if consumed. The LD50 of class 3 substances ranges

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from 50 mg/kg to 300 mg/kg, making them toxic if swallowed. The LD50 of class 4 substances is between 300 and 2000 mg/kg, making them dangerous if swallowed. A class 5 substance's LD50 ranges from 2000 mg/kg to 5000 mg/kg, making it potentially hazardous. A chemical with an LD50 of 5000 mg/kg or higher is considered non-toxic.

The toxicity analysis results for the 6 ligands showed that 1 ligand is non-toxic, 2 ligands are possibly harmful, 1 ligand is harmful if swallowed, and 2 ligands are toxic if swallowed. This indicates that the compounds contained in cogon grass roots include relatively safe compounds, such as umbelliferone. Cyclovalone complies with Lipinski's rule of five (MW = 367 Da, logP = 4.34, H-bond donors = 2, acceptors = 5), implying favorable oral bioavailability (Chahal et al., 2023). Its LD50 value of 5000 mg/kg points to relatively low acute toxicity, offering a notable safety advantage over diclofenac. This aligns with ongoing research emphasizing scaffold optimization to balance efficacy and safety (Elewa et al., 2024).

Amino acid residues interaction

To evaluate the binding affinity between the active compounds and the target enzymes, COX-1 and COX-2, we identify the amino acid residues that interact with each ligand. An active compound is estimated to have a strong bond with the target enzyme if it can form strong hydrogen bonds with the same amino acid residues, compared to the control drug, diclofenac. The hydrogen bond interactions between the ligands and amino acids at the COX-1 receptor's active site suggest that only the ligands quercetin and brucine exhibit similar interactions with the control diclofenac. This is because these ligands form hydrogen bonds with the amino acid residue Gln44. Meanwhile, binding to the COX-2 receptor does not show a ligand that binds to the same amino acid as the diclofenac control. The similarity of these residues suggests that the active compounds in the ethanol extract of cogon grass roots resemble the comparison compound, making them potential candidates for anti-inflammatory agents. These chemicals are thought to have the capacity to prevent the overexpression of the proteins COX-1 and COX-2, which are the principal causes of inflammation.

Cyclovalone formed hydrogen bonds with key residues of COX-1 (Glu465, Cys47, Asn34) and COX-2 (Ala156, Asn43; Table 6; Figure 3), which likely contributes to its binding stability and enhanced COX-2 affinity. Diclofenac, in contrast, formed three hydrogen bonds with COX-1 (Tyr130, Arg469, and Gln44) and two hydrogen bonds with COX-2 (Asn104 and Arg109). Although diclofenac engages multiple residues in COX-1, its overall docking score was weaker than cyclovalone, suggesting that binding affinity depends not only on the number of hydrogen bonds but also on their orientation, strength, and involvement with key catalytic residues. This aligns with structural insights highlighting how COX-2 selective inhibitors exploit unique binding pockets absent in COX-1 (Mohsin et al., 2022; Ju et al., 2022).

Taken together, these findings suggest that cyclovalone is a promising candidate as a COX-2 selective, low-toxicity anti-inflammatory agent, outperforming diclofenac in in silico affinity and safety metrics. However, limitations remain: docking cannot fully account for pharmacokinetics, metabolism, or chronic toxicity. Thus, further validation via ADMET modeling, in vitro enzymatic assays, and in vivo evaluations is essential. Additionally, rational design strategies, such as scaffold hybridization or linker modification to optimize selectivity and pharmacokinetic properties, could further enhance the therapeutic profile of cyclovalone (Chahal et al., 2023; Bokhtia et al., 2023).

Table 6. Hydrogen bond interactions between ligands and COX-1/COX-2 residues

Ligand	COX-1 Residue	COX-2 Residue	Hydrogen Bond Interaction with COX-1	Hydrogen Bond Interaction with COX-2
Diclofenac	Tyr130, Arg469, Gln44	Asn104, Arg109	Yes	Yes
Quercetin	Cys47, Gly45, Gln44, Cys41, Glu465, Gln461	Gln461	Yes	Yes
Cyclovalone	Glu465, Cys47, Asn34	Ala156, Asn43	Yes	Yes
Umbelliferone	-	Thr206	No	Yes
Isorhamnetin	Asn382	Pro154, Gly45, Cys47	Yes	Yes
Brucine	Gln44, Arg83	Asn43	Yes	Yes
Annohexocin	Arg79, Arg120	Tyr130, Asn39, Gln461, Gln42, Lys468	Yes	Yes

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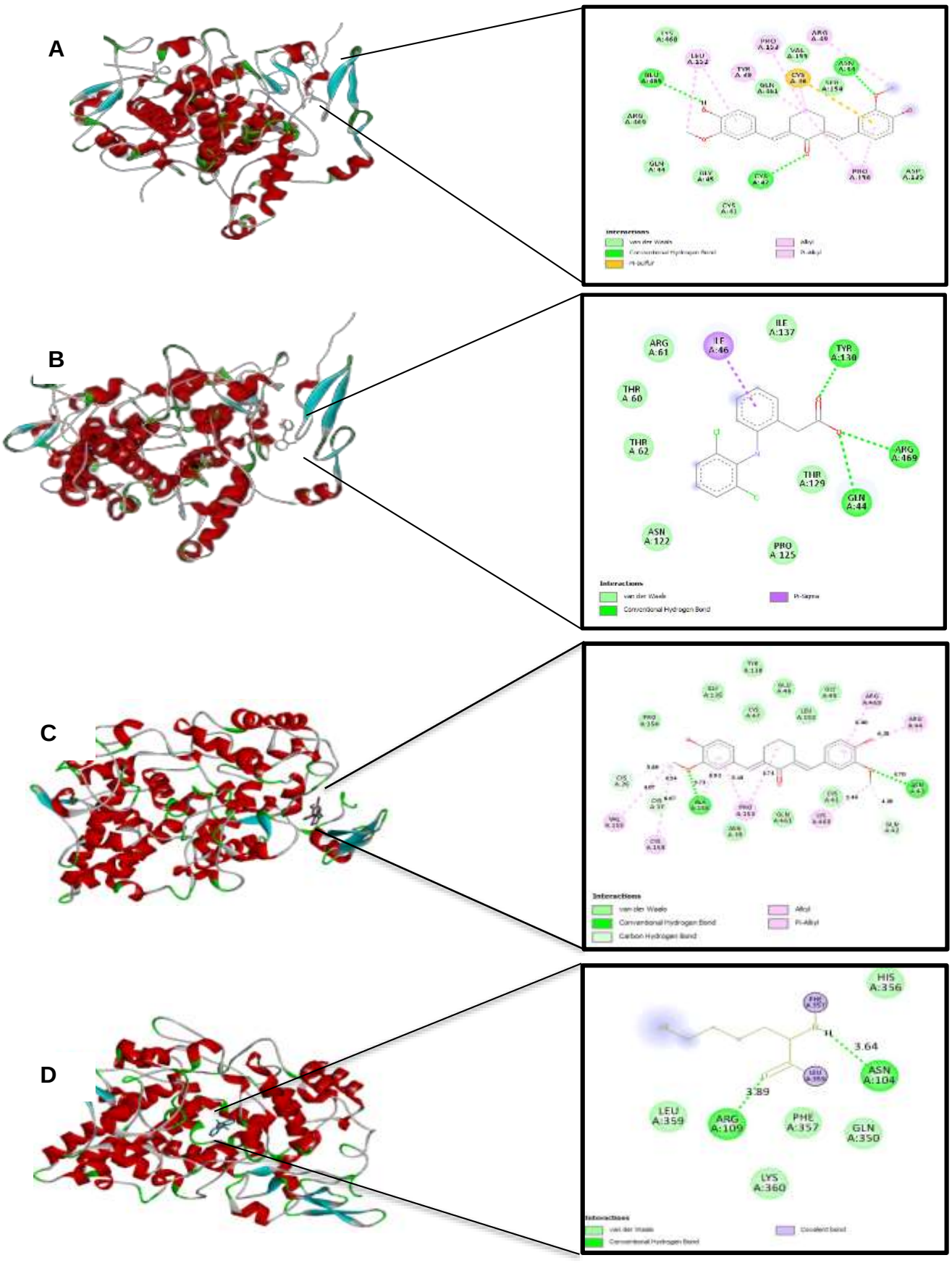

Figure 3. Docking of (A) cyclovalone to COX-1; (B) diclofenac to COX-1; (C) cyclovalone to COX-2; and (D) diclofenac to COX-2; and its hydrogen bond interactions are shown in green dotted lines

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CONCLUSION

The study's findings reveal that the ethanol extract from cogon grass roots includes cyclovalone, a promising ligand with anti-inflammatory properties. In-silico experiments have shown that cyclovalone interacts with COX-2 and exhibits a higher binding score compared to COX-1. Furthermore, a 30% ethanol extract of cogon grass roots exhibited anti-inflammatory activity with an IC50 value of 71.79 µg/mL, according to *in vitro* testing using the BSA protein denaturation method. Further research is required to confirm the potential of cogon grass roots as a promising anti-inflammatory candidate. This can be achieved through *in vitro* testing, utilizing the COX-2 enzyme inhibition method.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.W., U.H., S.J.S.; Methodology, S.J.S., D.W.; Software, S.W.; Validation, A.H.M., D.W., U.H.; Formal Analysis, U.H., A.H.M.; Investigation, D.W., S.W.; Resources, A.H.M., D.W.; Data Curation, S.W., A.H.M., S.J.S.; Writing - Original Draft, S.W., U.H., S.J.S., A.H.M., D.W.; Writing - Review & Editing, S.W., U.H., S.J.S.; Visualization, S.W., S.J.S.; Supervision, A.H.M., D.W.; Project Administration, U.H.; Funding Acquisition, S.W.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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Acute Oral Toxicity and Histophatological Study of Ethanol Extract and Fractions of *Etlingera elatior* Flowers in Mice

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Abstract

Background: *Etlingera elatior (Jack) R.M. Sm or Kecombrang Flower had been used traditionally to enhance the taste of food. Some studies reported its pharmacological activities such as antioxidant, anti-inflammatory, antimicrobial, and anticancer. However, its safety has not yet been reported explicitly.* ***Objective****: To investigate the acute oral toxicity, macropathological and histopathological changes of 96% ethanol extract, n-hexane, ethyl acetate and methanol-water fractions of Etlingera elatior flowers in Balb/C mice.* ***Methods****: The 96% ethanol extract, n-hexane, ethyl acetate and methanol-water (3:7) fractions were given to mice with 4 dose levels (75, 150, 300, and 600 mg/Kg body weight). Single oral administration of them was done on the first day of the test and the mice were then observed in 14 consecutive days. The control group received Na-CMC 0,3%. Changes in behavior, mortality rate, body weight, macropathology and histopathology of kidneys and liver were assessed.* ***Results****: No signs of toxicity or mortality were observed when mice were exposed to the 96% ethanol extract, n-hexane, ethyl acetate and methanol-water fractions. The were no significant changes in the body weight. Macropathological examination of the liver and kidneys showed normal results with a brownish red color, smooth surface and rubbery consistency. Histopathological examination revealed mild, moderate, and severe damage to the liver and kidneys of mice, however the level of damage was not followed by an increase in dose. The oral lethal dose was higher than 600 mg/Kg.* ***Conclusion****: Etlingera elatior (Jack) R.M. Sm did not produce toxic effects in mice after acute treatment.*

Keywords: *acute toxicity, Etlingera elatior, kecombrang*

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INTRODUCTION

Herbal medicines are usually considered safe or have low toxicity based on their long history of use by humans. However, recent studies showed that many herbal medicines have side effects (Bent, 2008). The safety issues are major problem in the use of medicinal plants, so it is important to carry out studies on toxicity to ensure their safety profile. In the context of developing and using standardized herbal medicines, ensuring effectivy and toxicity are necessasy issues. Therefore, evaluating the toxicological effects of each medicinal plant extract is an important aspect before the drug is used for humans.

Etlingera elatior (Jack) R.M. Sm, commonly known as Kecombrang, is one of the medicinal plants. It is a shrub that grows annually and has fronds similar to banana plants, it forms rhizomes and is green in color. This plant produces flowers, fruit and seeds, and is useful as a vegetable. The flower is a hump-shaped, top-shaped compound flower with a stem length of between 40 and 80 cm. This plant belongs to the Zingiberaceae family (Saudah et al., 2022).

Etlingera elatior flowers are usually used as a spice for food flavouring and for ornamental purposes. In traditional medicine, *Etlingera elatior* was used to treat fever by roasting or burning the shoots, and then consuming the inner side or contents. This plant could also be used as a medicine for skin-related diseases, including measles (Lachumy et al., 2010). Previous studies have demonstrated the pharmacological effects of *Etlingera elatior* flowers as an antioxidant (Jackie et al., 2011), anti-inflammatory (Juwita et al., 2020; Nurhayatun et al., 2023), antibacterial (Ferreira et al., 2023), and anti-cancer (Zan et al., 2011). According to Naufalin et al. (2021), the secondary metabolite compounds of *Etlingera elatior* flowers were alkaloids, flavonoids, tannins, terpenoids, saponins, steroids, polyphenols and essential oils. The diverse pharmacological activities are linked to these secondary metabolites such as phenolics and flavonoids, diarylheptanoids, terpenoids, and curcumin (Juwita et al., 2018).

The safety and side effects of medicinal plants can be determined through toxicity tests, one of which is the acute toxicity test. Acute toxicity is interpreted as Lethal Dose 50 (LD_{50}), the dose of a substance that causes death in 50% of a test population (usually rodents) after a single exposure. The higher the LD_{50} value, the lower the toxicity of the active ingredient (Ayun et al., 2021). The main purposes of acute oral toxicity testing are to obtain the presence of toxic effects on the tested animals after administration of a single dose or repeated doses within 24 hours. When a substance is suspected of causing harm, histopathological examination is crucial to evaluate its toxic effects on cells and tissues. Histopathology is the examination that aims to see the structure of damaged tissues and cells under a microscope (BPOM, 2022).

Acute toxicity evaluation of ethanol extract and methanol fraction of *Etlingera elatior* flowers had been studied. The acute toxicity test of the ethanol extract of *Etlingera elatior* flowers in mice at doses of 1000, 1500, 2000 mg/KgBW showed that the extract was not toxic (Sungthong & Srichaikul, 2018). The acute toxicity test of methanol extract of *Etlingera elatior* flowers using the Brine Shrimp Lethality Test (BSLT) method also showed no toxicity (Lachumy et al., 2010). Meanwhile, the evaluation of the ethyl acetate and hexane fraction have not yet been conducted by any research group. This study, thus, was aimed at examining the acute toxicity of *Etlingera elatior* flowers in Balb/C mice and histopathological examination of the liver and kidneys, so that the maximum limit for consuming these flowers safely could be determined.

MATERIALS AND METHODS

Materials

Etlingera elatior flowers were harvested from Pangandaran, West Java. Mature flowers were collected from plants that took around two years to flower. The flowers sample was authenticated by a botanist at the Biology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret with a certificate of determination with the letter number 131/UN27.9.6.4/Lab/2022.

Male BALB/C mice (2-3 months old, weighing 20-25 grams) were acclimated for 7 days before testing. Prior to the test, mice were fasted for 3-4 hours, during which water remained available. After the test substance was administered, food was withheld for an additional 1 to 2 hours. This short period allowed for the absorption and initial effects of the administered substance without interference from food intake. This study protocol had obtained Ethical Clearance from the RSUD dr. Moewardi Solo with the letter number 1.726/XII/HREC/2022.

Method

Collection and processing of plant material

The flowers that were still in bud and pink were cut crosswise with a thickness of 1-2 cm and then washed with tap water to clean off the extraneous materials. After sorting, they were dried in the oven at temperature

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of 50º C. The dried flowers were then ground in a blender to fine powder and sieved using a 60 mesh sieve to get size uniformity.

Extraction and fractinations

Etlingera elatior flowers powder was extracted for 24 hours in 96% ethanol by maceration technique. The powder and solvent ratio was 1:5. The extract was then filtered through the filter paper. Remaceration with fresh solvent was carried out twice with powder and solvent ratio of 1:3. The remaceration step used a smaller solvent-to-material ratio compared to the initial maceration to maximize the extraction of remaining active compounds from the plant material. By using a more concentrated ratio in the second step, the gradient for diffusion was improved, allowing for further extraction and a higher overall yield of the desired compounds.

The filtrate of maceration and remaceration was evaporated using a rotary evaporator and followed by a water bath to concentrate. Fractination was carried out with liquid-liquid extraction. Ten grams of thick ethanol extract was dissolved in 100 mL of methanol-water (3:7), then liquid-liquid partition was carried out using n-hexane which formed two layers (methanol-water and hexane phases). The n-hexane solution was separated, while the remain filtrate was partitioned with ethyl acetate. The ethyl acetate solution was separated, and remain methanol-water solution. The three fractions obtained were methanol-water, ethyl acetate and hexane fractions which were then evaporated and concentrated to achieve the thick extract. To ensure all solvent had evaporated, the sample was heat in a controlled way to a constant weight by repeatedly heating and weighing the sample until the weight no longer changes.

Acute toxicity test

The number of tested animals was 5 mice in each 4 dose levels, so that 20 mice were needed. There were 4 extract treatment group (96% ethanol extract, n-hexane, ethyl acetate, and methanol-water fraction), so that 80 mice were needed. The negative control group (Na-CMC 0.3%) consisted of 5 mice, so a total of 85 mice were used in this study. Each extract was given orally once with dose I (75 mg/KgBW), dose II (150 mg/KgBW), dose III (300 mg/KgBW), and dose IV (600 mg/KgBW). The extract and fractions were given to mice at fixed dose levels according to OECD Guideline 423: *Acute Toxic Class Method*. This study protocol had obtained Ethical Clearance from the RSUD dr. Moewardi Solo with the letter number 1.726/XII/HREC/2022.

Observation of animal behavior, body weight, macropathology and histopathology of liver and kidneys

Toxic symptoms were observed in the first 30 minutes and continued for 4 hours after administration of the extract. Observations were continued for 14 days, once a day (BPOM, 2022). Animal behavior observed was fast heartbeat, decreased or increased breathing, excessive licking, skin (itching), changes in fur color, hair loss, body shaking, convulsions, excessive salivation (salivation), diarrhea, sleep, weakness or decreased activity, and death (Fithria et al., 2018); (BPOM, 2022). On the 15th day, surgery was performed on 1 mouse for each dose, then the liver and kidneys were observed macropathologically and histopathologically.

Body weight was monitored at least once a week after administration of the test preparation (BPOM, 2022). In this study, weighing was carried out 3 times: 0 day, 7th day, and 13th day.

Observations were made using the H and E (Hematoxcylline and Eosin) staining method and observed using a microscope at 400x magnification. Observation of liver damage was carried out thoroughly in the hepatocyte cells near the central vein and those that far from the central vein. Observation of kidney damage was carried out thoroughly in the cortex and medulla of the kidney. Fatty degeneration and inflammation in the liver and kidneys of mice were scored, score 0 if there was no damage, score 1 (mild damage) if there was local or focal inflammation or fatty degeneration, score 2 (moderate damage) if there was multifocal inflammation or fatty degeneration, score 3 (severe damage) if there was even or diffuse inflammation or fatty degeneration (Darmayanti et al., 2020).

Data analysis

Body weight was analyzed statistically using One Way Anova, followed by Tukey test. Toxic symptoms, macropathology and organ histopathology were analyzed qualitatively. The LD_{50} was calculated using the Thomson and Weil method. According to Siswadi & Saragih (2018), the Thomson and Weil formula is:

$\text{Log } LD_{50} = \text{Log } D + d\ (f + 1)$

Note: D: smallest dose, d: logarithm of dose multiples, f: a factor in Weil's LD_{50} calculation list.

RESULTS AND DISCUSSION

Extraction and fractinations

Etlingera elatior flowers used in this study were still in bud and pink in color with the aim of ensuring

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that the secondary metabolite content, especially flavonoids, was optimal. The flowers were dried at a temperature of 50º C because this temperature was considered safe to prevent damage to flavonoid compounds. The extraction was carried out by maceration with 96% ethanol as a solvent because it can dissolve flavonoid and polyphenol compounds found in *Etlingera elatior* flowers (Suwarni & Cahyadi, 2016).

The thick ethanol extract was then continued to the fractionation process. The purpose of fractionation was to separate secondary metabolites based on their polarity, with each fraction enriched in compounds of similar chemical properties, such as nonpolar or semi-polar compounds. This is crucial for identification of which specific compounds or groups of compounds were responsible for the toxic effects observed. Thick ethanol extract might contains a complex mixture of hundreds of compounds, while a single fraction might contain a compound or a mixture of compounds that is more potent (e.g., more toxic or more active) than the original whole extract, as it concentrates the active molecules. The yield of ethanol extract and fractions can be seen in Table 1.

Behavioral responses and mortality rate

Mice that were given Na CMC, 96% ethanol extract, and fractions at all doses did not die during 14 days of observation (Table 2). In this study, the highest dose (600 mg/Kg body weight) did not cause death. Therefore, if the highest dose given did not cause death for 14 days, then the preparation was declared non-toxic (BPOM, 2022). The LD_{50} value in this experiment could not be calculated because there were no deaths in the tested animals.

The results of toxicological observations were compared between the negative control and the treatment group. After administration of ethanol extract and fractions, several toxic symptoms were observed, including sleepiness, decreased activity, excessive licking, and rapid heartbeat (Table 3). The mice in the negative control group, ethanol extract and fractions treatment were experienced sleeping behavior. This was normal behavior because mice are nocturnal animals that are active at night. According to Lestari et al. (2019), if these symptoms also occur in the control group, they cannot be concluded as symptoms of poisoning or toxic symptoms.

Table 1. Yield of ethanol extract and fractions of *Etlingera elatior* flowers

Extraction / Fractination solvent	Yield (% *w/w*)
96% ethanol extract	14.55
Methanol:water (3:7)	7.14
Ethyl acetate	2.08
n-Hexane	57.9

Table 2. The number of deaths in mice given Na CMC, extract, and fraction of *Etlingera elatior* flowers

Treatment	Number of mice	Number of dead mice
Negative control (Na CMC 0.3%)	5	0
Ethanol extract dose 75 mg/KgBW	5	0
Ethanol extract dose 150 mg/KgBW	5	0
Ethanol extract dose 300 mg/KgBW	5	0
Ethanol extract dose 600 mg/KgBW	5	0
Methanol-water dose 75 mg/KgBW	5	0
Methanol-water dose 150 mg/KgBW	5	0
Methanol-water dose 300 mg/KgBW	5	0
Methanol-water dose 600 mg/KgBW	5	0
n-Hexane dose 75 mg/KgBW	5	0
n-Hexane dose 150 mg/KgBW	5	0
n-Hexane dose 300 mg/KgBW	5	0
n-Hexane dose 600 mg/KgBW	5	0
Ethyl acetate dose 75 mg/KgBW	5	0
Ethyl acetate dose 150 mg/KgBW	5	0
Ethyl acetate dose 300 mg/KgBW	5	0
Ethyl acetate dose 600 mg/KgBW	5	0

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Table 3. Toxic symptoms after administration of Na-CMC, extract and fractions

Treatment group	Behavioral responses
Negative control (Na-CMC 0.3%)	Sleeping
96% ethanol extract	Sleeping, decreased activity, excessive licking
Methanol-water fraction	Sleeping, decreased activity, faster heart beats
N-hexane fraction	Sleeping, decreased activity
Ethyl acetate fraction	Sleeping, decreased activity, excessive licking, faster heart beats

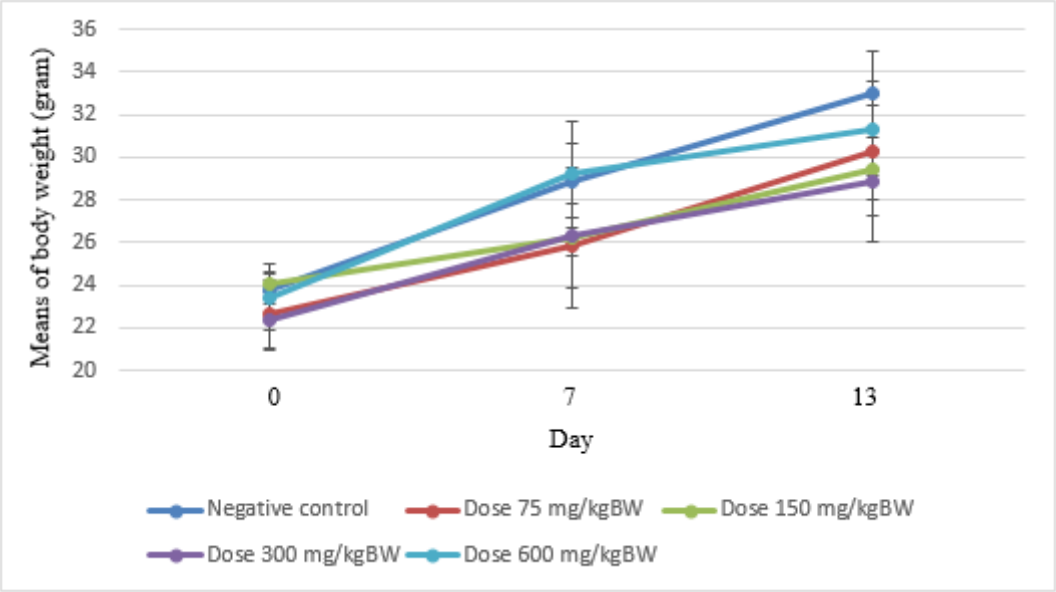

Figure 1. Effect of 96% ethanol extract on mice's body weight

During 14 days of observation, the behavior was evaluated such as excessive licking, decreased activity, and a faster heartbeat. Faster heart beat in a mouse was observed visually. After administering the extract, the mouse's chest was observed for increased visible heart pulsation. These symptoms were not found in all mice and were not found after day 2 to day 14, so this behavior could be caused by the different response abilities of mice. According to Rauf (2018), mice could experience stress which was characterized by changes in behavior such as frequently licking their bodies, their hearts beating faster, and being often silent. Apart from that, changes in mouse behavior that did not occur in all mice could be caused by other factors; different physiological conditions in each mouse (Fithria et al., 2018).

Previous studies have shown that ethanol extract of *Etlingera elatior* flower were not acutely toxic, with oral administration of ethanol extracts at doses 1000, 1500, and 2000 mg/KgBW to mice showing no fatalities (Sungthong & Srichaikul, 2018). According to Lachumy et al. (2010) methanol extract of *Etlingera elatior* flowers using the BSLT method also found the extract to be non-toxic to *Artemia salina*, showed an LD_{50} value was 2.52. This recent study also found that the methanol:water, ethyl acetate, and n-hexane fractions did not cause toxicity in mice. Therefore, the results of this study complement previous studies. These consistent results from different studies and testing models support the conclusion that *Etlingera elatior* flower extracts are safe in terms of acute toxicity at the tested doses.

Body weight measurement

Mice treated with 96% ethanol extract at doses of 75, 150, 300 and 600 mg/KgBW showed an increase in body weight at the 1st, 2nd, and 3rd weighing. The 96% ethanol extract did not affect mouse growth, as both the extract-treated groups and the negative control group showed weight gain after the 1st, 2nd, and 3rd weighings. In the One Way Anova test, a Sig. value of > 0.05 was obtained, which means there was no significant difference in body weight of mice between the negative control group and ethanol extract-treated group. The findings in this study, that 96% ethanol extract of *Etlingera elatior* did not cause toxic effects, as shown by increa sing body weight in experimental animals that was not significantly different from the control group.

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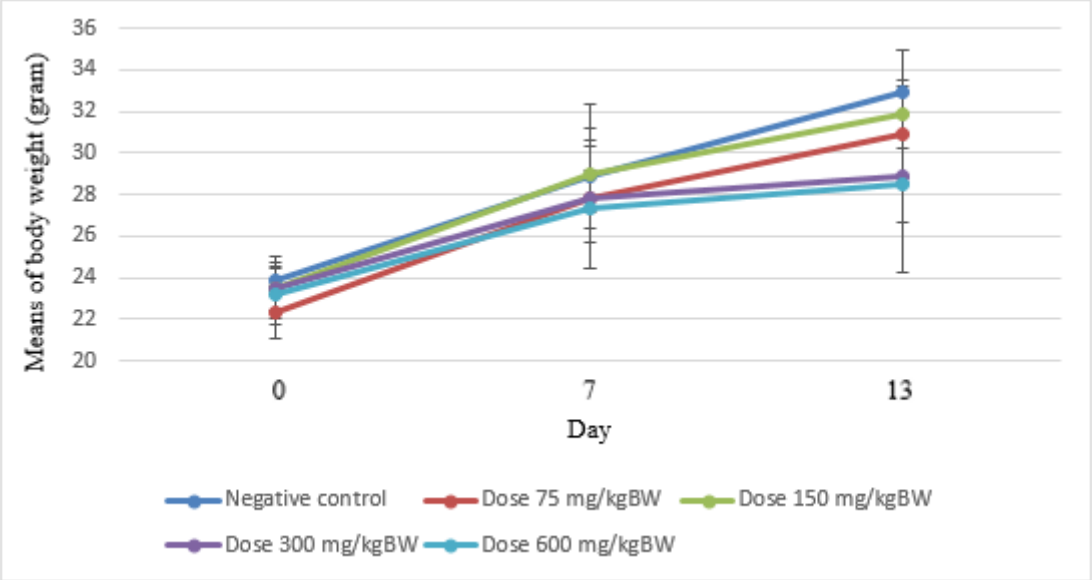

Figure 2. Effect of methanol-water fraction on mice's body weight

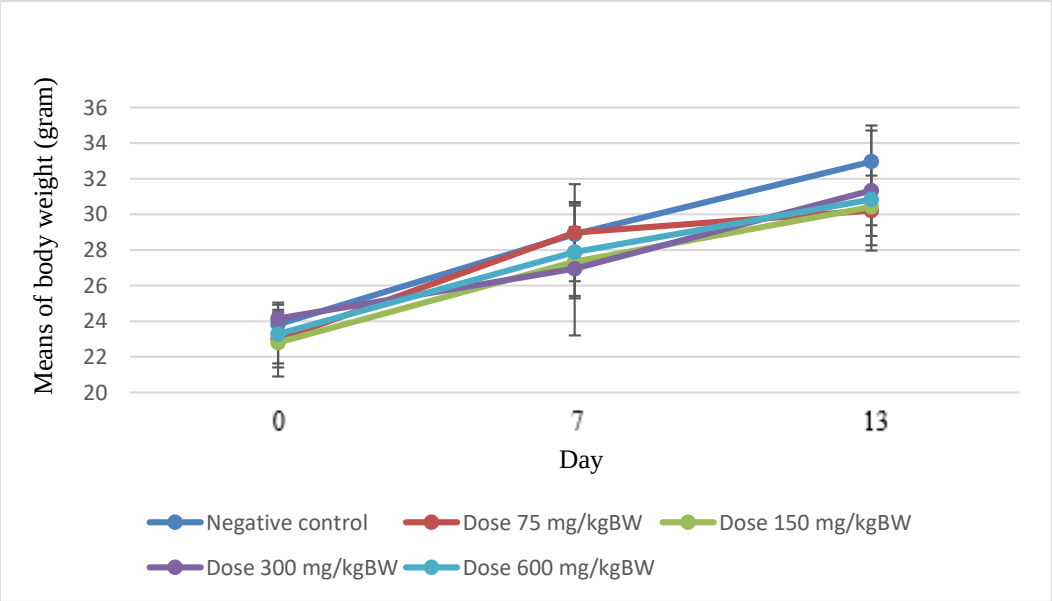

Figure 3. Effect of n-hexane fraction on mice's body weight

Mice treated with methanol-water fraction at doses of 75, 150, 300 and 600 mg/KgBW showed an increase in body weight at the 1st, 2nd, and 3rd weighing. The methanol-water fraction did not affect mouse growth, as both the fraction-treated group and the negative control showed weight gain after the 1st, 2nd, and 3rd weighings. In the One Way Anova test, a Sig. value of > 0.05 was obtained, which means there was no significant difference in body weight of mice between the negative control and methanol-water group. This indicated that methanol-water fraction of *Etlingera elatior* did not cause toxic effects, as shown by increasing body weight in experimental animals that was not significantly different from the control group.

Mice treated with n-hexane fraction at doses of 75, 150, 300 and 600 mg/KgBW showed an increase in body weight at the 1st, 2nd, and 3rd weighing. The n-hexane fraction did not affect mouse growth, as both the n-hexane fraction treated and the negative control group showed weight gain after the 1st, 2nd, and 3rd weighings. In the One Way Anova test, a Sig. value of > 0.05 was obtained, which means there was no significant difference in body weight of mice between the negative control and n-hexane group. This indicated that n-hexane fraction of *Etlingera elatior* did not cause toxic effects, as shown by increasing body weight in experimental animals that was not significantly different from the control group.

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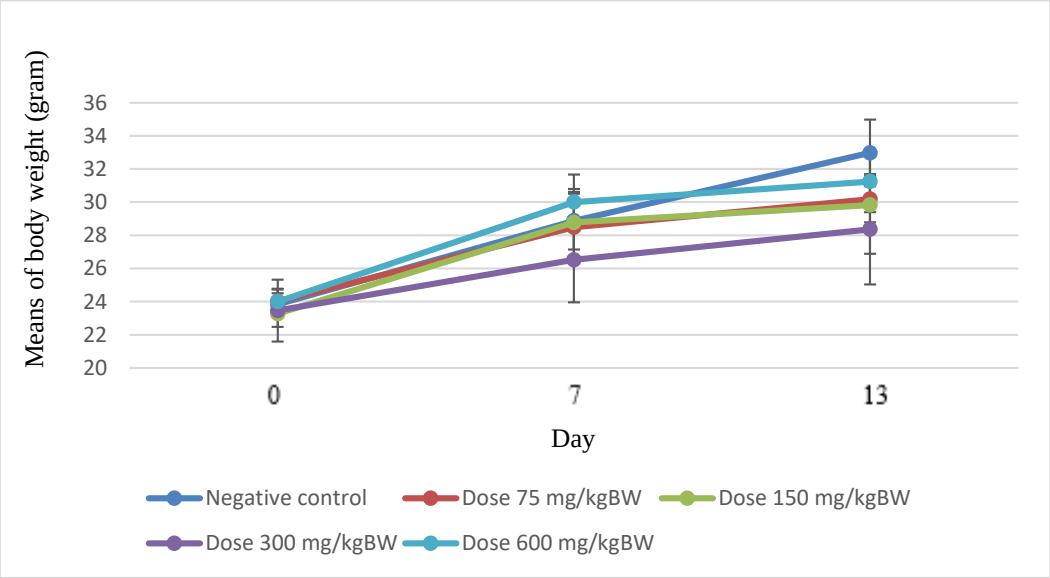

Figure 4. Effect of ethyl acetate fraction on mice's body weight

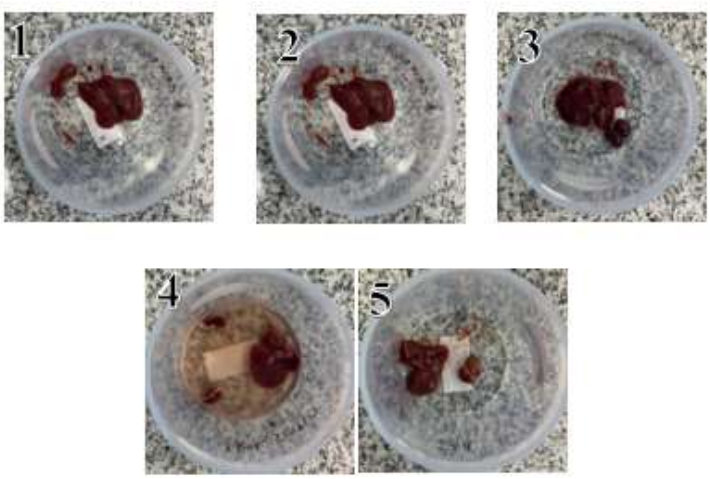

Figure 5. Liver macropathology after administration of Na CMC, extract and fractions of *Etlingera elatior* flowers
Notes: (1). Negative control Na-CMC 0.3%, (2). Ethanol extract dose-I (75 mg/KgBB), (3). Methanol-water fraction dose-I (75 mg/KgBB), (4). N-Hexane fraction dose-I (75 mg/KgBB), (5). Ethyl acetate fraction dose-I (75 mg/KgBB)

Mice treated with ethyl acetate fraction at doses of 75, 150, 300 and 600 mg/KgBW showed an increase in body weight at the 1st, 2nd, and 3rd weighing. The ethyl acetate fraction did not affect mouse growth, as both the fraction treated and the negative control group showed weight gain after the 1st, 2nd, and 3rd weighings. In the One Way Anova test, a Sig. value of > 0.05 was obtained, which means there was no significant difference in body weight of mice between the negative control and ethyl acetate fraction group. This indicated that ethyl acetate fraction of *Etlingera elatior* did not cause toxic effects, as shown by increasing body weight in experimental animals that was not significantly different from the control group.

After administration of ethanol extract and fractions of *Etlingera elatior* flowers, mice experienced weight gain which was marked by an increase in the graph. According to Tangkere et al. (2022), if the tested animals experience weight loss, this could be due to the administration of the extract. While an increase in body weight indicates that the mice are healthy, which is characterized by eating regularly. In toxicological studies, a healthy animal will either maintain or increase its body weight. If a substance is toxic, it will typically show a significant decrease in body weight.

Mice in all treatment groups experienced increased body weight. These increases were not statistically significant when compared to the negative control group ($p>0.05$), meaning there were no significant differences between the weight gain in the treatment groups and the control group at the end of the experiment. Based on this statistical results, it could be concluded that

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administration of ethanol extract and fractions did not affect the body weight of mice. The evidence suggested that the ethanolic extract and fractions of *Etlingera elatior* flower were safe and did not induce harmful effects, as shown by these physiological markers.

Macropathology of liver

Macropathological observations were used to determine toxic effects visibly or visually. Organ damage can be seen from changes in color, consistency and surface of the organ. The results of observations of the liver and kidney organs of mice in the negative control group and the treatment groups can be seen in Figure 5. Based on Figure 5, it can be seen that the liver and kidney organs of mice are brownish red in color, have a smooth surface, and have a chewy consistency, so that the liver and kidneys of mice are normal. According to Takapaha et al. (2022), a normal liver has a brownish red color, while an abnormal liver changes color. Apart from that, a normal liver has a surface that has a smooth texture and is somewhat hard when pressed (Dorland, 2002), whereas a normal kidney is characterized by a smooth surface, springy consistency, has a bean shape, and is brownish red in color because it receives 22% of the blood volume pumped by the heart (Guyton & Hall 2007).

Histological features of liver

The scoring for liver damage in mice can be seen in Table 4. Observations of the liver after administration of ethanol extract and fractions resulted in fatty degeneration and inflammation in hepatocyte cells. Normal liver cells are characterized by polyhedral cells arranged radially around the central vein (Surasa et al., 2014). Fatty degeneration in hepatocytes is characterized by small vacuoles in the cytoplasm, which can then develop into larger vacuoles, resulting in the nucleus being compressed to the periphery (Andreas et al., 2015). Further damage, inflammation, is characterized by cells that are very purple in color, have an indeterminate size, and lack of distance between the cytoplasm and the nucleus (Nazarudin et al., 2017).

Liver damage can be seen in Figure 6-10. The negative control group showed no damage in the form of inflammation or fatty degeneration. The choice of Na CMC was based on its non-toxic or inert nature and its inability to react with the extract. Administration of 96% ethanol extract caused moderate liver damage in the form of inflammation and fatty degeneration. Inflammation occurred at all doses, while fatty degeneration only occurred at dose 2 (150 mg/KgBW).

Administration of the methanol-water fraction caused mild liver damage in the form of inflammation. Fatty degeneration did not occur with the methanol-water fraction, indicating that this fraction did not disrupt fat metabolism in the liver. Inflammation occurred only at dose 3 (300 mg/KgBW).

The ethyl acetate fraction caused mild inflammation in the livers of mice, but no fatty degeneration occurred after administration of the ethyl acetate fraction. Inflammation occurred only at dose 1 (75 mg/KgBW), while no inflammation occurred at doses 2 (150 mg/KgBW), 3 (300 mg/KgBW), and 4 (600 mg/KgBW).

Administration of the n-hexane fraction caused mild to moderate liver cell damage in the form of fatty degeneration and inflammation. Dose 1 (75 mg/KgBW) resulted in more inflammation and fatty degeneration than dose 4 (600 mg/KgBW), while dose 2 (150 mg/KgBW) only caused fatty degeneration and in greater amounts than dose 4 (600 mg/KgBW). Dose 3 (300 mg/KgBW) only resulted in inflammation, while dose 4 (600 mg/KgBW) resulted in both fatty degeneration and inflammation. The level of liver damage that occurred was not accompanied by an increase in dosage.

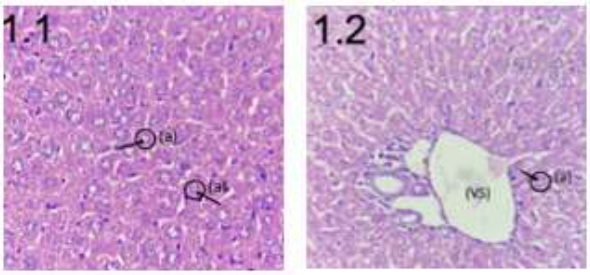

Figure 6. Liver histpathology after administration of Na CMC 0.3%

Note: (a) Normal hepatocytes, (vs) central vein, (1.1). Far from central vein, (1.2). Near central vein

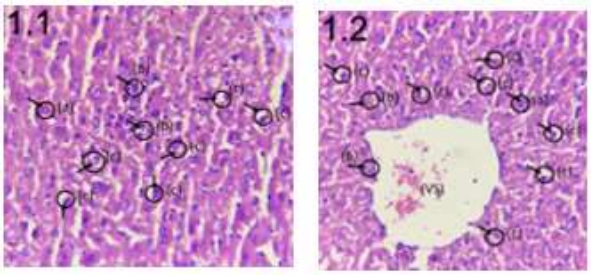

Figure 7. Liver histpathology after administration of 96% ethanol extract

Note: (a) Normal hepatocytes, (b) Inflammation cells, (c) Fatty degenerations, (vs) central vein, (1.1). Far from central vein, (1.2). Near central vein

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Table 4. Scoring of liver damage in mice after administration of Na-CMC, extract and fractions

Preparate code	Fatty degeneration score	Number of locations		Inflammation score	Number of locations	
		Near central vein	Far from central vein		Near central vein	Far from central vein
Negative control (Na CMC 0.3%)	0	0	0	0	0	0
Ethanol extract dose 75 mg/KgBW	0	0	0	2	2	0
Ethanol extract dose 150 mg/KgBW	3	Difuse	Difuse	2	2	1
Ethanol extract dose 300 mg/KgBW	0	0	0	2	4	0
Ethanol extract dose 600 mg/KgBW	0	0	0	2	2	1
Methanol-water dose 75 mg/KgBW	0	0	0	0	0	0
Methanol-water dose 150 mg/KgBW	0	0	0	0	0	0
Methanol-water dose 300 mg/KgBW	0	0	0	1	1	0
Methanol-water dose 600 mg/KgBW	0	0	0	0	0	0
n-Hexane dose 75 mg/KgBW	0	0	0	1	0	1
n-Hexane dose 150 mg/KgBW	0	0	0	0	0	0
n-Hexane dose 300 mg/KgBW	0	0	0	0	0	0
n-Hexane dose 600 mg/KgBW	0	0	0	0	0	0
Ethyl acetate dose 75 mg/KgBW	2	3	0	1	1	0
Ethyl acetate dose 150 mg/KgBW	2	4	0	0	0	0
Ethyl acetate dose 300 mg/KgBW	0	0	0	1	0	1
Ethyl acetate dose 600 mg/KgBW	1	1	0	1	1	0

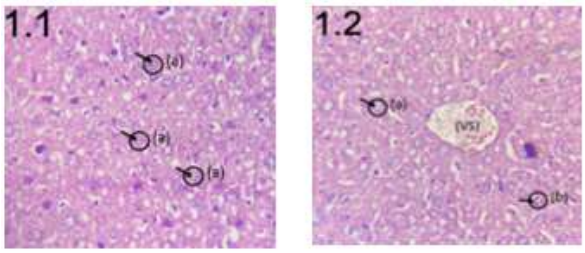

Figure 8. Liver histpathology after administration of methanol-water fraction

Note: (a) Normal hepatocytes, (b) Inflammation cells, (vs) central vein, (1.1). Far from central vein, (1.2). Near central vein

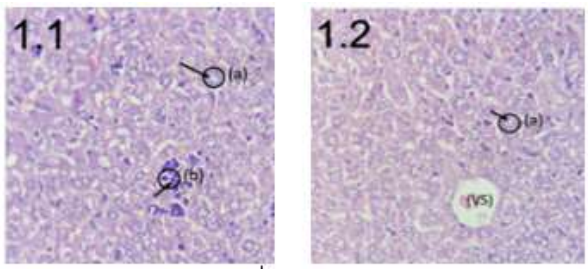

Figure 9. Liver histpathology after administration of ethyl acetate fraction

Note: (a) Normal hepatocytes, (b) Inflammation cells, (vs) central vein, (1.1). Far from central vein, (1.2). Near central vein

Histological features of kidneys

The scoring for kidney damage in mice can be seen in Table 5. In this study, the kidney damage in mice showed inflammation, characterized by very purple cells, an uncertain size, and no distance between the cytoplasm and the cell nucleus. Inflammation is a crucial defense mechanism for the body against various hazards and compounds that can disrupt its balance. Tissues experiencing resistance will show signs of inflammatory cell infiltration, such as an accumulation of white blood cells and immune cells like lymphocytes, plasma cells, and macrophages, in a histopathological examination. This infiltration is a typical sign of the inflammatory response to tissue damage or infection and can be observed and quantified under a microscope after staining tissue samples.

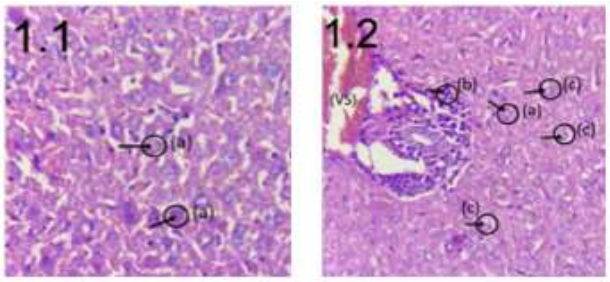

Figure 10. Liver histpathology after administration of n-hexane fraction

Note: (a) Normal hepatocytes, (b) Inflammation cells, (c) Fatty degeneration, (vs) central vein, (1.1). Far from central vein, (1.2). Near central vein

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Table 5. Scoring of kidneys damage in mice after administration of Na-CMC, extract and fractions

Preparate code	Fatty degeneration score	Number of locations		Inflammation score	Number of locations	
		Cortex	Medula		Cortex	Medula
Negative control (Na CMC 0.3%)	0	0	0	0	0	0
Ethanol extract dose 75 mg/KgBW	0	0	0	3	Difuse	Difuse
Ethanol extract dose 150 mg/KgBW	0	0	0	3	Difuse	Difuse
Ethanol extract dose 300 mg/KgBW	0	0	0	3	Difuse	Difuse
Ethanol extract dose 600 mg/KgBW	0	0	0	3	Difuse	Difuse
Methanol-water dose 75 mg/KgBW	0	0	0	2	3	0
Methanol-water dose 150 mg/KgBW	0	0	0	0	0	0
Methanol-water dose 300 mg/KgBW	0	0	0	0	0	0
Methanol-water dose 600 mg/KgBW	0	0	0	2	3	0
n-Hexane dose 75 mg/KgBW	0	0	0	3	Difuse	Difuse
n-Hexane dose 150 mg/KgBW	0	0	0	3	Difuse	Difuse
n-Hexane dose 300 mg/KgBW	0	0	0	3	Difuse	Difuse
n-Hexane dose 600 mg/KgBW	0	0	0	3	Difuse	Difuse
Ethyl acetate dose 75 mg/KgBW	0	0	0	2	6	4
Ethyl acetate dose 150 mg/KgBW	0	0	0	2	2	2
Ethyl acetate dose 300 mg/KgBW	0	0	0	2	4	5
Ethyl acetate dose 600 mg/KgBW	0	0	0	2	12	8

Kidney damage can be seen in Figure 11-15. Administration of 96% ethanol extract at all doses caused severe inflammation in the kidneys of mice, but did not cause fatty degeneration.

Administration of the methanol-water fraction caused inflammation in the kidneys of mice. Moderate inflammation was observed in the kidneys of mice at doses 1 (75 mg/KgBW) and 4 (600 mg/KgBW), while no inflammation was observed in doses 2 (150 mg/KgBW) and 3 (300 mg/KgBW).

Administration of ethyl acetate fraction at all doses caused severe inflammation, while administration of n-hexane fraction at all doses caused moderate inflammation in the kidneys of mice.

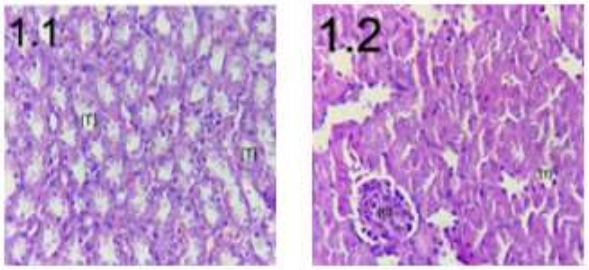

Figure 11. Kidneys histopathology after administration of Na-CMC 0.3%

Note: (T) Tubulus, (G) Glomerulus, (1.1). Medula, (1.2). Cortex

Histopathological examination was carried out to determine the presence of organ damage that was not visible macroscopically due to the administration of the test preparation. In this study, histopathological observations were carried out on the liver and kidneys of mice. This is because the liver is the place for drug metabolism, while the kidneys are a site for excretion of metabolites. The liver is the major place where a drug is metabolized when it enters the body. The results of metabolism are called metabolites, where the resulting metabolites can be toxic even though the initial compound is not toxic (Rollando, 2017). In addition, a histopathological examination of the kidneys was carried out, which functions to filter and excrete metabolic waste. A toxic compound that enters the body will undergo several processes before being excreted in the body, the process of entering the compound is the exposition, toxokinetic and toxodynamic phase (Rahayu & Solihat, 2018).

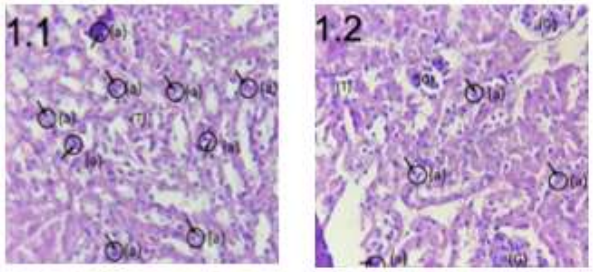

Figure 12. Kidneys histopathology after administration of 96% ethanol extract

Note: (a) Inflammation cell, (T) Tubulus, (G) Glomerulus, (1.1). Medula, (1.2). Cortex

The damage that occured to the mice's liver was mild to moderate inflammation and fatty degeneration, while the damage to the mice's kidneys was moderate to severe inflammation. Mild or focal damage (score 1) indicated minor or localized problem where only one

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inflammatory lesion or fatty degeneration was found in one location. A score of 2 indicates moderate or multifocal damage, if more than one inflammatory lesion or fatty degeneration was found. A score of 3 indicates severe or diffuse damage, if inflammation or fatty degeneration was found evenly in several locations.

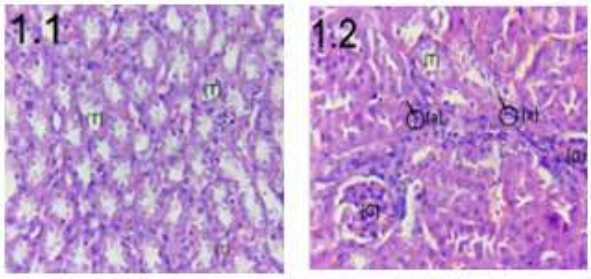

Figure 13. Kidneys histopathology after administration of methanol-water fraction

Note: (a) Inflammation cell, (T) Tubulus, (G) Glomerulus, (1.1). Medula, (1.2). Cortex

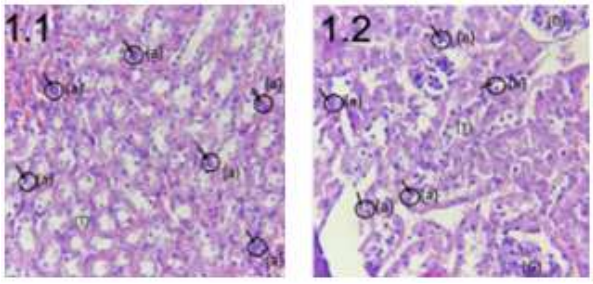

Figure 14. Kidneys histopathology after administration of ethyl acetate fraction

Note: (a) Inflammation cell, (T) Tubulus, (G) Glomerulus, (1.1). Medula, (1.2). Cortex

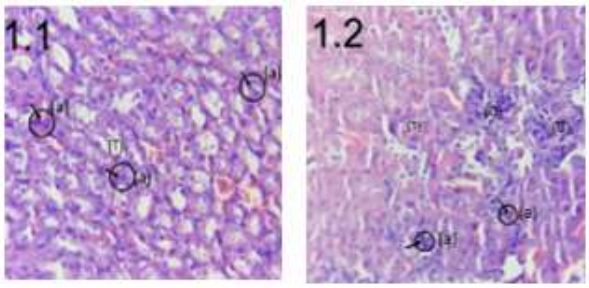

Figure 15. Kidneys histopathology after administration of n-hexane fraction

Note: (a) Inflammation cell, (T) Tubulus, (G) Glomerulus, (1.1). Medula, (1.2). Cortex

The inflammation in the kidney tent to be higher than in the liver. The kidneys are highly susceptible to toxic damage due to high blood flow, concentration of toxicants during filtration and secretion, high metabolic activity, the ability to metabolize chemicals, and prolonged residence time of substances within the tubules. Kidney's renal tubules are responsible for reabsorbing and secreting substances, which can concentrate toxins in the kidneys, unlike the liver where biotransformation can detoxify them. Toxic substances are filtered from the blood and processed in the liver, then re-enter the blood to be excreted by the kidneys (Lee et al., 2018).

Inflammation is characterized by cells having a very purple color, there is no distance between the cytoplasm and the cell nucleus, and the size of the cells is uncertain (Nazarudin et al., 2017). Fatty degeneration is characterized by cells having small vacuoles, which can then become larger so that the nucleus is compressed and pushed to the edge (Andreas et al., 2015). Scoring of damage that occurred to the liver and kidneys of these mice was not followed by an increase in dose. This could happened by various factors. Different metabolic processes, internal infections, nutritional deficiencies, cell aging, and lack of oxygen can significantly impact mice's health and physiology, contributing to varied responses in experimental studies. These factors can trigger physiological shifts like chronic inflammation, altered immune function, and oxidative stress, affecting diverse biological processes and ultimately complicating scientific interpretations of mouse models (Li et al., 2023). According to Apriliani et al. (2015), this also might be occured because each mouse has a different metabolic process and response, resulting in significant fatty degeneration even at lower doses. This results also could be due to the mice's endurance and stress factors, which vary from individual to individual. These factors can trigger increased oxidative stress, leading to the production of free radicals or reactive oxygen species exceeding the body's defenses. The resulting free radicals, which the body cannot neutralize, can cause cell damage (Ervina & Sukarjati, 2017).

The inflammation and fatty degeneration that occured could also be caused by the secondary metabolite content of the flowers. According to Naufalin et al. (2021), *Etlingera elatior* flowers contain alkaloids, flavonoids, tannins, polyphenols, terpenoids, steroids, saponins, and contain essential oils. The methanol-water fraction is polar and contains flavonoid and tannin compounds, while the ethyl acetate fraction is semi-polar and contains saponin, tannin, flavonoid and steroid compounds. The hexane fraction is non-polar and contains terpenoid compounds, namely dodecanal, 1-dodecanol, dedecanoic acid, 1-hexadecanol, 1-hexadecene, and 17-pentatriacontene which are volatile

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compounds resulting from GC-MS analysis of the n-hexane fraction of *Etlingera elatior* plants (Maimulyanti & Prihadi, 2015). The flavonoid compounds contained in *Etlingera elatior* flowers are quercetin, apigenin, kaempferol, luteolin. Rutin, quercetin, kaempferol, and kaempferol-3-O-glucoside, are polar flavonoids (Ghasemzadeh et al., 2015).

Inflammation and fatty degeneration in the liver and kidneys of mice were thought to be caused by the compounds kaempferol, quercetin and tannins in *Etlingera elatior* flowers. This was in accordance with several previous studies. In the study of Su et al. (2018), *Etlingera elatior* flowers contained kaempferol and quercetin, thus causing death in *Spodoptera litura*. Apart from that, in research by Suryanto et al. (2018), the kaempferol compound in *Etlingera elatior* leaves caused death in *Culex quinquefasciatus* mosquito larvae. According to Koraag et al. (2016), *Etlingera elatior* flowers contained tannins, which caused death to *Aedes aegypti* larvae. The mechanism of inflammation is that plasma protein fluid and leukocytes will go to the area experiencing infection, tissue that fights inflammatory cells will be characterized by infiltration of inflammatory cells (Baratawidjaja, 2002). Fatty degeneration can occur due to an increase in free fatty acids, then a decrease in triglyceride export due to a deficiency in fat-binding apoproteins, and a reduction in free fatty acid oxidation (Dancui et al., 2009).

The result of histopathology examination of liver and kidney in this study confirmed the results of toxicity result. If a high dose of a substance results in death in mice, histopathological analysis of tissues can confirm whether the death was due to specific damage (e.g., liver necrosis, renal failure). Even if no obvious symptoms of toxicity were observed, histopathology might reveal microscopic organ damage (such as cell death, inflammation, or fatty degeneration) that would otherwise go unnoticed. In this study, there were no toxic symptoms or death in mice treated with ethanol extract and fractions of *Etlingera elatior* flowers. But the administration of ethanol extract and fractions of *Etlingera elatior* flowers caused mild to moderate damage to the liver, and moderate to severe damage to kidney. This suggested the ethanol extract and its fractions caused internal organ harm despite the absence of outward signs of toxicity or mortality in the animal model. Futher research is needed to identify the toxic components, elucidate the exact mechanisms of organ damage, determine the dose-response relationship for toxicity, investigate potential interactions with other substances, and establish safe and effective dosage limits for human use of the ethanol extract and its fractions.

CONCLUSION

Administration of 96% ethanol extract, n-hexane, ethyl acetate and methanol-water (3:7) fractions of *Etlingera elatior* flowers did not cause toxic symptoms and death in mice, so they were categorized as non-toxic. Macropathological examination of the liver and kidneys showed normal results with a brownish red color, smooth surface and rubbery consistency. Effect of administration of ethanol extract and fractions of *Etlingera elatior* flowers in the liver of mice caused mild and moderate damage, while in the kidneys of mice, it caused moderate and severe damage.

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AUTHOR CONTRIBUTIONS

Conceptualization, I.S.; Methodology, I.S., N.H.; Software, S.B.N.; Validation, I.S.; Formal Analysis, S.B.N.; Investigation, I.S., N.H., S.B.N.; Resources, N.D.I.; Data Curation, I.S., S.B.N.; Writing - Original Draft, I.S., S.B.N.; Writing - Review & Editing, I.S., S.B.N.; Visualization, S.B.N.; Supervision, N.H.; Project Administration, N.H.; Funding Acquisition, N.H.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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Public Perception and Practices Towards Ethanol Content and Halal Assurances of Herbal Syrup Products

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Abstract

Background: *Herbal syrup is known to be an alternative medicine due to its safe material. However, the presence of ethanol content in herbal syrup as the extraction residue can be crucial in determining the halalness of the product.* ***Objectives:*** *The study aims to evaluate public perceptions and practices regarding the ethanol content and halal assurances of herbal syrup products in Yogyakarta City, Indonesia.* ***Methods:*** *A cross-sectional study using convenience sampling was conducted with 300 respondents for perception and 250 respondents for practices among the general public in 14 sub-districts across Yogyakarta City. A validated and self-administered questionnaire was developed and distributed both online and paper-based.* ***Results:*** *This study found that 51% of the respondents had positive perceptions and 62.80% of the respondents had positive practices regarding the ethanol content and halal assurances of herbal syrup products. Age ($p<0.001$) was found to have an association with perception, while religion ($p=0.0013$) was found to be associated with practices. The correlation between perception and practices was also found to be moderate ($p<0.001$; $r=0.340$).* ***Conclusion:*** *The majority of general public perceptions and practices in Yogyakarta City were found to be positive regarding the ethanol content and halal assurances of herbal syrup products and identified a correlation between perception and practices as well as sociodemographic characteristics with perception and practices. These findings can be used by various stakeholders, including the government and manufacturers, to improve the halal certification of herbal syrup products and raise public awareness about the ethanol content of herbal syrup products.*

Keywords: *ethanol, halal, perception, practices, syrup*

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INTRODUCTION

Herbal medicine is becoming increasingly popular around the world. The World Health Organization (WHO), reports that 80% or approximately 4 billion people rely on herbal medicine, prioritizing them in healthcare. Herbal medicines are medicinal products that are made from one or more plant parts that contain active ingredients and are then packaged and labeled as finished products (Embassy of The Republic of Indonesia in Brussels, 2021). Herbal medicines can be found as non-prescription medicines, which makes them easily accessible. People also believed that herbal medicines were safer, more easily accessible, and less expensive than modern medicines (Suryawati et al., 2023). Consumption of herbal medicines can help to alternatively treat various types of diseases, such as cough, fever, common cold, nausea, vomiting, and even lung diseases (Poursaleh et al., 2022; Sultana et al., 2016).

Among the various forms of herbal medicines, herbal syrup is a common liquid formulation that is easily absorbed orally (Goswami & Srivastava, 2016). Herbal syrups are often formulated from plant extracts, a process that commonly uses ethanol as a solvent. While ethanol is generally considered safe, the presence of its residue can pose a significant health concern. This is particularly true for vulnerable populations such as pediatrics, geriatrics, and pregnant women, as excessive consumption of ethanol can lead to toxicity (Neo et al., 2017). In addition, alcohol consumption can impair brain health and disrupt the blood-brain barrier at certain levels (Aziza et al., 2025; Haorah et al., 2005; Laksitorini et al., 2021). The Food and Drug Administration (FDA) and WHO have recommended limits for ethanol content in oral medicines, with level less than 10% for adults, less than 5% for children aged 6-12 years, and less than 0.5% for children under 6 years . Indonesian regulations from the Food and Drug Administration (BPOM) limit the ethanol content in traditional liquid medicines to no more than 1% and the presence of alcohol content must be stated on the packaging as a percentage (%). According to the research conducted by Rs et al. (2021) two samples of herbal medicine were discovered to contain 0.07 percent and 0.21 percent ethanol (Rs et al., 2021). Additionally, Neo et al. (2017) still found pediatric herbal cough syrup contained ethanol without any warning of its presence in the package (Neo et al., 2017). This showed that the regulations related to ethanol content in herbal syrups are still not adequately implemented in the market.

Furthermore, the presence of ethanol is also a crucial consideration in determining and ensuring the halal status of products. Halal is an obligation in Islam, as stated in the holy Quran and hadith, means permitted to consume. As a form of religious obedience, Muslims are required to consume only halal products and avoid haram products. Various countries with majority of Muslim population such as Malaysia and Indonesia surely taken product halalness seriously. Indonesia has the largest Muslim population in the world, with around 13% of the total number of Muslims in the world, product halalness is of utmost importance. In 2020, the Muslim population in Indonesia was around 229.12 million, accounting for 87.2% of the total population, and is expected to continue to increase (Djunaidi et al., 2021). The growing Muslim population has led to an increase in demand for halal-certified products in the country (Hamid et al., 2019). Halal assurance is provided through the inclusion of the Halal label and the granting of Halal certification. As in Indonesia, halal assurance is provided by the Indonesian Ulema Council (MUI). All products circulating in Indonesia must have certification and labeling as halal assurances, including traditional medicines, as stated in Law No. 33 of 2014 on Halal Product Assurances (Sembiring & Arpangi, 2023).

There is a common misconception that halal products only exclude non-halal ingredients like pork and alcohol. However, the concept of halal products encompasses not only the ingredients but also the preparation and production process that must meet halal requirements (Nayeem et al., 2020). Therefore, it is important to assess public perception and practices to determine the extent to which people understand and implement the concept of halal certification and labels also the possible content of alcohol in herbal syrup products. Similar studies regarding halal have been conducted in different settings around the world, for instance in Malaysia (Sadeeqa, Sarriff, Masood, & Farooqui, 2013; Sadeeqa, Sarriff, Masood, Saleem, *et al*., 2013; Sadeeqa & Sarriff, 2014; Santa *et al*., 2019; Xuan *et al*., 2022) and Palestine (Eid *et al*., 2022). The studies used different groups of respondents, including students (Sadeeqa et al., 2013; Santa et al., 2019; Xuan et al., 2022), healthcare workers (Eid et al., 2022; Sadeeqa & Sarriff, 2014), and the general public (Sadeeqa et al., 2013). Previous studies also evaluated pharmacist and community knowledge of the ethanol content in medication, but were more focused on pediatric medications and did not include herbal medicine (Wulandari et al., 2025).

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The study presented herein offers significant novel contributions to herbal medicine research, examining the public's perceptions and practices regarding the ethanol content and halal certification of herbal syrup products in Yogyakarta, Indonesia. This research is distinguished by its specific emphasis on herbal syrups, a subset of herbal medicine that previous literature has largely overlooked. In contrast to prior studies that have either broadly addressed the halal status of pharmaceutical products or explored the safety concerns associated with the ethanol content in medications, this investigation uniquely merges these two critical considerations. It thoroughly investigates the impact of ethanol content on the halal status of herbal syrups and assesses public awareness and regulatory compliance in this context. Furthermore, the choice of Yogyakarta as the geographical focus of the study—a region noted for its significant Muslim population and a rich tradition of herbal medicine use—provides insightful nuances that have the potential to extend beyond the Indonesian setting. This geographical lens provides a detailed exploration of how cultural and religious values influence consumer behaviors toward herbal syrups, offering implications relevant to similar contexts worldwide.

A notable innovation of this study lies in its comprehensive evaluation of public perceptions and practices. Through assessing the knowledge, attitudes, and perceptions (KAP) concerning the halalness and ethanol content in herbal syrups and correlating these insights with actual consumer practices, the research offers a holistic view of the societal implications of these factors. This analysis is further deepened by examining the role of sociodemographic variables on KAP, illuminating how different segments of the population understand and engage with the complexities of halal certification and ethanol content in herbal syrups. Additionally, the research provides pivotal insights that could significantly influence policy-making and regulation. Identifying lapses in regulatory compliance and gaps in public awareness concerning the ethanol content and halal certification of herbal syrups emphasizes the necessity for more rigorous policies and enhanced educational initiatives in this area. Moreover, this study broadens the scope of halal research to encompass herbal medicines, specifically herbal syrups, thus addressing a substantial gap in scholarly discussion regarding the halal status of drugs.

The studies conducted in Malaysia similarly assessed the knowledge, attitude, and perception (KAP) regarding halalness of pharmaceutical products with different population showed that the majority of the respondents have good score each domain. The studies also found the correlation between attitude and knowledge, perception and knowledge, knowledge and perception. It was found that general public knowledge regarding alcohol content present in pharmaceutical products still low, meanwhile hospital pharmacist, pharmacy student, and academicians had better knowledge and perception regarding alcohol content. Sociodemographic factors such as religion, age, gender, occupation, and level of education also had correlation with KAP (Sadeeqa et al., 2013; Sadeeqa & Sarriff, 2014; Santa et al., 2019; Xuan et al., 2022). However, research conducted in Indonesia is still rarely developed. Furthermore, studies on drug halalness have only been conducted on conventional drugs, with no studies on herbal drugs, particularly herbal syrups. Additionally, research on perceptions and practices related to ethanol content and halal assurance, such as halal certification and halal labeling of herbal syrup products, is still uncommon. Therefore, this study aims to evaluate public perception and practices regarding the ethanol content and halal assurances of herbal syrup products in Yogyakarta City, Indonesia.

MATERIALS AND METHODS

Study design and participants

A cross-sectional study was conducted, and data were collected among the general public based in Yogyakarta City. Yogyakarta City is located in Indonesia, specifically on Java Island, which has 14 sub-districts. The sampling method used was convenience sampling to obtain respondents based on the predetermined criteria. The inclusion criteria include individuals residing in Yogyakarta city, aged 18 years and older, and willing to participate and complete the questionnaire. An additional criterion for the practices data was applied exclusively to respondents with prior experience using herbal syrup. Data collection took place from November 2023 to December 2023.

Questionnaire structures

A self-administered questionnaire was developed specifically for this study by synthesizing and adapting relevant questions from existing academic literature, as well as adding a few adjustment, specific questions to address our research objectives. This approach allowed for a tailored instrument that precisely aligned with our research aims. The questionnaire then translated into Bahasa Indonesia and underwent a rigorous validation process mentioned in the Pilot Study section below to ensure all questions demonstrated validity and

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reliability. The survey was made available in both an online format and a paper-based format to maximize accessibility and ensure a robust data set from a diverse range of respondents (Sadeeqa & Sarriff, 2014; Xuan et al., 2022). The questionnaire consisted three sections. Section one involved collecting the respondent's sociodemographic data, such as gender, age, occupation, education, and religion. Respondents were also questioned about their experiences with herbal syrup consumption, with the intention of using this information for further analysis of practical data. Section two examined perceptions regarding ethanol content and halal assurances in herbal syrup products. Section three examined practices regarding the usage of herbal syrup products with ethanol content and halal assurances (Larasati, 2024).

Scoring method

There are total 14 statements to evaluate perception and practices with four-point Likert Scale: "Strongly Agree" (SA), "Agree" (A), "Disagree" (D), and "Strongly Disagree" (SD). The statements include favorable and unfavorable point. Favorable statements was scored as follow: SA = four (4) marks; A = three (3) marks; D = two (2) marks; and SD = one (1) marks. Meanwhile unfavorable statements was scored as follow: SA = one (1) marks; A = two (2) marks; D = three (3) marks; and SD = four (4) marks.

Ethical consideration

The Gadjah Mada University Ethics Commission granted permission to conduct this study by issuing an ethical clearance letter with the number KE/UGM/067/EC/2023.

Pilot study

The translated questionnaire was then tested for further validation through a pilot study conducted with 36 respondents outside the study sample before data collection. Public feedback was obtained from the pilot study to validate and improve the questionnaire. All questions demonstrated validity and reliability tests. The Pearson correlation test was used to determine the validity of the questionnaire. The reliability test scored 0.72 for perception and 0.68 for practices indicating that Cronbach's alpha exceeded 0.6 thus the questionnaires were reliable to be used.

Data analysis

The study utilized SPSS version 23 (IBM Corporation, America) for data collection. Descriptive analysis was conducted on sociodemographic characteristics. Perception and practice-related data were also presented descriptively based on the distribution of answers of each question. Perceptions and practices are categorized into positive and negative categories. Kolmogorov-Smirnov normality tests were conducted to categorize perceptions and practices. Furthermore, the relationship between sociodemographic characteristics with respondents' perceptions was assessed through crosstab analysis with the Chi-Square Test and also relationship between perception and practices was assessed through the Rank Spearman Test with p-values of less than 0.05 were regarded as statistically significant.

RESULTS AND DISCUSSION

Respondent's sociodemographic characteristics

Sociodemographic characteristics of respondents are displayed in Table 1. A total 300 respondents from 14 sub-districts in Yogyakarta city participated in the survey. Age range was between 18 – 88 years with a mean ± Standard Deviation of 43.5 ± 20.81 years.

Most of the respondents were female 220 respondents (73.3%), mostly aged 18 – 25 years with 114 respondents (38%) and the majority of them were students with 104 respondents (34.7%). The level of education can be seen in Table 1 that 153 respondents (51.3%) have graduated from senior high school, this indicates more than half of the respondents have completed their 12-year compulsory education in compliance with the regulations in Indonesia (Kusumah, 2021). Meanwhile for religion, the majority of respondents identify as Islam are 266 respondents (88.7%) which is consistent with the religion of the majority of the Indonesian population.

As for experiences of herbal syrup consumption, 250 respondents (83.3%) have previously consumed herbal syrup products, whereas the remaining 50 respondents (16.7%) have not. This also relevant with the result of the Indonesian Basic Health Research conducted in 2013 stated that 60% of people in Indonesia over the age of 15 have already used herbal medicine (Suryawati et al., 2023). This distribution is utilized for the practices analysis in this study. Since it is more relevant to focus on subjects who have consumed herbal syrup for practices analysis.

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Table 1. Sociodemographic characteristics of respondents (n = 300)

Variable	Category	n(%)
Gender	Male	80 (26.7)
	Female	220 (73.3)
Age	18 - 25 years	114 (38.0)
	26 - 35 years	17 (5.7)
	36 - 45 years	20 (6.7)
	46 - 55 years	41 (13.7)
	56 - 65 years	56 (18.7)
	> 65 years	52 (17.3)
Occupation	Students	104 (34.7)
	Self-employed	37 (12.3)
	Private sector employee	28 (9.3)
	Civil servants	7 (2.3)
	Retired	26 (8.7)
	Housewife	91 (30.3)
	Other	7 (2.3)
Level of education	No formal education	8 (2.7)
	Elementary school	16 (5.3)
	Junior high school	31 (10.3)
	Senior high school	153 (51.3)
	Diploma/Bachelor degree	79 (26.3)
	Master degree	9 (3.0)
	Doctoral Degree	3 (1.0)
Religion	Islam	266 (88.7)
	Christian	16 (5.3)
	Catholic	17 (5.7)
	Buddhist	1 (0.3)
Experiences of herbal syrup consumption	Yes	250 (83.3)
	No	50 (16.7)

Table 2. Perception towards ethanol content and halal assurances of herbal syrup products (n = 300)

No	Statement	SD* n (%)	D* n (%)	A* n (%)	SA* n (%)
1.	The public has the right to know the halal status of a herbal syrup product	12 (4.0)	4 (1.3)	121 (40.3)	163 (54.4)
2.	Not all people need detailed information about the halal status of a herbal syrup product	63 (21.0)	130 (43.3)	90 (30.0)	17 (5.7)
3.	Herbal syrup products that are certified and labeled halal have relatively expensive prices	32 (10.7)	177 (59.0)	70 (23.3)	21 (7.0)
4.	Guaranteeing the halalness of herbal syrup products should be determined by religious institutions	8 (2.7)	29 (9.7)	150 (50.0)	113 (37.7)
5.	The absence of halal certification and labels can still guarantee the safety of a herbal syrup product	68 (22.7)	145 (48.3)	71 (23.7)	16 (5.3)
6.	The inclusion of a halal label without being proven by halal certification is sufficient to guarantee the halalness of a product	81 (27.0)	150 (50.0)	57 (19.0)	12 (4.0)
7.	Herbal syrup products that contain alcohol in accordance with Indonesian Ulema Council (MUI) regulations can be categorized as halal	26 (8.7)	73 (24.3)	148 (49.3)	53 (17.7)
8.	Only people who want to know about halal should be educated	74 (24.7)	130 (43.3)	75 (25.0)	21 (7.0)
9.	Considering the halalness of herbal syrup products is an important step in maintaining consumer confidence in the products they consume	13 (4.3)	15 (5.0)	143 (47.7)	129 (43.0)

* SD is strongly disagree, D is disagree, A is agree, SA is strongly agree

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Table 3. Practices towards ethanol content and halal assurances of herbal syrup products (n = 250)

No	Statement	SD* n (%)	D* n (%)	A* n (%)	SA* n (%)
1.	I always choose to use halal herbal syrup products	2 (0.8)	17 (6.8)	125 (50.0)	106 (42.4)
2.	I always use herbal syrup products that already have the halal logo	4 (1.6)	21 (8.4)	131 (52.4)	94 (37.6)
3.	I always use halal herbal syrup products without considering the price	11 (4.4)	82 (32.8)	106 (42.4)	51 (20.4)
4.	I just need to look at the halal logo on the packaging to ensure there is halal certification for the herbal syrup product	5 (2.0)	44 (17.6)	142 (56.8)	59 (23.6)
5.	I did not notice the presence of alcohol when using herbal syrup products	34 (13.6)	116 (46.4)	75 (30.0)	25 (10.0)

* SD is strongly disagree, D is disagree, A is agree, SA is strongly agree

Perception towards ethanol content and halal Assurances of herbal syrup products

The frequency of respondents' perception towards ethanol content and halal assurances of herbal syrup products is shown in Table 2. There are 9 statements with minimum and maximum potential score is 9 and 36.

Regarding the right to know about halal status, 284 respondents (94.7%) of the respondents Strongly Agreed and Agreed that the public has the right to know halal status. More than 60% of respondents believed that the public needs detailed information about the halal status of herbal syrup products, and that herbal syrup products that have already obtained halal assurances such as certification and halal label are not relatively expensive, despite the fact that the process of obtaining the halal assurance can costs money (Santoso et al., 2021). Regarding the perception of halal assurances determination, total of 87.7% respondents both Strongly Agreed and Agreed that halal assurances should be determined by religious institutions.

Respondents also emphasized the importance of halal certification and labeling to ensure the safety of herbal syrup products for public consumption and that halal certification is required in order to obtain a halal label. In terms of alcohol content in herbal syrup products, more than half of the respondents believed that herbal syrup products containing alcohol in the formula could be considered halal if they met the criteria established by The Indonesian Ulema Council or *Majelis Ulama Indonesia* (MUI). Most of them also believed that the public should be educated about halal, not just those who wanted to be educated. Lastly, 90.7% of the respondents consider the halalness of herbal syrup products an important step in maintaining consumer confidence in the products they consume.

Practices towards ethanol content and halal assurances of herbal syrup products

Table 3 shows the respondents' practices towards ethanol content and halal assurances of herbal syrup products. There are 5 statements with minimum and maximum potential score is 5 and 20.

It can be seen that 231 respondents (92.4%) of the respondents have always chosen to use herbal syrup products that have already stated halal and 225 respondents (90%) of the respondents have always used products that have halal logo. In terms of the price of herbal syrup products, more than 60% of respondents did not mind or did not consider the price of herbal syrup products labeled halal. Meanwhile, only 49 respondents (19.6%) out of 250 respondents are aware that the halal logo cannot ensure halal certification presence directly, implying that they didn't double-check for halal label and the presence of halal certification. Regarding the presence of alcohol content, 60% of the respondents noticed the presence of alcohol or the alcohol labeling when using herbal syrup products, but many people still do not pay attention to the presence of alcohol or alcohol labeling on herbal syrup products.

Categorization of general public perceptions and practices

Based on normality test with Kolmogorov-Smirnov, the results revealed that the data exhibited a normal distribution in perception statements ($p=0.200$), whereas it did not show normal distribution in practices statements ($p<0.001$). Therefore, for perception categorization the mean value is used, while for practices categorization the median value is used. The mean value of the perception score is 27, and if the score obtained is greater than or equal to 27, it is in the positive perception category; otherwise, it is in the negative perception category. The results show that 153

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respondents scored greater than or equal to 27, while 147 scored less than 27.

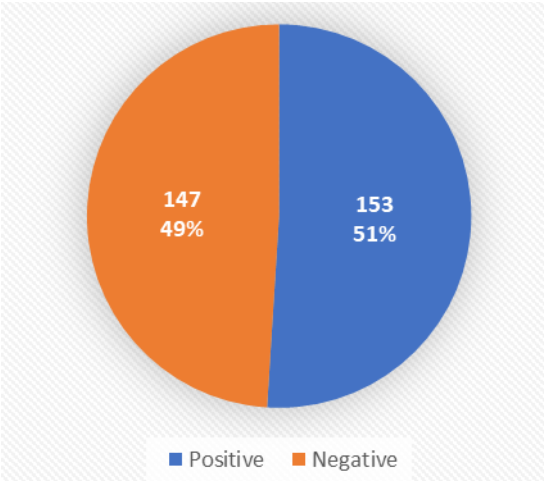

Figure 1. Distribution of general publics' perception (n = 300)

Figure 1 summarizes the category of respondents based on their perception. Overall, the perception of the general public in Yogyakarta City, Indonesia, demonstrated a positive stance (51%) regarding their beliefs in ethanol content and halal assurances for herbal syrup products. Meanwhile, practices categorization based on median value. The median value of the practices score is 14, and if the score obtained is greater than or equal to 14, it is in the positive practices category; otherwise, it is in the negative perception category. The results show that 157 respondents scored greater than or equal to 14, while 93 scored less than 14.

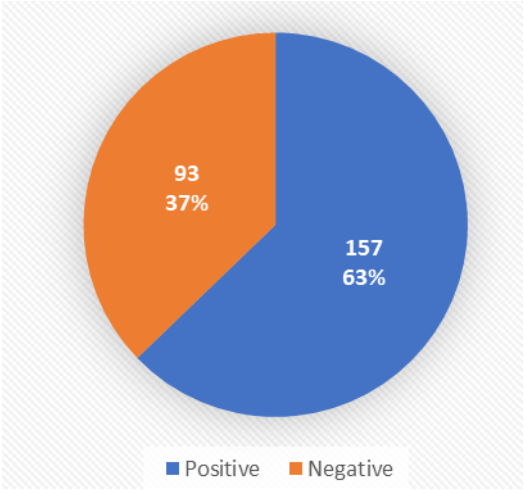

Figure 2. Distribution of general publics' practices (n = 250)

Similar to perception but slightly higher, Figure 2. summarizes the practices of the general public in Yogyakarta City, Indonesia showcased positive actions (62.80%) aligning with their beliefs in ethanol content and halal assurances for herbal syrup products.

Association between sociodemographic characteristics of respondents versus perception and practices

Table 4 shows the correlation between perception and sociodemographic characteristics including gender, age, occupation, education, and religion. The association was analyzed using Chi-Square test.

Table 4. Association between perception and sociodemographic characteristics

Variable	Perception Category		Total	p-*value*
	Positive	Negative		
Gender				
Male	41 (51.2%)	39 (48.8%)	80	0.958
Female	112 (50.9%)	108 (49.1%)	220	
Age				
Adults (≤45 years)	104 (68.9%)	47 (31.1%)	151	<0.001
Elderly (>45 years)	49 (32.9%)	100 (67.1%)	149	
Occupation				
Unemployed	115 (52.0%)	106 (30.8%)	104	0.548
Employed	38 (40.3%)	41 (38.7%)	79	
Education				
Non-College education	104(49.8%)	105 (50.2%)	209	0.515
College education	49 (53.8%)	42 (46.2%)	91	
Religion				
Muslim	136 (51.1%)	130 (48.9%)	266	0.901
Non-Muslim	17 (50%)	17 (50%)	34	

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Table 5. Association between practices and sociodemographic characteristics

Variable	Practices Category		Total	p-*value*
	Positive	Negative		
Gender				
Male	53 (71.6%)	21 (28.4%)	74	0.061
Female	104 (59.1%)	72 (40.9%)	176	
Age				
Adults (≤45 years)	95 (65.5%)	50 (34.5%)	145	0.296
Elderly (>45 years)	62 (59%)	43 (41%)	105	
Occupation				
Unemployed	166 (61.7%)	72 (38.3%)	188	0.532
Employed	41 (66.1%)	21 (33.9%)	62	
Education				
Non-College education	102 (60.4%)	67 (39.6%)	169	0.248
College education	55 (67.9%)	26 (32.1%)	81	
Religion				
Muslim	147 (65.3%)	78 (34.7%)	225	0.013
Non-Muslim	10 (40%)	15 (60%)	25	

If the p-value is less than 0.05, the result is statistically significant, indicating that there is an association between variables. Sociodemographic characteristics of the general public in Yogyakarta City, Indonesia which had a significant association with practices were religion (p=0.013), whereas gender (p=0.061), age (p=0.296), occupation (p=0.532), and level of education (p=0.248) showed p-value greater than 0.05 indicating that there are no association between the variables and practices.

Correlation between perception and practices

Assessing the correlation between perception and practice was using Rank Spearman test. If the significance value is less than 0.05, it indicates that there is a significant relationship between the variables. The result of the correlation test between perception and practices showed a significance value (2-tailed) of <0.001 which indicates that there is a significant relationship between the two variables. The correlation coefficient obtained indicates that the level of strength of the relationship or correlation between the perception and practice variables is 0.340, which means that the relationship or correlation between the perception and practice variables is moderate (0.3 – 0.7).

DISCUSSION

The methodological choices in this study were specifically designed to provide a comprehensive evaluation of public perceptions and practices. The use of a convenience sampling method enabled to gather a diverse sample of respondents that was essential for our comparative analysis. This approach was crucial for identifying the significant association between sociodemographic variables and consumer perceptions, a key finding that would be difficult to establish with a homogenous sample. Furthermore, the selection of the Chi-Square and Rank Spearman tests was deliberate, as these statistical methods are appropriate for analyzing the categorical and ordinal data collected via our questionnaire. This allowed us to rigorously assess relationships between consumer demographics, perceptions, and actual practices. Ultimately, our chosen methodology provided the necessary framework to address a notable gap in the literature by measuring the public's understanding of both ethanol content and halal assurances in herbal syrups.

This study examined the perception and practices of the general public in Yogyakarta City, Indonesia towards ethanol content and halal assurances of herbal syrup products. The main findings are: (1) The positive perception of general public in Yogyakarta City is quite high towards ethanol content and halal assurances of herbal syrup products (2) The positive practices of general public in Yogyakarta City is high towards the presence of ethanol content and halal assurances of herbal syrup products (3) The majority of the residents in Yogyakarta City still lack understanding of how to verify halal certification (4) Some residents in Yogyakarta City are still unaware of the alcohol content in herbal syrup products (5) Sociodemographic characteristics have a significant association with public perception and practices (6) There are positive correlation between public perception and practices. There is currently no research in Indonesia that specifically discusses the issue of halal assurances especially in herbal syrup regarding perception and practices of the general public.

As in Indonesia, all products in circulation must be halal certified required by Law No. 31 of 2014, which is part of the government's effort to ensure halal assurance

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and is mandatory. Regarding the situation, not only the manufacturer should be aware of and follow the regulations, but so should the general public. The general public's perception and practices play a critical role in how they perceive, accept, and implement halal assurances in their daily lives, particularly in relation to residual ethanol content in products such as herbal syrups, which are subject to limitations set by national and international organizations (Neo et al., 2017). A positive perception of halal assurance encourages the community to be more mindful and selective in their product selection. How the public perceives manufacturers' compliance with halal standards influences consumer decisions, creating pressure on the industry to adhere the existing regulations.

This study identified that positive perceptions regarding ethanol content and halal assurances of herbal syrup products are quite high. More than 90% of the respondents perceived that everyone has the right to know halal status and also the presence of halal assurance can give consumers confidence in the products they consume, this topic correlates with study by Sadeeqa et al. (2013) in which the respondents agreed that patient has a right to ask information about sources of ingredients in the medicine they are about to consume. Furthermore, the majority of respondents had a positive perception of halal assurances such as halal certification and halal labeling. They understood that in order to be able to include the halal logo on the packaging of the product, the manufacturer was required to show halal certification. Most of them believed that halal assurances should be determined by religious institutions. In Indonesia, religious institutions managing halal assurances are MUI along with BPJPH and LPH (Wajdi, 2021). More than 60% of the respondents also believed that herbal syrup products containing alcohol could be considered halal if they complied with MUI regulations. According to MUI Fatwa, regarding alcohol or ethanol content in medicines, the use of alcohol in medicine can be considered permissible (halal) if it is not harmful and given in proper doses (Indonesian Ulema Council, 2018).

Consistent with perception, the practices of respondent also showed to be positive regarding the presence of ethanol content and halal assurances in herbal syrup products. A majority of participants expressed a preference for herbal syrups labeled as halal and actively sought products with the halal logo on the packaging. The halal label will help especially Muslim consumers feel more confident in consuming products that meet their need (Maulana et al., 2022). Over 60% of respondents indicated that the price of herbal syrup was not a significant consideration as long as it carried a halal label. However, there was still a misunderstanding among 80.4% respondents regarding the process of checking for halal certification, as it may not be directly indicated on the packaging, such as the halal logo. It is recommended that individuals utilize the MUI website to verify the validity of halal certification. In terms of alcohol or ethanol content, exactly 60% of respondents said they would check for alcohol or ethanol content in herbal syrup products. Nonetheless, some participants did not test for alcohol or ethanol content, possibly due to a lack of awareness about the presence of alcohol in herbal preparations, which are frequently perceived as natural and safe for consumption. The study from Sadeeqa et al. (2013) also said that general public tend to have lack of awareness regarding alcohol content that present in pharmaceutical preparation.

Additionally, the association between sociodemographic characteristics and the perception of the respondents showed that age is significantly associated. Age is one of the factors that can influence perception of individual. In the settings, the age range is quite large, this is due to no presence of upper age restriction applied. As for the association between sociodemographic characteristics and the practices of the respondents showed that religion is significantly associated. Furthermore, Utami et al. (2022) discovered a link between religiosity and perceptions in order to encourage intentions for behavior change in the use of halal medicines in Indonesia. Meanwhile, Annisa et al. (2022) discovered that non-Muslims believe halal products are safe and quality guaranteed, but some non-Muslims do not consider halal certification and labeling to be important because it is not part of their religious teachings. Correlations between perception and practices were also examined for 250 participants in the study who filled out both the perception and practices sections. It was discovered that there is a significant positive relationship between variables, with a moderate level of relationship strength or correlation between perception and practice variables. This means that the better the perception, the better the community practice. The concept that actions/practices tend to be reflected in how people understand and assess things (perceptions) is relevant in relation to the issue of ethanol content and halal assurance.

Therefore, the perception and practices of general public in Yogyakarta City showed to be positive, with 51% of the respondents have positive perception, and

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slightly higher with 62.80% of the respondents have positive practice. However, the percentage reaches nearly half of the respondents, particularly in the perception section. This is likely due to the statements presented to the public, which are considered to require thoughtful consideration before responding. Additionally, the unfavorable statements in the perception section outnumber the favorable ones. Respondents may require additional comprehension to understand the intent behind these statements, contributing to the prolonged decision-making process.

In summary, this research emphasizes the crucial connection between ensuring the halal status of herbal syrup products and the presence of ethanol content. While the significance of halal considerations and the specific attention given to ethanol align with Islamic teachings, the broader concept of halalness serves as an important framework for ensuring consumer safety, particularly in the realm of medicinal products. The findings of this study highlight the general public preference for products with strong halal assurances, including certifications and clearly visible halal labels on packaging. Consequently, there is a compelling need for manufacturer to enhance the optimization of halal product guarantees in circulation throughout Indonesia, catering to the evident public preference for products with clear halal certification and labeling.

CONCLUSION

This study findings show that the perception and practices regarding the presence of ethanol content and halal assurances such as halal certification and halal label among general public in Yogyakarta City, Indonesia were found to be positive. This study also discovered the association between age with perception ($p<0.001$) and also religion with practices ($p=0.013$). Correlation between perception and practices was also found to be positive with moderate strength ($p<0.001$; $r = 0.340$). The study's findings are expected to provide information and strengthen regulations concerning alcohol residue content in herbal syrups. Furthermore, it aims to encourage herbal syrup manufacturers to ensure halal assurances through the process of obtaining halal certification, as well as prominently displaying the halal logo on their products. Additionally, active participation from the community, halal assurance organizations, and the government is crucial in educating the public about the presence of alcohol or ethanol content, as well as halal assurance through certification and halal labeling.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.D.L, D.E.; Methodology, D.E., M.D.L., S.W.L.; Validation, S.W.L., N.V.U.; Formal Analysis, S.W.L., M.D.L., D.E.; Investigation, S.W.L., N.V.U.; Resources, M.D.L.; Data Curation, S.W.L., M.D.L., N.V.U.; Writing - Original Draft, S.W.L., M.D.L.; Writing - Review & Editing, D.E., M.D.L.; Visualization, S.W.L.; Supervision, D.E., M.D.L.; Project Administration, M.D.L.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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