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EDITORIAL WORDS

apt. Mohammad Rizki Fadhil Pratama, S.Farm., M.Si.

Editor in Chief
Borneo J Pharm

Assalamu'alaikum Wr. Wb.

Alhamdulillahirabbil 'alamin. The next edition of **Borneo Journal of Pharmacy** (*Borneo J Pharm*), has been published at February 2024. This edition contains ten articles: Pharmacognosy-Phytochemistry, Analytical Pharmacy-Medicinal Chemistry, and Clinical-Community Pharmacy. This edition includes writings from four countries: India, Indonesia, South Africa, and Thailand. The authors come from several institutions, including Universitas Muhammadiyah Kalimantan Timur, Universitas Gadjah Mada, Khon Kaen University, Universitas Muhammadiyah Surakarta, University of the Witwatersrand, Universitas Sanata Dharma, Universitas Halu Oleo, Universitas Bhakti Kencana, Universitas Negeri Gorontalo, Sekolah Tinggi Farmasi Mahaganesha, Universitas Tanjungpura, National Research and Innovation Agency of the Republic of Indonesia, Universitas Surabaya, Universitas Airlangga, Universitas Indonesia, M. S. Ramaiah University of Applied Sciences, and Universitas Muhammadiyah Yogyakarta.

Editorial boards are fully aware that there are still room for improvement in this edition, hence with all humility willing to accept constructive suggestions and feedback for improvements to the publication for the next editions. The editorial board would like to thank all editors and reviewers, and contributors of the scientific articles who have provided the repetoire in this issue. We hope that all parties, especially the contributors, could re-participate for the publication in the next edition on May 2024.

Wassalamu'alaikum Wr. Wb.

Palangka Raya, February 2024

Editor-in-Chief

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1. Writing is the result of research or review of critical analysis study in the field but not limited to Pharmacology-Toxicology, Pharmacognosy-Phytochemistry, Pharmaceutical, Analytical Pharmacy-Medicinal Chemistry, Natural Product Development, Clinical-Community Pharmacy, Management Pharmacy, and other Pharmacy aspects which have never been published in other scientific publication media.
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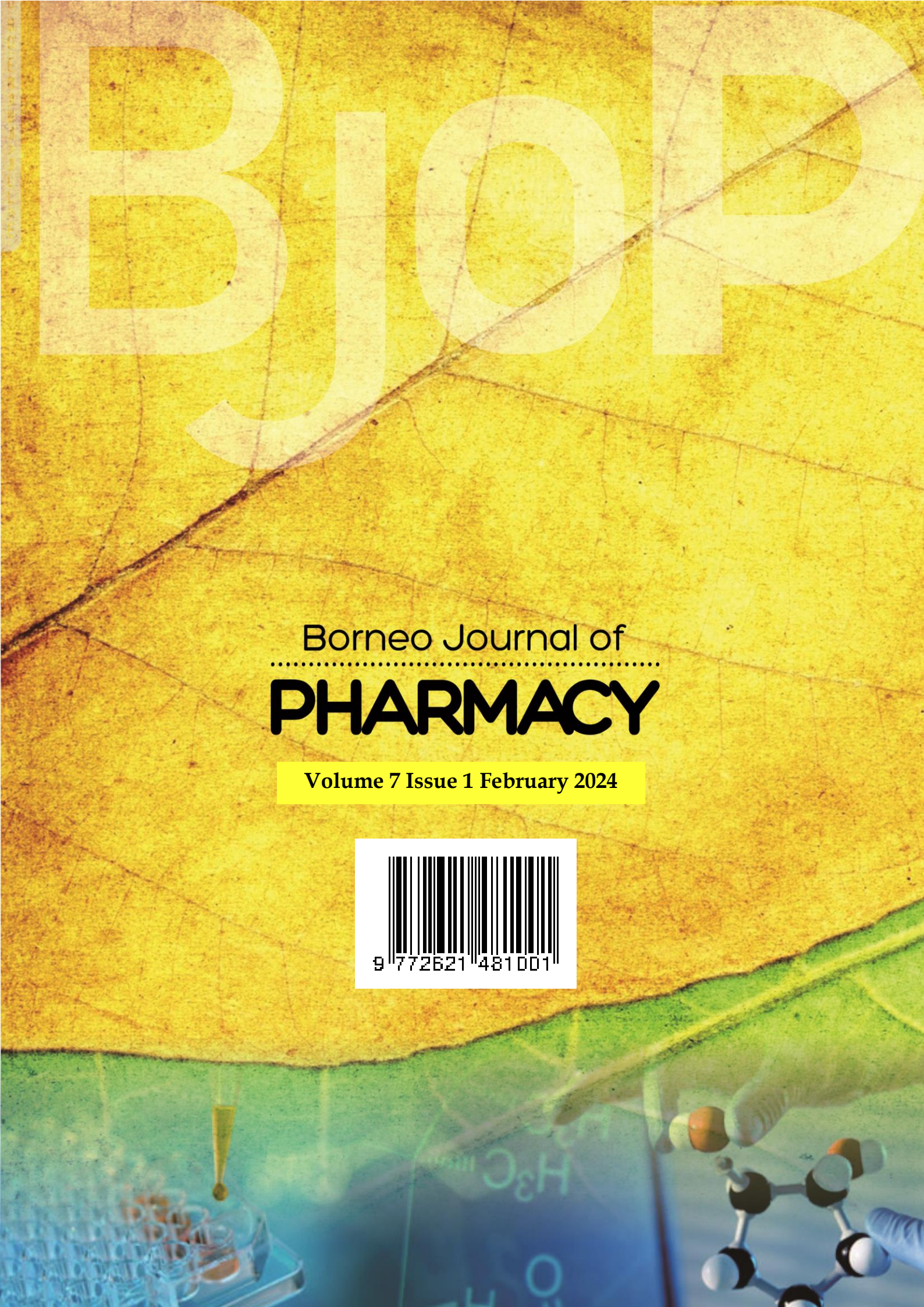
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Research Article

Nanoemulsion Mouthwash Formulation of Bajakah Tampala (*Spatholobus littoralis* Hassk.) Skin Extract Against *Candida albicans*

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Abstract

Candida albicans can cause two infections in humans: superficial and systemic. The ability of *C. albicans* to infect the host is influenced by virulence factors and character changes so that it can fool the immune system. From the character change factor, *C. albicans* can form a biofilm. This study aims to determine the good concentration in inhibiting and determine the antifungal and antibiofilm activity of nanoemulsion mouthwash formulation of bajakah tampala (*Spatholobus littoralis* Hassk) skin extract against *C. albicans*. This research was conducted with an experimental method. The formulation used a spontaneous magnetic stirrer technique to make nanoemulsion preparations. Antifungal and antibiofilm tests were carried out by dilution method using a 96-well plate and a microplate reader with a wavelength of 620 nm to determine the percentage inhibition against *C. albicans* and determine MIC_{50} and $MBIC_{50}$. The results showed that the nanoemulsion mouthwash formulation of *S. littoralis* inhibited the planktonic and biofilm of *C. albicans*. The concentration of 1% is expressed as MIC_{50} and $MBIC_{50}$. Therefore, the nanoemulsion formulation of *S. littoralis* extract could inhibit the growth of *C. albicans* in the oral cavity.

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INTRODUCTION

Based on the results of the Basic Health Research in 2018, the percentage of Indonesian people who have problems with their teeth and mouth increased from 25.9% in 2013 to 45.3% in 2018, meaning that in 5 years, the percentage of dental and oral problems is still experiencing problems enhancement, one of which is caused by *Candida albicans* infection[1]. *Candida albicans* is one of the microorganisms that can form biofilms to protect themselves from external attacks the formation of this biofilm is influenced by saliva and food eaten daily. *Candida albicans* form mycelia in a transformed environment in stem cells, then adapt to the ecological microenvironment. The three forms differ in cell morphology, function, and growth

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conditions[2]. The potential of *C. albicans* to alter yeast morphology and filamentous form is a condition that influences its pathogenic potential on the mucosal surface of the host[3]. The ability of *C. albicans* to infect various hosts is influenced by virulence factors and character changes so that it can escape the immune system.

Candida albicans can cause two main infections in humans: superficial infections such as oral and vaginal candidiasis and systemic infections that endanger life[4]. Some characteristics of *C. albicans* are changes in the shape between yeast and hyphae, expression of adhesins and invasions on the cell surface, thigmotropism, formation of biofilms, and secretion of hydrolytic enzymes, which are infectious factors[3]. The infection factor of *C. albicans* is caused by hydrolytic enzymes: phospholipase and proteinase[5]. Of these two factors, there are other enzymes: extracellular hydrolytic enzymes SAPs (Secreted aspartyl proteinases) are one of the leading infectious factors that contribute to the proliferation of *C. albicans* because these enzymes provide an entry point for adhesion, penetration, and invasion of tissues[3]. Yeast cells, hyphae, and pseudohyphae can be involved in the formation of biofilms that are usually found in the oral cavity, skin, and vagina[2]. *Candida albicans* consumes glucose as a carbon source and amino acids as a nitrogen source[6].

Biofilm is a complex network of several types of microorganisms surrounded by an extracellular polymeric matrix consisting of nucleic acids, proteins, and carbohydrates. This structured arrangement of cells provides various advantages for *C. albicans*, such as protection against environmental stressors, manipulation of the immune system stem cells, and the central part can increase resistance to antimicrobial drugs[7]. Protection of *C. albicans* can be through the formation of biofilms. The ability of *C. albicans* to form biofilms can be formed through three steps: adhesion and colonization of cells on the host surface; cell growth and proliferation; and formation of a basal layer, hyphae, and pseudohyphae with the secretion of extracellular matrix[8].

Ordinary emulsion preparations have a shape that is less pleasing to the eye because they have a larger particle size. Meanwhile, nanoemulsion preparations have low turbidity, so they are excellent for mouthwash because they look like water[9]. Nanoemulsion is also a solution for making clear, stable mouthwash and making it easier for substances to be absorbed into the mouth because of their small particles. Several studies have also shown that nanoemulsions enhance antimicrobial activity[10,11]. Currently, the use of mouthwash as a form of nanoemulsion still uses a lot of chemical-based ingredients. Seeing from this, the risk of drug side effects is quite considerable. Some mouthwashes have an alcohol percentage of 25% or more; this can cause the risk of mouth, throat, and pharyngeal cancer with a percentage of 50%[12]. This can be prevented with natural ingredients. Therefore, research related to herbal medicines must continue to be carried out to obtain safe treatment and minimal side effects[12-14].

The use of mouthwash against fungi in the oral cavity still needs to be improved. Therefore, researchers are interested in using the bajakah tampala (*Spatholobus littoralis*) as an alternative to mouthwash made from natural ingredients. *Spatholobus littoralis* is a plant that grows in the Kalimantan region[12-14]. *Spatholobus littoralis* is used by the local community by drinking boiled water from the trunk of *S. littoralis*[15]. Several species of the genus Spatholobus are found in the interior of the tropical forests of Indonesia. *Spatholobus littoralis* is often found in the interior forests of Kalimantan and usually propagates on tall and large wooden trees[16]. Phytochemical screening results showed that the ethanol extract of *S. littoralis* contained saponins, tannins, and flavonoids[17]. Research by Kumar *et al.*[18] reported that the active substance above can be an antifungal against *C. albicans*. The compounds above have the potential as antifungals, especially alkaloids, saponins, and flavonoids, which are also helpful as antioxidants, antiinflammations, and antibacterials[19-21].

Researchers are actively investigating the formulation of a nanoemulsion mouthwash utilizing *S. littoralis* skin extract as a potent antifungal and antibiofilm agent against *C. albicans*. This pioneering research aims to develop a natural and effective nanoemulsion mouthwash capable of inhibiting the growth of *C. albicans* in the oral cavity. The study is breaking new ground as, until now, research has yet to be conducted on this specific formulation.

MATERIALS AND METHODS

Materials

The tools used include a pycnometer (Iwaki, Japan), Erlenmeyer (Iwaki, Japan), Laminar airflow (LAF), water bath (Faithful, Australia), rotary vacuum evaporator (Buchi, Switzerland), digital scale (Ohaus, USA), Ostwald viscometer (Iwaki, Japan), vortex mixer (DLab, China), magnetic stirrer (DLab, China), hot plate (Maspion), micropipette (DLab, China), Particle size

analyzer (Microtac, Germany), pH meter (Ionix, Thailand), autoclave (All American, USA), incubator, microplate 96 well flat bottom (Iwaki, Japan), microplate reader (HiPo, Germany) and oven (LabTech, Hungary). The ingredients include *S. littoralis* skin (**Figure 1**) collected from Loa Kulu Forest, Samarinda, East Borneo, Indonesia. Determination was carried out at the Faculty of Forestry, Universitas Mulawarman, with the identification number 04/UN17.4.08/LL/2022, identified as *Spatholobus littoralis* Hassk. Other materials include Listerine® Mouthwash, tween 80, PEG 400, virgin coconut oil (VCO; Al Afiat), peppermint oil, sorbitol, sodium benzoate, 96% ethanol, potato dextrose agar (Oxoid), potato dextrose broth (HiMedia), pure culture of *C. albicans* ATCC 10231 from the Microbiology Laboratory of the Medical Education Study Program, Universitas Islam Negeri Maulana Malik Ibrahim, sterile distilled water, crystal violet, Mayer's, Wagner's, and Dragendorff's reagents, NaOH, concentrated H_2SO_4, concentrated Mg-HCl, as well as 1% and 5% $FeCl_3$.

Figure 1. *Spatholobus littoralis* skin.

Methods

Nanoemulsion mouthwash formulation

The procedure of mouthwash nanoemulsion preparation resulted from a combination of previous research[22,23] with modifications. The oil phase was made with VCO mixed with tween 80, put into a 250 mL beaker glass, and stirred with a magnetic stirrer for 2 minutes at 800 rpm. The oil phase mixed with surfactant was then added with PEG 400 as a co-surfactant and stirred for 10 minutes at 1000 rpm. Both *S. littoralis* skin extract and sodium benzoate were dissolved in 10 mL of distilled water. The aqueous phase was made with *S. littoralis* skin extract into another 250 mL beaker glass, mixed with the remaining distilled water, and stirred for 2 minutes at 1000 rpm. The aqueous phase formed was mixed with sorbitol and sodium benzoate, then five drops of peppermint oil were added and stirred for 10 minutes at 1000 rpm. The aqueous phase was added to the oil phase drop by drop using a dropper until it ran out, then stirred for 10 minutes at 1000 rpm so that a mouthwash nanoemulsion of *S. littoralis* skin extract would be formed.

pH test

The test was carried out with a pH meter. The pH meter was calibrated using pH 4 and 7 buffer liquid. Then, the lower end of the pH meter was immersed in the test sample, remained until the pH on the instrument was constant, and recorded the results obtained[24]. The pH test is part of the mouthwash preparation's physical and chemical examination criteria and must be based on the quality requirements of the herbal mouthwash, which are 5 to 7[25,26].

Particle size distribution, polydispersity index, and zeta potential

Particle size distribution, polydispersity index, and zeta potential were measured using the particle size analyzer. The nanoemulsion formula should have an average droplet size of <100 nm[27]. The polydispersity index indicates uniform particle size in the mouthwash nanoemulsion formulation. The lower the polydispersity index, the more homogeneous the resulting nanoemulsion[28].

Candida albicans antifungal test

The antifungal test was carried out by the microdilution method. The test was carried out on microplate 96 well flat bottom with 0%, 0.5%, 1%, and 2% concentrations. Positive control was Listerine® Mouthwash, and fungal suspension was used for negative control. Subsequently, incubation was carried out at 37°C for 72 hours. Then, the absorbance value test was carried out with a microplate reader at a wavelength of 595 nm. The data obtained was in optical density (OD) values; the data was then calculated as % inhibition in the **Equation 1**. The sample concentration inhibiting at least 50% biofilm formation is expressed as MIC_{50} (minimal inhibition concentration)[29].

$$\%\ inhibition = \frac{\text{Average OD of negative control} - \text{Average OD of test samples}}{\text{Average OD of negative control}} \text{x} 100\% \quad [1]$$

Candida albicans antibiofilm test

In the antibiofilm test, 100 L of fungal suspension was added to each hole of the required 96 wells plate. For the attachment phase, the suspension was incubated at 36-37°C for 90 minutes. Then, the plate was washed thrice with 150 L of sterile distilled water to remove nonadherent cells. A total of 100 L samples with concentrations of 0%, 0.5%, 1%, and 2% were added to the required wells and washed. Fungal suspension was used as a negative control. The drug was given a microbial suspension added with Listerine® Mouthwash as a positive control. Furthermore, incubation is carried out at 36-37°C for 24 hours for the middle phase and 48 hours for the ripening phase. Then, the plate was washed with distilled water three times, rinsed, then dried at room temperature for 5 minutes. A total of 125 L of 1% crystal violet was inserted into each well, which was used to color the biofilm formed. Then, the plate was incubated at room temperature for 15 minutes. After that, it was washed with running water three times to remove crystal violet on the plate and then added with 200 L of 96% ethanol to each well used to dissolve the biofilm. The absorbance value was read using a microplate reader with a wavelength of 595 nm. The test was carried out with three duplications. The data obtained was in the form of OD values; the data was then calculated as % inhibition in the **Equation 2**. The sample concentration inhibiting at least 50% biofilm formation is expressed as $MBIC_{50}$ (minimal biofilm inhibition concentration)[29].

$$\%\ inhibition = \frac{\text{Average OD of negative control} - \text{Average OD of test samples}}{\text{Average OD of negative control}} \text{x} 100\% \quad [2]$$

Data analysis

Data analysis in this study used Microsoft Excel software and the Statistical Package for the Social Sciences (SPSS). The OD value was obtained in the microplate reader antifungal and antibiofilm test results, which were later calculated in Excel and SPSS. The absorbance value results were analyzed using the Shapiro-Wilk method and the Levene test on SPSS to determine the distribution of normal and homogeneous data. The results were normal and homogeneous if the significance value was >0.05. Furthermore, the one-way ANOVA test was carried out to test for differences in fungal growth after being given the test solution, indicated by a p-value of <0.05. Then, a follow-up test was carried out using post-hoc Tukey to determine the significant difference in the resistance of each test solution. The percentage of inhibition in the antifungal and antibiofilm tests was calculated using Excel. Both MIC_{50} and $MBIC_{50}$ values are determined by looking at % inhibition of >50% with the smallest concentration; the MIC_{50} and $MBIC_{50}$ values.

RESULTS AND DISCUSSION

Nanoemulsion mouthwash formulation

The formulation used in manufacturing mouthwash nanoemulsion of *S. littoralis* skin extract is a modification of the existing formulation. The formulation of the ethanol extract of *S. littoralis* skin can be seen in **Table I**. Mixing with a magnetic stirrer is a spontaneous nanoemulsion (spontaneous emulsification) method in which the energy used is low so that the particle size is less uniform. After being magnetic, it should be continued with ultrasound using a sonicator to reduce the particle size of the nanoemulsion further and make it more stable, but due to limited tools, only use a magnetic stirrer to make nanoemulsions. The mixing method using magnetic alone is not optimal in making nanoemulsions because the droplet size

and polydispersity index during storage have increased[30]. The ultrasonication method has the advantage of a simple, fast, and more efficient process in producing nanoparticles than using conventional methods[31].

Table I. Nanoemulsion mouthwash formulation of *S. littoralis* skin extract.

Ingredients	Formulations (in %)			
	F1	F2	F3	F4
Spatholobus littoralis skin extract	0	0.5	1	2
VCO	2	2	2	2
Tween 80	20	20	20	20
PEG 400	10	10	10	10
Sorbitol	10	10	10	10
Natrium benzoat	0.02	0.02	0.02	0.02
Peppermint oil (drops)	5	5	5	5
Distlled water	ad 100	ad 100	ad 100	ad 100

pH test

The pH testing has been performed using a pH meter. The results of the pH test of the mouthwash nanoemulsion can be seen in **Table II**, which was observed at weeks 0, 1, 2, 3, and 4. We are examining the pH of the mouthwash nanoemulsion preparation and obtained data on F1 to F4 in 4 weeks. From weeks 1 to 4, the pH range in F1 is 6.3 – 5.9; F2 is 5.9 – 5.7; F3 is 6.0 – 5.5 F3; and F4 is 5.9 – 5.7. Changes in the pH value that occur during storage indicate a reaction in the components that make up the preparation so that it can increase or decrease pH[32]. According to Numberi *et al*[33], changes in pH during storage indicate a less stable preparation. Changes in pH value are influenced by sorbitol, with an acidity level of 4.5[34]. Then changes in pH can also be caused by temperature, poor storage, and it could also be due to the preparation process[35].

The formulations given the extract with a predetermined concentration experienced a decrease in pH at the beginning of the test, and after being tested for four weeks, the four formulas experienced changes in pH, but the pH obtained was still within the standard quality of herbal mouthwash. The quality requirement for herbal mouthwash is pH 5 to 7[36]. Based on the results in **Table II**, the mouthwash nanoemulsion formulation has met the standard requirements for herbal mouthwash.

Table II. pH of nanoemulsion of *S. littoralis* skin extract.

Formulation	Testing time (week)				
	0	1	2	3	4
F1	6.3	6.2	6.1	6.0	5.8
F2	5.9	5.9	5.8	5.8	5.7
F3	6.0	6.0	5.9	5.9	5.5
F4	5.9	5.8	5.8	5.7	5.7

Particle size distribution, polydispersity index, and zeta potential

The results of the measurement of the particle size distribution and the polydispersity index of the mouthwash nanoemulsion can be seen in **Tables III** and **IV**. The particle size distribution (droplet) test was carried out to determine whether the nanoemulsion preparation of *S. littoralis* skin mouthwash had a particle size that was by the ideal standard of nanoemulsion particle size, which was <100 nm[37]. It is necessary to know the particle size of the nanoemulsion tested to determine drug absorption and release rate—the smaller the particle size, the faster the absorption process and the resulting pharmacological effects[38]. Tests were only carried out on F2 and F4 because F1 and F3 used the same ingredients, only differing in the concentration of the extract used and expected to have particle sizes that are not much different from F2 and F4 because of the use of the same ingredients. Based on the particle size distribution carried out in three repetitions, the results from the first to the third test in a row were 26.7, 27.41, and 27.54 nm for F2 and 26.2, 25.22, and 25.67 nm for F4. The data obtained are by the theory of Kumar *et al*.[39] that the droplet size in the nanoemulsion has a size of <200 nm and is by the statement of Sonneville-Aubrun *et al*.[24] that the ideal standard of nanoemulsion particle size is <100 nm. To get the results above, a reasonably high focus is needed in the manufacture because, at the time of mixing, it should not be too slow; this will affect the nanoemulsion's homogeneity level. This is to the statement of Nirmalayanti[40] that mixing using a magnetic stirrer should not be too slow or too fast because if it is too slow, it will not form a nanoemulsion preparation, and if it is too fast, it can cause turbulence so that the particle size is not evenly dispersed and this results in the formation of a larger particle

size. Then, the ultrasonication method required fast and relatively long stirring and treatment to get the maximum nanoparticle size. This is to the research of Delmifiana and Astuti[41] that the longer the stirring, the smaller the size of the nanoparticles because the more particles that break into nano. The sonification time in the optimal range will provide a more homogeneous and stable droplet size[42].

The polydispersity index indicates uniform particle size in the mouthwash nanoemulsion formulation. The lower the polydispersity index value, the higher the uniform particle size in the mouthwash nanoemulsion formulation[28]. The polydispersity index obtained from three repetitions in a row on F2 has a polydispersity index of 0.088, 0.0561, and 0.0789, while F4 has a polydispersity index of 0.1477, 0.1127, and 0.364. This shows that the two formulas tested, F2 and F4, produced a more uniform particle size. This is by the statement of Prihantini *et al.*[43] that the polydispersity index has a value range from 0 to 1; the droplet size is declared uniform if the polydispersity index value obtained is close to a value of 0, which indicates a homogeneous dispersion. The polydispersity index can affect drug delivery and release and the stability of nanoparticles. The polydispersity index provides information about the physical stability of the dispersion system, which is more stable in the long term[44].

Table III. Particle size distribution of nanoemulsion of *S. littoralis* skin extract.

Formulation	Nanoparticle size duplication			Average
	1	2	3	
F2	26.97	27.41	27.54	27.30
F4	26.20	25.22	25.67	25.69

Table IV. Polydispersity index measurement of nanoemulsion of *S. littoralis* skin extract.

Formulation	Polydispersity index duplication			Average
	1	2	3	
F2	0.088	0.0561	0.0789	0.0743
F4	0.1477	0.1127	0.364	0.2081

The measurement of the zeta potential value of the mouthwash nanoemulsion can be seen in **Table V**. Zeta potential is a repulsive force between particles, indicated by a zeta potential value. The zeta potential value is used to determine the charge and stability of nanoparticles[45]. The zeta potential data repeated three times showed that the zeta potential value in F2 is 38.2, 28.6, and 30.7, while F4 is 16.2, 11.1, and 11.6. These results indicate that the nanoemulsion of *S. littoralis* mouthwash has good stability at F2 because it has a value of >+30 mV, while at F4, it has poor stability because the potential zeta value falls into the range of +30 to -30 mV. This is by the statement of Nugroho *et al.*[46] that the nanoemulsion preparation is declared to have a higher degree of stability if the charge is more than +/- 30 mV. that the zeta potential value >+30 or <-30 mV has a higher degree of stability. The positive-negative sign indicates that the particles in the nanoemulsion formulation have a charge. From the results of the zeta potential, which got a positive value, it means that most of the nanoemulsion formulations have a positive charge, so there is a repulsive force between the particles. The repulsion that occurs makes the nanoemulsion formulation not settle quickly[47].

Several factors, such as the type of surfactant, medium concentration, particle size, and pH, can influence zeta potential value. This is to the research of Huda and Wahyuningsih[48] that the difference in the type of surfactant and the volume of the surfactant is one of the factors causing the difference in the zeta potential value. Tween 80 was used as a surfactant in the formulation. Tween 80 is a non-ionic surfactant, so it has no charge on the hydrophobic groups[49].

Table V. Zeta Potential value of nanoemulsion of *S. littoralis* skin extract.

Formulation	Zeta potential value (mV)			Average
	1	2	3	
F2	38.2	28.6	30.7	32.5
F4	16.2	11.1	11.6	12.96

Candida albicans antifungal test

The antifungal test results of the mouthwash nanoemulsion found that all the concentrations tested and the positive control Listerine® Mouthwash showed inhibition of *C. albicans*, as shown in **Figure 2**. The wavelength used is 620 nm. This is to the

research of Maghfirah *et al*.[50], in which absorbance was measured using a microplate reader with a wavelength of 620 nm. They also used the same wavelength for antifungal and biofilm assays[50].

The value of OD indicates the high and low growth of *C. albicans* in the media. The value seen is the OD value of 620 nm; the smaller the OD value of 620 nm, the better the sample inhibits the growth of *C. albicans*. From the three replications, the average OD value of 620 nm at a concentration of 0% was 0.90156, 0.5% of 0.692767, 1% of 0.4573, 2% of 0.3785, the negative control was 1.2976, while Listerine® Mouthwash was 0.094667. Calculations using **Equation 1** obtained that the % inhibition of *C. albicans* with control at a concentration of 0% was 31.52%, 0.5% was 46.61%, 1% was 64.75%, 2% was 70.83%, while Listerine ® Mouthwash by 92.70%. At a concentration of 0%, there was an inhibition of *C. albicans* because the VCO and sodium benzoate have antifungal activity. Based on research conducted by Burhanuddin *et al*.[51], VCO has the potential to be used as an alternative treatment for *C. albicans* infection. VCO can be used orally or applied directly to the infected skin with fungi. According to Mroz *et al*.[52], benzoate is a natural element found in some plants and is used as an antibacterial and antifungal to preserve food.

The results obtained are by the statement of Hamzah *et al*.[29] that the higher the concentration the more remarkable the inhibition given. The percentages obtained at the given concentration variations were still under positive control, but at concentrations of 1% and 2%, the percentages were above 50%, indicating a relatively significant inhibition of *C. albicans*. Then, MIC_{50} of the nanoemulsion formulation of *S. littoralis* skin extract was a concentration of 1% with % inhibition of 64.75%. Determination of the MIC_{50} was carried out to determine the minimum concentration of *S. littoralis* skin extract that was able to inhibit the growth of *C. albicans*. This is to Hamzah *et al*.[29] that the sample content that can inhibit at least 50% of fungal growth is considered a MIC_{50}. If the antifungal test is known as MIC_{50}, it differs from the antibiofilm test known as $MBIC_{50}$.

Several compounds that inhibit *C. albicans* are flavonoids, saponins, tannins, terpenoids, and alkaloids. Flavonoids increase protein denaturation, disrupting the fat layer and causing damage to cell walls[53]. Saponins work by lowering the surface tension of the sterol membrane of the cell wall of *C. albicans* so that its permeability increases, which can cause intracellular fluid to come out of the cell so that later enzymes, proteins, nutrients, and metabolic substances come out of *C. albicans*, causing death in *C. albicans*[54]. Tannins work by inhibiting the synthesis of chitin, which is used in forming cell walls in *C. albicans*, and damaging cell membranes to disrupt fungal growth[55]. Terpenoids work by inhibiting the growth of fungi through the cytoplasmic membrane or by interfering with the growth and development of *C. albicans* spores[56]. And lastly, alkaloids work by damaging the cell walls of microbes[57].

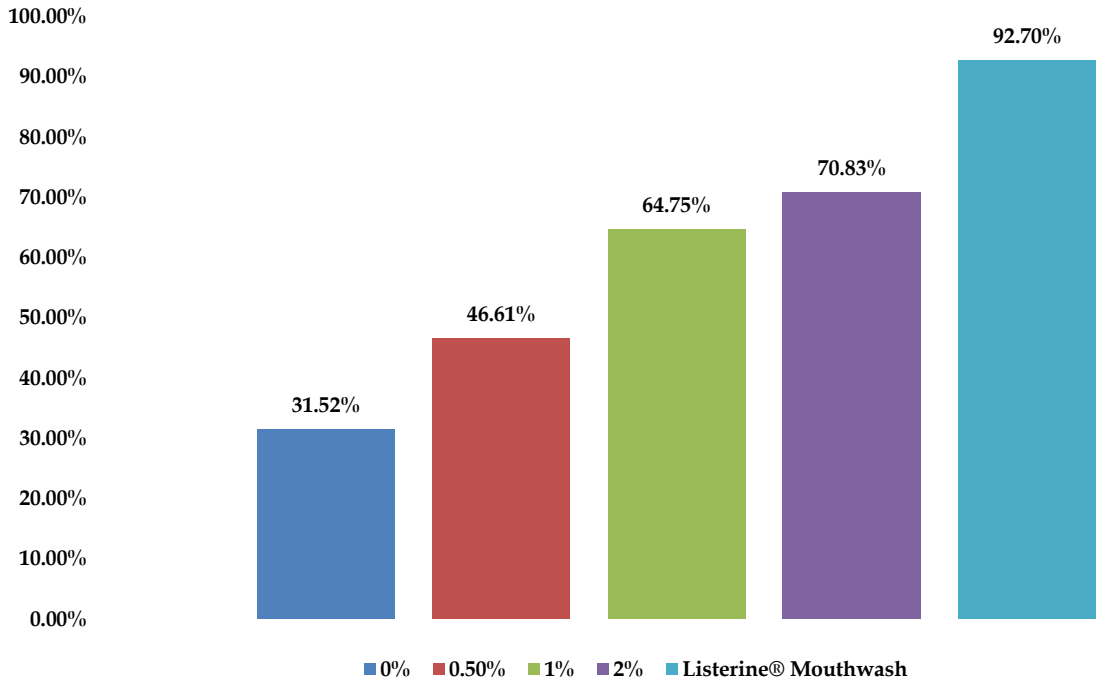

Figure 2. % inhibition of *C. albicans* from nanoemulsion of *S. littoralis* skin extract.

Candida albicans antibiofilm test

The mouthwash nanoemulsion antibiofilm test found that all the concentrations tested and Listerine® Mouthwash inhibited *C. albicans* biofilm growth, as shown in **Figure 3**. From the three replications, the average OD value of 620 nm at 0% concentration was 0.0908, 0.5% of 0.083367, 1% of 0.0605, 2% of 0.0487, the negative control was 0.1469, and Listerine® Mouthwash was 0.0322. After getting the average of OD 620 nm, the percentage of inhibition of the biofilm produced is calculated using the formula for the % inhibition (**Equation 2**). From the calculation, the % inhibition of *C. albicans* biofilm at concentrations of 0% of 38.18%, 0.5% of 43.24%, 1% of 58.81%, 2% of 66.84%, and Listerine® Mouthwash of 78.08%.

The % inhibition obtained at the given concentration variations was still under positive control, but at concentrations of 1% and 2%, the percentages were above 50%, indicating a relatively significant inhibition of the *C. albicans* biofilm. Then, $MBIC_{50}$ from the nanoemulsion formulation of *S. littoralis* skin extract was a concentration of 1% with a percentage of inhibition of 58.81%. $MBIC_{50}$ is the lowest concentration of the test sample that can inhibit biofilm growth with a percentage of >50%[58]. Hamzah *et al.*[29] state that the test sample concentration that can inhibit at least 50% of biofilm formation is $MBIC_{50}$.

Several compounds that act as antibiofilms are flavonoids, phenols, and tannins. Flavonoids, phenols and tannins have a biofilm inhibition mechanism by inhibiting intercellular adhesion (icaA and icaD). Both icas mediate the formation of polysaccharide intercellular adhesin (PIA), an essential component in forming biofilms. Intercellular adhesion, after being inhibited, will also have an inhibitory effect on the formation of PIA, and this causes the formation of biofilms to be disrupted or damaged[59,60].

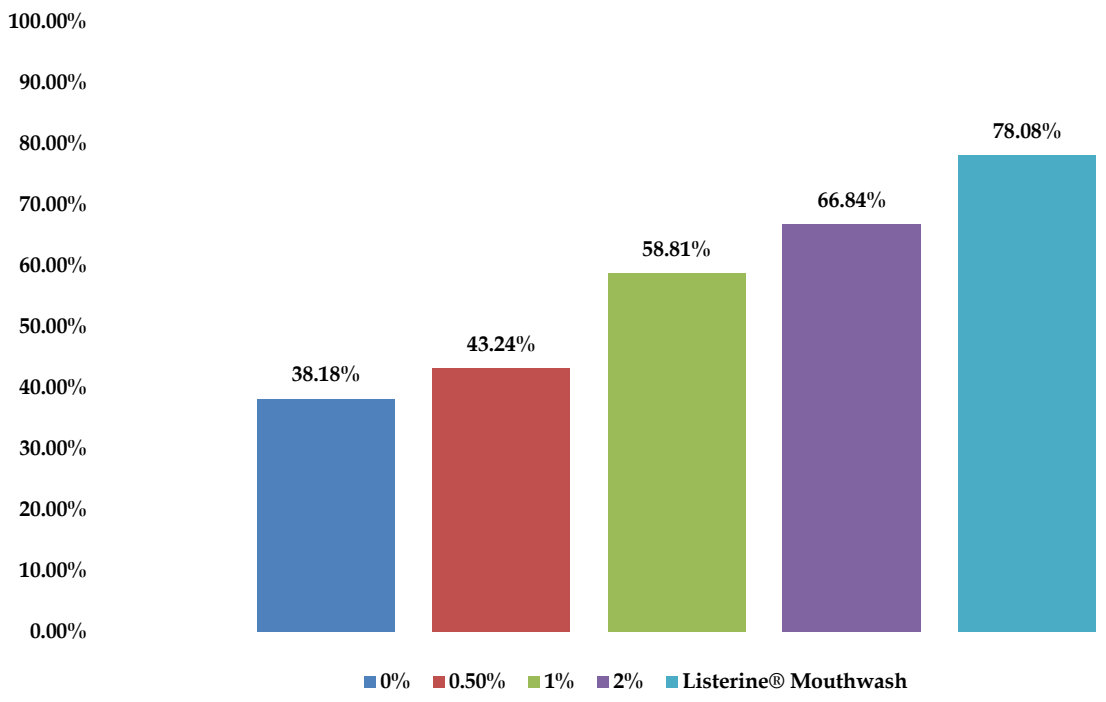

Figure 3. % inhibition of *C. albicans* biofilm from nanoemulsion of *S. littoralis* skin extract.

CONCLUSION

In summary, the nanoemulsion mouthwash of *S. littoralis* skin extract demonstrates excellent pH stability, small nanoparticle size, and homogeneity. Both formulations show antifungal and antibiofilm efficacy against *C. albicans*, with the 2% concentration inhibiting *C. albicans* by 70.83% and biofilm formation by 66.48%. The 1% concentration also inhibits fungal growth and biofilm formation (>50% inhibition). This underscores the promising antimicrobial potential of *S. littoralis* skin extract nanoemulsion mouthwash.

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DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Comparative Phytochemical Profiling and Biological Activities in the Flowers and Stalks of *Tulbaghia violacea*

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Phytochemical screening
Tulbaghia violacea

Abstract

Tulbaghia violacea is indigenous to Southern Africa and has been used extensively in traditional medicine in this region. Extensive research has been documented on the bioactive compounds found in the leaves and roots but not in the flowers and stalks. Thus, this study assessed the phytochemical profile and biological activities in the flowers and stalks of *T. violacea*. Methanolic and aqueous extracts of the air and freeze-dried *T. violacea* were screened for phytochemicals, and then antioxidant and antibacterial assays were performed. Phytochemicals such as phenols, tannins, flavonoids, coumarins, and terpenoids are present in either of the tested plant parts. The flowers contain most of the phytochemicals being tested and a higher total phenolic, tannin, and proanthocyanidin content than the stalks. The flowers exhibit the strongest scavenging activity against 2,2-diphenylpicryhydrazyl radicals and metal oxidants. The hydrogen peroxide scavenging activities show that the aqueous flower extracts have a higher radical scavenging activity than stalks. In contrast, the methanolic stalk extracts have a higher antioxidant activity than the flowers. Antibacterial activity is only exhibited in the flowers, showing resistant and intermediate inhibition zones of *Escherichia coli* and *Staphylococcus aureus* growth, respectively. This study validates the use of *T. violacea* in traditional medicine, and these results are significant for conserving the species as specific plant parts can be harvested to treat specific ailments. This study suggests the potential application of *T. violacea*, particularly the flowers and stalks, in the pharmaceutical and cosmetic sectors.

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INTRODUCTION

Medicinal plants have been widely used since prehistoric times[1]. Their usage is common in most African homes as they are easily accessible and less expensive than Western medicine[2]. Ethnomedicine studies how ethnic groups have survived and continue using traditional medicine[3]. Ethnomedicine and ethnobotany go hand in hand as it is how different ethnic groups view and approach health-related issues, especially with preventing and curing diseases by using plants that contain bioactive compounds[4,5]. As per the World Health Organization (WHO), approximately 80% of the global population depends on ethnomedicine practices as their primary source of health care. Plants that are utilized in South African traditional medicines include *Tulbaghia violacea*[6].

Commonly known as the 'wild' or 'society' garlic, *T. violacea* is a monocotyledonous plant of the Amaryllidaceae family[6,7]. It is one of the species native to Southern Africa[8]. It is a fast-growing perennial plant native to the Eastern Cape, Kwazulu-Natal, and Limpopo provinces of South Africa[9]. It thrives under the full sun and resists environmental stresses such as droughts[7]. This species is characterized by its small tubular violet or lilac flowers[10]. These flowers are usually in clusters of

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10-15, resting on a green stalk that can grow as long as 30 cm[11] (**Figure 1**). Its flowers are in full bloom during the hottest times, around January to April[7]. Its green leaves are long and leathery, producing a strong garlic-onion-like scent when bruised[9]. *Tulbaghia violacea* has triangular-shaped capsules that split open when ripe, releasing black seeds for propagation[12]. Its brightly colored, sweetly scented, and nectar-rich flowers allow the plant to be pollinated by bees and butterflies during the day and moths at night[11].

Most parts of *T. violacea* (i.e., leaves, bulbs, roots) are documented to have medicinal importance[6]. This includes the treatment of esophageal cancer, sinus headaches, stomach aches, asthma, fever, colds, high blood pressure, and tuberculosis[13]. *Tulbaghia violacea* is also one of the medicinal plants that have shown antimicrobial activities against pathogens that result in infection in individuals with HIV and AIDS[14]. This plant can have all these functions as it contains phytochemicals and secondary metabolites produced naturally by plants to resist stressors such as herbivory and pathogens[15]. Distinct parts of *T. violacea* have biologically active compounds such as flavonoids, saponins, terpenoids, tannins, phenolics, and cardiac glycosides[16]. These phytochemicals allow it to have antioxidant, antibacterial, antifungal, anticancer, and anthelmintic properties[14].

Extensive research has been done on this plant's bulbs, leaves, and roots with substantial scientific documentation. However, although both the leaves and flowers are edible and have been used traditionally in ethnomedicine, there is no scientific documentation on the phytochemical profile and biological activities in flowers and stalks of *T. violacea*[2]. Therefore, the present study was to comparatively assess the phytochemical profile and biological activities in the flowers and stalks of *T. violacea*.

a **b**

Figure 1. (**a**) *Tulbaghia violacea* in natural habitat and (**b**) their flowers and stalks.

MATERIALS AND METHODS

Materials

The stalks and attached flower heads of *T. violacea* were collected at the University of the Witwatersrand (26.1929 °S, 28.0305 °E), Johannesburg, South Africa, in March 2023, when the flowers were in full bloom. Fresh aerial parts were authenticated by Dr. Ida Risenga at the same university. The voucher specimen (IR/2023/01) and the plant species were deposited at the university's medicinal plant laboratory.

Methods

Preparation of plant material

Collected flowers and stalks were washed with distilled water (H_2O_d) before being separated cautiously. These were dried using the hot air drier (40°C) and freeze-drying (-83°C). Dried plant materials were ground into fine powder and kept in separate containers at room temperature.

Preparation of plant extracts

The extraction of the separate ground powder was prepared using two solvents: 80% methanol and H_2O_d. About 3 g of each plant powder was extracted using 25 mL of each solvent inside 100 mL Schott bottles. The mixture was agitated on an orbital shaker at 150 rpm for 48 hours and centrifuged for 5 minutes at 3500 rpm. Samples were then filtered through Whatman® No.1 filter paper.

Qualitative analysis of phytochemicals

Recommended laboratory procedures[17,18] were followed to carry out preliminary phytochemical screening of methanolic and aqueous extracts of *T. violacea*.

Saponins (froth test): About 0.5 mL of the plant extract was added to 5 mL of H_2O_d and then shaken vigorously for 15 minutes. A foam layer confirmed the presence of saponins.

Terpenoids (chloroform test): In a test tube, 0.5 mL of chloroform was mixed with 1 mL of the plant extract and three drops (~150 µL) of concentrated H_2SO_4. A red-brown precipitate indicated terpenoids.

Glycosides: In a test tube, 2 mL of H_2SO_4 was added to 0.5 mL of the plant extract. A red-brown color confirmed the presence of glycosides.

Steroids: To 1 mL of the plant extract, 10 drops of chloroform and five drops of H_2SO_4 were added. A blue-brownish ring confirmed the presence of steroids.

Volatile oils: About 1 mL of the plant extract was mixed with 0.2 mL of 10% NaOH. The formation of a precipitate indicated that volatile oils were present.

Coumarins (NaOH test): About 1 mL of 10% NaOH was mixed with 1 mL of the plant extract; the formation of a yellow top layer was indicative of the presence of coumarins.

Phlobatannins (HCl test): Five drops (~250 µL) of 2% HCl was added to 1 mL of the plant extract. A red precipitation indicated the presence of phlobatannins.

Alkaloids (Mayer's test): A drop (~50 µl) of Mayer's reagent was added to 1 mL of the plant extracts. A creamy precipitate confirmed alkaloids as present.

Phenolics (Ferric chloride test): In a test tube, 1 mL of the plant extract was mixed with three drops (~150 µL) of 10% $FeCl_3$. A dark blue-green or violet color confirmed the presence of phenolics.

Tannins (Bromine water test): In a test tube, 10 mL of bromine water was added to 1 mL of the plant extract. A decolorization of the mixture indicated the presence of tannins.

Quinones (H_2SO_4 test): About 1 mL of the plant extract was added to 1 mL of concentrated H_2SO_4. The presence of quinones was indicated by the formation of a red color.

Cardiac glycosides (Keller-Killani test): About 2 mL of glacial acetic acid, a ml of concentrated H_2SO_4, and a single drop (~50 µL) of 5% $FeCl_3$ were added to 0.5 mL of the plant extract. A brown ring confirmed the presence of cardiac glycosides.

Flavonoids (Alkaline reagent test): In a test tube, 2 mL of 2% NaOH was added to 1 mL of the plant extract. A color change from yellow to colorless after adding a few drops of diluted HCl was indicative of the presence of flavonoids.

Carbohydrates: The presence of carbohydrates was confirmed by a formation of purple color when two drops of Molisch's reagent were added to 2 mL of the plant extract and 1 mL of concentrated H_2SO_4.

Fixed oils and fats (Stain/spot test): The plant extract was filtered through a filter paper. An oil stain confirmed that fixed oils and fats were present.

Gums and mucilage (Alcohol test): About 1 mL of H_2O_d and 2.5 mL of concentrated H_2SO_4 were added to 1 mL of the plant extracts. A white precipitate showed the presence of gums and mucilage.

Resins: Three drops of glacial acetic acid and 1 mL of concentrated H_2SO_4 were added to 1 mL of the plant extract. An orange/yellow color confirmed the presence of resins.

Triterpenoids and phytosterol: About 1 mL of chloroform and three drops of concentrated H_2SO_4 were added to 1 mL of the plant extract. The solution was shaken vigorously and left to set for a few seconds. A yellow or red color confirmed the presence of triterpenoids or phytosterols, respectively.

Anthocyanins: About 1 mL of 2 N HCl was added to 1 mL of the plant extract. A color change from reddish pink to violet after adding a few drops of ammonia indicated the presence of flavonoids.

Cholesterol: About 1 mL of chloroform, five drops of glacial acetic acid, and two drops of H_2SO_4 were added to 1 mL of the plant extract. A red color was indicative of the presence of cholesterol.

Quantitative analysis of phytochemicals

Total phenolic content: About 0.3 mL of the prepared plant extracts were added to a solution of 7.5% sodium carbonate (Na2CO3). To this mixture, 0.75 mL of Folin-Ciocalteu's (FC) phenol reagent was added then the entire mixture was diluted with H2Od to a final volume of 7 mL. The mixture was then left to incubate for 2 hours in the dark. Using a Genesys 10s UV-Vis spectrophotometer, the absorbance of the sample was taken at 765 nm. The total phenolic content (TPC), expressed in milligrams of gallic acid equivalents (GAE) per gram of dry weight (mg GAE/g), was calculated using the following linear regression obtained from the gallic acid standard curve graph (**Figure 2**).

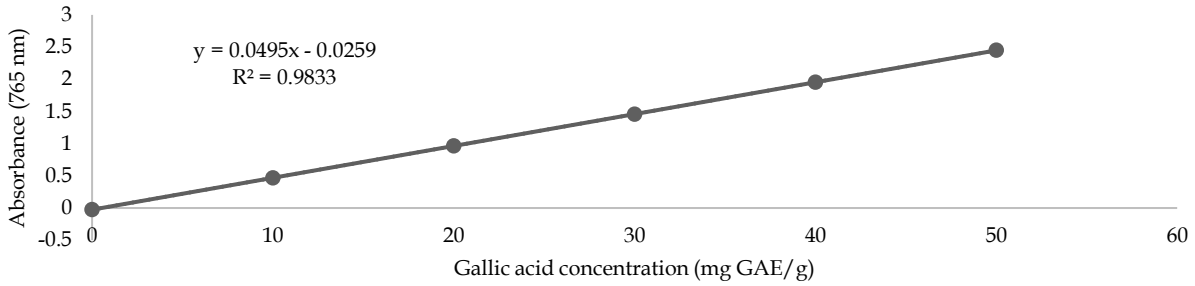

Figure 2. Standard curve for total phenolic content.

Total flavonoid content: The aluminum chloride ($AlCl_3$) colorimetric assay was followed. A 5% (w/v) sodium nitrate ($NaNO_3$) solution was prepared by adding 100 mL of H_2O_d to 5 g of $NaNO_3$. In a test tube, 0.3 mL of the prepared plant extracts were combined with the prepared 5% $NaNO_3$, which was then left to set for 5 minutes. About 3 mL of 10% (w/v) $AlCl_3$ solution (prepared by dissolving 10 g of $AlCl_3$ in 100 mL of H_2O_d) was added to the test tube that contained the extract and $NaNO_3$. This test tube was then left to rest for 6 minutes. After that, 2 mL of 7.5% sodium hydroxide (NaOH) was added to the test tube. To the entire mixture, 0.75 mL of diluted FC reagent and H_2O_d were to reach a final volume of 10 mL and were then left to incubate for 1 hour in the dark at room temperature. As described earlier, the absorbance readings were measured at 510 nm against the blank, which was 80% methanol. Total flavonoid content (TFC), expressed in milligrams of quercetin equivalents per gram of dry weight (mg QE/g), was calculated using the following linear regression obtained from the quercetin standard curve graph (**Figure 3**).

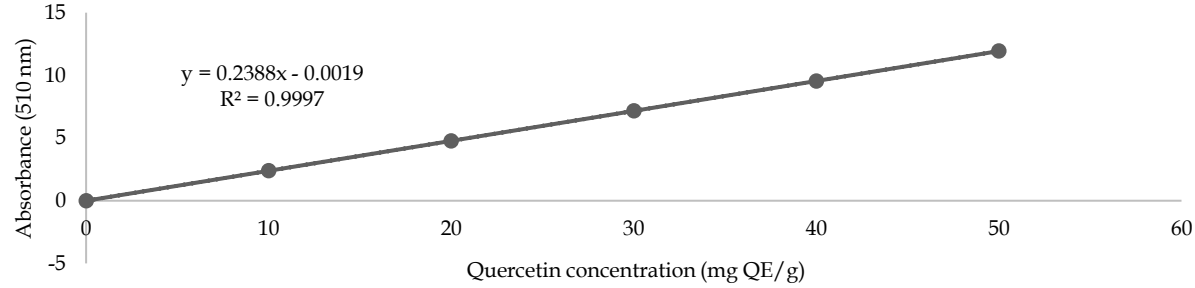

Figure 3. Standard curve for total flavonoid content.

Total tannin content: About 0.1 mL of the prepared plant extracts were diluted with 7.5 mL of H_2O_d before adding 0.5 mL of Folin-Ciocalteu's phenol reagent. About 0.1 mL of 35% (w/v) sodium carbonate (Na_2CO_3) solution (prepared by adding 10 mL of H_2O_d to 3.5 g of Na_2CO_3) was added to the mixture of extract and FC phenol reagent. The entire mixture was made up of 10 mL with H_2O_d. The absorbance of the mixture was measured at 725 nm, as described earlier, against the blank, which was 80% methanol. Total tannin content (TTC), which was expressed in mg GAE/g, was calculated using the following linear regression obtained from the gallic acid standard curve (**Figure 4**).

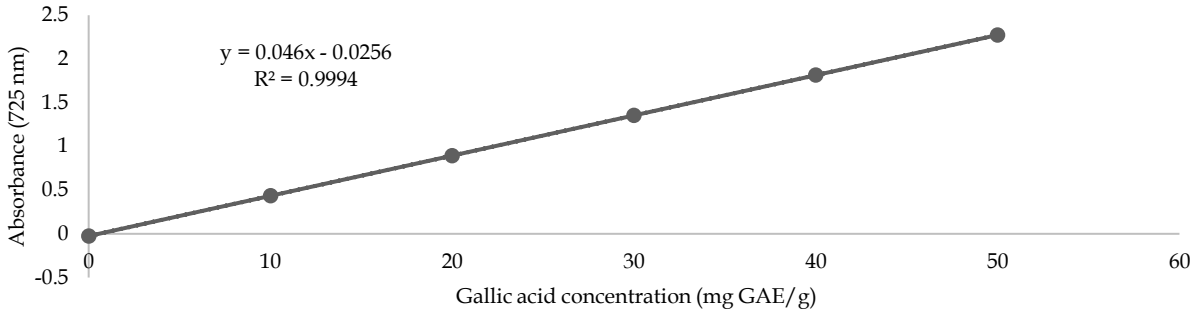

Figure 4. Standard curve for total tannin content.

Total proanthocyanidin content: About 3 mL of 4% vanillin-methanol (w/v) (prepared by adding 100 mL of water to 4 g of vanillin-methanol) was mixed with 0.5 mL of the prepared plant extracts, then 1.5 mL of HCl was added. This mixture was vortexed and then left to incubate for 15 minutes in the dark at room temperature. The absorbance of the mixture was measured at 500 nm as described earlier against the blank which was 80% methanol. Total proanthocyanidin content (TPAC), which was expressed in milligrams of catechin equivalents per gram of dry weight (mg CE/g) was calculated using the following linear regression obtained from the catechin standard curve (**Figure 5**).

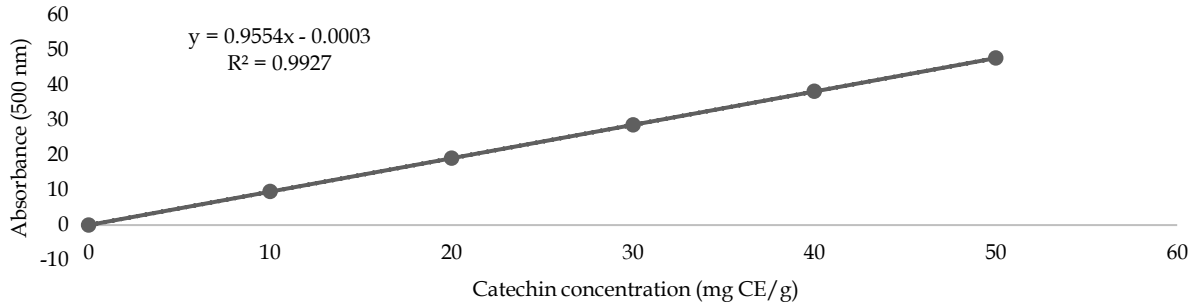

Figure 5. Standard curve for total proanthocyanidin content.

Antioxidant assays

2,2-diphenylpicrylhydrazyl (DPPH) scavenging assay: To determine the DPPH scavenging activities of the plant extracts, the DPPH solution was prepared by mixing 50 mg of DPPH and 100 mL of 80% methanol, which was then shaken vigorously (stock solution). This solution was diluted 1 : 5 times with 80% methanol (work solution). About 70 µL of the work solution was added to the different volumes (10, 20, 30, 40, and 50 µL) of the plant's extracts. The work solution without the plant extracts was used as a control. The extract and DPPH solution mixture was left to incubate for 45 minutes in the dark at room temperature. The absorbance of the mixture was measured at 517 nm, as described earlier. **Equation 1** was used to calculate the DPPH scavenging percentage of the extract, in which Âcc was the absorbance of the control, and Âss was the absorbance of the test compound (plant extract).

$$\%\ DPPH = \frac{Âcc - Âss}{Âcc} x100\% \qquad [1]$$

Hydrogen peroxide assay: A 30% H_2O_2 solution was prepared by mixing 30 mL of concentrated H_2O_2 with 70 mL of H_2O_d. Then, a 40 mM H_2O_2 solution was prepared by mixing 4.53 mL of the 30% H_2O_2 solution with 995.47 mL of phosphate buffer (pH 7.4). About 600 µL of 40 mM H_2O_2 solution was added to the different volumes (10, 20, 30, 40, and 50 µL) of the plant extracts, and these were left to set for 10 minutes. About 40 mM H_2O_2 served as a control. The absorbance of the mixture was measured at 230 nm as described earlier, against the blank which was the phosphate buffer without the H_2O_2. **Equation 2** was used to calculate the percentage of H_2O_2 reducing the power of the extract, in which Âcc was the absorbance of the control, and Âss was the absorbance of the test compound (plant extract).

$$\% H_2O_2 = \frac{Âcc - Âss}{Âcc} x100\% \quad [2]$$

Metal chelating assay: A 2 mM iron chloride solution was prepared by dissolving 0.03244 g of $FeCl_3$ in 100 mL of H_2O_d. For determining the iron-reducing power of *T. violacea*, different volumes (10, 20, 30, 40, and 50 µL) of the plant extracts were mixed with 0.05 ml of the 2 mM $FeCl_3$. As a reaction initiator, 200 µL of 5 Mm ferrozine solution (prepared by adding 0.246 g of ferrozine to 100 mL of H_2O_d) was added to the mixture of the plant extracts and 2 mM $FeCl_3$. The mixed solution was shaken vigorously and left to set for 10 minutes in the dark at room temperature. The mixed $FeCl_3$ and ferrozine solution without the extracts served as a control. The absorbance of the mixture was measured at 562 nm, as described earlier. **Equation 3** was used to calculate the percentage metal chelating effect of the extract, in which Âcc was the absorbance of the control, and Âss was the absorbance of the test compound (plant extract).

$$\% Chelating = \frac{Âcc - Âss}{Âcc} x100\% \quad [3]$$

Preliminary antibacterial assays

An agar well diffusion method was followed to determine the antimicrobial activity of flowers and stalks of *T. violacea*. This was assessed from gram-negative (*Escherichia coli*) and gram-positive bacteria (*Staphylococcus aureus*). The Mueller-Hinton (MH) and Baird-Parker (BP) agar were used to culture the *E. coli* and *S. aureus*, respectively. The bacteria strains were inoculated on cooled petri dishes with the MH and BP agar, respectively, before incubating for 24 hours at 37°C, which is the normal human body temperature. Subsequently, holes were punched into the agar plates using sterilized 6 mm diameter pipette tips. About 100 µL of the plant extracts were then added to the punched holes, and the Petri dishes were left to set for 10 minutes before being incubated for 48 hours at 37°C in a binder oven. The 80% methanol was used as a negative control, while the antibiotic rifampicin (100 µg/mL) was used as a positive control. After 48 hours, zones of inhibition (ZOI) on the plates were measured in mm to determine the antibacterial activity of the plant extracts.

Data analysis

The results are expressed in mean ± SD with n = 3. All experiments were done in triplicates. The quantitative analysis and antioxidant activity results were analyzed using paired t-tests ($p \leq 0.05$). Pearson correlations were conducted to determine the relationship between phytochemical constituents and antioxidant activity. All statistical analyses were conducted on R studio version 4.12.

RESULTS AND DISCUSSION

Qualitative analysis of phytochemicals

A qualitative analysis was used to evaluate the presence or absence of phytochemicals in the flowers and stalks of *T. violacea*. Phytochemical screening results at varying intensities (Strong presence, moderate presence, weak presence, and absent) are displayed in **Table I**. Both the methanolic and aqueous extracts of both plant parts showed the absence of saponins, volatile oils, alkaloids, carbohydrates, and resins and this detection was consistent in both drying methods. The absence of alkaloids and carbohydrates coincides with a study performed by Madike *et al*.[13] for other plant parts of *T. violacea*.

Table I. Phytochemical screening analysis of freeze- and air-dried methanolic and aqueous extracts of flowers and flower stalks of *T. violacea*.

	Plant Part	Flower		Stalk		Flower		Stalk	
	Drying method	Air Dried				Freeze-dried			
	Solvent	M	W	M	W	M	W	M	W
Phytochemicals	Saponin	-	-	-	-	-	-	-	-
	Terpenoid	+++	+++	-	-	++	+++	-	-
	Glycosides	++	+++	++	++	+++	+++	++	++
	Steroids	-	++	-	+++	+++	+++	-	-
	Volatile oils	-	-	-	-	-	-	-	-
	Coumarins	+++	+++	-	+++	+++	-	-	-
	Phlobatannins	++	+	-	-	+	+	+++	+++
	Alkaloids	-	-	-	-	-	-	-	-
	Phenolics	+++	+++	+++	+++	+++	+++	+++	+++
	Tannins	+++	+++	++	++	+++	+++	+	+
	Quinones	+	-	+	-	-	-	+++	++
	Cardiac glycosides	-	+++	+++	+++	+++	++	+++	+++
	Flavonoids	+	+++	+	+	++	+++	+	+
	Carbohydrates	-	-	-	-	-	-	-	-
	Fixed oils and fats	+	+	++	++	+	+	++	++
	Gums and Mucilage	-	++	-	-	-	+	-	-
	Resins	-	-	-	-	-	-	-	-
	Triterpenoids	-	-	++	+++	-	-	+++	+
	Phytosterols	+++	-	-	-	+++	++	-	-
	Anthocyanin	+	-	-	-	+	-	-	-
	Cholesterol	++	+	-	-	+++	+	-	-

+++ Strong presence
++ Moderate presence
+ Weak presence
- Absence

M : methanol; W : water

Despite the solvents or the drying methods, glycosides, phenolics, tannins, flavonoids, and fixed oils and fats were extracted from both plant parts. Glycosides, which were very strongly detected in the flowers, are known for their antinociceptive and anti-inflammatory properties and have the potential for treating diabetes mellitus[19,20]. Phenolic compounds, known to have anti-inflammatory, antimicrobial, and antioxidant properties, showed a strong presence in both plant parts and for both drying methods[21]. Tannins, which showed a more substantial presence in the flowers, are known for their antiparasitic, antiviral, and antimicrobial properties and can be used to stop the replication of HIV[13]. Previous research has also shown that tannins can treat kidney-related ailments[20]. Flavonoids, the largest group of phenolic compounds, were strongly detected in the methanolic flower extracts across the two drying methods. Flavonoids have been shown to exhibit antioxidant, analgesic, antidiarrhea, and antimicrobial properties and have been used in cancer and Alzheimer's disease treatments[22,23]. Therefore, this data suggests that the stalks of *T. violacea* can potentially treat the above-mentioned deceases. Fixed oils and oils were more strongly detected in the stalks than in flowers. They possess antifungal and antibacterial properties and can be used as an insect repellent[24], suggesting that stalks could have antifungal, antibacterial, and insect-repellent properties.

Terpenoids, gums, mucilage, phytosterol, anthocyanidins, and cholesterol were only detected in flowers. Terpenoids have anticancer, anti-inflammatory, and antioxidant properties[25,26]. Gums and mucilage, only present in the aqueous extracts, can treat irritated mucous membranes in the throat and digestive tract[27]. Phytosterols and cholesterol, which fluctuated in their strength of presence, can be used to lower cholesterol levels[28,29]. Anthocyanidins, only present in the methanolic extracts, have antioxidant, anticancer, anti-obesity, and anti-inflammatory properties. Triterpenoids were the only compounds that were detected in the stalks and not flowers. These compounds have antiviral, anti-inflammatory, and antitumor properties[30]. More phytochemicals were detected in the flowers as compared with stalks. The detected phytocompounds are natural chemicals that can be used in pharmacological fields or the production of bioactive compounds, and these are preferred and have fewer side effects than synthetic drugs[31]. Therefore, the presence of these phytochemicals supports the use of *T. violacea* flowers and stalks in ethnomedicine.

Quantitative analysis of phytochemicals

The quantitative phytochemical analysis of air and freeze-dried flowers and stalks are displayed in **Tables II** and **III**, respectively. Phenolic compounds have numerous pharmacological effects, including their antioxidant, anti-inflammatory, and antidiabetic properties[21]. Gallic acid possesses antioxidant and anti-inflammatory properties, thus increasing the health benefits with limited side effects compared to modern-day medicine[32]. The results in this study show that for both air and freeze-dried methanolic and aqueous extracts, the flowers had a significantly higher total phenolic content as compared with the stalks ($p < 0.001$).

Table II. Quantitative phytochemical analysis of methanolic and aqueous extracts of air-dried flowers and stalks of *T. violacea* ($p \leq 0.05$).

Air-dried samples		
Phytochemical Constituents	**Flowers**	
	Methanol	**Water**
Phenol (mgGAE/g)	40.6±0.013	41.41±0.015
Flavonoid (mgQE/g)	8.9±0.068	7.58±0.19
Tannin (mgGAE/g)	40.07±0.017	34.03±0.012
Proanthocyanidin (mgCE/g)	0.7±0.013	0.36±0.0091
Phytochemical Constituents	**Stalks**	
	Methanol	**Water**
Phenol (mgGAE/g)	27.26±0.017	28.52±0.016
Flavonoid (mgQE/g)	5.79±0.067	9.29±0.14
Tannin (mgGAE/g)	22.64±0.02	28.07±0.013
Proanthocyanidin (mgCE/g)	0.46±0.0057	0.2±0.0015

Table III. Quantitative phytochemical analysis of methanolic and aqueous extracts of freeze-dried flowers and stalks of *T. violacea* ($p \leq 0.05$).

Freeze-dried samples		
Phytochemical Constituents	**Flowers**	
	Methanol	**Water**
Phenol (mgGAE/g)	34.62±0.02	37.29±0.0027
Flavonoid (mgQE/g)	2.54±0.022	2.42±0.085
Tannin (mgGAE/g)	44.27±0.011	33.37±0.065
Proanthocyanidin (mgCE/g)	0.66±0.002	0.46±0.015
Phytochemical Constituents	**Stalks**	
	Methanol	**Water**
Phenol (mgGAE/g)	26.27±0.037	24.82±0.0058
Flavonoid (mgQE/g)	2.5±0.067	2.52±0.19
Tannin (mgGAE/g)	28.89±0.18	13.87±0.46
Proanthocyanidin (mgCE/g)	0.24±0.0062	0.36±0.28

Flavonoids, a phenolic compound, possess antimicrobial, analgesic, and antioxidant properties, among other pharmacological uses[22]. Quercetin is a type of flavonoid that has antioxidant properties thus increasing the health benefits with fewer side effects[33]. The results in this study show that for the methanolic extracts of the air-dried extracts, the flowers had a significantly higher total flavonoid content than the stalks ($p < 0.001$). For both air and freeze-dried aqueous extracts, the stalks had a significantly higher total flavonoid content as compared with the flowers ($p < 0.05$). The low TFC for freeze-dried extracts coincides with a study performed by Madike *et al.*[13] for other plant parts of *T. violacea*. Tannins possess antiparasitic, antiviral, and antimicrobial properties, among other functions. This study's results show that for air and freeze-dried methanolic and aqueous extracts, the flowers had a significantly higher total tannin content than the stalks ($p < 0.001$). Proanthocyanidins have been documented to possess anti-allergic, antioxidant, and antimicrobial properties. They also have pharmacological uses such as the improvement of eyesight[23]. Catechin has been reported to possess antioxidant properties and can be used in the prevention of congestive heart failures thus increasing the health benefits with fewer side effects[34]. The presence of all these phytochemicals at varying concentrations can be used in pharmaceutical industries to promote human health. The results in this study show that for both air and freeze-dried methanolic and aqueous extracts, the flowers had a significantly higher total proanthocyanidin content as compared with the stalks ($p < 0.001$).

Analysis of antioxidant activity

The DPPH radicals, H_2O_2, and iron oxidant scavenging activity of air and freeze-dried flowers and stalks are displayed in **Figures 6** to **8**, respectively. Free radicals are unstable and reactive molecules produced during metabolism[35]. Oxidative stress can result from over-accumulating free radicals in the body, which can be fatal to cells and thus cause illnesses[36]. Medicinal plants contain phytochemicals that have antioxidant activities. The plant extracts' ability to scavenge for and neutralize free radicals in the body gives us a general idea of their antioxidant properties[37]. The samples' ability to scavenge the DPPH, H_2O_2, and metal radicals is expressed by IC_{50} values. An IC_{50} value, the "half-maximal inhibitory concentration", indicates how much of an extract is needed to inhibit a detrimental biological activity by 50%. Low IC_{50} values indicate high antioxidant activities[38].

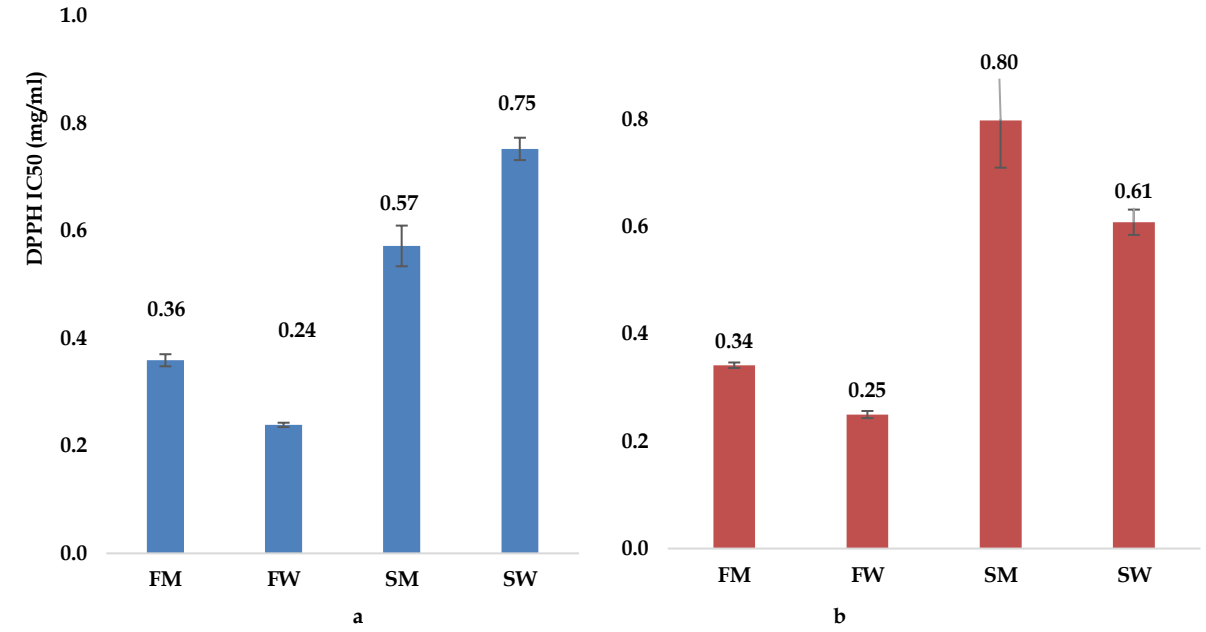

Figure 6. DPPH IC_{50} values of (**a**) air-dried and (**b**) freeze-dried flowers and flower stalks of *T. violacea* ($p \leq 0.05$). FM: flower-methanol; FW: flower-water; SM: stalk-methanol; SW: stalk-water.

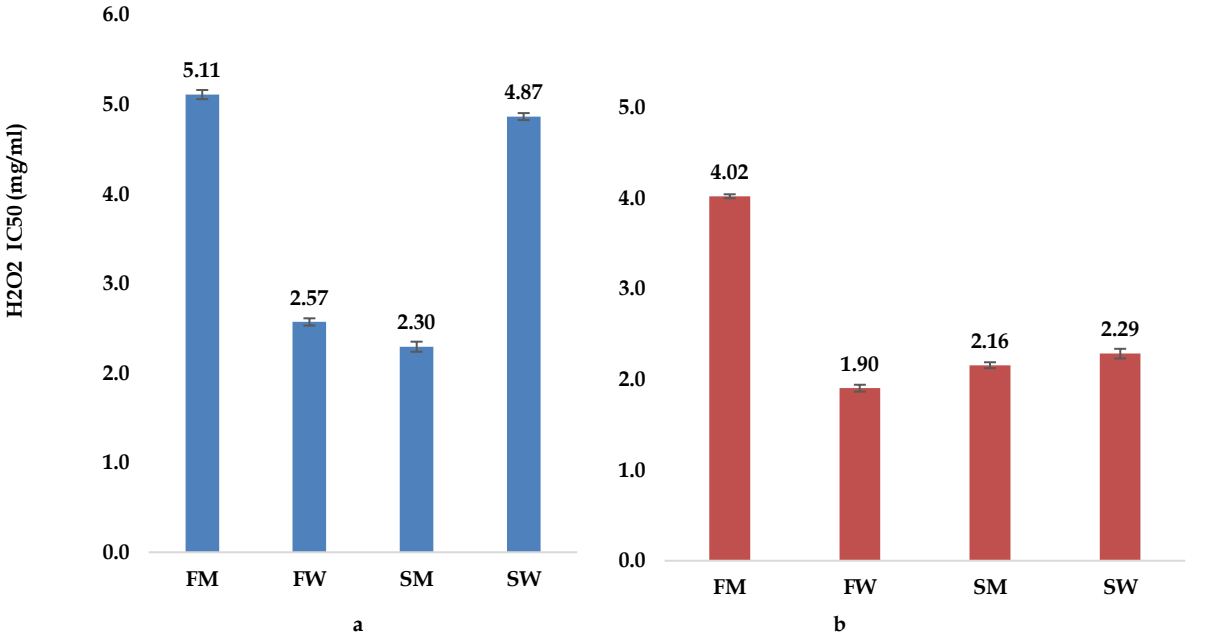

Figure 7. H_2O_2 IC_{50} values of (**a**) air-dried and (**b**) freeze-dried flowers and flower stalks of *T. violacea* ($p \leq 0.05$). FM: flower-methanol; FW: flower-water; SM: stalk-methanol; SW: stalk-water.

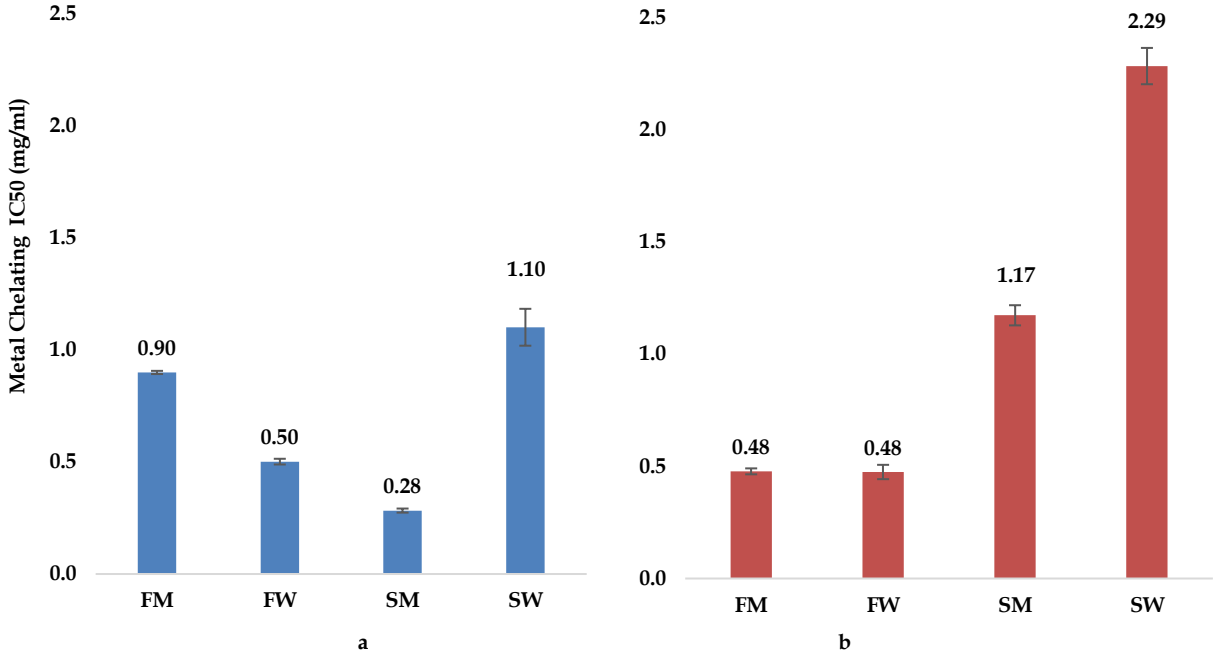

Figure 8. Metal chelating IC_{50} values of (**a**) air-dried and (**b**) freeze-dried flowers and flower stalks of *T. violacea* ($p \leq 0.05$). FM: flower-methanol; FW: flower-water; SM: stalk-methanol; SW: stalk-water.

For both drying methods, the methanolic and aqueous extracts of the flowers and stalks of *T. violacea* had IC_{50} values below 1 mg/mL, thus indicating an excellent scavenging activity against DPPH radicals[38]. The very low IC_{50} values coincide with a study performed by Takaidza *et al.*[39], where *T. violacea* as a whole plant was used. The results in this study show that the flowers had significantly lower IC_{50} values than the stalk ($p < 0.001$). This was consistent in both drying methods and solvents that were used. The higher IC_{50} values in the stalks can be attributed to the lower phytochemical presence (**Table I**). The DPPH scavenging activity exhibited strong positive correlations with most of the quantified phytochemicals, and perfect strong positive correlations ($r = 1$) are exhibited between the DPPH scavenging activity and TPAC for air-dried methanolic extracts of the flowers and the TFC for freeze-dried aqueous extracts of the stalks (**Tables IV** and **V**). This shows that the tested plant parts' ability to scavenge for and neutralize DPPH radicals can be attributed to the presence of phytochemicals as they possess antioxidant activities.

For both drying methods, the methanolic and aqueous extracts of the flowers and stalks had IC_{50} values below 10 mg/mL (upper limit of IC_{50}), thus indicating a strong scavenging activity against H_2O_2. The results of this study show that the stalks had significantly lower IC_{50} values than the flowers ($p < 0.001$). For the aqueous extracts, the results in this study showed that the flowers had significantly lower IC_{50} values as compared with the stalks ($p < 0.001$). The scavenging activity of H_2O_2 is dependable on the solvent used for extraction. Thus, since water and methanol have different polarities, this then affects the tested plant parts' scavenging power against H_2O_2[40]. Apart from the freeze-dried aqueous extracts, for both drying methods, the H_2O_2. Scavenging activity exhibited strong positive correlations with the quantified phytochemicals (**Tables IV** and **V**). There were perfect strong positive correlations ($r = 1$) between the H_2O_2 scavenging activity and the TFC, TTC, and TPAC for the air-dried aqueous flower extracts; the TTC for the air-dried methanolic stalk extracts; and the TPAC of the air-dried aqueous stalk extracts (**Tables IV** and **V**). This shows that the tested plant parts' ability to scavenge for and neutralize H2O2 can be attributed to the presence of phytochemicals.

For both drying methods, the methanolic and aqueous extracts of the flowers and stalks had IC_{50} values below 10 mg/mL (upper limit of IC_{50}), thus indicating a strong scavenging activity against iron oxidants. Except for the air-dried methanolic extracts, the flowers had significantly lower IC_{50} values than the stalks ($p < 0.01$). Apart from the freeze-dried methanolic extracts, the iron oxide scavenging activity exhibited very strong positive correlations with most of the quantified phytochemicals for both drying methods. This shows that the tested plant parts' ability to chelate iron oxidants can be attributed to the presence of phytochemicals.

Table IV. Pearson correlation coefficients (r) between TPC, TFC, TTC, TPAC, and antioxidant activities of the methanolic and aqueous extracts of air-dried flowers and stalks of *T. violacea* ($p \leq 0.05$).

Air-dried samples				
Antioxidant assay	**TPC**	**TFC**	**TTC**	**TPAC**
		Flower-Methanol		
DPPH	0.737	0.808	0.808	1*
H_2O_2	0.95	0.979	0.979	0.911
MC	0.907	0.948	0.949	0.953
		Flower-Water		
DPPH	0.737	0.994	0.994	0.993
H_2O_2	0.808	1*	1*	1*
MC	0.938	0.962	0.962	0.962
		Stalk-Methanol		
DPPH	0.563	0.847	0.673	0.616
H_2O_2	0.99	0.963	1*	0.997
MC	0.977	0.98	0.997	0.989
		Stalk-Water		
DPPH	0.778	0.945	0.993	0.693
H_2O_2	0.992	0.891	0.771	1*
MC	0.994	0.972	0.898	0.973

*: Perfect strong correlation

Table V. Pearson correlation coefficients (r) between TPC, TFC, TTC, TPAC, and antioxidant activities of the methanolic and aqueous extracts of freeze-dried flowers and stalks of *T. violacea* ($p \leq 0.05$).

Freeze-dried samples				
Antioxidant assay	**TPC**	**TFC**	**TTC**	**TPAC**
		Flower-Methanol		
DPPH	0.845	0.95	0.881	0.737
H_2O_2	0.941	0.995	0.963	0.866
MC	0.442	0.648	0.507	0.277
		Flower-Water		
DPPH	0.908	0.945	0.778	0.804
H_2O_2	0.648	0.721	0.442	0.481
MC	0.908	0.945	0.778	0.804
		Stalk-Methanol		
DPPH	0.59	0.786	0.721	0.661
H_2O_2	0.751	0.901	0.854	0.808
MC	0.997	0.941	0.97	0.986
		Stalk-Water		
DPPH	0.951	1*	0.932	0.994
H_2O_2	0.99	0.985	0.98	0.998
MC	0.916	0.995	0.981	0.978

*: Perfect strong correlation

Preliminary antibacterial assays

Escherichia coli is a common bacteria strain and is linked with urinary infections[41]. *Staphylococcus aureus* is linked with skin conditions such as skin and soft tissue infections (SSTI), a common infection[21]. Flowers were the only tested plant part that showed the inhibition of *E. coli* and *S. aureus* (**Table VI**). Zones of inhibitions for *E. coli* were only exhibited in the flower methanolic extracts, which fall under the resistant category. Zones of inhibitions for *S. aureus* were only exhibited in the aqueous extracts for flowers, and these fall under the intermediate category. This shows that the aqueous extracts have the potential to be used to cure skin conditions such as SSTI.

Table VI. Zone of inhibition (mm) of the methanolic and aqueous extracts of freeze-dried flowers and stalks of *T. violacea* against *E. coli* and *S. aureus*.

Drying method	**Air-dried**		**Freeze-dried**	
	E. coli	*S. aureus*	*E. coli*	*S. aureus*
Flower-methanol	10±0.1	-	11±0.1	-
Flower-water	-	12±0.2	-	13±0.2
Stalk-methanol	-	-	-	-
Stalk-water	-	-	-	-
Antibiotic	12.67±0.58	12.67±0.58	12.67±0.58	12.67±0.58

CONCLUSION

Consuming *T. violacea* would be beneficial as it contains phytochemicals that allow the plant to have therapeutic properties. Flowers had more phytochemicals present, higher antioxidant activity (DPPH, H_2O_2, and metal chelating) than stalks, and were the only plant part with antibacterial activity. The results of this study can be highly beneficial to communities that rely on medicinal plants as their source of health care as they can use each plant part for specific ailments. This can also be beneficial to pharmaceutical industries for the promotion of human health.

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DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Genetic *CYP2A6* Polymorphism May Worsen Glycohemoglobin Levels: Study among Javanese Indonesian Smokers

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CYP2A6*9
HbA1c levels

Abstract

We have examined the inactive *CYP2A6* alleles gene, including CYP2A6*4, CYP2A6*7, and CYP2A6*9, associated with glycohemoglobin levels among Javanese Indonesian smokers. There are 106 smokers participating in this study. Due to the number of cigarettes smoked per day, there are three groups of smokers: light, intermediate, and heavy smokers, with 98.7% being light and intermediated smokers while the rest are heavy smokers. All participants had smoked for more than 10 years, indicating they had been exposed to nicotine for a long time. Based on their genotype, there were four groups of smokers, including fast, intermediate, slow, and poor metabolizers. Most fast and intermediate metabolizers have HbA1c levels in the normal range (<5.7). On the other hand, most slow metabolizers have Hb1c levels >5.7, and all fast metabolizers have HbA1c levels >5.7, indicating that they the prediabetes and diabetes. The chi-square test showed a relationship between CYP2A6 polymorphism and HbA1c levels among the participants (P-value 0.000 <0.005 and χ^2=54.6, df=1). The presence of an inactive allele will worsen the HbA1c levels in smokers.

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INTRODUCTION

Diabetes mellitus (DM), a chronic disease, was the third leading cause of death in Indonesia, with a percentage of 6.7% after stroke (21.1%) and coronary heart disease (12.9%). The DM prevalence in Indonesia has increased substantially from 6.9% in 2013 to 8.5% in 2018[1]. Other data has estimated that approximately 30% of Indonesia's population (30 million people) with diabetes remains undiagnosed. The diabetics in Indonesia were estimated could reach 30 million people in 2030 if lifestyles including unhealthy diet, obesity, lack of physical activity, alcohol consumption, and smoking are not a concern[2]. In line with this report, The International Diabetes Federation (IDF) found that people with diabetes in Indonesia have increased precipitously in the last ten years from 2021. Without proper management, people with diabetes will jump to a staggering 28.57 million in 2045, or 47% greater than 19.47 million in 2021[3].

Type 2 diabetes mellitus (T2DM) is the most common in adults and accounts for 90% of all diabetes cases. In past years, T2DM typically develops in adulthood. However, in recent years, it has been increasingly seen in children and adolescents partially due to lifestyle, including rising obesity rates, unhealthy diet, lack of physical inactivity, alcohol consumption, and smoking[4]. The Basic Health Research of Indonesia (*Riset Kesehatan Dasar, RISKESDAS*) 2018 reports that T2DM prevalence in the Daerah Istimewa Yogyakarta (DIY) Province was second among provinces in Indonesia[5]. About 74,668 DIY people have been diagnosed with diabetes, but only 55,190 patients have received standard health services or the equivalent of 73.9%[6].

Several studies have suggested that poor smoking behavior is associated with chronic complications of T2DM compared to non-smokers[7,8]. Another study has reported that smoking can increase glycohemoglobin (HbA1c) blood levels[9]. This HbA1c value can accurately reflect glucose control 2-3 months ago. HbA1c levels are normal if <5.7%, prediabetes 5.7 to 6.4%, and

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diabetes ≥6.5%[10]. Nicotine, the main compound in cigarettes, was considered most responsible for increasing blood sugar levels due to insulin resistance[11].

Nicotine is primarily metabolized by the CYP2A6 enzyme to cotinine and excreted in the urine[12]. The CYP2A6 enzyme encoded by the *CYP2A6* gene is a polymorphic gene. The active allele gene is *CYP2A6*1*, and the inactive is CYP2A6*4, CYP2A6*7, and CYP2A6*9. Due to their genotype, a person with having CYP2A6*4, CYP2A6*7, and CYP2A6*9 allele genes is associated with a slow metabolizer or poor metabolizer[13]. Furthermore, according to Liu *et al*.[14], reduced metabolism function *CYP2A6* in smokers appears to be associated with a higher risk of T2DM.

Our preliminary study[15] revealed a high-frequency CYP2A6*4 allele gene among smokers and non-smokers in Javanese Indonesian. We have also reported that smoking can increase diabetes risk factors. Prediabetes was developing in smokers who had smoked for at least 25 years with 25 cigarettes per day[16]. Furthermore, in our recent study on diabetic patients, both smokers and non-smokers, high-frequency CYP2A6*4, the inactive allele gene of *CYP2A6*, was detected[17]. In high frequency, the other inactive alleles, CYP2A6*7 and CY2A6*9, have also been found among Javanese Indonesian smokers[13]. So, in this research, we study the association of the CYP2A6*4, CYP2A6*7, and CYP2A6*9 on glycohemoglobin levels in Indonesian Javanese smokers.

MATERIALS AND METHODS

Materials

A Norudia® N HbA1c Immunoassay Method using the Architect 600 instrument, calibrated using Diabetes Control and Complications Trial (DCCT) standards with a coefficient of variation <2.5% was used to analyze total HbA1c in the Clinical Pathology Laboratory, Bethesda Hospital, Yogyakarta. Genomic DNA was extracted using a DNA Mini Kit from Bioron GmbH (Germany). The CYP2A6*4, *7, and *9 allele genes were analyzed using the Polymerase Chain Reaction (PCR) method. The forward and reverse primers used in this study were 5' CCT CAT CAC ACA CAA CTT CCT C 3' and 5' TGC AGG TAC TGG GTG CTT GGT AG 3' for CYP2A6*4; 5′-CTC CCA GTC ACC TAA GGA CAC-3' and 5′-AAA ATG GGC ATG AAC GCC C-3' for CYP2A6*7; as well as 5′-GAT TCC TCT CCC CTG GAA C-3' and 5′-GGC TGG GGT GGT TTG CCT TTC-3' for CYP2A6*9.

The PCR mixture contained 12.5 µL Promega Go Taq Green Master Mix, 1.25 µL forward primer, 1.25 µL reverse primer, 5.0 µL genomic DNA, and 5.0 µL nuclease-free water in a final volume of 25 µL. This mixture was run using a PCR machine (Thermal cycler Perkin Elmer 2400) to amplify the genomic DNA. The PCR conditions used are shown in **Table I**.

Table I. PCR condition used.

PCR Condition	Allele gene		
	CYP2A6*4	**CYP2A6*7**	**CYP2A6*9**
Initial denaturation	95°C (5′)	95°C (5′)	94°C (3′)
Denaturation	98°C (20″)	95°C (20″)	94°C (30″)
Annealing	64°C (15″)	56.5°C (30″)	60°C (30″)
Extention	72°C (30″)	72°C (30″)	70°C (25″)
Cycle	30	35	35
Final extention	72°C (5′)	72°C (5′)	72°C (5′)

Methods

Research subject

It is an observational study using a cross-sectional design to analyze the *CYP2A6* polymorphism among Javanese Indonesian Smokers associated with glycohemoglobin blood levels, the main predictor for diabetes disease. Participants were enrolled between July and August 2022. They live in Yogyakarta, as indicated by their identity card. A preliminary survey was conducted to find respondents who smoked using a self-reported smoking questionnaire adopted from the Fagerström Test for Nicotine Dependence (FTND) questionnaire[18]. The participants had to meet the study's inclusion criteria: active smokers who had smoked for at least ten years, Javanese Indonesians with at least three grandparents of Javanese descent due to their self-reported, male, aged 20-50 years, weight between 46 to 75 kg, with a varying height between 150-170 cm. This study excluded the participant who had an infection at the blood sampling and was taking an

anticoagulant. All participants had agreed to participate in this study indicated by signing the informed consent. The study was approved by the Ethics Commission for General Medicine Research, Universitas Duta Wacana, Yogyakarta (No. 1413/C.16/FK/2022).

Blood sample collection

Three mL of blood was sampled from a cubital vein in all participants who had met the inclusion and exclusion criteria. Blood samples were collected in a vacutainer containing EDTA (1.8 mg/mL blood) and immediately stored in the refrigerator at 4°C.

PCR analysis

The PCR products were analyzed using electrophoresis with 1.5% agarose and evaluated using a UV transilluminator. Expressed PCR products are documented using a Polaroid camera.

Data analysis

To describe the study population and evaluate data, we used Microsoft Excel 2019. All values are displayed as the mean ±SD or number (%). We assumed $p < 0.05$ indicated significant differences. Using a box plot diagram, we also described the distribution of HbA1c levels among the subjects based on their *CYP2A6* allele gene. The chi-square test was used to analyze the relationship between *CYP2A6* polymorphism and HbA1c levels.

RESULTS AND DISCUSSION

A total of 106 participants were participating in this study. There are three groups of test subjects, based on the number of cigarettes per day (CPD) they smoked: light smokers (CPD: 1-10), intermediate smokers (CPD: 11-20), and heavy smokers (CPD: 21-30)[19]. All the respondents were smoking a white filter cigarette containing 12 mg of nicotine/cigarette. **Table II** below shows the respondent characteristics participating in this study. Based on **Table II**, 88.7% of the respondents are light and intermediate smokers, while 11.3% are heavy smokers. The Ministry of Health of the Republic of Indonesia has reported that the average CPD by Indonesian adults was 13 cigarettes or the equivalent of one pack[6]. Some of the respondents started smoking at the age of under ten years. Several factors influence smoking behavior among children and adolescents, including easy access to cigarettes, family and peer environment, and cigarette promotion/advertising[20]. All respondents had smoked for at least ten years, indicating that they had been exposed to nicotine for a long time.

Table II. Respondent characteristics.

Characteristics	Smoking Status			Total
	Light	Intermediate	Heavy	
Number (%)	43 (40.6)	51 (48.1)	12 (11.3)	106
Age				
Mean ± SD	44.4 ± 9.5	43.6 ± 11.7	45.0 ± 8.2	47.2 ± 12.9
Range	32 - 71	29 - 78	37 - 62	29 -78
First age smoking				
Mean ± SD	18.5 ± 3.8	17.2 ± 3.0	14.1 ± 6.2	17.3 ± 3.4
Range	13 - 30	13 - 27	10 - 16	10 - 27
Smoking duration				
Mean ± SD	26.3 ± 9.8	26.5 ± 11.7	29.8 ± 7.1	30.1 ± 12.5
Range	14 - 51	13 - 63	24 - 46	13 - 63
CPD				
Mean ± SD	8 ± 2	14 ± 2	24 ± 3	13 ± 5
Range	3 - 10	11 - 20	21 - 30	3 - 30

Several studies have proven that cigarette dependence can trigger the occurrence of T2DM[21,22]. Compared to non-smokers, active smokers have a 76% higher risk of developing T2DM[23,24]. Nicotine in cigarette smoke was responsible for the development of T2DM in smokers[25-27]. Nicotine in cigarettes has caused insulin resistance and reduced insulin secretion[28]. Xie *et al.*[29] has revealed that nicotine exposure in the long term will decrease insulin secretion through the activation of nAChRs present in pancreatic cells. Furthermore, Xie *et al.*[29] also mentioned that nicotine exposure for a short period (24

hours) will inhibit insulin release from the pancreas. Other studies have shown that nicotine exposure can cause pancreatic cell dysfunction and increased cell apoptosis[30,31]. Eventually, it will cause an increase in blood glucose levels and the T2DM risk factor in smokers[7].

Our study assesses the T2DM risk factor in smokers using the HbA1c blood level. Several studies have used the HbA1c parameter to control blood glucose levels[8,16,23,32]. Indonesian Endocrinology Society (*Perkumpulan Endokrinologi Indonesia, PERKENI*) stated that people with HbA1c levels <6.5 have a normal glucose level. People with HbA1c levels between 5.7% and 6.4% have prediabetes and a higher chance of getting diabetes. The diabetes condition is established if the HbA1c levels are higher than 6.5%[33]. Akkuzulu *et al.*[23] has reported a positive correlation between nicotine dependence and HbA1c levels in smokers. Several other previous studies have also revealed that compared to non-smokers, smokers have higher HbA1c levels and a 30-40% higher risk of T2DM[8,34]. Somm *et al.*[31] has revealed that nicotine administration in low doses will increase HbA1c levels by 8.8%, and at high doses, after being given nicotine for two days, increase HbA1c levels by 34.5%.

Figure 1 describes the distribution of HbA1c levels among the respondents. According to **Figure 1**, 16.04% of the respondents participating in this study had diabetes, and 13.16% were pre-diabetic. They are mainly distributed among intermediate and heavy smokers with smoking for more than 20 years. It is in line with our previous study that prediabetes among Javanese smokers will occur at a minimum CPD of 20 cigarettes with a minimum smoking duration of 25 years. Meanwhile, diabetes will occur at a minimum CPD of 20 cigarettes with a minimum smoking duration of 29 years[16]. Therefore, it is possible for respondents whose HbA1c levels <5.7 will still develop T2DM if they continue to smoke. Diabetes was an underdiagnosed disease. Approximately 30% of diabetics are often unaware of their condition, resulting in 25% of people with diabetes being diagnosed with microvascular complications. The average delay from onset to diagnosis is about seven years[35]. This study has also supported the report issued by *RISKESDAS* 2018, that only about 25% of diabetics in Indonesia know that they have diabetes[36].

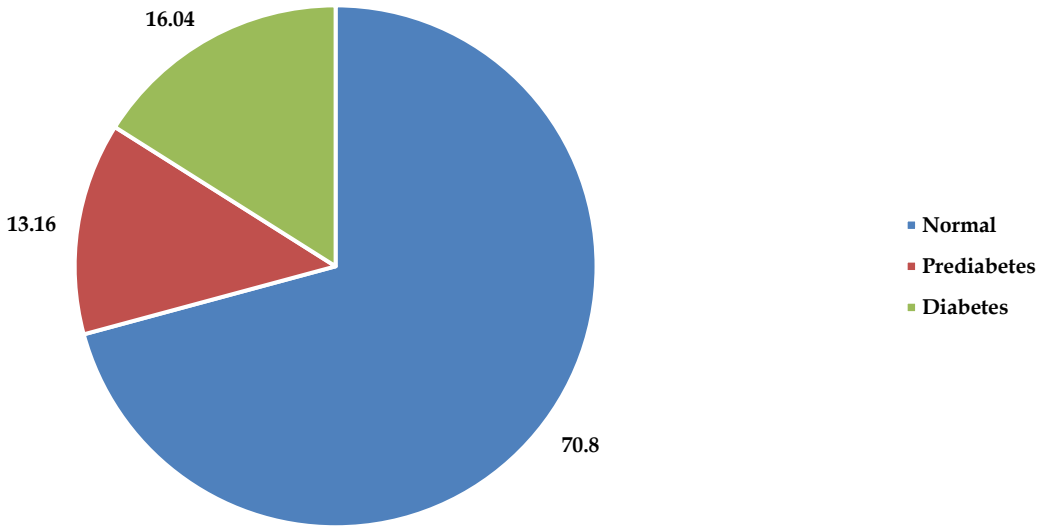

Figure 1. The HbA1c distribution among participants.

In addition, another factor that can increase the T2DM risk in a smoker is the *CYP2A6* polymorphism. The three *CYP2A6* inactive allele genes have been identified in this study: CYP2A6*4, *7, and *9. The CYP2A6*4, a whole gene deletion, due to the unequal crossover junction with CYP2A7. CYP2A6*7 occurred due to the Single Nucleotide Polymorphism (SNPs) in the 8454th nucleotide base sequence (T>C). The CYP2A6*9 allele formed due to the SNPs in the TATA box in the *CYP2A6* promoter region at the -48T>G point[37]. These three allele genes will decrease the CYP2A6 enzyme activity, either intermediate, slow, or poor metabolizer. Smokers with slow or poor metabolizers are more susceptible to suffering T2DM than fast metabolizers[38].

Table III shows that the CYP2A6*4, CYP2A6*7, and CYP2A6*9 allele frequency were 50.9%, 4.3%, and 3.8%, respectively. It is consistent with our previous studies[15,16], where the CYP2A6*4 allele frequency in Javanese was high. These allele genes will decrease the CYP2A6 enzyme activity. Several studies[39-41] have revealed that smokers with the inactive allele would slowly metabolize the nicotine compared to the active allele. Consequently, the nicotine blood level becomes higher, and the

CPD and nicotine dependence become lower. Based on the three allele genes, Peamkrasatam *et al.*[42] and Malaiyandi *et al.*[43] classified the *CYP2A6* phenotype into four groups: fast metabolizer (CYP2A6*1/*1), intermediate metabolizer (CYP2A6*1/*4; CYP2A6*1/*7, CYP2A6*1/*9), slow metabolizer (CYP2A6*4/*7; CYP2A6*4/*9, CYP2A6*7/*9), and poor metabolizer (CYP2A6*4/*4). As shown in **Table III**, only four (3.8%) smokers are fast metabolizers, and most smokers are intermediate metabolizers (74.5%), while the rest are slow and poor metabolizers (21.7%).

Table III. *CYP2A6* genotype and allele frequency among respondents.

Allele	Frequency (n = 212)	Genotype	Frequency (n = 106)
*CYP2A6*1*	41% (87)	*CYP2A6*1/*1*	3.8% (4)
*CYP2A6*4*	50.9% (108)	*CYP2A6*1/*4*	74.5% (79)
*CYP2A6*7*	4.3% (9)	*CYP2A6*4/*7*	8.5% (9)
*CYP2A6*9*	3.8% (8)	*CYP2A6*4/*9*	7.5% (8)
		*CYP2A6*4/*4*	5.7% (6)
Total	**100%**	**Total**	**100%**

Figure 2 describes the distribution of HbA1c levels among the respondent based on their phenotype. **Figure 2** shows that all participants with fast metabolizers and most intermediate metabolizers had HbA1c levels <5.7. There are only 10 participants with intermediate metabolizers had HbA1c >5.7. In the slow metabolizer, two people have HbA1c values <5.7, and the rest have >5.7. On the other hand, all participants with poor metabolizers have HbA1c levels >5.7, indicating that they have diabetes condition. It is in line with another study[44] that heavy smokers with slow and poor metabolizers would have a high risk of developing T2DM compared to light smokers with fast and intermediate metabolizers. Furthermore, we used a chi-square test to analyze the effect of the inactive alleles on the HbA1c levels among the participants.

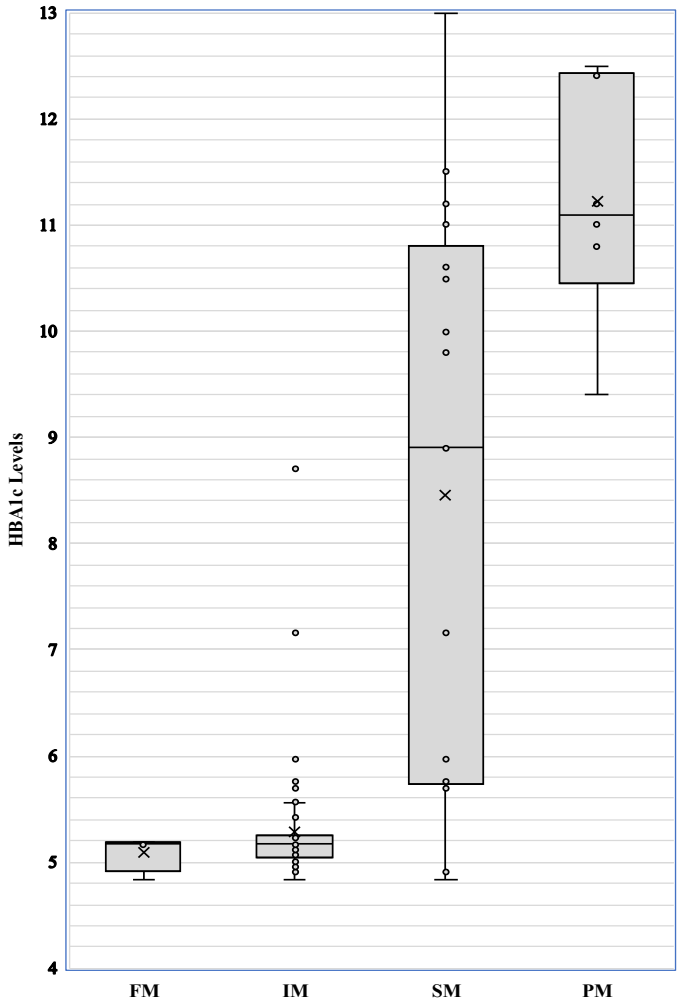

Figure 2. HbA1c levels distribution among the test subjects according to their genotype. **FM**: fast metabolizers; **SM**: slow metabolizers; **IM**: intermediate metabolizers; **PM**: poor metabolizers.

As shown in **Table IV**, due to its p-value (0.000 <0.005) and χ^2 (54.6) with df=1, it is known that *CYP2A6* polymorphism could have affected the HbA1c levels among the participants. The homozygous and heterozygous *4, *7, and *9 among smokers would increase the risk of HbA1c levels in smokers. CYP2A6 enzyme encoded by *CYP2A6* is the enzyme corresponding to nicotine inactivation. The inactive metabolites of nicotine excreted in the urine are cotinine and trans-3-hydroxycotinine[45]. Therefore, heavy smokers with slow or poor metabolizers tend to have higher nicotine plasma levels than light smokers with fast or intermediate metabolizers. Several studies have revealed that smokers may increase the risk of T2DM, indicated by an increase in HbA1c levels. It is due to pancreatic β cell dysfunction and insulin resistance[34,46].

Table IV. The relationship between *CYP2A6* polymorphism to HbA1c values among participants.

***CYP2A6* polymorphism**	**HbA1c levels (n, %)**		**Total**	**p-value (*V*)**	χ^2 **(df)**
	<5.7	**>5.7**			
Homozigote **1/*1* and heterozigote **1/*4*	73 (88%)	10 (12%)	83 (100%)	0.000 (0.718)	54.6 (1)
Homozigote and heterozigote **4, *7, *9*	2 (8.7%)	21 (91.3%)	23 (100%)		
Total	**75 (70.8%)**	**31 (29.2%)**	**106 (100%)**		

CYP2A6 is also known as the enzyme responsible for nitrosamine metabolic activation, the pre-carcinogen compound in tobacco smoke, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), *N*'-nitrosonornicotine (NNN), *N*'-nitrosoanabasine (NAB), and *N*'-nitrosoanatabine (NAT)[47]. Therefore, smokers with slow or poor metabolizers could reduce the hepatic first-pass clearance of tobacco nitrosamines, resulting in greater exposure to other organs, such as the pancreas, due to its higher systemic levels[48]. The increased exposure of nitrosamine in pancreatic islet cells could lead it's the metabolic activation by other cytochrome P450 enzymes (CYPs), including CYP2E1[47], resulting in inflammation and apoptosis of pancreatic cells, which is furthermore might decrease insulin secretion and the increased risk of developing T2DM[49].

According to Bergman *et al.*[50], insulin sensitivity will recover in a smoker who has quit smoking; therefore, to prevent diabetes, a smoker must stop smoking. It is also supported by other studies[51-53] on preventing T2DM among smokers through smoking cessation strategies. Several studies[54-56] have also shown that smokers who have inactive alleles tend to quit smoking more easily. Therefore, to increase efforts to reduce the prevalence of diabetes in Indonesia, cooperation from various parties is needed to reduce cigarette consumption in Indonesia. *RISKESDAS* in Indonesia has reported that the number of smokers over 15 years of age was 33.8%, of which 62.9% were male and 4.8% were female[57]. In addition, The Southeast Asia Tobacco Control Alliance (SEATCA) in The Tobacco Control Atlas has reported that the number of smokers in Indonesia was 65.19 million, placing Indonesia as the highest number in Southeast Asia[58]. Therefore, based on our study, we suggest promoting smoking cessation campaigns is the best effort to reduce cigarette consumption and diseases related to cigarettes, such as T2DM, stroke, and coronary heart disease.

Quite a few limitations of our study are: this was a cross-sectional study, the causal association between *CYA2A6* polymorphism and HbA1c levels should be interpreted carefully; we used self-report surveys to collect the data regarding smoking behavior, thus it might have been caused bias data; the other inactive allele of *CYP2A6* might be reduced *CYP2A6* activity resulting in the alteration of phenotype, primarily on fast and intermediate metabolizers; and some confounding factor, including obesity, physical activity, and dietary factors have not fully accounted in our analysis.

CONCLUSION

In conclusion, this study reveals that the heterozygote CYP2A6 alleles, including *4, *7, and *9, corresponding to slow and poor metabolizers, may worsen HbA1c levels among Javanese Indonesian smokers. Furthermore, due to our result, it may be crucial for the government to encourage smoking cessation programs in Indonesia, which are trusted to prevent various health problems, especially diseases related to smoking behavior, including T2DM, stroke, and coronary heart disease.

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AUTHORS' CONTRIBUTION

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DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Marine Sponge *Xestospongia sp.*: A Promising Source for Tuberculosis Drug Development - Computational Insights into Mycobactin Biosynthesis Inhibition

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Abstract

Mycobacterium tuberculosis (MTB) remains the leading cause of infection, with a significant fatality rate, owing primarily to drug resistance. MTB contains the enzyme salicylate synthase, which regulates mycobactin production to bind iron ions from the host cell, facilitating the bacteria to grow and reproduce. This study investigates the potential of marine sponges to inhibit the MTB salicylate synthase by exploiting a computational approach combining molecular docking and dynamics simulations. Forty-six compounds from *Xestospongia sp.* were chosen from the Marine Natural Products database. The docking results selected four compounds (CMNPD15071, CMNPD7640, CMNPD26706, and CMNPD7639) from this sponge, which provide more negative binding energy than their inhibitors (RVE). After reclassifying their interactions, such as hydrophobic and hydrogen bonds, CMNPD15071 (Sulfuric acid mono-(8-methoxy-12b-methyl-6-oxo-2,3,6,12b-tetrahydro-1H-5-oxa-benzo[k]acephenanthrylen-11-yl) ester) and CMNPD7640 (secoadociaquinone B) performed molecular dynamics simulations to assess their stability. These two compounds show a promising stability profile compared to RVE based on RMSD, RMSF, SASA, and gyration analysis. Furthermore, the binding affinity prediction of these two compounds using the MM/GBSA calculation method reveals that CMNPD15071 (-38.48 kJ/mol) had the highest affinity for binding to MTB salicylate synthase compared to RVE (-35.36 kJ/mol) and CMNPD7640 (-26.03 kJ/mol). These findings demonstrate that compounds from *Xestospongia sp.* can block MTB mycobactin biosynthesis by inhibiting salicylate synthase.

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INTRODUCTION

Mycobacterium is a worldwide endemic bacterium involving non-pathogenic and pathogenic species associated with infection in many organisms, especially humans and animals[1,2]. *Mycobacterium tuberculosis* (MTB) is a respiratory infection thought to infect one-quarter of the world's population, and as a bacteria, it has killed more individuals than any other in human history[3]. Around 10.6 million tuberculosis (TB) cases will occur in 2021, with up to 1.6 million people dying globally[4,5]. Tuberculosis therapy is still a challenge, in part due to drug resistance[6]. As a response, discovering and developing drugs to overcome this disease remains urgently needed[7]. Salicylate synthase from MTB is one of the most appealing targets for developing and identifying new anti-TB drugs[8].

Salicylic synthase is responsible for the biosynthesis of mycobactin MTB by converting chorismate to salicylic acid[9]. Mycobactin is a small molecule (siderophore) synthesized by MTB that binds iron ions from host proteins such as transferrin

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and lactoferrin. MTB requires iron ions to replicate hence inhibiting salicylate synthase activity in mycobactin production is a promising target for anti-TB drugs[10,11]. In addition, mycobactin is not present in host cells, so inhibiting this enzyme can be a potential drug target[12]. A recent study revealed the potential for inhibition of MTB salicylate synthase by 5-phenylfuran-2-carboxylate and chromane derivatives with inhibition concentrations (IC_{50}) of 250 µM and 55 µM, respectively[13,14]. However, due to a lack of information on inhibitors of this enzyme, the search for compounds that can inhibit salicylate synthase from MTB is still essential, particularly those derived from natural sources.

The marine is a natural resource with high biodiversity and rich in active chemicals spread in various marine ecosystems and can be developed into medicine[15,16]. Marine natural resources such as mollusks, algae, and sponges offer a high potential for development as pharmaceutical raw materials[17]. *Xestospongia sp*. is a species of sponge that has been shown to have anti-inflammatory, antioxidant, antibacterial, antifungal, antiviral, and anticancer activities[18]. Because the activity of *Xestospongia sp*. against MTB is still relatively limited, this investigation was conducted to explore and study the anti-TB activity of bioactive compounds from *Xestospongia sp*. molecularly utilizing a computational study approach. This research is also expected to obtain the lead compounds as inhibitors of the salicylate synthase enzyme from MTB.

MATERIALS AND METHODS

Materials

Enzyme structures

The structure of MTB salicylate synthase (PDB ID: 3ST6) crystallized with native ligand (RVE) was considered due to its high resolution (1.75 Å) (https://www.rcsb.org/)[19]. The crystallographic structure was created by omitting the protein's B, C, and D chains and the associated residues, such as water molecules, to verify the quality of the docking procedure[20]. Lastly, polar hydrogen atoms and Kollman charges were adjusted to the target protein using AutoDock Tools v.1.5.6[21].

Test compounds structures

Forty-six *Xestospongia sp*. compounds were collected from the Comprehensive Marine Natural Products Database (https://www.cmnpd.org/)[22]. The test compounds were selected based on the 400-500 g/mol molecular weight range. All test compounds' structures were converted into *pdbqt format using Open Babel[23]. Bound inhibitor (RVE) was employed as a control to compare with the test compounds.

Methods

Molecular docking study

Molecular docking was performed using Autodock software to determine the binding affinity and interactions between compounds from *Xestospongia sp*. against MTB salicylate synthase[24]. The docking process was confirmed to the enzyme binding site using the redocking method by calculating the root mean square deviation (RMSD) of the RVE conformation, which must be less than 2 Å[25]. The test compounds were docked following the RVE binding coordinates with a cubic conformational search area of 40 Å. The docking technique involves the Genetic Algorithm, performed up to 100 times run. The population was limited to 150, with a maximum of 2,500,000 number evaluations. The other docking algorithm and parameters were left as default settings.

Enzyme-compound interactions analysis

The best compound's conformation from the docking process was continued to the interaction analysis stage. Discovery Studio Visualizer v17.2.0.16349 software was used to study and depict hydrogen bonds and hydrophobic interactions produced between enzymes and the best compounds.

Molecular dynamics simulation

Molecular dynamics (MD) simulations were carried out using the GROMACS 2022 package[26]. Protein topology was prepared using AMBER99SB-ILDN[27], and ligand topology was designed using the General AMBER Force Field (GAFF)[28] generated with the help of ACPYPE[29]. This simulation was carried out in an aqueous environment as a cubic box using the TIP3P water molecule model. The neutral system was obtained after adding Na^+ and Cl^- ions[30,31]. An equilibrium system

consisting of protein, ligand, solvent, and ions was received after simulating NVT and NPT at 300 K with a pressure of 1 bar[32]. The production system lasts for 50 ns. The simulation results were analyzed using the RMSD, root mean square fluctuation (RMSF), solvent accessible surface area (SASA), and radius of gyration (Rg) criteria. Lastly, the binding affinity was calculated using the MM/GBSA method approach.

Data analysis

For molecular docking, the RMSD value was calculated by measuring the distance of RVE's heavy atom between the crystal conformation overlapped with the conformation after the redocking process[33]. For molecular dynamics, the analysis focused on assessing the stability of the salicylate synthase and compound complexes based on RMSD and RMSF criteria. The measurement of Rg was used to examine the protein folding during the simulation, which correlates with the complex's stability[34]. A lower SASA value indicates a more stable ligand-receptor complex. Lastly, the effectiveness of chemical constituents from *Xestospongia sp.* in binding with MTB salicylate synthase was assessed by calculating their binding energy using MM/GBSA approach.

RESULTS AND DISCUSSION

Molecular docking study

Mycobacterium tuberculosis remains a primary infectious disease with a high mortality rate in every country. Therefore, searching for compounds that can be candidates for anti-tuberculosis drugs is still very much needed. Computational studies using molecular docking methods are essential in accelerating drug development, especially in finding lead compounds[35]. This study tries to reveal the potential of *Xestospongia sp*. to inhibit mycobactin biosynthesis in MTB.

Based on the validation results, the redocking technique obtained an RMSD RVE value of 0.47 Å (**Figure 1**). This RMSD value was calculated by measuring the distance of RVE's heavy atom between the crystal conformation overlapped with the conformation after the redocking process. The best RVE conformation from the redocking results has a bond energy of -9.17 kcal/mol. This conformation's hydroxyl and carbonyl groups form hydrogen bonds with residues Gly270, Tyr385, Arg405, Gly419, Gly421, and Lys438 on the active site of MTB salicylate synthase[19]. In addition, the RVE benzene ring exhibits hydrophobic interactions with Leu268 and His334.

A total of 30 compounds from *Xestospongia sp*. gave a binding energy range from -0.15 to -9.98 kcal/mol, and as many as 16 compounds do not provide binding affinity. This binding affinity indicates the stability of the binding between the ligand and the target protein[36]. Two potential compounds from this marine sponge are CMNPD7640 (secoadociaquinone B) and CMNPD15071 (sulfuric acid mono-(8-methoxy-12b-methyl-6-oxo-2,3,6,12b-tetrahydro-1H-5-oxa-benzo [k]acephenanthrylen-11-yl) ester) (**Figure 2**) has a better binding affinity than RVE and binds to the active site of salicylate synthase with energies of -9.98 and -9.93 kcal/mol, respectively. In addition, these two compounds are also estimated to have an inhibition constant of 52.29 nM for CMNPD7640 and 48.59 nM for CMNPD15071.

Interestingly, these two compounds share a similar basic framework, being quinone derivatives, and exhibit a similar interaction pattern with RVE. The CMNPD7640 and CMNPD15071 compounds exhibit similar interactions, including hydrogen bonding with residues Lys205, Gly270, Thr271, His334, Glu431, and Lys438, as well as hydrophobic interactions with two residues Ala269 and Ile423 on the binding site of MTB salicylate synthase. Furthermore, unique hydrogen bonding was observed in the CMNPD7640 sulfonate groups that interacted with Arg405. Meanwhile, the sulfonate group of CMNPD15071 interacts with Ser301 (**Figure 2**). The 2D structure of the best-identified compound from *Xestospongia sp*. is depicted in **Figure 3**, as determined by molecular docking results.

Molecular dynamics simulation

The stability of the top two hit compounds obtained from the molecular docking process was verified through a 50 ns MD simulation. The MD trajectory was utilized to compute the RMSD of the entire complex system[37] and the corresponding graph is depicted in **Figures 4A-C**. During the simulation, it was observed that the salicylate synthase complex with the RVE inhibitor exhibited the highest stability, as indicated by an average RMSD of 0.606 nm. When the salicylate synthase interacted with the compound CMNPD15071, it demonstrated even better stability than CMNPD7640, with average RMSD

values of 0.648 and 0.689 nm, respectively. Interestingly, the compound CMNPD15071 derived from *Xestospongia sp*. exhibited exceptional stability with a low RMSD of 0.163 nm. Moreover, the RVE inhibitor and CMNPD7640 also displayed reasonably stable interactions with RMSD average values of 0.179 and 0.325 nm, respectively.

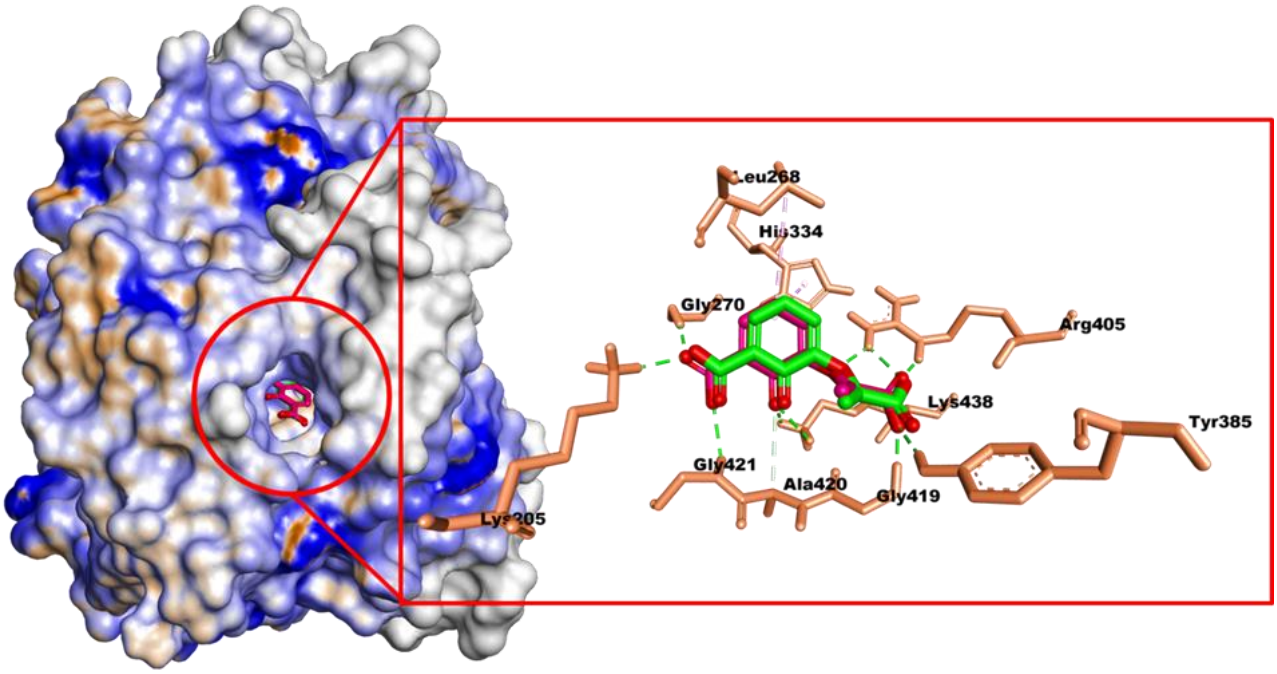

Figure 1. Visualization of the RVE crystallographic conformation (green) overlapping with the redocking conformation (pink) on the active site of MTB salicylate synthase. Dashed lines in green and pink indicate hydrogen bonds and hydrophobic interactions.

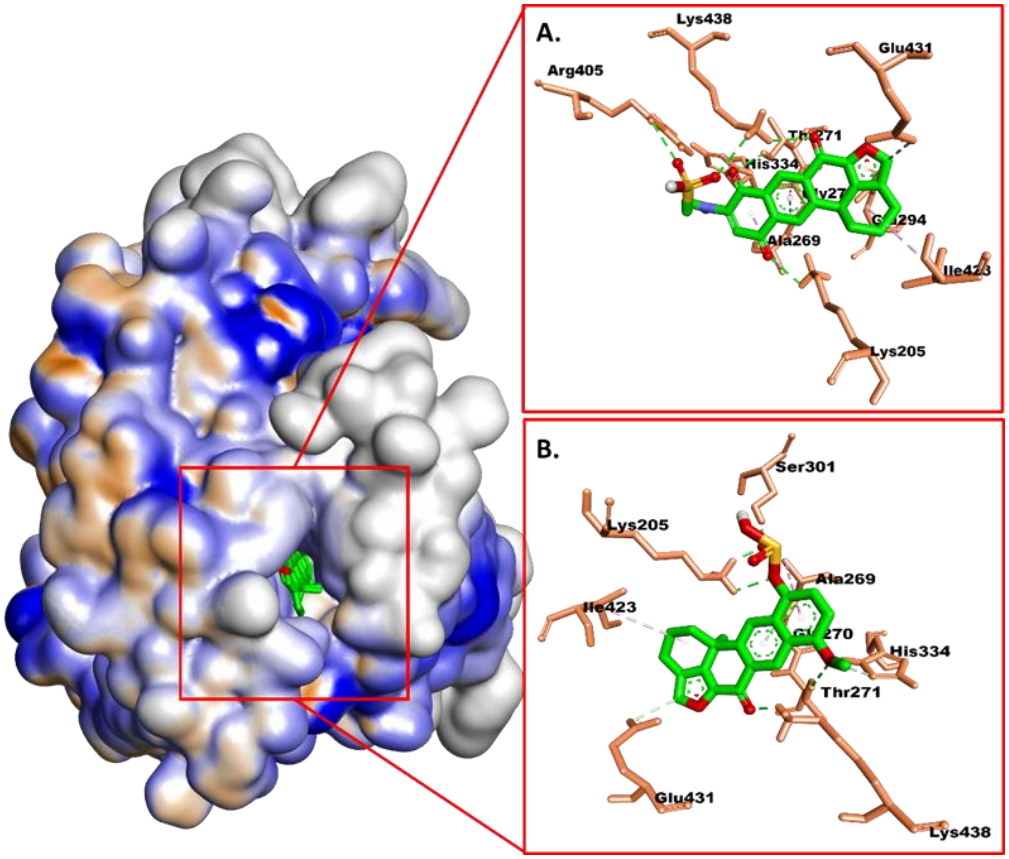

Figure 2. Molecular interactions of the best compounds from the *Xestospongia sp*. on the active site of the MTB salicylate synthase. (**A**) CMNPD7640 and (**B**) CMNPD15071.

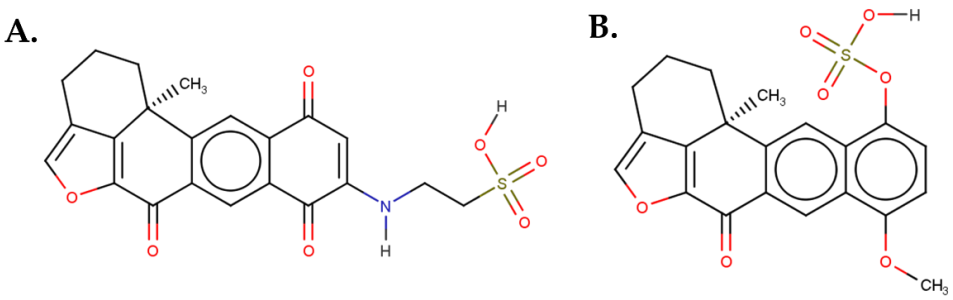

Figure 3. 2D structures representation of (**A**) CMNPD7640 and (**B**) CMNPD15071.

Meanwhile, during the simulation, the RMSF value for each complex was recorded, and the corresponding graph is shown in **Figure 4D**. This RMSF graph provides insights into the fluctuation of amino acid residues in the salicylate synthase over the 50 ns simulation period. Notably, all complexes exhibited a similar trend of fluctuating amino acid residues, with certain residues showing high-intensity oscillations. Specifically, residues Ala7, Pro278, Lys293, Ser331, and Gly412 displayed significant fluctuations. Notably, residues 293-304 demonstrated higher fluctuations than other compounds. However, the RMSF graph also highlighted a compelling observation: CMNPD15071 exhibited the ability to stabilize the amino acid residues of MTB salicylate synthase, resulting in lower fluctuations compared to other compounds. This finding suggests that CMNPD15071 may form more stable interactions with the salicylate synthase, potentially contributing to its inhibitory potential against MTB.

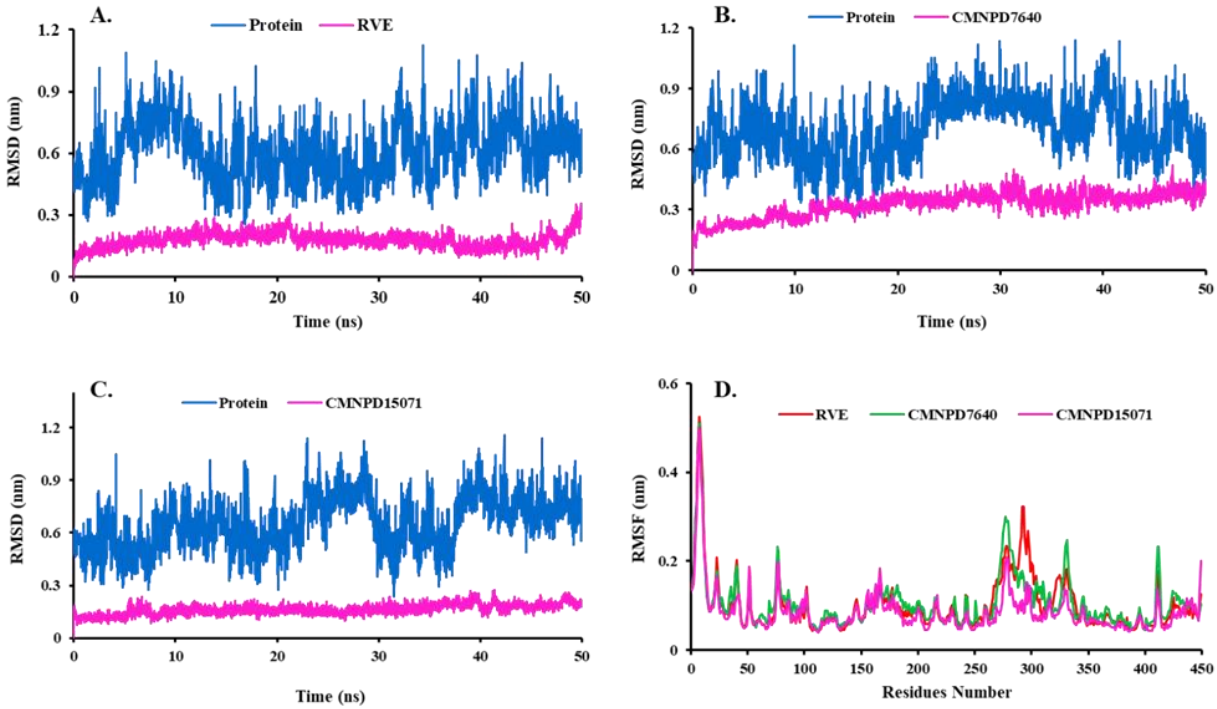

Figure 4. Evaluation of the RMSD criteria for the salicylate synthase-compounds complex of (**A**) protein-RVE, (**B**) protein-CMNPD7640, (**C**) protein-CMNPD15071, and (**D**) RMSF MTB salicylate synthase backbone during 50 ns MD simulation.

The plot of Rg is presented in **Figure 5A**. The lowest Rg value indicated the most stable compound in the complex with salicylate synthase. The best-hit compound was observed to have the same protein folding stability during the simulation[38]. Based on the analysis results, the average Rg values of the CMNPD15071 and CMNPD7640 complexes were 2.228 and 2.229 nm, respectively, lower than the RVE Rg value of 2.234 nm. These findings show that the CMNPD15071 complex has the lowest Rg value and higher cohesiveness than other hit compounds.

To comprehensively investigate the stability of each hit compound complex, we conducted a SASA analysis for each ligand. This analysis provides valuable insights into the complex's folding and stability[39] by monitoring variations in the protein solvent area during the simulation (**Figure 5B**). The SASA analysis graph revealed that all hit compounds exhibited a similarly wide range of areas accessed by solvent molecules. The average SASA values for each complex were 196.87, 195.24, and 194.15 nm^2 for RVE, CMNPD7640, and CMNPD15071, respectively. Interestingly, CMNPD15071 demonstrated exceptional stability compared to the other compounds, as evidenced by its SASA area remaining relatively unchanged throughout the experiment. This suggests that CMNPD15071 forms vigorous interactions with the salicylate synthase, enhancing its stability within the complex.

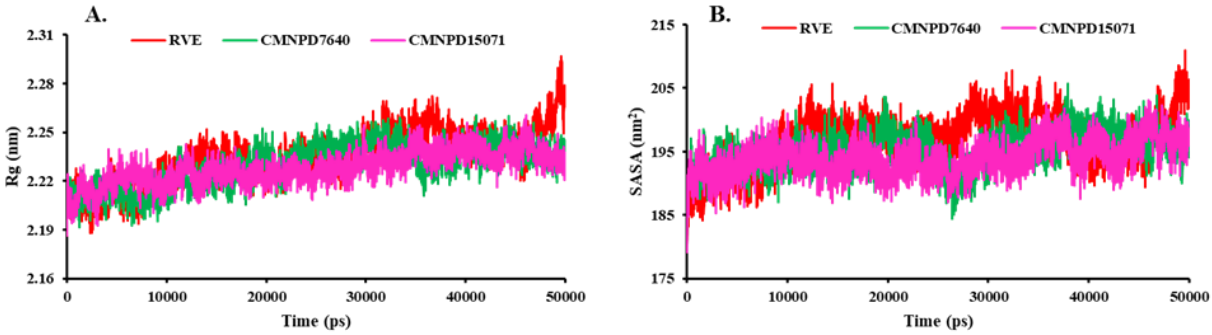

Figure 5. Evaluation of (**A**) Rg and (**B**) SASA graph during 50 ns MD simulations.

The effectiveness of chemical constituents from *Xestospongia sp*. in binding with MTB salicylate synthase was assessed by calculating their binding energy using the MM/GBSA approach. This influential computational tool delves into the molecular-level interactions between ligands and the receptor's active site, providing valuable insights into the stability and affinity of potential drug candidates as anti-TB agents[40]. The total binding energy (ΔE_{Bind}) for the RVE system, which serves as a known inhibitor, was determined to be -35.36 kJ/mol. This value reflects the totality of various interactions, including electrostatic, van der Waals, and solvation energies, which contribute to the overall stability of the ligand-receptor complex[41]. Interestingly, the energy of CMNPD15071 was more negative at -38.48 kJ/mol, indicating that this compound may have a stronger binding affinity to MTB salicylate synthase than the RVE inhibitor, suggesting that it may form highly stable interactions with the target enzyme. On the other hand, CMNPD7640 exhibited a more positive binding energy of -26.03 J/mol than RVE. In **Table I**, we presented a comprehensive set of calculated binding energies for each system, providing a detailed comparison of the potential inhibitory capabilities of the compounds from *Xestospongia sp.* against MTB salicylate synthase.

To understand the molecular-level details, we analyzed the individual energy components contributing to the overall binding stability. The electrostatic energy (ΔE_{ELE}) arises from the electrostatic interactions between charged residues in the active site of MTB salicylate synthase and the ligand[42]. In the RVE system, this energy was slightly positive at 70.05 kJ/mol, suggesting a net repulsion between the ligand and the receptor. However, CMNPD15071 exhibited a significantly more negative value of -47.56 kJ/mol, indicating attractive electrostatic interactions with specific residues in the active site. Likewise, CMNPD7640 displayed a highly negative electrostatic energy of -74.75 kJ/mol, indicating strong attractive forces between the ligand and the enzyme. This result may be attributed to interactions formed by *Xestospongia sp*. compounds with positively charged residues of Lys205 and His334, as well as negatively charged Glu431, contributing to the electrostatic energy when binding to MTB salicylate synthase.

Conversely, the electrostatic contribution to the solvation energy (ΔE_{GB}) considers the interactions of the ligand with the solvent molecules in the surrounding environment[43]. The RVE system demonstrated a relatively negative value of -79.07 kJ/mol, indicating a favorable solvation effect that promotes binding. However, both CMNPD15071 and CMNPD7640 showed positive values (61.3 and 89.91 kJ/mol, respectively), suggesting that these compounds may experience less favorable solvation effects when binding to MTB salicylate synthase. Nevertheless, the positive solvation contribution does not negate their potential as inhibitors, as other strong interactions contribute to their overall binding affinity[44].

Furthermore, the van der Waals energy (ΔE_{VDW}) was pivotal in the favorable binding energy[45]. CMNPD7640 and CMNPD15071 displayed highly negative van der Waals energies (-35.6 and -45.69 kJ/mol, respectively), indicating strong

attractive forces between the ligand and the enzyme's hydrophobic pockets. These values were significantly more negative than the RVE system's van der Waals energy (-22.33 kJ/mol), implying that the *Xestospongia sp*. compounds may form tighter and more stable interactions within the active site of MTB salicylate synthase.

Table I. The MM/GBSA binding energy calculated for the RVE and chemical constituents from *Xestospongia sp*.

Energies (kJ/mol)	RVE	CMNPD7640	CMNPD15071
ΔE_{VDW}	-22.33	-35.60	-45.69
ΔE_{ELE}	70.05	-74.75	-47.56
ΔE_{GB}	-79.07	89.91	61.30
ΔE_{SURF}	-4.01	-5.59	-6.53
ΔE_{Bind}	-35.36	-26.03	-38.48

In summary, this comprehensive computational analysis provides valuable molecular insights into the potential inhibitory capabilities of chemical constituents from *Xestospongia sp*. against MTB salicylate synthase. The study highlights CMNPD15071 and CMNPD7640 as promising candidates for further investigation and development as potential therapeutic agents against tuberculosis. Moreover, these findings underscore the significant potential of marine natural products in the quest for novel anti-TB drugs, setting the stage for further experimental validations in drug development.

CONCLUSION

This study succeeded in identifying CMNPD15071 (sulfuric acid mono-(8-methoxy-12b-methyl-6-oxo-2,3,6,12b-tetrahydro-1H-5-oxa-benzo[k]acephenanthrylen-11-yl) ester) and CMNPD7640 (secoadociaquinone B) from *Xestospongia sp*. which can inhibit mycobactin biosynthesis based on their affinity and interaction to MTB salicylate synthase. However, further research based on molecular dynamics studies showed that the CMNPD15071 has the potential as a lead compound for the salicylate synthase inhibitor of MTB. This finding can be an impetus for future investigations for antimicrobial agents against MTB.

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AUTHORS' CONTRIBUTION

Conceptualization: Arfan, Aiyi Asnawi, La Ode Aman
Data curation: Arfan
Formal analysis: Arfan
Funding acquisition: Arfan, Aiyi Asnawi, La Ode Aman
Investigation: Arfan, Aiyi Asnawi, La Ode Aman
Methodology: Aiyi Asnawi, La Ode Aman
Project administration: Arfan, Aiyi Asnawi, La Ode Aman
Resources: Aiyi Asnawi, La Ode Aman
Software: Aiyi Asnawi, La Ode Aman
Supervision: Aiyi Asnawi, La Ode Aman
Validation: Arfan
Visualization: Arfan
Writing - original draft: Arfan, Aiyi Asnawi, La Ode Aman
Writing - review & editing: Arfan, Aiyi Asnawi, La Ode Aman

DATA AVAILABILITY

All data related to this study are included herein.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Total Flavonoid Levels in *n*-hexane and Ethyl Acetate Fractions of *Rosmarinus officinalis* L. Leaves and Their Antibacterial and Antioxidant Activities

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Abstract

The rosemary (*Rosmarinus officinalis* L.) is a plant of the Lamiaceae tribe that has not been widely studied regarding its pharmacological activity, known from previous studies to contain secondary metabolites of flavonoids. Flavonoids are phenol compounds with many pharmacological activities, including antibacterials and antioxidants. This study aims to determine the total flavonoid levels in *R. officinalis* leaves and their effect on antibacterial and antioxidant activities. This research began with the preparation of ethanol extract from *R. officinalis* leaves, then the fractionation of the extract produced *n*-hexane and ethyl acetate fractions. Total flavonoid levels were determined against both fractions by UV-Vis spectrophotometry. A test of the fraction's antibacterial activity against *Staphylococcus aureus* was performed using the disc diffusion method. The antioxidant test is carried out by the DPPH method. The total flavonoid content of the ethyl acetate fraction is 47.437 ± 1.947%, higher than the *n*-hexane fraction. Test antibacterial and antioxidant activity showed more significant results in the ethyl acetate fraction than in the *n*-hexane fraction. In conclusion, the total flavonoid levels of ethyl acetate fraction are directly proportional to the antibacterial and antioxidant activities of *R. officinalis* leaves.

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INTRODUCTION

Rosemary (*Rosmarinus officinalis* L.) is one of the herbal plants belonging to the Lamiaceae family. In Indonesia, *R. officinalis* leaves have yet to be widely studied and are only known as a seasoning and food preservative. However, in several studies that have been carried out on *R. officinalis* leaves, it is known that the content of secondary metabolites is owned, one of which is flavonoids[1-3]. Flavonoid is one of the secondary metabolite compounds that belong to the phenol group and are found in all parts of the plant and have a C6-C3-C6 core structure[4].

Generally, flavonoids will bind to sugars, forming glycosides, which cause these compounds to dissolve more easily in polar solvents, such as methanol, ethanol, butanol, and ethyl acetate. In the form of glycans of a less polar nature, flavonoids will be more easily soluble in non-polar solvents, such as chloroforms and ethers[5,6]. The pharmacological activities of flavonoid compounds are very diverse, among which the most common is their activity as antibacterial and antioxidant. Flavonoids inhibit bacterial growth by damaging the cytoplasmic membrane, inhibiting protein synthesis from bacterial cell walls, nucleic acid synthesis, and energy metabolism[7].

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Staphylococcus aureus bacteria are one type of Gram-positive bacteria with a round and colonized shape, so they resemble the shape of grapes. This bacterium is also a normal skin and upper respiratory tract flora that can cause infectious diseases[8]. To treat bacterial infections, medical personnel will provide antibiotic therapy. However, the risk of antibiotic resistance events also increases with the increase in the number of infections that occur every year and the improper use of antibiotics. This is associated with the presence of factors such as improper use of the drug and improper length of use[9]. So, in this study, the antibacterial activity of *R. officinalis* leaves was tested against *S. aureus* growth.

A free radical is a compound or molecule with one or more unpaired electrons on its outer orbital. The presence of unpaired electrons causes the compound to be very reactive in looking for a partner, by attacking and binding to the electrons of the molecules around it. If the electrons bound by the free radical compound are ionic, the impact that arises is not so dangerous, but if free radicals from covalent bond compounds bind the electrons, it will be very dangerous[10]. The number of free radicals exceeding antioxidants will cause an imbalance between free radicals and endogenous antioxidants, known as oxidative stress. Oxidative stress conditions cause the body to need an intake containing a compound, antioxidants, that can capture and neutralize these free radicals so that further reactions that cause oxidative stress can stop and cell damage can be avoided or the induction of a disease can be stopped. The content of plants, such as flavonoids, can be used as a substitute for antioxidants that are disturbed in the body[11]. This research did qualitative and quantitative measurements of flavonoid presence in *R. officinalis* leaves and tested its antioxidant activity.

However, from the studies that have been carried out on *R. officinalis* leaves, no one has tested the pharmacological activity of *R. officinalis* leaves fractions. Therefore, in this study, fractionation of *R. officinalis* leaves extract was carried out using semi-polar solvents (ethyl acetate) and non-polar solvents (*n*-hexane). Research on herbal plant fractions showed more significant pharmacological activity than plant extracts. Guleria *et al*.[12] found that total phenolic and total flavonoid content was higher in ethyl acetate fraction than in ethanol extract of *Terminalia chebula*, and antibacterial activity was also higher in ethyl acetate fraction with a more expansive zone of inhibition against the bacteria. Aisyah *et al*.[13] found that ethyl acetate fraction and hexane faction had a higher antioxidant activity than the ethanol extract of *Angiopteris ferox* Copel. This study aimed to determine the total flavonoid levels of the n-hexane fraction and the ethyl acetate fraction of *R. officinalis* leaves extract from Bali, Indonesia. It also aimed to determine the influence of flavonoid levels on the antibacterial and antioxidant activity of each fraction.

MATERIALS AND METHODS

Materials

Plant materials were obtained from Bali (Bedugul-Tabanan Regency). Identification of the plant was carried out by the Indonesian Institute of Science, Bali Botanic Garden, Bali, Indonesia, with the voucher specimen number B-305/IPH.7/AP/XI/2020. *Staphylococcus aureus* FNCC 0047 isolates from Universitas Gadjah Mada, Indonesia. Another materials including Nutrient Agar (NA; Merck), Nutrient Broth (Merck), distilled water, 96% ethanol, *n*-hexane, ethyl acetate, NH3, CH3COOH, *n*-butanol, Mayer's reagent, KI, HCl, NaOH, chloroform, Na_2CO_3, K2Cr2O7, H_2SO_4, $FeCl_3$, $AlCl_3$, potassium acetate, violet crystalline solution, iodine solution, safranin solution, immersion oil, quercetin, 5% tween 80, and 3% amoxicillin. Instrument used was glassware (Iwaki), Petri dish, test tube (Iwaki), digital balance (Ohaus), split funnel, thin-layer chromatography (TLC) plate, TLC chamber (Camag), oven (Binder), autoclave, incubator (Binder), UV lamp, LAF Cabinet, and UV-Vis spectrophotometry.

Methods

Preparation of ethanol extract

A total of 600 g of simplicia was macerated with 96% ethanol, then allowed to stand 3 x 24 hours, which was occasionally stirred, filtered using flannel, and then the first filtrate was evaporated. The maceration residue was added 96% ethanol solvent back, allowed to stand for 3 x 24 hours, and stirred occasionally. The results of the re-maceration were filtered with a flannel cloth, and then the second filtrate was evaporated, and later, a thick extract of *R. officinalis* leaves ethanol was obtained[14].

Ethanol-free by color test

A total of 2 g of extract was added to 2 drops of concentrated H_2SO_4 and 1 mL of potassium dichromate solution. The ethanol content in the extract was indicated by a color change from orange to bluish-green[15].

Fractionation of ethanol extract from R. officinalis leaves

A total of 10 g of *R. officinalis* leaves ethanol extract was dissolved in 50 mL of distilled water, then added to 20 mL of 96% ethanol solvent, and filtered using filter paper. The filtration results were put into a split funnel, 100 mL *n*-hexane was added, shaken slowly for 5 minutes, and allowed to stand until the *n*-hexane and water layers were separated. The *n*-hexane layer in the upper layer was taken, and then the water layer at the bottom was repartitioned until the layer was color-cleared using *n*-hexane solvent. The process was then repeated with ethyl acetate solvent[16].

Phytochemical screening of flavonoid test by color reaction test

The extract was weighed at 0.2 g, then 10 mL of distilled water was added. The solution was heated for 5 minutes, cooled, then filtered. Five mL of filtrate was taken and put into a test tube, then added 0.1 g of magnesium powder, 1 mL of concentrated HCl, and 2 mL of amyl alcohol, then shaken and separated[17].

Flavonoids screening by TLC

The *n*-hexane and the ethyl acetate fractions were dissolved with solvents according to each fraction. The fraction was spotted on the TLC plate with a size of 4 x 10 cm at a distance of 1.5 cm from the bottom edge of the TLC plate using capillary pipes. The TLC plate was dried and diluted using an eluent of *n*-butanol : glacial acetic acid : distilled water with a ratio of 4 : 1 : 5 and a total volume of 20 mL. The elusion TLC plate was detected under a UV lamp of 254 and 366 nm, and then the appearance of spots using ammonia vapor was carried out[18,19].

Total flavonoid levels of n-hexane and ethyl acetate fractions

The mother liquor was prepared by weighing 10 mg of quercetin and then dissolving it with 10 mL of ethanol. Furthermore, a concentration series solution was made using a pipetted mother liquor of 0.2, 0.4, 0.6, 0.8, 1, and 1.2 mL, respectively, into a 10 mL measuring flask. Ethanol was added to the limit mark to make a solution with a concentration of 20, 40, 60, 80, 100, and 120 mg/L. Each standard solution of 20, 40, 60, 80, 100, and 120 mg/L was pipetted by 0.5 mL and fed into a 5 mL measuring flask, then added with 1.5 mL of ethanol, 0.1 mL of 10% aluminum chloride, 0.1 potassium acetate 1 M, and distilled water to the limit mark to obtain concentrations of 2, 4, 6, 8, 10, and 12 mg/L. The solution was allowed to stand at room temperature for 30 minutes then absorbance absorption was measured with a UV-Vis spectrophotometer at maximum wavelength. The *n*-hexane and ethyl acetate fractions of *R. officinalis* leaves extract were weighed as much as 25 mg and dissolved in ethanol on a 25 mL measuring flask until the limit mark. It was repeated three times with 0.5 mL of pipetted each, then added with 1.5 mL of ethanol, 0.1 mL of 10% $AlCl_3$, 0.1 mL of potassium acetate 1 M, and distilled water up to a volume of 5 mL. This solution was then allowed to stand at room temperature 25-30°C for 30 minutes and measured its absorbance on UV-Vis spectrophotometry at maximum wavelength[20].

Gram coloring of bacteria

The glass of the object was taken out with tongs and sterilized on the fire of the bunsen. The ose needle was heated with bunsen fire until the ose needle looked red; a drop of aqua dest was taken with an ose needle and placed on the glass of the object. The mouth of the Petri dish containing bacterial cultures was sterilized by aseptic means using bunsen fire, the ose needle was re-sterilized and the bacterial culture was taken. Bacterial cultures on the object glass were laid out in evenly circular motions; the object glass was dried with bunsen fire by passing it until it was dried and placed on a rack or dye container. Bacterial staining was carried out with four reagents: violet crystal, iodine, 95% alcohol, and safranin solutions. Each coloring was rinsed using distilled water. Areas that were not growing bacteria were cleaned using tissue to facilitate the drying process; The object glass was dried by rolling it until dry[21].

Antibacterial activity test against S. aureus by disc diffusion method

The bacterial suspension was applied with a sterile cotton swab on NA media in a Petri dish using the scratching method performed near the bunsen fire. Disc paper was soaked in each test solution for 30 minutes to 1 hour and placed on NA media containing bacterial cultures in a Petri dish using pre-sterilized tweezers. The antibacterial activity test of the *n*-hexane fraction and the ethyl acetate fraction of *R. officinalis* leaves ethanol extract used four concentration series: 3%, 6%, 9%, and

12%. Also, a media control containing only NA media in a Petri dish was created to observe whether mold or mold growth on NA media could damage the test results. All growth media were incubated at 37°C for 24 hours[22].

Antioxidant activity test by DPPH method

A total of 1000 mg/L for *n*-hexane fraction of *R. officinalis* leaves ethanol extract, ethyl acetate fraction of 100 mg/L, and quercetin solution of 100 mg/L were prepared. The amount of each *n*-hexane fraction 1000 mg/L was pipetted as much as 0.2, 0.6, 0.8, 1, and 1.2 mL were then put into a 10 mL volumetric flask to obtain concentrations of 20, 40, 60, 80, 100, and 120 mg/L, respectively. For ethyl acetate fraction and quercetin solution, 100 mg/L were pipetted as much as 0.1, 0.2, 0.3, 0.4, 0.5, and 0,6 mL were then put into a 10 mL volumetric flask to obtain concentrations of 1, 2, 3, 4, 5, and 6 mg/L, respectively. Ethanol 96% was used as the solvent. An amount of 5 mL of DPPH solution 40 mg/L was added to each volumetric flask, and then the volume was filled with ethanol to the marked line, allowed to stand in the dark for 30 minutes, then the absorbance was measured on a spectrophotometer with a wavelength of 517 nm[23].

Data analysis

Observation of bacteria was carried out using a microscope, which had previously been dripped with immersion oil. The inhibitory diameter of bacteria was measured on a clear zone formed around the disc paper using a ruler. The data from the DPPH assay were obtained from three repeated experiments (n = 3) and presented as the mean ± standard deviation (SD)[24].

RESULTS AND DISCUSSION

Extraction yield, ethanol-free, and R. officinalis leaves fractionation

As a result of the extraction of *R. officinalis* leaves by the maceration method, a dry extract with a percentage of amendments of 18.21% was obtained. The extract was tested ethanol-free to ensure that the *R. officinalis* leaves extract no longer contains ethanol, indicated by the absence of discoloration from orange to bluish-green (**Figure 1**)[15]. This indicates that the extract has been freed from ethanol content. Fractionation of *R. officinalis* leaves ethanol extract resulted in *n*-hexane and ethyl acetate fractions with a percentage yield of fractions of 3.7% and 15.3%. Results showed that the ethyl acetate fraction attracted more compounds from the ethanol extract of *R. officinalis* leaves.

Figure 1. The ethanol-free test result of *R. officinalis* extract.

Phytochemical screening and identification of flavonoids using TLC

Based on the test results, *n*-hexane and ethyl acetate fractions of *R. officinalis* leaves positively contain flavonoid compounds indicated by a change in color to orange (**Table I**). Mg and HCl will reduce the benzopyran nucleus in the flavonoid structure so that a red, yellow, or orange flavylium salt is formed[25].

Table I. The flavonoids screening.

Compound	*n*-hexane fraction	Ethyl acetate fraction
Flavonoids	+	+

+ : presence of compound

As a result of the fractionation of *R. officinalis* leaf extract, the *n*-hexane fraction and the ethyl acetate fraction were obtained. The TLC method then identified the two fractions using a quercetin comparison. The solvent is used because it includes a phase of motion commonly used in TLC to identify flavonoid compounds glycans and aglycone. Evaporation of TLC plates with ammonia can emphasize the intensity of color, according to the properties of flavonoid compounds that can glow when viewed with a UV light of 365 nm[26]. The results of flavonoid identification showed that the *n*-hexane fraction and the ethyl acetate fraction contained flavonoid compounds seen from the spots on the TLC plate (**Figure 2**). In addition, the Rf value of the *n*-hexane fraction and the ethyl acetate fraction are close to the Rf value of the quercetin standard of 0.98 (**Table II**).

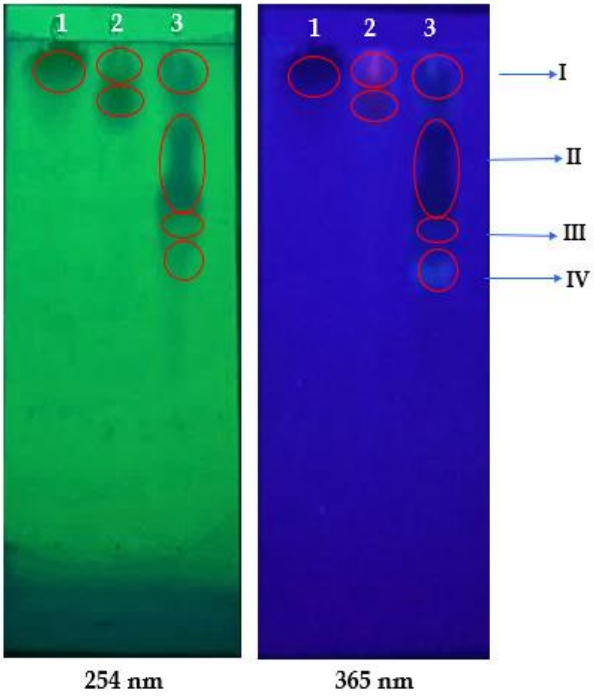

Figure 2. The chromatogram of (**1**) quercetin, (**2**) *n*-hexane, and (**3**) ethyl acetate fractions of *R. officinalis* leaves extract after evaporation with ammonia.

Table II. TLC-bioautography of *n*-hexane and ethyl acetate fractions of *R. officinalis* leaves.

Samples	Spot	Rf	The color at 254 nm	The color at 365 nm	Suspected types of flavonoids
Quercetin	I	0.98	Dim yellow-green fluorescence	Dark mauve	Flavonols
n-hexane fraction	I	0.97	Brownish-yellow	Red	Flavonols, chalcones
	II	0.87	Brownish-yellow	Red-black	Flavonols, chalcones
Ethyl acetate fraction	I	0.93	Blue-green fluorescence	Purple-blue fluorescence	Flavonols, flavones
	II	0.85	Blue-green fluorescence	Dark mauve	Flavonols
	III	0.62	Invisible	Purple blue	Isoflavones
	IV	0.56	Invisible	Blue-green fluorescence	Isoflavones

Total flavonoid levels of a fraction of R. officinalis leaves extract

Quercetin is used as a comparison standard in determining the total flavonoid levels against the *n*-hexane and the ethyl acetate fractions. Quercetin is included in the flavonoid group of flavonols, which has a keto group on the C-4 atom and a hydroxyl group in the C-3 or C-5 atom, which can bind to form a complex with $AlCl_3$[27]. The maximum wavelength measurement result obtained is 430 nm, similar to other study by Krisyanella *et al.*[28] that determined flavonoid levels using quercetin. Standard determination of the standard curve aims to determine the relationship between the concentration of the solution and its absorbance value so that a linear regression equation is obtained. The linear regression equation obtained from the absorbance value of the quercetin series solution (**Figure 3**) is $y = 0.0067x + 0.0225$ with a correlation coefficient value (R^2) of 0.9993. A value of R^2 close to 1 indicates that the standard curve is linear and there is a correlation between the solution concentration and the absorbance value; the higher the concentration, the higher the absorbance obtained[29].

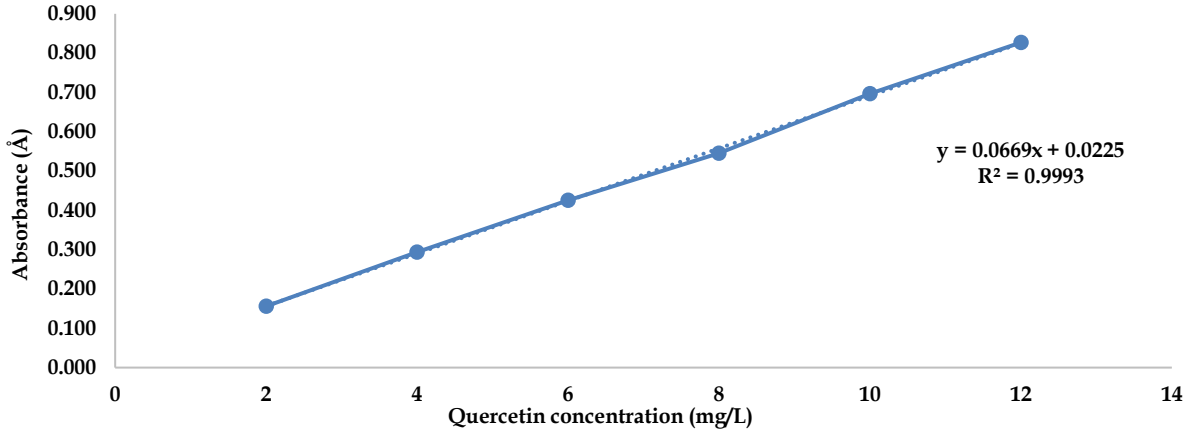

Figure 3. Quercetin standard curve.

In determining total flavonoid levels, adding potassium acetate aims to detect the presence of a 7-hydroxyl group[30,31]. Based on the results, total flavonoid levels of the *n*-hexane fraction were obtained by 19.129 ± 3.243%, and the ethyl acetate fraction by 47.437 ± 1.947% (**Table III**). These results show that ethyl acetate solvents can attract more flavonoid compounds than n-hexane as a non-polar solvent. The results are similar to another study by Suoth *et al*.[32], stating that the ethyl acetate fraction has higher flavonoid levels than the n-hexane fraction.

Table III. Flavonoid levels of total fractions of *n*-hexane and ethyl acetate fractions of *R. officinalis* leaves.

Fractions	Absorbance (Å)	Total flavonoid levels (mgQE/g)	Total flavonoid levels (%)
n-hexane	0.151 ± 0.022	191.290 ± 32.433	19.129 ± 3.243
Ethyl acetate	0.340 ± 0.013	474.373 ± 19.477	47.437 ± 1.947

Antibacterial activity of the fractions of R. officinalis extract against S. aureus

The Gram staining of bacteria showed that the bacteria were indeed *S. aureus*. From **Figure 4**, it can be seen that the bacteria are purple and have a colonized round shape. Gram-positive bacteria can bind to the primary paint because they have a thicker peptidoglycan layer than Gram-negative, and their cell walls are composed of heteropolymer proteins and sugars known as mureins. Mureins will become a barrier so the violet-iodine crystal complex cannot escape during the staining process[33,34]. In testing antibacterial activity, the disc diffusion method is used because the method is easy, simple, does not require a long time, does not require special equipment, and costs less[22]. The positive control was 3% amoxicillin, and the negative control was 5% tween 80. The selection of positive controls is based on its broad-spectrum nature with a mechanism of action preventing crosslinking of peptidoglycans in the late stages of cell wall synthesis, which is one of the antibacterial mechanisms by flavonoid compounds[35]. Tween 80 was used as a negative control following another study by Turahman and Sari[36] that stated that tween 80 did not produce an inhibitory zone in *S. aureus*. The selection of tween 80 is also because when manufacturing a series solution, the concentration is not all fractions can dissolve in polar solvents; the addition of tween 80 as a surfactant in the solution can lower the interface voltage of the solution so that it will improve the solubility process when making concentration series.

The results of antibacterial testing on the *n*-hexane and the ethyl acetate fractions with concentrations of 3%, 6%, 9%, and 12% showed moderate-strong inhibition of bacterial growth at concentrations of 9% and 12% (**Table IV**). This is associated with the total flavonoid levels possessed by each fraction, which is directly proportional to the antibacterial activity shown. In the negative control, there is no visible inhibition zone, and the positive control shows the strongest inhibitory power. The results of antibacterial testing were analyzed statistically using the Kruskal-Wallis test, which showed a value of 0.001 ($p < 0.05$). The results signaled a significant difference between positive control, negative control, *n*-hexane, and ethyl acetate fractions. The magnitude of antibacterial activity in the ethyl acetate fraction is due to the ability of ethyl acetate as a semipolar solvent containing more complex chemical compounds when compared to polar and non-polar fractions so that it can attract more flavonoid compounds. Flavonoid compounds have a strong ability to inhibit the growth of bacteria[37].

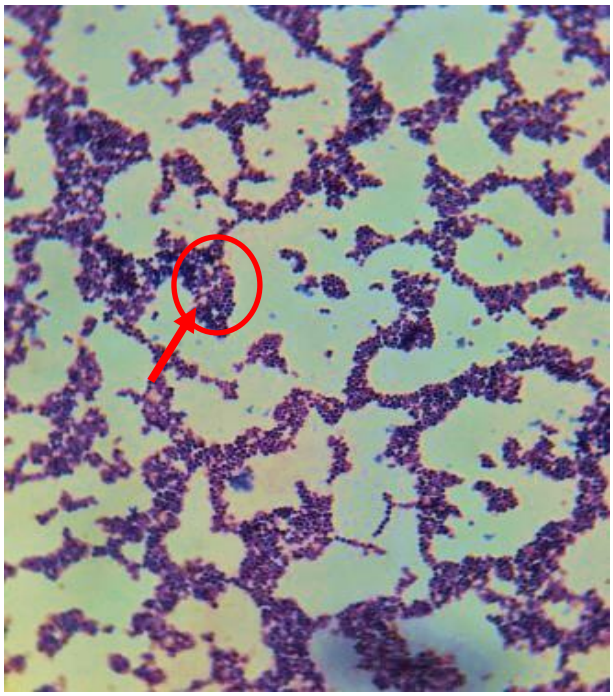

Figure 4. *Staphylococcus aureus* morphology. These bacteria included Gram-positive bacteria that were small and round in shape (coccus) and occurred as clusters appearing like a bunch of grapes on microscopy (red arrow).

Table IV. Inhibition zones diameter of *n*-hexane and ethyl acetate fractions against *S. aureus* FNCC 0047.

No.	Samples	Concentration (%)	$\bar{x}$ diameter of inhibition zones ± SD	Classification[38]	Sig.*
1	*n*-hexane	3	1.667 ± 0.577	Weak	0.001
		6	1.667 ± 0.577	Weak	
		9	6 ± 0.866	Medium	
		12	9.5 ± 1.322	Medium	
2	Ethyl acetate	3	2.166 ± 0.288	Weak	0.001
		6	3.5 ± 0.866	Weak	
		9	10 ± 1.322	Medium-strong	
		12	11.883 ± 0.577	Strong	
3	Positive control		14.166 ± 3.055	Strong	
4	Negative control		0 ± 0	No inhibition	

* Kruskal-Wallis statistical test, there are significant differences ($p < 0.05$)

Antioxidant activity of the fractions of R. officinalis extract by DPPH methods

Table V shows that the higher concentration of fraction (as an antioxidant) made an increase in inhibition of the DPPH oxidant. The increasing inhibition percentage for each fraction of *R. officinalis* extract as well as quercetin can be seen in **Figures 5** to **7**. This graph uses a linear regression equation to calculate the IC_{50} value, the concentration required to reduce the initial DPPH concentration by 50%. The ethyl acetate and *n*-hexane fractions of *R. officinalis* leaves extracts needed 5.486 and 66.293 mg/L, respectively, to decrease 50% of the DPPH oxidant. The antioxidant activity category of *R. officinalis* leaves extract based on IC_{50} value was very strong for ethyl acetate and strong for n-hexane fractions. The classification of antioxidant activities is divided into five: <50 (very strong), 50-100 (strong), 100-150 (moderate), 150-200 (weak), and >200 mg/L (very weak)[39].

In this research, quercetin was used as a positive control and is simply a known antioxidant. This will clearly show that this amount of sample has antioxidant activity compared to a certain amount of the control. A negative control is a mixture of methanol and DPPH 40 mg/L. Quercetin had high antioxidant activity, with a lower IC_{50} value of 3.751 mg/L. **Table V** showed ethyl acetate fraction of *R. officinalis* leaves extract had a higher IC_{50} value than the *n*-hexane, and its antioxidant activity was as strong as quercetin. This proved that fractions of *R. officinalis* leaves extract could scavenge free radicals of DPPH. The mechanism for scavenging DPPH radicals by antioxidant compounds is through donating hydrogen atoms where the incubation period of the sample mixed with the DPPH reagent is 30 minutes, causing the DPPH color to change from purple to yellow[23]. The color change was caused by the reduction of the conjugated double in DPPH due to the

presence of one electron by the antioxidant compound, which caused the unavailability of the electronic place to resonate where the change could be measured. The reaction process between antioxidant compounds and DPPH radicals occurs through hydrogen atom donation[40].

Table V. Absorbance value and inhibition percentage of *R. officinalis* leaves extract fractions.

Samples	Concentration (mg/L)	Absorbance (Å)	Percentage of inhibition (%)	IC_{50} (mg/L)	Category[39]
Quercetin	1	0.535 ± 0.025	16.822 ± 3.912	3.751	Very strong
	2	0.497 ± 0.02	22.682 ± 3.123		
	3	0.368 ± 0.005	42.802 ± 0.857		
	4	0.294 ± 0.004	54.314 ± 0.589		
	5	0.21 ± 0.015	67.382 ± 2.309		
	6	0.149 ± 0.004	76.82 ± 0.678		
Ethyl acetate fraction	1	0.4 ± 0	14.894 ± 0	5.486	Very strong
	2	0.392 ± 0.002	16.667 ± 0.325		
	3	0.343 ± 0	27.021 ± 0		
	4	0.324 ± 0	31.064 ± 0		
	5	0.272 ± 0.002	42.057 ± 0.325		
	6	0.181 ± 0.008	61.56 ± 1.784		
n-hexane fraction	20	0.368 ± 0.02	19.474 ± 4.442	66.293	Strong
	40	0.343 ± 0.008	24.872 ± 1.755		
	60	0.264 ± 0.015	42.231 ± 3.179		
	80	0.166 ± 0.005	63.53 ± 1.205		
	100	0.09 ± 0.026	80.16 ± 5.627		
	120	0.062 ± 0.023	86.360 ± 5.11		

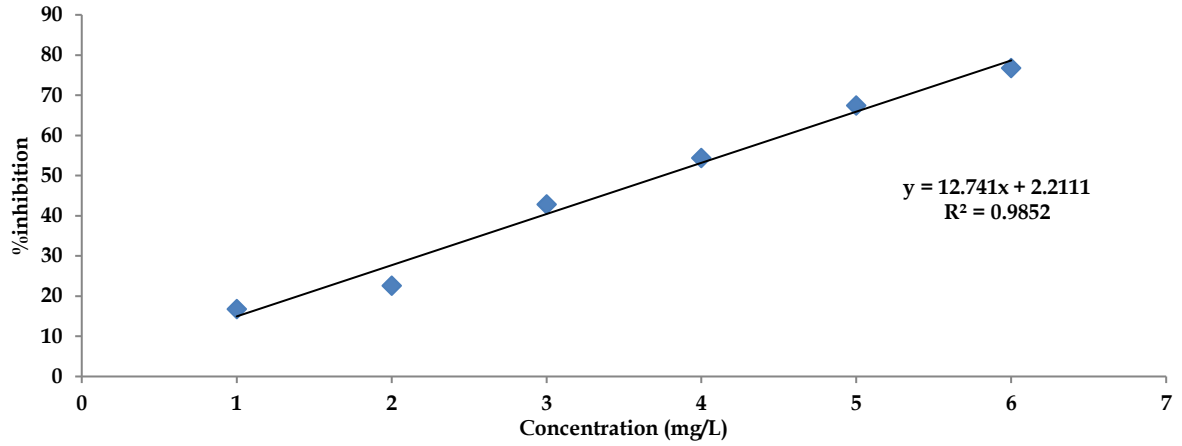

Figure 5. Inhibition percentage and concentration of quercetin.

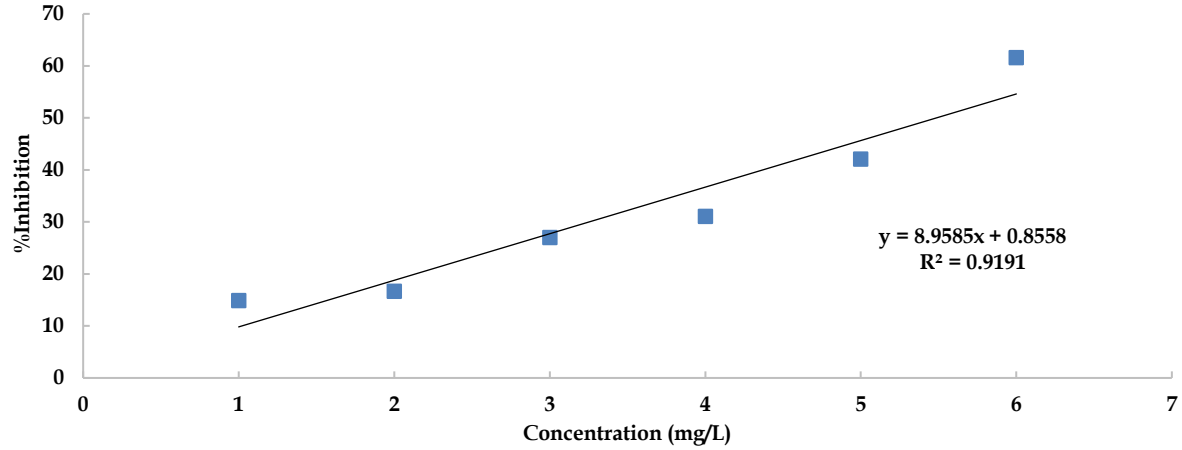

Figure 6. Inhibition percentage and concentration of ethyl acetate fraction.

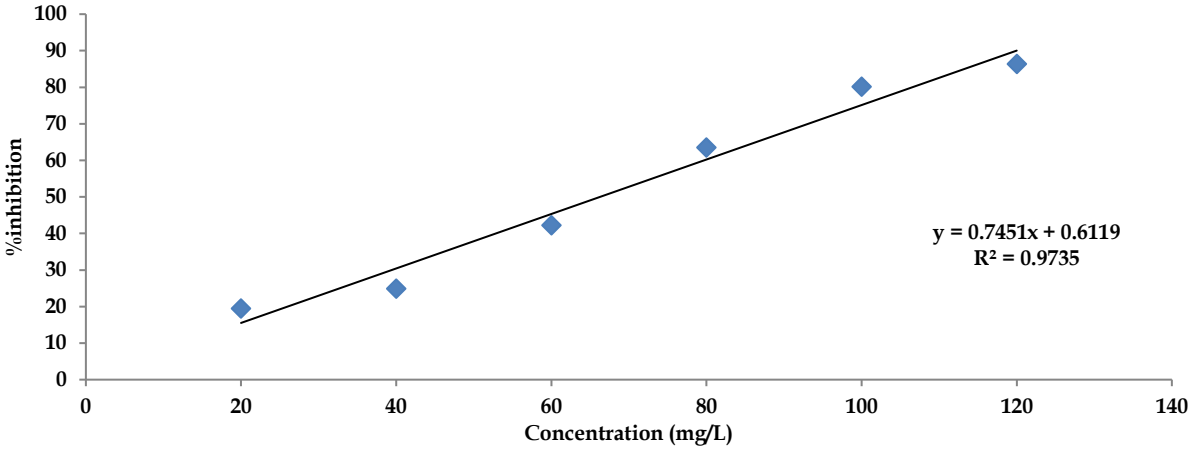

Figure 7. Inhibition percentage and concentration of *n*-hexane fraction.

CONCLUSION

The ethyl acetate fraction of *R. officinalis* has higher total flavonoid levels of 47.437 ± 1.947% than the *n*-hexane fraction. These are directly proportional to the antibacterial activity of the ethyl acetate fraction at a concentration of 12%, showing a more significant inhibition zone of 11.883 ± 0.577 mm than the *n*-hexane fraction. Antioxidant activity of ethyl acetate fraction with IC_{50} value of 5.486 mg/L, which is classified as very strong.

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Data curation: Ni Putu Sudiasih, Ni Nyoman Dina Saniasih
Formal analysis: Ni Putu Sudiasih, Ni Nyoman Dina Saniasih
Funding acquisition: -
Investigation: Ni Putu Sudiasih, Ni Nyoman Dina Saniasih
Methodology: -
Project administration: -
Resources: -
Software: -
Supervision: Ni Ketut Esati, Elisabeth Oriana Jawa La
Validation: -
Visualization: -
Writing - original draft: Ni Putu Sudiasih
Writing - review & editing: Ni Ketut Esati, Elisabeth Oriana Jawa La

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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Review Article

Potential of Indonesian Plants as Polymicrobial Anti-Biofilm

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Keywords:
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Quorum sensing

Abstract

Biofilm infection occurs in 80% of chronic infections caused by 60% of biofilms from plankton cells and polymicrobial biofilms. Due to synergistic interactions between species, infections caused by polymicrobial biofilms are more virulent than monospecies biofilm infections. New anti-biofilm candidates are constantly being developed by tracing the content of active compounds from medicinal plants native to Indonesia. The need to find new plant sources that have the potential as anti-biofilms is increasingly needed along with increasing microbial resistance. Various studies show that active compounds that have anti-biofilm potential are polyphenols, quercetin, curcumin, gallic acid, and ferulic acid. The mechanism of action of anti-biofilms is through the prevention of attachment and formation of biofilms, inhibition of quorum sensing, and inhibition of gene expression in microbes.

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INTRODUCTION

Antimicrobial resistance represents a significant global health challenge. According to statistics from the World Health Organization (WHO), the utilization of antibiotics is expected to surge by 200% by 2030[1]. The ineffectiveness of antibiotic treatments, a phenomenon observed in 65% of chronic human infections, can be attributed to microbial infections linked to biofilms[2,3]. Bacteria forming biofilm layers can communicate through a quorum sensing (QS) system, forming polymicrobial microcolonies that may include bacteria, fungi, or viruses. This collective interaction makes these microorganisms approximately 10,000 times more resistant to antibiotic treatments[4]. Infections originating from polymicrobial biofilms exhibit greater virulence than those from monospecies biofilms. This heightened virulence is attributed to the synergistic interactions among different species, leading to an elevated fatality risk in acute and chronic infectious diseases[5,6]. Polymicrobial-induced infections can be observed in various health conditions, including lower lung infections characterized by chronic inflammation in individuals with cystic fibrosis. These infections are also evident in inner ear infections (otitis media), where polymicrobial biofilm complexes pose a risk of both partial and complete hearing loss[7].
Furthermore, urinary tract infections can also be attributed to polymicrobial biofilms. Notably, a significant percentage, ranging from 60% to 90%, of infections occurring in the oral cavity, such as dental caries, originate from polymicrobial infections. Another condition associated with polymicrobial biofilms is diabetic foot wounds, which lead to tissue damage,

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chronic inflammation, and the formation of a biofilm layer. This biofilm formation contributes to delayed wound healing, potentially necessitating amputations and elevating the risk of mortality[8,9]. The need to find anti-biofilm candidates, especially from active compounds native to Indonesia, is increasingly needed along with increasing microbial resistance[10].

The growing necessity for discovering potential anti-biofilm agents, particularly among active compounds of plant origin, is escalating in response to the rising levels of microbial resistance[11]. Plants have a long history of use in traditional medicine, contain diverse natural compounds, and offer a promising source for discovering new antibiofilm agents. Their potential therapeutic benefits, lower resistance development, and relative safety make them an attractive avenue for research in the fight against biofilm-related infections[12].

Indonesia, an equatorial archipelago comprising more than 17,000 Southeast Asian islands, is recognized for its remarkable biodiversity. It ranks as the world's second-most biodiverse country, trailing only Brazil, and boasts a diverse array of 47 unique natural ecosystems teeming with plant and animal resources[13]. Additionally, Indonesia is home to a substantial number of island-specific species. The country is renowned for its invaluable biological heritage, deeply rooted cultural traditions, and sizable population, which underscore its significant role in the herbal medicine industry[14].

Indonesia boasts an impressive flora, with over 38,000 plant species, of which approximately 9,600 are classified as medicinal plants[15]. Despite this wealth of botanical resources, the Indonesian National Agency of Drug and Food Control (NADFC or *Badan Pengawas Obat dan Makanan Republik Indonesia*, BPOM RI) has scientifically examined only around 300 plant species to assess their medicinal properties, officially registering them for commercial use in traditional medicine. A substantial portion of the plant species remains unexplored, necessitating further research and screening to uncover their potential medicinal attributes[16]. Many studies have been conducted to explore the biological properties of commonly used plants in Indonesian traditional medicine. However, it's worth noting that there has been limited exploration of Indonesian medicinal plants concerning their potential for anti-biofilm activity[17,18].

POLYMICROBIAL BIOFILM

A biofilm represents an assembly of microbial cells firmly adhered to a surface and enclosed within a matrix of extracellular polymeric substances (EPS) that cannot be easily dislodged[19]. Microorganisms proficient in forming biofilm structures often demonstrate heightened resistance to antibiotics, disinfectants, and phagocytosis and are the primary culprits behind human infections, presenting considerable treatment challenges[20,21]. Biofilm-related infections are responsible for 80% of chronic infections, including persistent wounds. Biofilms also possess an inherent adaptability that can give rise to both acute infections and long-lasting inflammation. Traditional antimicrobial agents like antibiotics are typically designed to eliminate or hinder the growth and division of planktonic cells, but they lose their effectiveness when dealing with infections linked to biofilms[22]. While a combination of antibiotic treatments may effectively address up to 60% of biofilms originating from planktonic cells, the success rate drops significantly to just 22% when dealing with infections associated with polymicrobial biofilms[23].

The EPS layer in biofilms leads to persistent infections, rendering treatment considerably more challenging. The biofilm matrix layer accounts for half of the biofilm's composition and possesses a thickness ranging from 0.2 to 1.0 mm. The production of EPS plays a pivotal role in facilitating microbial adhesion to cell surfaces and cell-to-cell adhesion. This polymer matrix is responsible for imparting mechanical stability to the biofilm. Additionally, The EPS layer serves as a shield, protecting microorganisms from hostile environments that are inhospitable to microbial growth, as well as from chemical toxins and antimicrobial agents. This layer can bind cations along with antimicrobial agents, such as chlorhexidine and antimicrobial peptides, preventing their penetration into the deeper layers of the biofilm, consequently diminishing the efficacy of antimicrobial treatments[24]. The formation of the biofilm structure is determined by various factors, including the microorganisms' capacity to adhere to surfaces, the production of EPS, inter-microbial signaling, and the dispersion of microbes as planktonic cells. Bacteria, fungi, and viruses can coexist in clinical scenarios, forming intricate communities within polymicrobial biofilms. This complexity poses challenges when selecting suitable antibiotic treatments, particularly in cases involving antibiotic-resistant microorganisms[25,26].

ACTIVE COMPOUNDS OF INDONESIAN PLANTS AS POLYMICROBIAL ANTI-BIOFILM POTENTIALS

Indonesia boasts a rich diversity of medicinal plants, representing a significant wellspring for drug discovery. The outcomes of a literature review focused on Indonesian plant species with the potential for combating polymicrobial biofilms are outlined in **Table I**.

Table I. Indonesian medicinal plants with anti-biofilm activity.

No	Local name (Scientific name)	Polymicrobial bacteria	Chemical compounds
1	*Masoyi (Mossaia aromatica)*[27]	Degradation of *Candida albicans, Pseudomonas aeruginosa,* and *Staphylococcus aureus* biofilm formation	Essential oil, massoia lactone
2	White turmeric/*temu rapet/kunci pepet (Kaempferia rotunda)* [27]	Degradation of *P. aeruginosa* AO1 and *S. aureus* Cowan I biofilm formation	Flavonoids, quercetin, flavonols
3	*Secang/sapang (Caesalpinia sappan)* [27]	Degradation of *P. aeruginosa* AO1 and *S. aureus* Cowan I biofilm formation	Triterpenoids, flavonoids, and phenolic compounds
4	Cinnamomum/*kiamis/ holim manis/ kanigar (Cinnamomum burmannii)* [27]	Degradation of *P. aeruginosa* AO1 and *S. aureus* Cowan I biofilm formation	Essential oils cinnamaldehyde and cinnamyl acetate, ethyl cinnamate, beta-caryophyllene, linalool, and methyl chavicol
5	*Sintok/huru sintok/madang lawang (Cinnamomum sintoc)* [27]	Degradation of *P. aeruginosa* AO1 and *S. aureus* Cowan I biofilm formation	Essential oil eugenol
6	Lotus/*tunjung (Nymphaea nouchali)* [27]	Degradation of *P. aeruginosa* AO1 and *S. aureus* Cowan I biofilm formation	The flowers contain quercetin, luteolin, isoquercetin, kaempferol, galuteolin, and alkaloids. The seeds contain quercetin and isoquercitrin
7	Cinnamomum verum/*manis jangan/huru mentek (Cinnamomum zeylanicum)*[28]	Inhibits communication between bacteria to inhibit the formation of *Escherichia coli* and *Pseudomonas* sp. biofilms	Essential oils, cinnamaldehyde, eugenol
8	Curcuma/*koneng/temu tombak (Curcuma xanthorrhiza)* [28]	The ethyl acetate fraction can inhibit plankton and biofilm in dental caries, destroy cell walls, inhibit enzymatic activity, and prevent bacterial attachment	Phenolic, Xanthorrhizol
9	*Melinjo/maninjo/tangkil (Gnetum gnemon)*[29]	Leaves water extract can inhibit cell attachment through the penetration of compounds into the EPS layer biofilm	Saponins, alkaloids, tannins and steroids
10	Clove/*cangke/lawang/ singke (Syzygium aromaticum)* [28]	Degradation of biofilm formation	Essential oils, eugenol
11	Ginger/*jahe/halia/tipakan (Zingiber officinale)*[21]	Degradation of biofilm formation	Essential oils, terpenoids
12	Mangosteen/*manggu/manggus/manggista* (*Garcinia mangostana*)[30]	Prevents communication between biofilm-forming bacteria and reduces the ability to form antibiotic resistance genes	Flavonoids
13	Clove leaves/*cangke/lawang/singke* (*Syzygium aromaticum*)[28,30]	Inhibit the growth of *E. coli*, *Salmonella enteritidis*, and *S. aureus* by inhibiting communication between bacteria in the formation of biofilms	Essential oils, eugenol
14	Henna/*pacar kuku/pacar petok/inai parasi (Lawsonia inermis)* [28]	Inhibit biofilm formation. Leaves ethyl acetate extract inhibits *Streptococcus pneumoniae* ATCC 49619 >90% and inhibits >98% MDRSP 2506	Phenolics, benzene derivatives, naphthoquinones, saponins, flavonoids, steroids
15	Lemongrass/*sere/bubu* (*Cymbopogon citratus*) [28]	Degradation of biofilm formation	Essential oils
16	*Senggani/senduduk* (*Melastoma candidum*)[17]	Ethanol and ethyl acetate extract from leaves can inhibit quorum sensing of *Aeromonas hydrophila* infection in fish	Tannins, flavonoids

17	*Kersen/seri/ceri/talok* (*Muntingia calabura*) [17]	Ethanol and ethyl acetate extract from leaves can inhibit quorum sensing of *Aeromonas hydrophila* infection in fish	Flavonoids, polyphenols, flavonols, steroids, triterpenoids, alkaloids and tannins
18	Turmeric/*kunyit/kunir/koneng/hunik* (*Curcuma longa*)[31]	0.5% pure curcumin isolates can destroy polymicrobial EPS biofilm layers of *S. aureus, P. aeruginosa, E. coli*, and *C. albicans* on catheters. 1% curcumin can inhibit biofilm formation in the middle and maturation phases by 62.23%	Curcumin
19	Creeping woodsorrel/small sour/*calincing/lela* (*Oxalis corniculata*)[32]	1% ethanol extract of *O. corniculata* leaves can inhibit *C. albicans* biofilm formation in the intermediate phase by 68.23%	Flavonoids, tannins, essential oils, saponins
20	Areca nuts/*pineng/ jambe/pining* (*Areca catechu*)[33]	Ethanol extract and water fraction can inhibit the biofilm-forming bacterium of *S. aureus*	Flavonoids, tannins, alkaloids, quinones, terpenoids and saponins
21	Papaya leaves/*kates/gedang/betik* (*Carica papaya*)[29]	This extract can inhibit cell attachment and degrade biofilms by 41.176% and 49.02%, respectively	Alkaloids, tannins, flavonoids, steroids/terpenoids
22	*Kepayang/kluwek/keluak* (*Pangium edule*)[34]	Ethanol extract can destroy *Streptococcus sanguinis* ATCC 10556 biofilm	Phenols, tannins, flavonoids, alkaloids, saponins, and fatty acids
23	*Bawang tiwai/bawang dayak/bawang sabrang/berlian/bawang lubak* (*Eleutherine bulbosa*)[35]	Ethanol extract and methanol soluble fraction can degrade biofilm	Phenolics and naphthoquinones
24	Coriander seeds/*ketumbar/hatumbar* (*Coriandrum sativum*)[36]	Ethanol extract inhibits the formation of *Streptococcus mutans* ATCC 25175	Linalool, phenolics, and flavonoids
25	Jackfruit/*nongko/langge/anane* (*Artocarpus heterophyllus*)[37]	*Artocarpus heterophyllus* leaves extract can destroy *S. sanguinis* ATCC 10556 biofilm	Saponins, flavonoids, and tannins
26	A combination of *S. aromaticum* leaves and *C. verum* bark[38]	Combination of 27 : 73 can inhibit *S. mutans* biofilm formation	Polyphenols, essential oils
27	*Melinjo leaves/maninjo/tangkil* (*Gnetum gnemon*)[39]	A concentration of 25% could inhibit the growth of 43.09% and degrade 43.04% of *P. aeruginosa*	Phenolics, alkaloids, tannins, saponins, and steroids
28	Mangosteen/*manggis/manggu/manggus/manggista* (*Garcinia mangostana*)[30]	Minimum biofilm inhibitory concentration (MBIC) of 0.78% against *S. mutans*	Flavonoids

Mossaia aromatica

Indonesian people use *M. aromatica* to treat several diseases, such as diarrhea, fever, vaginal discharge, stomach cramps, and postpartum. Existing research states that *M. aromatica* contains essential oils that can be obtained from the bark, stems, and fruit of *M. aromatica*[40]. Massoia lactone (**Figure 1**; 92.05%) was the main constituent of *M. aromatica* essential oil[41]. *Mossaia aromatica* has been proven to be able to inhibit the formation of biofilm by *S. aureus* by 50% with a concentration of 0.03%, while the use of essential oil from the bark of *M. aromatica* with a concentration of 0.12% can disrupt the biofilm that has already been formed by 50%[42]. The mechanism of action of massoia lactone is to damage the EPS so that the cell and nutrient communication pathways between microbes are cut off so that microbes that previously wanted to form biofilm ultimately cannot form biofilm, causing these microbes to lyse or die because of loss of nutrients as a component of biofilm formation[41].

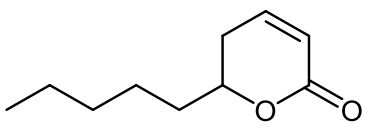

Figure 1. Massoia lactone (PubChem CID 39914)[41].

Kaempferia rotunda

Kaempferia rotunda is commonly known as white ginger by Javanese and Malay people in Indonesia. *Kaempferia rotunda* ethanol extract has been proven to have antibacterial and antibiofilm effects, with the mechanism of action inhibiting eDNA

production in *S. aureus* biofilm biomass. *Kaempferia rotunda* extract can reduce biofilm formation at concentrations ranging from 0.019 to 0.625 mg/mL. Curcumin is the active compound that plays a vital role in this effect (**Figure 2**)[44].

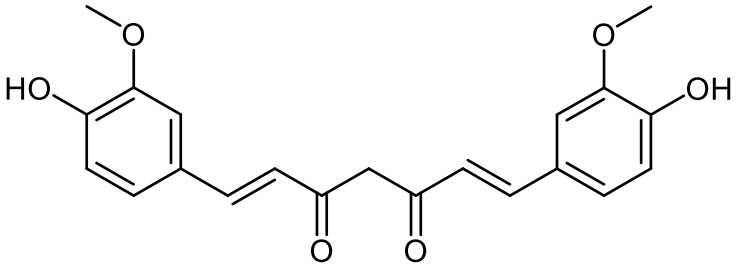

Figure 2. Curcumin (PubChem CID 969516)[44].

Caesalpinia sappan

Caesalpinia sappan is a plant from the Caesalpiniaceae family that has many benefits and is often consumed by Indonesian people as a medicine for wounds, stopping bleeding, anti-diarrhea, pain relief, and increasing body stamina. *Caesalpinia sappan* wood contains compounds like brazilin, sappanin, brazilein, and essential oils[45]. Brazilin (**Figure 3**) showed antibacterial activity, including minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 0.5 mg/mL[46]. Based on research conducted by Utami *et al*.[47], the leaves and stems of *C. sappan* were proven to have antibacterial and antibiofilm activity against *S. mutans*. The inhibitory ability of bacterial biofilms is thought to be the role of the flavonoid compounds in *C. sappan*. The mechanism of action of this substance is to inhibit bacterial infections, which is related to its ability to form bonds with proteins, thereby inhibiting bacterial adhesion. The brazilin content of *C. sappan* stem can be well absorbed into the bacterial walls, resulting in leakage of the cytoplasmic membrane and inhibiting bacterial growth and is responsible for inhibiting the formation of bacterial biofilm mass.

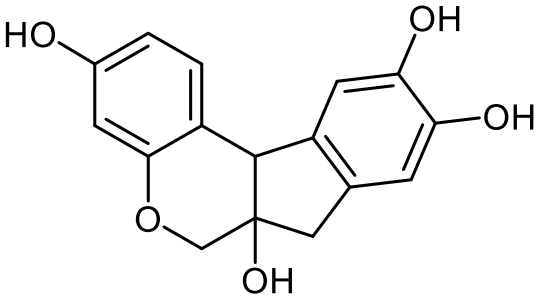

Figure 3. Brazilin (PubChem CID 73384)[48].

Cinnamomum burmannii

Cinnamomum burmannii, known as *kiamis*, *holim manis*, and *kanigar* by Indonesian people. *Cinnamomum burmannii* bark has a distinctive smell and is widely used for various purposes, such as flavoring food or cakes. Al-Dhubiab[49] states that the most significant chemical components in *C. burmannii* are cinnamic alcohol, coumarin, cinnamic acid, cinnamaldehyde, anthocyanin, and essential oils containing sugar, protein, simple fat, pectin, and others. The main contents of *C. burmannii* essential oil are cinnamaldehyde (**Figure 4**) and eugenol[50]. Cinnamon oil has several benefits such as antimicrobial, antioxidant, antidiabetic, and antiallergenic effects. The active compounds contained in cinnamon have been proven to have antibacterial and antibiofilm effects through a mechanism of action in the form of inhibiting the formation of bacterial biofilm mass. As an anti-biofilm agent, *C. burmannii* has an MBIC value of 0.01%[51]. The active compound cinnamaldehyde can dissolve well in the polar groups of bacterial walls so that phospholipid molecules will break down into glycerol, carboxylic acid, and phosphoric acid. This change will cause leakage of the cytoplasmic membrane and inhibit bacterial growth. The eugenol content can also be dissolved well in the polar group of bacterial glucosyltransferase enzymes to interfere with the formation of bacterial biofilm mass[47].

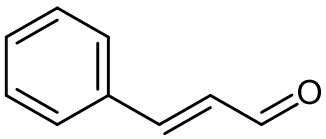

Figure 4. Cinnamaldehyde (PubChem CID 637511) [50].

Cinnamomum sintoc

Cinnamomum sintoc, known as *sintok* by Indonesian people, is used for traditional medicine such as diarrhea and wound healing. Based on research conducted by Muhamad[52], methanol extract of *C. sintoc* leaves (50 mg/mL) has activity against *S. aureus* and *P. aeruginosa*. As an anti-biofilm agent, *C. sintoc* has an MBIC value of 0.06%[51]. The active compounds detected in *C. sintoc* leaves extract include terpenoids, phenols, and tannins. One of the active compounds that plays a major role as an antibacterial is terpenoids, which react by forming strong polymer bonds, destroying porins in bacteria. This damage will reduce the permeability of the bacterial walls and result in bacterial cells lacking nutrition so that bacterial growth is hampered and they die[53].

Nymphaea nouchali

Nymphaea nouchali or lotus is known as *tunjung* by Indonesian people. Based on research by Dash *et al*.[54], the methanol extract of *N. nouchali* flowers has antibacterial activity against *Bacillus subtilis*, *Sarcina lutea*, *E. coli*, and *Klebsiella pneumoniae*. The antibacterial activity is believed to be due to secondary metabolite compounds such as alkaloids, tannins, steroids, phenols, saponins, and flavonoid compounds, which were previously reported to have antimicrobial properties.

Cinnamomum zeylanicum

Cinnamomum zeylanicum, or cinnamon, is a plant that has a distinctive aroma. This species has been used in traditional medicine for its properties, including its activity as an astringent, aphrodisiac, antiseptic, aperitif, aromatic, carminative, digestive, stimulant, hypertensive, sedative, tonic, vasodilator, antidiabetic, antinociceptive, astringent, and diuretic. Essential oil components include cinnamaldehyde (65-78%) and eugenol (4-10%)[55]. The research results prove that cinnamon has antibacterial activity against *P. aeruginosa*, *E. coli*, and *S. aureus* strains. This research also shows anti-biofilm activity through a mechanism that prevents the formation of biofilms and planktonic cells, making bacterial biofilms unstable[56].

Curcuma xanthorrhiza

Koneng or *temu tombak* is another name for *C. xanthorrhiza* for Indonesian people. This plant has antibacterial and antifungal effects. Phytochemically, *C. xanthorrhiza* was detected to have active components in the form of alkaloids, flavonoids, phenolics, saponins, triterpenoids, and glycosides. According to research, *C. xanthorrhiza* has an antimicrobial effect against several microorganisms, especially against *B. subtilis*, *E. coli*, and *S. aureus*. The active substances in *C. xanthorrhiza* that can be antibacterial are curcumin (curcuminoids) and essential oils. Essential oils can lyse bacterial cell membranes, and curcumin can inhibit the proliferation of bacterial cells[57]. When present at a concentration of 1%, polyphenolic compounds like curcumin have demonstrated the ability to impede approximately 62.23% of the intermediate phase and 59.43% of the maturation phase in biofilm development. Furthermore, curcumin exhibits a remarkable eradication rate of 55.79% against polymicrobial biofilms involving *S. aureus*, *P. aeruginosa*, *E. coli*, and *C. albicans*. It also achieves a 50% inhibition of polymicrobial biofilm formation on catheters through the disruption of the polymer-based EPS[31].

Gnetum gnemon

Gnetum gnemon or melinjo/maninjo/tangkil contains active compounds such as alkaloids, flavonoids, steroids, and tannins. Based on Kinning *et al*.[58], *G. gnemon* leaves extract inhibited biofilm cell attachment by 49.8%. This inhibitory process is attributed to direct antibacterial activity, in others to QS disruption or unknown causes, perhaps inhibition of sortases or adhesins. Tannins and flavonoids are compounds contained in *G. gnemon* leaves extract that are thought to inhibit biofilm

formation by binding to one of the bacterial adhesin proteins, which is used as a bacterial surface receptor, resulting in a decrease in bacterial adhesion and inhibition of protein synthesis for cell wall formation.

Syzygium aromaticum

Syzygium aromaticum is known for its use as a spice in cooking. *Syzygium aromaticum* essential oil exhibits anti-inflammatory, cytotoxic, and anesthetic activities in addition to antimicrobial, antifungal, antiviral, antioxidant, and insecticidal properties. The research results prove that *S. aromaticum* can influence the homeostasis of the formed bacterial biofilm. The eugenol compound (**Figure 5**) contained in *S. aromaticum* oil provides a bactericidal effect that decreases the number of planktonic cells and changes the ability of bacterial cell attachment[59].

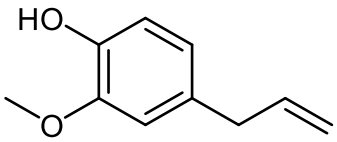

Figure 5. Eugenol (PubChem CID 3314)[59].

Zingiber officinale

Phytochemical studies of *Z. officinale* show that *Z. officinale* has anti-inflammatory and antioxidant properties and can potentially prevent cancer. Their active compound components are polyphenols, such as 6-gingerol (**Figure 6**), and their derivative compounds. Other compounds directly related to antibiofilm and antibacterial activity are curcumin, 6-shogaol, and zingerone[60].

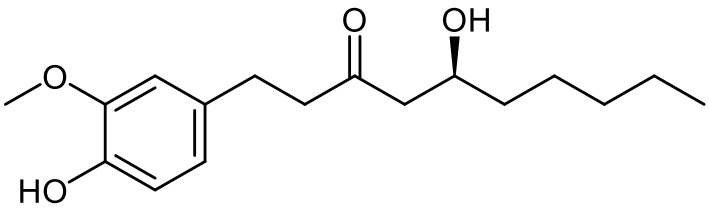

Figure 6. Eugenol (PubChem CID 442793)[60].

Garcinia mangostana

The main chemical content in the roots, bark, and rind of the *G. mangostana* fruit is saponin. The stems contain flavonoids and polyphenols, and the pericarp contains flavonoids, steroids/terpenoids, and quinones. Research results prove that *G. mangostana* fruit can inhibit biofilm with a percentage of 48.8% to 84%. Flavonoids in fruit skin can damage bacterial cell membranes by destroying the lipid layer on the bacterial membrane and causing obstruction of cell membrane function[61]. Flavonoid compounds in the phenol group inactivate a bacterial enzyme that stimulates the activity of the glucosyltransferase enzyme used by bacteria to synthesize sucrose in the medium into glucan. As a result, biofilm formation is hampered because of the amount of glucan as a medium for attaching small or limited bacteria[62].

Lawsonia inermis

Lawsonia inermis is known as *pacar petok* or *inai* by the Indonesian people. This plant was proven to have antibiophilic activity, with inhibition reaching 84.9%. Research results prove that the active compounds that play a role in this effect are the presence of glycosides, phytosterols, steroids, saponins, tannins, and flavonoids[63].

Cymbopogon citratus

Cymbopogon citratus or lemongrass essential oils have been widely used as traditional medicine and are famous for their antimicrobial properties. *Cymbopogon citratus* oil has been proven to reduce biofilm formation by 45-76% in *Candida tropicalis* strains by inhibiting the formation of planktonic cells in bacterial biofilms[64,65].

Melastoma candidum

Acetone extract of *M. candidum* showed a good bactericidal effect. The MIC and MBC for the acetone extract were 0.02 to 0.64 mg/mL and 0.08 to 2.56 mg/mL, respectively, while 95 mL/100 mL for the ethanol extract[66]. Antibacterial substances, especially flavonoids, can destroy bacterial cell walls and cytoplasmic membranes, causing leakage from the cytoplasm[67].

Muntingia calabura

Compounds that have been isolated from *M. calabura* include three flavones and one chalcone: 5,7-dihydroxy-3,8-dimethoxyflavone, 2',4'-dihydroxychalcone, 5-hydroxy-3,7-dimethoxyflavone, and 3,5,7-trihydroxy-8-methoxyflavone. *Muntingia calabura* leaves have been proven to respond as an antibiofilm against *P. aeruginosa*. The antibacterial activity of *M. calabura* leaves is thought to come from the active compound 2',4'-dihydroxychalcone (**Figure 7**)[68]. The mechanism of action of the methanol extract of *M. calabura* leaves anti-quorum sensing in bacteria so that biofilm formation can be inhibited[69].

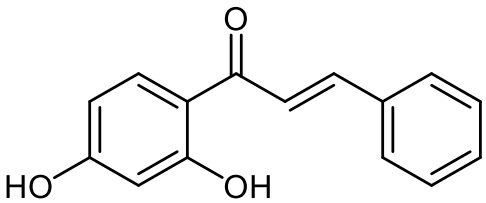

Figure 7. 2',4'-dihydroxychalcone (PubChem CID 6433293) [68].

Curcuma longa

Curcuma longa, known as turmeric, has been proven in the methanol fraction at a concentration of 0.5-5% to inhibit biofilm formation, so it can be concluded that this extract has anti-biofilm activity. One of the main components detected from the methanol fraction of *C. longa* is curcumin, an active substance that plays a vital role in inhibiting bacterial biofilm. Curcumin can inhibit biofilm formation by reducing the expression of genes involved in quorum-sensing mechanisms that cause biofilm maturation in bacteria[70].

Oxalis corniculata

Oxalis corniculata, known as calincing/lela by Indonesian people, has empirical properties as a medicine for stomach aches and coughs. Scientifically, data has been obtained that the leaves of this plant have an antimicrobial effect. Hamzah *et al*.[71] research results prove that 1% ethanol extract of *O. corniculata* provides anti-biofilm activity on *S. aureus* of 69.333%.

Areca catechu

Areca catechu, or *pinang* by the Indonesian people, has various activities, including methanol extract, which is proven to have anti-bacterial activity. The active compounds detected in the methanol extract of *A. catechu* palm were flavonoids, tannins, alkaloids, quinones, terpenoids, and saponins. *Areca catechu* nuts contain catechins, tannins (15%), gum, and alkaloids[72]. Methanol extract from *A. catechu* nuts has been proven to have anti-biofilm activity by forming complex compounds with proteins through hydrogen bonds, causing changes in the structure of proteins and nucleic acids. These structural changes can cause the proteins that makeup EPS and biofilms to be degraded. The high phenolic content of *A. catechu* nuts, gallic acid (**Figure 8**), is important in anti-biofilm activity[33,73]. When isolated at a 100 µg/mL concentration, gallic acid exhibited the highest level of inhibition in biofilm formation. The findings from the anti-biofilm assessment indicated a dose-dependent effect, meaning that the anti-biofilm activity increased as the concentration of gallic acid increased[74]. Gallic acid derivatives like methyl gallate belong to the phenolic compounds category and can act through various mechanisms, including enzyme inhibition via oxidized compounds, reactions involving sulfhydryl groups, or non-specific interactions with microbial proteins[75,76].

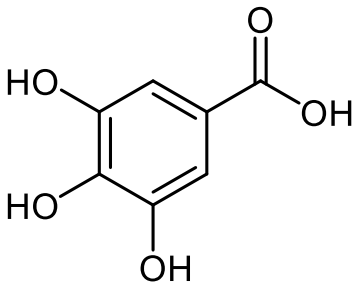

Figure 8. Gallic acid (PubChem CID 370) [74].

Carica papaya

Carica papaya or *kates* leaves contain the active compounds carpainin alkaloids, carpaine, ferulic acid, vitamins C and E, choline, papain proteolytic enzymes, saponins, flavonoids, and tannins. With a concentration of 25%, the extract of *C. papaya* leaves can inhibit the growth of biofilms by 39.837%[77]. *Carica papaya* leaves extract has been proven to have anti-biofilm activity with a mechanism in the form of binding to one of the bacterial adhesin proteins, which is used as a bacterial surface receptor, resulting in a decrease in bacterial adhesion and inhibition of protein synthesis for cell wall formation[78]. The stems, leaves, and young fruit of *C. papaya* contain white sap, which contains a protein-breaking enzyme or proteolytic enzyme called papain. Papain is thought to play an important role in degrading the EPS layer in the bacterial biofilm that forms[29]. Another compound that plays a vital role in anti-biofilm activity is ferulic acid (**Figure 9**). Research has reported that gallic acid and ferulic acid display potent preventive effects on biofilms formed by *P. aeruginosa*, *Listeria monocytogenes*, and *S. aureus*, with significant activity observed at concentrations exceeding 1 mg/mL[79]. Ferulic acid, in particular, disrupts the exopolymeric matrix of biofilms, impacting various cellular targets, including adhesion proteins, cell surface proteins, exopolymers, and communication pathways among biofilm cells during the maturation phase[80].

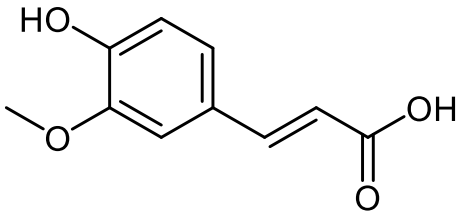

Figure 9. Ferulic acid (PubChem CID 445858) [79].

Pangium edule

Pangium edule, or *kluwek* by Indonesian people, especially on the island of Java, contains tannins, natural polyphenolic compounds that inhibit microbial growth by changing the permeability of their cell walls. An extract concentration of 4-8% effectively inhibits the growth of *S. aureus*, *E. coli*, and *P. aeruginosa*[81]. *Pangium edule* seeds has antibiofilm properties toward *S. sanguinis* biofilm ability of extract concentration 1.25% and 2.5%[34].

Eleutherine bulbosa

Eleutherine bulbosa has active compounds in the form of phenolic and flavonoid derivatives, naphthalene, anthraquinones, and naphthoquinones. Scientific investigations reveal that various pharmacological activities of *E. bulbosa* include anticancer, antidiabetic, antibacterial, antifungal, antiviral, anti-inflammatory, dermatological problems, antioxidant, and antifertility. *Eleutherine bulbosa* tuber extract shows good microbial inhibition against pathogenic bacteria such as *S. aureus*. *Eleutherine bulbosa* ethanolic extract has been proven to inhibit forming and degrade biofilms in *S. aureus*. Methanol soluble fraction at concentration 0.01 - 0.5 mg/mL can degrade biofilms from tested bacteria, while the ethanolic extract can only degrade the biofilms on *S. aureus*[35]. Microbial inhibition is caused by interference from alkaloid compounds and the formation of peptidoglycan components, thereby disrupting microbial cell walls[82,83].

Coriandrum sativum

Coriandrum sativum or coriander is often used as a kitchen spice and traditional medicine. *Coriandrum sativum* extracts and essential oils have been shown to exhibit antibacterial, antioxidant, free radical, antidiabetic, and anticancer activities. The strongest anti-biofilm activity of *C. sativum* essential oil was found against *Stenotrophomonas maltophilia* and *S. aureus* with a mechanism of action in the form of preventing the formation of biofilms and planktonic cells so that the bacterial biofilm becomes unstable[84]. The $MBIC_{50}$ and $MBIC_{90}$ of *S. maltophilia* of *C. sativum* essential oils were 7.49 and 7.96 µL/mL, respectively, while for *B. subtilis* were 7.42 and 6.95 µL/mL, respectively[59].

Artocarpus heterophyllus

Artocarpus heterophyllus or jackfruit is believed to help treat fever, boils, skin diseases, anti-diarrhea, analgesics, and immunomodulators[85]. The results of the phytochemical screening of *A. heterophyllus* leaves extract contained several compounds: phenolics, flavonoids, alkaloids, saponins, steroids, and tannins. Based on research by Khan *et al*.[86], *A. heterophyllus* leaves have antibacterial activity against *S. aureus*, *P. aeruginosa*, and *E. coli*. Extract from *A. heterophyllus* completely inhibited the growth of primary cariogenic bacteria at 3.13–12.5 µg/mL[87]. The MIC of *A. heterophyllus* leaves extract against *S. aureus* was found at 320 µg/mL[88].

Combination of Syzygium aromaticum leaves and Cinnamomum verum bark

The combination of *S. aromaticum* and *C. verum* essential oils was proven to be an effective antibacterial agent by showing high antibacterial activity against extended-spectrum beta-lactamases (ESBL)-producing *E. coli* and *K. pneumoniae* isolates, as evidenced by the diameter of the inhibition zone and MIC values. The MBC values of *S. aromaticum* and *C. verum* essential oils ranged from 0.078% to 0.156% for all bacteria tested. Morphological changes in each test bacterial cell were observed via scanning electron microscopy. Every test bacterium treated with *S. aromaticum* and *C. verum* essential oils showed cell shrinkage and lysis. *Syzygium aromaticum* leaves and *C. verum* bark contain various essential oils with different antibacterial activities. *Syzygium aromaticum* contains 15-20% essential oils, dominated by eugenol (70-85%), eugenyl acetate (15%), and β-caryophyllene (5-12%). Eugenol is a bioactive compound with bactericidal activity that causes damage, such as holes in the envelope and deformation of bacterial cells. *Cinnamomum verum* contains 0.5-1% essential oils consisting of cinnamaldehyde (63.69%), cinnamyl acetate (9.93%), and 1,8-cineole (8.75%). Cinnamaldehyde also has bactericidal activity which can affect membrane permeability and integrity as well as bacterial cell morphology[89].

CONCLUSION

Bacteria capable of forming biofilm layers are driving an escalation in antibiotic resistance. Numerous studies have indicated the presence of various active compounds with potential as anti-biofilm agents, including flavonoids, tannins, and polyphenols like gallic acid, ferulic acid, and curcumin. These compounds' modes of action against microorganisms encompass hindering attachment and biofilm formation, impeding quorum sensing, and suppressing gene expression in microbes.

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DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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Research Article

An Initial Investigation of the Potential of Robusta Coffee, Arabica Coffee, and Caffeine in Asthma Treatment through the Evaluation of 5-Lipoxygenase Inhibition Activity

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Abstract

Numerous studies have documented the potential of coffee to aid in asthma prevention. Nevertheless, research into how coffee influences asthma management has not been available. One known mechanism by which asthma medications work involves inhibiting 5-lipoxygenase (5-LOX) activity. This study aims to determine the potency of *Coffea canephora* var. Robusta extract (CRE), *Coffea arabica* extract (CAE), and caffeine are the primary isolates against 5-LOX activity. Extraction was performed by a reflux procedure using 96% ethanol with a sample-total solvent ratio of 1 : 10, an extraction time of 1 hour, and the extraction was conducted in triplicate. Fractionation was carried out by liquid-liquid partition using a chloroform-water system. Caffeine further purification was performed by the sublimation method, and the inhibition of 5-LOX activity was evaluated using the spectrophotometric method at λ = 234 nm. Apigenin was used as a positive control. From the experiment conducted, the IC_{50} of the CRE, CAE, caffeine, and apigenin against 5-LOX was 32.2 ± 1.4, 42.1 ± 2.3, 14.3 ± 1.6, and 7.4 ± 1.7 µg/mL, respectively. Continued efforts to isolate bioactive compounds from coffee extract led to the discovery of caffeine, which exhibited a more potent inhibitory effect on 5-LOX. The inhibition of 5-LOX activity by caffeine occurs in a non-competitive manner.

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INTRODUCTION

Respiratory disorders are one of the most prevalent diseases in the world. According to the World Health Organization (WHO), 262 million people worldwide suffer from asthma, and this number will increase to 400 million in 2025, where 80% of deaths from asthma occur in developing countries[1]. Asthma is characterized by bronchoconstriction, mucus hypersecretion, and airway inflammation[2]. Products from the 5-lipoxygenase (5-LOX) activity, such as leukotrienes B4 and 5-hydroxy-eicosatetraenoic acid (5-HETE), are responsible for triggering bronchoconstriction and excessive mucus secretion in response to inflammation[3]. One approach used in the treatment of asthma is through the inhibition of 5-LOX activity. Lipoxygenase (LOX) is an oxidative enzyme that contains non-heme iron (Fe) in its active site. This enzyme initiates inflammatory reactions by triggering the formation of proinflammatory mediators known as leukotrienes. Lipoxygenase catalyzes the addition of oxygen (O_2) to poly-unsaturated fatty acids (PUFAs) such as arachidonic acid and linoleic acid[4,5].

5-lipoxygenase is known to have an essential role in acute inflammation and trigger cardiovascular disorders through increased leukocyte chemotaxis, blood vessel inflammation, and increased permeability of the respiratory tract membrane[6]. 5-lipoxygenase is also known to play an essential role in triggering asthma responses. Activated immune cells will first

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produce arachidonic acid as a result of a reaction catalyzed by phospholipase A2 in the plasma membrane then, followed by the formation of 5-hydroperoxyeicosatetraenoic acid (5-HPETE) by 5-LOX to produce further leukotrienes, which have strong potential to cause bronchoconstriction via binding to the cysteinyl leukotriene receptor 1[7,8].

Zileuton (trade name Zyflo) is the only 5-LOX inhibitor available for over 25 years[9,10]. Along with research assessing the effectiveness of 5-LOX inhibitors in treating asthma, research to find potential 5-LOX inhibitor candidates is ongoing, especially research on natural compounds derived from plant extracts[11,12]. Coffee is one of the exciting plants to be further developed as a 5-LOX inhibitor. Coffee, a significant agricultural product, yields over seven million tons of green beans annually and is the second most traded commodity worldwide. The two main species cultivated throughout the tropical world are *Coffea arabica* and *Coffea canephora* var. Robusta represents 70% and 30%, respectively, in world production[13]. Coffee has a positive effect on reducing the inflammatory reaction that triggers asthma. Coffee consumption has an inverse relationship with mortality due to respiratory disorders[14,15].

Coffee studies in treating asthma have been carried out at the extract level. Coffee extract (CE) provides a weak bronchodilation effect and reduces muscle fatigue in the airways. Coffee extract from Ethiopia, Kenya, and Brazil inhibited LOX activity with EC_{50} values of 2750 to 2940 µg/mL[16]. The compound responsible for producing the anti-asthma effect in CE is predicted to be caffeine, the dominant compound in coffee. Chemically, caffeine is similar to theophylline used as an asthma medication. One of the results of caffeine metabolism in the body is also theophylline. From this relationship, it is predicted that caffeine can provide anti-asthma effects like theophylline[17]. Clinical trials on 55 patients showed that caffeine consumption at doses of less than 5 mg/kg BW could improve lung function for 2 hours after use. Based on the study, caffeine in CE can inhibit the NFκB signaling pathway, vital in producing various proinflammatory cytokines and chemokines (TNF-α and IL-6) by suppressing cyclooxygenase-2 (COX2) expression[18]. Meanwhile, studies on caffeine activity in inhibiting LOX have never been done.

Although clinically proven, the mechanism of action of CE in the treatment of asthma is still being studied. We tested *C. canephora* var. Robusta extract (CRE), *C. arabica* extract (CAE), and caffeine as bioactive compounds on 5-LOX activity in this study. This investigation aims to determine whether CE and caffeine can hinder the creation of leukotrienes that initiate airway inflammation by targeting LOX activity. This study is intended to serve as preliminary research, contributing to our understanding of how caffeine, as the primary component in CE, is involved in anti-asthmatic effects, particularly concerning the suppression of 5-LOX activity.

MATERIALS AND METHODS

Materials

The materials and instruments used in this research include soybean 5-LOX (Sigma Aldrich-L7395, US), apigenin (Sigma Aldrich-10798, US), linoleic acid (Sigma Aldrich-1376, US), demineralized water (Brataco, Indonesia), 96% ethanol (Brataco, Indonesia), distilled water (Brataco, Indonesia), Robusta Gold and Arabica Gold coffee powders (commercially available product from Indonesia), microplate reader (BioTek ELX800), and thin-layer chromatography (TLC) scanner (CAMAG).

Methods

Coffee extraction and caffeine isolation

One hundred g of coffee powder (Robusta and Arabica) were extracted with 400 mL of 96% ethanol for 2 hours using the reflux method. The extract was concentrated using a rotary vacuum evaporator at 60±5°C and dried in a vacuum oven for 6 hours to form a thick extract. This extract was used as a sample in the LOX activity assay. The remained thick extract of Robusta coffee was then added with 50 mg of MgO and 300 mL of distilled water, heated for 1 hour at 90±10°C, and filtered. The residue was boiled again for 1 hour with 500 mL of distilled water; this process was repeated two times, and then the obtained extract was filtered using a Buchner funnel. The filtrate obtained was added with 50 mL of 10% H_2SO_4 and then concentrated until the volume was reduced to 250 mL. The liquid-liquid extraction was added with 250 mL of chloroform into the aqueous filtrate. The chloroform layer was taken and washed with 40 mL of 1% NaOH and shaken with 40 mL of hot water. The transparent-colored chloroform layer is evaporated to obtain a concentrated filtrate. The sublimation was

then carried out to the filtrate at a temperature of 180-200°C to obtain caffeine isolate in white needle crystals[19]. The caffeine isolate was used in the LOX activity assay as a sample.

Examination of isolate purity using TLC-densitometry

The isolate was dissolved in 96% ethanol until a concentration of 1000 µg/mL was obtained and then eluted with the mobile phase *n*-hexane : ethyl acetate : ethanol (2.5 : 1.5 : 0.4). The spot was compared to standard caffeine. The purity level was assessed from the number of visible spots under UV 254 and 366 nm and the instrument calculations[20].

Melting point determination

The melting point of the isolate was determined using the Fisher-Johns melting point apparatus with corrected temperature[21]. Isolate crystals obtained from the sublimation process were put in the capillary tube. The temperature when the crystals melt for the first time until they melt entirely was observed using a thermometer on the instrument. The melting point was then compared to the caffeine standard.

5-LOX inhibitory activity assay

5-LOX inhibitory activity assay was carried out based on previous studies[22-24] with slight modifications. As much as 50 µL of each CRE, CAE, caffeine isolate, and apigenin solution was put into the vial, then added with 1650 µL of 0.2 M borate buffer solution pH 9 and 1000 µL of 300 µM linoleic acid as substrate solution. The mixture was vortexed and then incubated for 10 minutes at 25°C. Then 300 µL of 1000 U/mL 5-LOX solution was added to the mixture, then incubated for 15 minutes at 25°C. After incubation, 1000 µL methanol was added to the mixture and vortexed. The absorbance of the solution was measured using a UV spectrophotometer under 234 nm.

Enzyme kinetics assay

The kinetics of the inhibitory enzyme activity was carried out by varying the concentration of the linoleic acid as substrate (final concentration = 50, 75, 100, and 125 µM) with constant inhibitor concentration (final concentration of caffeine = 18.75 µg/mL). The concentration of caffeine used for the enzyme kinetics assay was determined by the concentration of caffeine that could inhibit 50% of the enzyme activity (IC_{50}). To determine the type of inhibition of 5-LOX activity, an enzyme kinetics assay of 5-LOX inhibition by caffeine was performed using Michaelis–Menten kinetics, as shown in **Equations 1** to **3**, in which V_i was the initial velocity of an enzymatic reaction, V_{max} was the maximal velocity of the enzymatic reaction, Km was Michaelis constant, and [S] was substrate concentration.

$$V_i = \frac{V_{max}[S]}{Km+[S]} \quad [1]$$

$$\frac{1}{v_i} = \frac{K_m+[S]}{V_{max}[S]} \quad [2]$$

$$\frac{1}{Vi} = \frac{Km}{V_{max}} x \frac{1}{[S]} + \frac{1}{V_{max}} \quad [3]$$

Data analysis

Statistical analysis was performed using one-way ANOVA, and further multiple comparison between groups was analyzed using the Tukey Post Hoc Method.

RESULTS AND DISCUSSION

The coffee plant contains various bioactive compounds with antioxidant properties, specifically phenolic compounds such as caffeic acid, chlorogenic acid, coumaric acid, ferulic acid, and cinnamic acid[25]. In addition to phenolic compounds, coffee contains methylxanthine alkaloid compounds such as caffeine, theophylline, and theobromine. Both groups of phenolic compounds and methylxanthine alkaloids have good solubility in organic solvents[26]. Thus, to produce a yield with a high content of bioactive compounds, the solvent chosen for the extraction of coffee bean powder is ethanol—extraction of *C. canephora* var. Robusta and *C. arabica* using ethanol yielded 9.84% and 8.12% samples, respectively. The resulting extract had a dark brown color and emitted a coffee aroma. The subsequent isolation process was performed in CRE following the alkaloid extraction principle, involving adding a base and extraction from the organic solvent layer (chloroform). This

process yielded fractions containing multiple compounds. The crude fraction was then subjected to purification through sublimation, producing pure caffeine in the form of needle-like crystals, with a yield of 0.11% relative to the total ground sample (**Table I**). The purity of the caffeine crystals formed from the sublimation process was confirmed by comparing the melting point of the crystal to caffeine standards, observing the number of spots visible on TLC, and observing the spot purity level by TLC-densitometry.

Table I. Extraction yield.

Component	Weight (g)	Yield (%)
Total sample weight	100	-
Coffea canephora var. Robusta extract	9.84	9.84
Coffea arabica extract	8.12	8.12
Caffeine	0.11	0.11

The melting point test results found that the caffeine crystals isolated from CRE had a narrow melting point range between 234-236°C, indicating a high purity level. The melting point range of isolated caffeine was similar to the caffeine standard (**Table II**). The chromatographic profile was evaluated using the TLC technique. The isolated compound produced a single spot under UV 254 nm with the same color, Rf, and spectral profile as standard caffeine. Examination of the purity level using TLC-densitometry showed a purity level of 98.6%. The results indicated that the isolated crystal was pure and confirmed as caffeine.

Table II. Evaluation of the melting point range of isolated and standard caffeine.

Component	Melting point range (°C)
Isolated caffeine	234-236
Standard caffeine	234-237.5

All CRE, CAE, and caffeine isolate samples were tested for 5-LOX inhibitory activity. The assay was carried out in triplicate. The IC_{50} values of CRE, CAE, and caffeine against 5-LOX were 32.2 ± 1.4, 42.1 ± 2.3, and 14.3 ± 1.6 µg/mL respectively. The standard compound used as a positive control was apigenin, which showed an IC_{50} value of 7.4 ± 1.7 µg/mL. In **Figure 1**, it can be observed that there is a significant difference in activity between CRE, CAE, and caffeine. As a single compound isolated from coffee extract, caffeine demonstrates an inhibitory activity of 2.3 times stronger than CRE and three times stronger than CAE, as indicated by the smaller IC_{50} values. Interestingly, caffeine's inhibition of 5-LOX activity is not significantly different (ns) compared to apigenin, used as the positive control. CRE's activity is superior to CAE; we hypothesize that this difference is due to variations in caffeine content in the samples. Robusta coffee consistently shows higher caffeine levels in various studies compared to Arabica coffee (±2.54% vs ±1.22%)[27,28].

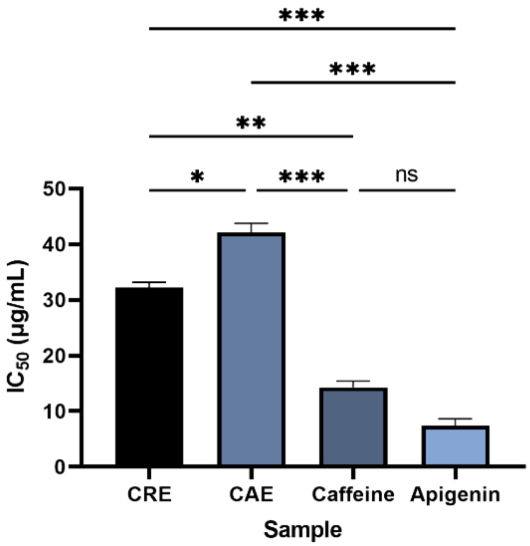

Figure 1. Inhibition of 5-LOX activity by CRE, CAE, caffeine, and apigenin represented by the IC_{50} value.

Data were obtained in the enzyme kinetics assay, as shown in **Table III**, and the Lineweaver-Burke graph shows the intersection on the *x*-axis (**Figure 2**). The results indicate that the type of inhibition is non-competitive. Non-competitive inhibition occurs when the inhibitor (Inh) binds to the enzyme at a different site than the substrate (S). There is no competition between the inhibitor (caffeine) and the substrate in non-competitive inhibition. The presence of non-competitive inhibitors decreases the V_{max} value with a relatively stable Km value.

Table III. Enzyme kinetic measurement data.

Parameter	With inhibitor (Caffeine)	Without inhibitor
a	1.907	1.0435
b	351.16	169.65
r	0.9992	0.9989
V_{max}	0.524	0.958
Km	184.143	162.578

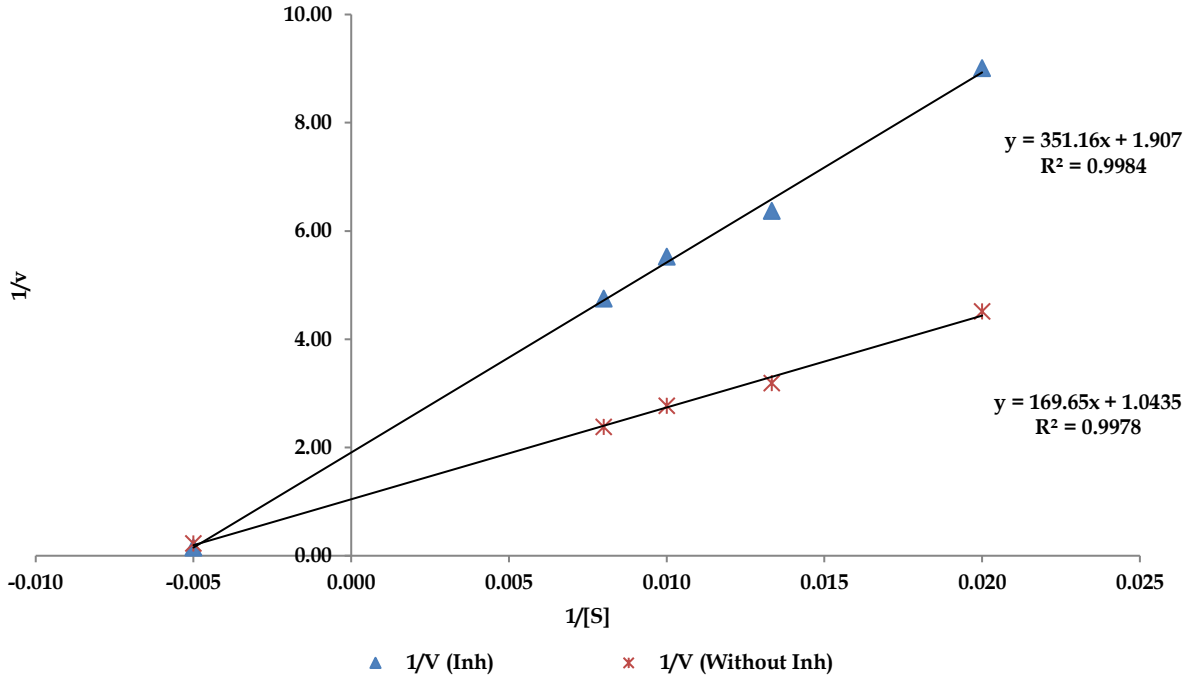

Figure 2. Enzyme kinetic profile of 5-LOX activity inhibition by inhibitors (caffeine).

Lipoxygenase is a metalloenzyme that has pro-oxidation and pro-inflammatory properties. Lipoxygenase generally catalyzes the oxidation of unsaturated fatty acids. In the human body, LOX metabolizes arachidonic acid to leukotrienes (a potent inflammatory mediator), making LOX a critical enzyme in the inflammatory pathway. Based on the relative oxidation position in the arachidonic acid structure, LOX is classified into 5-LOX, 12-LOX, and 15-LOX[29]. Inhibition of LOX activity can reduce oxidation and inflammation, which can trigger asthma. The studies for effective LOX inhibitors from plants are still ongoing. Compounds from plants that are known to inhibit LOX activity are phenolics and alkaloids, both of which are found in coffee[30]. It is known that coffee extract also contains alkaloid compounds of the methylxanthines type: caffeine (1,3,7-trimethyl xanthine), theophylline (3,7-dimethyl xanthine), and theobromine (1,3-dimethyl xanthine). Coffee also has theobromine and theophylline despite concentrations 20 times lower than caffeine[31]. The caffeine content in one cup of coffee depends on the variety of coffee plants used. The average caffeine content in brewed coffee grounds is 57 mg/100 mL[32]. Other research stated that coffee contains caffeine ± 1177 mg/g as the main compound[33].

Our study proves that caffeine plays a role in inhibiting LOX. The inhibitory potency of caffeine in inhibiting 5-LOX cannot be compared with Zileuton (a standard drug approved by the FDA as a 5-LOX inhibitor) due to access limitation; thus, in this study, apigenin was used as a positive control. However, from other studies, it was known that using a similar colorimetric method, Zileuton showed an IC_{50} of 2.08 µM or 32 times more potent in inhibiting 5-LOX activity compared to caffeine in this study. Zileuton is the ligand that inhibits iron binding to the LOX and has a weak potential reduction[34].

Although caffeine has a weaker 5-LOX inhibitory potency than Zileuton, caffeine in coffee may be used as an alternative in relieving asthma because of its pharmacokinetic profile. A comparative study showed that caffeine has a more rapid onset of action, lower fluctuations in plasma concentrations, a longer half-life, and fewer peripheral side effects compared to other methylxanthines[32,35,36]. In oral administration, gastrointestinal absorption of caffeine is rapid and complete, achieving almost 100% bioavailability. After reaching the bloodstream, caffeine binds to albumin and is distributed to all tissues by simple diffusion or carrier-mediated transport[32,37].

This study's *in vitro* enzymatic activity assay results might be used as preliminary data to assess the caffeine potency in inhibiting 5-LOX activity. For future research, more selective and stable methods might be developed, one of which is the colorimetric method using thiocyanate ions (SCN^-) to form a red ferric thiocyanate (FTC) complex measured at λ 480 nm. This colorimetric method is considered more selective and stable for measuring products formed from the enzymatic activity of 5-LOX[38].

CONCLUSION

Coffee exhibits potential inhibition activity against 5-LOX, with IC_{50} values of 32.2 ± 1.4 µg/mL for CRE, 42.1 ± 2.3 µg/mL for CAE, 14.3 ± 1.6 µg/mL for caffeine, and 7.4 ± 1.7 µg/mL for apigenin. Further bioactive compound isolation of coffee extract produced caffeine with a more substantial inhibitory potential against 5-LOX. Caffeine inhibits 5-LOX activity in a non-competitive manner.

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AUTHORS' CONTRIBUTION

Conceptualization: Tegar Achsendo Yuniarta, Rosita Handayani
Data curation: Tegar Achsendo Yuniarta, Rosita Handayani
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Funding acquisition: Rosita Handayani
Investigation: Tegar Achsendo Yuniarta, Rosita Handayani
Methodology: Tegar Achsendo Yuniarta, Rosita Handayani
Project administration: Rosita Handayani
Resources: Tegar Achsendo Yuniarta, Rosita Handayani
Software: -
Supervision: -
Validation: Tegar Achsendo Yuniarta, Rosita Handayani
Visualization: Tegar Achsendo Yuniarta, Rosita Handayani
Writing - original draft: Tegar Achsendo Yuniarta
Writing - review & editing: Rosita Handayani

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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Research Article

Ziziphus rugosa Leaf: Pharmacognostical Characters and Anti-Inflammatory Properties against Carrageenan-Induced Paw Edema

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Abstract

Ziziphus rugosa belongs to the family Rhamnaceae, which includes many flowering species, primarily trees and shrubs, and sometimes vines. This study aims to describe the pharmacognostic characteristics and potential anti-inflammatory properties of *Z. rugosa* leaf. The pharmacognostical and preliminary phytochemical studies were performed following standard procedures. Acetone, ethanol, and aqueous extracts were screened for anti-inflammatory potential using the carrageenan-induced paw edema model. *Ziziphus rugosa* was identified by its evergreen nature, recurved hooks, and drupe-type fruits. Leaves are elliptic/rounded with cordate base exhibiting a dark green glossy upper surface and pubescent lower surface. The leaf exhibited a dorsiventral nature in the transverse section, covering trichomes, collenchyma, sclerenchyma patch, and calcium oxalate crystals as key histological characters. Anamocytic stomata, covering trichomes, crystals, and fragments of vessels, are the imperative elements in powder. The extracts contain carbohydrates, alkaloids, glycosides, tannins, saponins, phenolic compounds, proteins, and flavonoids. The acetone extract at 400 and 200 mg/kg displays a maximum inflammation inhibition of 56.96% and 48.77% among the extracts, and the standard diclofenac sodium inhibits inflammation by 65.61% at 24 hours. The altered liver superoxide dismutase, glutathione, and malondialdehyde levels in the positive control group are significantly near normal in the treatment groups. The histopathological studies of treated animals show significant protection against paw and liver tissue damage. Pharmacognostical study outcomes aid in the identification of species along with ascertaining standardization parameters. Further fractionation of acetone extract followed by isolating compounds responsible for the anti-inflammatory activity would provide an alternative to managing inflammation.

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INTRODUCTION

Plants have been used from time immemorial for their therapeutic potential, with a belief that human well-being is dependent on natural sources[1]. This is evident from the wide usage of medicinal herbs in various folklore and indigenous systems of medicine across various regions. Thus, recognition of medicinal plants role in alleviating the disease conditions led to the search for plant-derived novel drugs from time to time[2]. The World Health Organization (WHO) report reinforces that around 80% of the population in developing countries satisfy their medical needs by relying on native systems of

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medicine owing to the non-accessibility of modern systems of medicine, increased healthcare costs, cultural beliefs, safety, and efficacy concerns with novel and available marketed drugs[3]. A considerable percentage of the population in India still relies on Ayurveda, Siddha, and Unani systems for treating their various health ailments, and the possible reason for this could be the significant biodiversity accommodating around 45,000 plant species[4]. These plants contain diverse bioactive compounds with various pharmacological activities such as antiepileptic, anti-inflammatory, antidiabetic, anticancer, antioxidant, antimicrobial, antiulcer, analgesic, hepatoprotective, wound healing properties, and other[5].

Ziziphus rugosa Lam. belongs to the family Rhamnaceae, which includes many flowering species, mostly trees and shrubs, and sometimes vines. Traditionally, various parts of this species, such as bark, flower, stem, fruit, and leaf, are used in conditions like diarrhea, menorrhagia, fever, mouth ulcer, cough, hypotension; treatment of wounds boils, rheumatism; as an aphrodisiac, astringent, and as a demulcent in treating broncho-pulmonary irritation[6,7]. Concurrently, the species is reported for its anti-inflammatory (root bark, root)[8], antidiabetic (bark)[9], antioxidant (stem)[10], anthelmintic, antibacterial, insecticidal, antioxidant, cytotoxicity, CNS depressant and anxiolytic (fruit, pericarp, seed)[11-14] and analgesic, CNS depressant, antioxidant, hepatoprotective, cytotoxic and antimicrobial activities (leaf)[15-17]. Therefore, this study aims to determine the anti-inflammatory activity of *Z. rugosa* leaves extract along with preliminary pharmacognostic and phytochemical characteristics.

MATERIALS AND METHODS

Materials

Fresh, healthy *Z. rugosa* leaves were collected from Yelahanka, Bangalore, in December 2020. The collected leaves were washed with water and dried under shade. The plant material was authenticated by Dr. K. Ravikumar, Emeritus Professor, The University of Trans-Disciplinary Health Sciences and Technology (TDU), Foundation for Revitalization of Local Health Traditions (FRLHT), Bangalore, India. The herbarium was prepared per the standard curatorial practices[18] with collection voucher number FPH-PG-55 and submitted to the crude drug museum, PG Department of Pharmacognosy, along with a few leaf samples for future reference. Carrageenan and diclofenac sodium were obtained from Sigma Aldrich and Yarrow Chem Products, respectively. All other chemicals and reagents used in the present study were analytical grade.

Methods

Pharmacognostical studies

The macroscopical characters were observed on intact *Z. rugosa* leaves and recorded. For microscopical studies, free-hand transverse sections of the midrib and petiole were obtained using a razor blade. The sections were stained with safranin, phloroglucinol, and concentrated HCl and observed for the histological characters. Powder microscopical characters were examined in coarse powder after clearing with chloral hydrate and stained with the aforementioned staining agents. Histochemical tests were performed to identify cell contents by treating the thin sections with phloroglucinol and concentrated HCl, Millon's reagent; Sudan III, iodine, ruthenium red, and ferric chloride solution[19,20].

Physicochemical parameters

Loss on drying, yield to solvents (95% ethanol and water), and ash values (total, water-soluble, and acid-insoluble) were determined following standard procedures. About 1 g of the powder sample was separately treated with 50% H_2SO_4, 50% HNO_3, 5% KOH, methanol, ethanol, acetone, 1 N HCl, 1 N methanolic NaOH, 1 N ethanolic NaOH, dilute ammonia solution, and visualized under 254, 365, and 425 nm for ultraviolet analysis. Successive solvent extraction was performed using solvents with varied polarity, such as *n*-hexane, chloroform, ethyl acetate, acetone, ethanol, and water. The obtained extracts were screened to identify the nature of contained phytochemicals[21,22].

Experimental design

The acetone, ethanol, and aqueous extracts were selected for the evaluation of *in vivo* anti-inflammatory activity based on the preliminary phytochemical screening.

FT-IR studies

The FT-IR spectra of *Z. rugosa* leaves extracts were recorded using Shimadzu FTIR-8400S based on diffuse reflectance spectroscopy. A background scan was run with potassium bromide and the extracts were mixed with potassium bromide in a ratio of 1 : 100 to obtain the spectra.

Experimental animals

Seventy-two albino Wistar rats of 150–200 g was procured from the faculty animal house. The rats were allowed to acclimatize to the animal house environment for 14 days with feed and water ad libitum. The research protocol was approved by the Institutional Animal Ethics Committee of the Faculty with approval number XXIV/MSRFPH/COG/M-009, dated 27th November 2020, and conducted by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines[23].

Acute toxicity study

The acute toxicity of the selected extracts was evaluated in Wistar rats following the Organization for Economic Co-operation and Development (OECD) 423 guidelines[24]. Animals were fasted overnight before dosing with water ad libitum. Six rats were administered 2000 mg/kg of each of the selected three extracts suspended in normal saline. Later, the animals were continuously monitored for 24 hours and subsequently for the next 14 days for any signs of abnormality or mortality.

In vivo anti-inflammatory activity study

Carrageenan-induced paw edema model was used to evaluate the *in vivo* potential of the extracts. The animals were randomly divided into nine groups with each group containing six animals. The treatment details are summarized in **Table I**. Vehicle-treated and carrageenan control group rats received only the vehicle, while standard and extract treatment groups received respective treatments for seven days. On the 7th day, zero-hour paw volume was measured after the last dose of vehicle/extracts and standard administration.

Consequently, paw edema was induced in each rat by injecting 0.1 mL of 1% carrageenan in 1% normal saline into the sub-plantar tissues of the left hind paw. A further change in paw volume at 1, 2, 4, 6, 8, and 24 hours post-carrageenan administration was measured using a digital plethysmograph. Later, the animals were sacrificed by euthanasia to excise paw and liver tissues. The antioxidant parameters such as superoxide dismutase (SOD), glutathione (GSH), and malondialdehyde (MDA) levels were determined in liver samples, while histopathological studies were performed in both liver and paw tissues[25-27].

Table I. Grouping and administration design.

Groups	Treatment
1	Vehicle control (normal saline)
2	Carrageenan control (0.1 mL, 1%)
3	Standard (Diclofenac sodium 10 mg/kg)
4	*Ziziphus rugosa* aqueous extract 200 mg/kg p.o.
5	*Ziziphus rugosa* aqueous extract 400 mg/kg p.o.
6	*Ziziphus rugosa* ethanol extract 200 mg/kg p.o.
7	*Ziziphus rugosa* ethanol extract 400 mg/kg p.o.
8	*Ziziphus rugosa* acetone extract 200 mg/kg p.o.
9	*Ziziphus rugosa* acetone extract 400 mg/kg p.o.

Histopathological studies

Liver and paw tissues were washed with normal saline and stored in 10% formalin. The tissues were dehydrated and embedded in paraffin wax for sectioning. The sections were stained with hematoxylin and eosin for histopathological studies.

Data analysis

The obtained results were statistically analyzed via one-way analysis of variance (ANOVA) and Tukey's Kramer multiple comparison tests using Graph-Pad InStat V-3 (https://www.graphpad.com/scientific-software/instat/). All the values were expressed as Mean ± SEM (n = 6).

RESULTS AND DISCUSSION

Ziziphus rugosa is a large evergreen straggling shrub/small tree of 3-6 m height with recurved hooks. The species is found distributed up to an altitude of 1800 m in dry deciduous forests. Flowers are in long-peduncled tomentose cymes, forming a panicle with globose buds and densely tomentose pedicels. Fruits are of drupe type, globose around 6-8 mm in diameter with one seed and white when ripe. Wood is moderately hard and red; while young branches were clothed with fulvous tomentum. The leaves are alternate, petiolate, elliptic/rounded with oblique or cordate base, and 5-7 cm long. The apex is rounded, retuse/mucronate, while the margin is serrulate. Fresh leaves are dark green, glossy on the upper surface, pale green to greyish-green, and pubescent on the lower side, while dry leaves are greyish-green. They possess three marked nerves almost traveling to the apex and are prominent on the lower surface. Petioles are tomentose and 6-12 mm in length. The taste of the leaf is mild, sweet, and with a characteristic odor (**Figure 1**).

Figure 1. Macroscopical characters of *Z. rugosa* (**a**: habit; **b**: leaf showing upper surface; **c**: leaf showing lower surface; **d**: flower; **e**: fruit; **f**: fresh leaf; **g**: dry leaf; **h**: twig showing recurved spine).

The transverse section of the leaf exhibited a dorsiventral nature with a prominent midrib on the lower side. The lamina comprises the upper epidermis, mesophyll, and lower epidermis. The upper epidermis comprises a single layer of small, tabular parenchyma cells covered with a cuticle. The mesophyll region is differentiated into single-layered upper palisade cells and spongy parenchyma. The lower epidermis comprises a single layer of small tabular parenchyma covering numerous trichomes. Vascular strands are found intermittently in the mesophyll regions of the lamina.

The upper epidermis is continuous in the midrib, while palisade cells are not continuous; instead, a patch of collenchyma appears in the midrib below the upper epidermis. The lower epidermis is continuous in the midrib region, with numerous unicellular covering trichomes. A patch of collenchyma is observed above the lower epidermis in the midrib region. "U" shaped vascular bundle is located in the midrib region with distinct phloem and xylem. The vascular bundle is surrounded by a patch of sclerenchyma cells. Spongy parenchyma cells are big, spherical, and located surrounding the sclerenchyma patch in the midrib. Druse and prismatic calcium oxalate crystals are found in collenchyma cells (**Figure 2**).

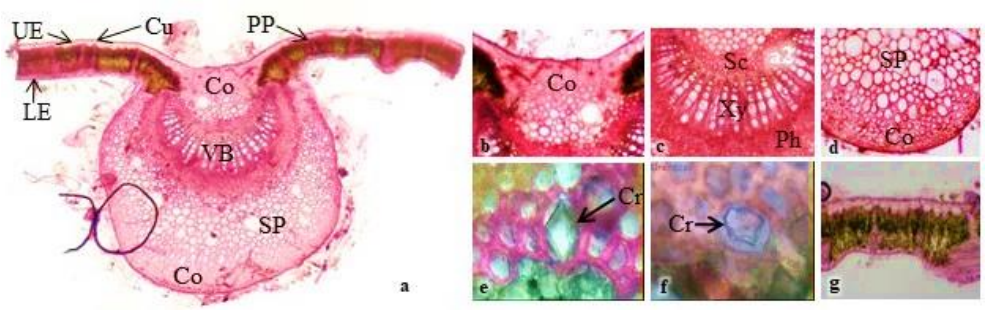

Figure 2. Microscopical characters of *Z. rugosa* leaf (**a**: transverse section showing midrib and lamina; **b**: midrib showing collenchyma below upper epidermis; **c**: midrib showing vascular bundle; **d**: midrib showing collenchyma and spongy parenchyma above lower epidermis; **e,f**: crystal; **g**: lamina (**Co**-collenchyma, **Cr**- crystal, **Cu**- cuticle, **LE**- lower epidermis, **Ph**- phloem, **PP**- palisade parenchyma, **Sc**-sclerenchyma, **SP**- spongy parenchyma, **UE**- upper epidermis, **VB**- vascular bundle, **Xy**- xylem).

The transverse section of the petiole shows an ovoid outline with a thin-walled single layer of epidermis covering trichomes. The cortex comprises 11-13 layers of thin-walled, loosely arranged compact parenchyma cells. One to two layers of thin-walled round to oval-shaped mucilage cells are found next to the cortex. Vascular bundles are collateral and rectangular, with a few layers of loosely arranged phloem cells. Loosely arranged spherical parenchyma, mucilage, and idioblast cells are observed at the center of the petiole (**Figure 3**). *Ziziphus rugosa* powder revealed the presence of parenchyma, covering trichomes, anomocytic stomata, fragments of vessels, and calcium oxalate crystals (**Figure 4**).

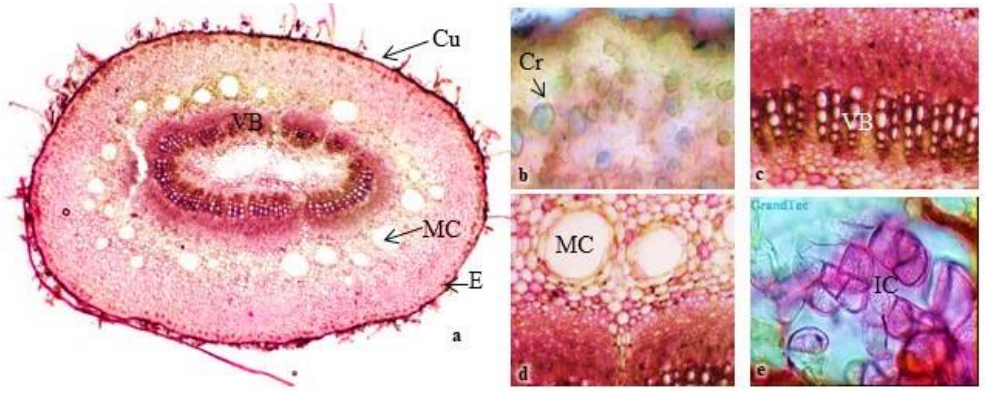

Figure 3. Microscopical characters of *Z. rugosa* petiole (**a**: transverse section showing epidermis, cortex, and vascular bundles; **b**: crystals in the cortical region; **c**: vascular bundle; **d**: mucilage cells; **e**: idioblast cells (**Cr**- crystal, **Cu**- cuticle, **E**-epidermis, **VB**- vascular bundle, **MC**-mucilage cell; **IC**-idioblast cell).

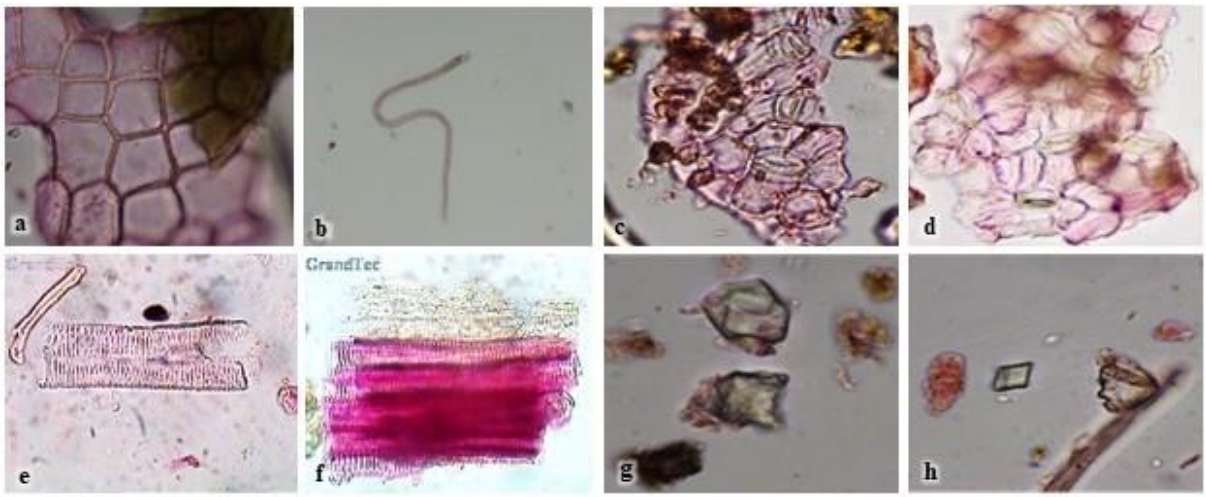

Figure 4. Powder characters of *Z. rugosa* leaf (**a**: parenchyma; **b**: covering trichome; **c,d**: stomata; **e,f**: fragments of vessels; **g,h**: crystals).

The contribution of plant-derived drugs in alleviating various diseased conditions is evident, and a resurgence in the usage of plant-based medicines has been observed in the recent past owing to efficacy and safety profile, accessibility, side effects of modern drugs, non-availability of a proper cure for specific disease conditions, microbial resistance, and other[28,29]. Acquiring maximum benefit from plant drugs depends on using the correct species claimed for particular indications. Hence, correctly identifying plant species by applying various scientific parameters is essential to garner the advantage of their possible and positive role in treating diseases. In this context, the first step towards identifying crude drugs is through macro- and microscopical characters[30] supported by herbarium specimen[31]. Macro and microscopical characters are essential as they provide insights into the diagnostic characters for identifying and differentiating plants belonging to diverse taxonomical hierarchies. *Ziziphus rugosa* can be identified by its evergreen nature with recurved hooks, long peduncled tomentose cymes, and drupe type of fruits, while leaves alternate with cordate base, pubescent, and with three prominent nerves on the lower side. Dorsiventral nature, covering trichomes, sclerenchymatous patch, druse, and prismatic calcium oxalate crystals are essential characteristics of its anatomical structure. Powder characters facilitate the establishment of the identity and quality of size-reduced plant materials. In this case, covering trichomes, stomata, crystals, and others, are the key characters to identify the leaf in powder form. Various physicochemical parameters determined will assist in ascertaining the Pharmacopoeial standards of the crude drug specimen[32].

Histochemical tests revealed the presence of starch, proteins, lipids, tannins, mucilage, lignin, and cellulose, as positive reactions were noted with iodine solution (blue), Millon's reagent (red), Sudan III (red), $FeCl_3$ (bluish-black), ruthenium red solution (pink mucilage), and iodine with 60% sulfuric acid (yellowish brown), respectively. The various physicochemical parameters, such as loss on drying, yield to solvents, and ash values, were determined and represented in **Table II**. Then, leaf powder treated with various reagents exhibited characteristic colors under 254, 366, and 425 nm. The results are tabulated in **Table III**. The extraction results of *Z. rugosa* leaves powder are based on several parameters, including color, nature, and yield obtained with various solvents are as follows: *n*-hexane: dark green, sticky mass, 1.36%; chloroform: greenish brown, solid mass, 0.66%; ethyl acetate: greenish brown, solid mass, 2.84%; acetone: brownish green, sticky mass, 2.14%; 95% ethanol: greenish brown, sticky mass, 5.96%, and water: brown, solid, 7.66%. The extracts possess various phytoconstituents such as alkaloids, carbohydrates, glycosides, proteins, phenolics, tannins, saponins, and flavonoids. The phytochemical study outlines the quantity and nature of phytoconstituents present in the sample, which can be further utilized to establish biomarker compounds that aid in proper identification and help to determine the purity of plant drug materials[33]. In addition to powder characters, fluorescence studies afford some diagnostic information on the identification and verification of the quality of the sample based on fluorescence exhibited under diverse wavelengths when treated with different reagents and solvents[34].

The spectra of various extracts of *Z. rugosa* leaves are illustrated in **Figure 5**. The FT-IR spectra signified the presence of diverse functional groups, and variations in peaks among all the extracts were also observed[35]. Some of the essential functional groups visualized were 1°, 2° amines (NH stretching, 3550-3230 cm^{-1}), amides (C=O stretching, 1652 cm^{-1}) aldehyde (CH stretching, 2900-2800 cm^{-1}) in *n*-hexane extract; aromatic CH (CH stretching, 1629-1427 cm^{-1}), 1° amines (NH stretching, 3294-3423 cm^{-1}), phenol (OH stretching, 3569-3529 cm^{-1}) in chloroform extract; aromatic (CH stretching out of plane bend, 966 cm^{-1}), amines (C-N stretching, 1764-1076 cm^{-1}), 1° amines (C=N stretching, 2252 cm^{-1}), amines (NH stretching, 3452-3404 cm^{-1}), alkanes (CH stretching, 2921 cm^{-1}) in ethyl acetate extract; alkane (CH stretching, 2927-2852 cm^{-1}), alkene (CH stretching, 2476-2619 cm^{-1}), ketone (C=O stretching, 1716-1458 cm^{-1}), aromatic alkene (C-C stretching, 1515-1458 cm^{-1}) in acetone extract; alcohol (OH stretching, 3851-3919 cm^{-1}), nitrile (C≡N stretching, 2354-2210 cm^{-1}), aldehyde (CHO stretching, 1955-1863 cm^{-1}), amines (C-N stretching, 1207-1035 cm^{-1}) in ethanol extract; and 1°, 2° amines (NH stretching, 3500-3100 cm^{-1}), alkyl (CH_3 bending, 1153 cm^{-1}) in the aqueous extract. The FT-IR spectra are considered a reliable option to detect the bio-molecular composition and provide valuable information on the various classes of compounds present in the extracts[36].

Table II. Physicochemical parameters of *Z. rugosa* leaves.

Loss on drying (%)	Ash value (%)			Yield to solvent (%)	
	Total ash	Water-soluble	Acid-insoluble	Water	95% ethanol
8.66	5.55	1.11	0.66	13.86	5.60

Table III. Fluorescence analysis of *Z. rugosa* leaves.

Reagents	Visible light	UV light	
		Short wave (254 nm)	Long wave (365 nm)
Powder as such	Sultry	Sultry	Mehandi N
50% H_2SO_4	Vivid green	Mehandi N	Dark drama
50% HNO_3	Copper leaf	Copper	Vivid green
5% KOH	Green gold	Meadow path	Mehandi N
Methanol	Mehandi N	Vivid green	Pine N
Ethanol	Mehandi N	Green gold	Pine N
Acetone	Dark drama	Amazon moss	Meadow path
1 N HCL	Sultry	Mehandi N	Green gold
1 N methanolic NaOH	Green gold	Dark drama	Vivid green
1 N ethanolic NaOH	Mehandi N	Mehandi N	Dark drama
Diluted NH_3 solution	Green gold	Meadow path	Amazon moss

Note: All color comparison is based on the "Asian paints" premium gloss enamel card, Asian Paints Limited, Mumbai

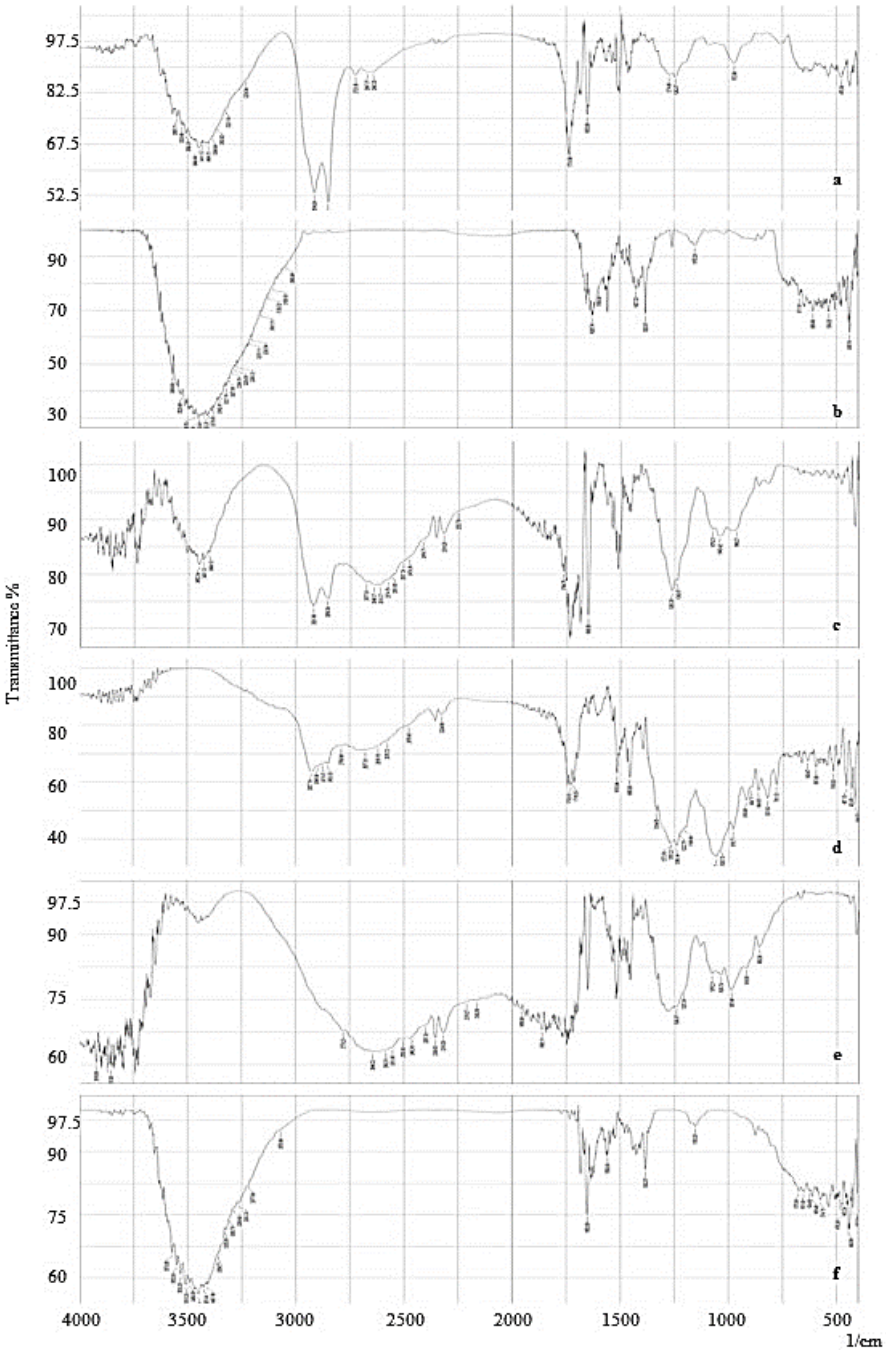

Figure 5. FT-IR spectra of various *Z. rugosa* leaves extracts (**a**: *n*-hexane extract; **b**: chloroform extract; **c**: ethyl acetate extract; **d**: acetone extract; **e**: ethanol extract; **f**: aqueous extract).

Acute toxicity studies of acetone, ethanol, and aqueous extracts of *Z. rugosa* leaves were performed following OECD 423 guidelines. None of the extracts exhibited any signs of abnormality or mortality at 2000 mg/kg dose either during short-term (24 hours) or long-term observation (14 days). Hence, $\frac{1}{5}^{th}$ (400 mg) and $\frac{1}{10}^{th}$ (200 mg) of the tolerated dose were used for *in vivo* evaluation[19]. An acute toxicity study is essential to understand the toxicological profile of the substance being administered to animals either as a single dose or over short-term exposure[37]. Administration of aqueous, acetone, and ethanol extract at 2000 mg/kg to animals did not exhibit any toxic symptoms or mortality over a 24-hour observation period. Hence 200 mg/kg and 400 mg/kg of these extracts were finalized for further *in vivo* anti-inflammatory activity study.

Carrageenan injection significantly developed the edema and a maximum increase in paw volume was noticed at four hours (23.98 ± 0.32%). The percentage increase in paw volume at 24 hours in carrageenan control animals was 8.55 ± 0.42%, while the diclofenac sodium treated animals exhibited a significant decrease in percentage increase of paw volume (2.94 ± 0.20%) representing the anti-inflammatory effect. The percentage increase in paw volume of the *Z. rugosa* leaves extract administered animals at 24 hours was as follows: aqueous extract (200 mg/kg: 6.82 ± 0.47%; 400 mg/kg: 5.28 ± 0.42%), ethanol extract (200 mg/kg: 5.62 ± 0.54%; 400 mg/kg: 5.25 ± 0.60%) and acetone extract (200 mg/kg: 4.38 ± 0.30%; 400 mg/kg: 3.68 ± 0.22%), as shown in **Figure 6**.

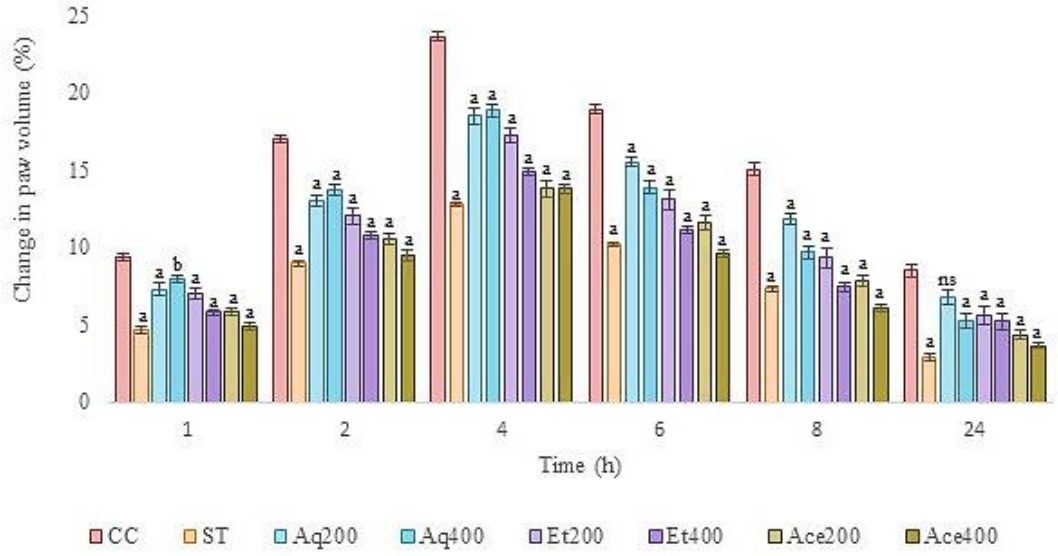

Figure 6. Change of paw volumes in carrageenan-induced paw edema at 1, 2, 4, 6, 8, and 24 hours (**CC**- carrageenan control; **ST**- standard treated; **Aq200**- aqueous extract 200 mg/kg; **Aq400**- aqueous extract 400 mg/kg; **Et200**- ethanol extract 200 mg/kg; **Et400**- ethanol extract 400 mg/kg; **Ace200**- acetone extract 200 mg/kg; **Ace400**- acetone extract 400 mg/kg). Values expressed as mean ± SEM (n = 6). ap <0.001, bp <0.05. standard and extract-treated group Vs. carrageenan control group.

Diclofenac sodium at 10 mg/kg inhibited the inflammation by 65.61% at 24 hours. Maximum percentage inhibition of inflammation was observed with acetone extract, 56.96% at 400 mg/kg and 48.77% at 200 mg/kg at 24 hours among the extract-treated groups. The percentage inhibition of inflammation noticed with ethanol extract was 38.6% (400 mg/kg) and 34.27% (200 mg/kg), while aqueous extract exhibited 38.25% and 20.23% of inflammation inhibition at the low and high doses, respectively.

Decreased liver GSH levels were observed in animals of the carrageenan control group compared to vehicle-treated animals, indicating an alteration in antioxidant status[38]. Administration of test substances (aqueous, ethanol, and acetone extracts) significantly increased the GSH levels. On the other hand, carrageenan control group animals exhibited an increase in liver MDA levels compared to vehicle-treated rats, and these elevated MDA levels were significantly decreased in various extract-treated animals at both doses. Liver SOD levels of carrageenan control animals were decreased as compared to control rats, and the trend was similar to that of liver GSH levels[39]. None of the extracts at low doses demonstrated a substantial increase in SOD. Yet, the animals administered with high doses (400 mg/kg) of all the extracts revealed a significant increase in SOD levels (**Figure 7**).

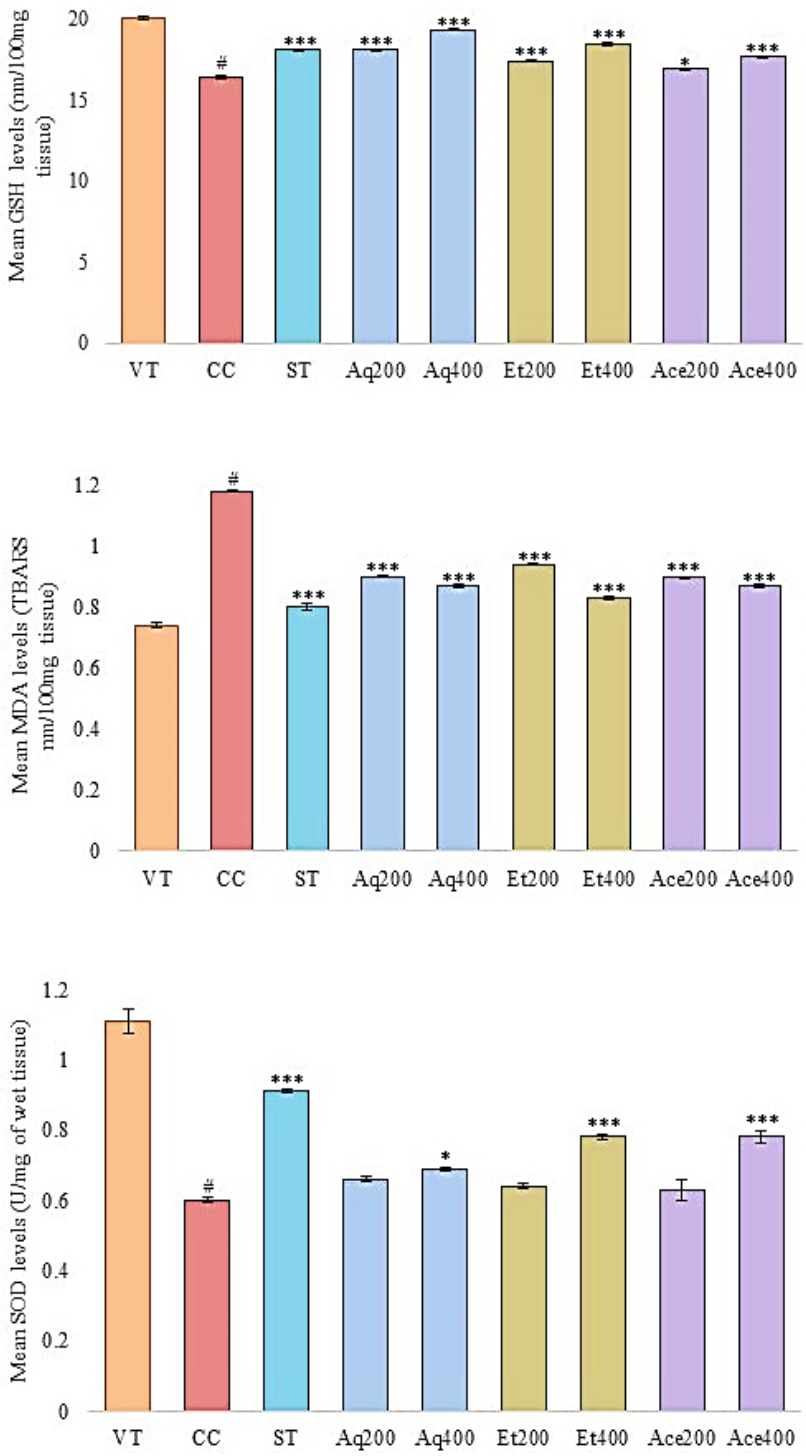

Figure 7. Mean liver GSH, MDA, and SOD levels (**VT**- vehicle-treated; **CC**- carrageenan control; **ST**- standard treated; **Aq200**- aqueous extract 200 mg/kg; **Aq400**- aqueous extract 400 mg/kg; **Et200**- ethanol extract 200 mg/kg; **Et400**- ethanol extract 400 mg/kg; **Ace200**- acetone extract 200 mg/kg; **Ace400**- acetone extract 400 mg/kg. Values expressed as mean ± SEM. (n=6). ***p <0.001; *p <0.05– standard and extract treated group Vs. carrageenan control group; #p <0.001– carrageenan control group Vs. vehicle-treated group).

Liver histopathological photomicrographs of animals about various groups are represented in **Figure 8**. Vehicle-treated group liver specimen confirms steady lobular architecture with usual hepatic cells and well-maintained cytoplasm exhibiting fine sinusoidal lines. The perivenular area was accompanied by a nucleus with complete architecture. Normal central veins, Kupffer cells, and endothelial cells with no structural alterations are visible. Histology of carrageenan control group livers exhibited degenerative sequence, blood-filled sinusoids, and nuclei with varied sizes and shapes. Cloudy swelling and large or focal necrosis of hepatocytes with granulated cytoplasm were detected, along with some fatty changes.

The nuclei of some cells were pyknotic or karyorrhectic, while megalocytosis with marginal hyperchromasia was also observed. Standard drug-treated group liver illustrated intact architecture with few neutrophil infiltrations and mild hepatocyte degeneration. Bi-nucleated hepatocytes with cystic lesions and perivascular inflammation were noticed. Vacuolization in the cytoplasm and focal nucleomegaly were also observed. Animals administered with 200 mg/kg aqueous extract demonstrated epithelial hyperplasia and increased connective tissues in portal gaps. Hepatocytes revealed vacuolar degeneration and signs of necrosis. Concomitantly, intrahepatic cholestasis, absence of lesions in the interface portal space with normal hepatocytes, and an increase in the number of perisinusoidal cells were noticed in the liver histopathology of animals treated with 400 mg/kg aqueous extract. The hepatic structure of rats administered with 200 mg/kg of ethanol extract illustrated hemorrhage between sinusoids and fibrocystic lesions with inflammatory cells. No structural loss was observed with periportal inflammatory cells. Ethanol extract 400 mg/kg treated group animal liver specimen demonstrated dilated sinuses filled with erythrocytes and congested blood vessels. Cystic lesions, focal areas of nuclear enlargement, and pyknosis were observed. Very large bi-nucleated hepatocytes with prominent nucleolus revealed mild hepatic damage. Animals of the group that received a low dose of acetone extract (200 mg/kg) displayed an intact architecture but with inflammatory infiltration, while the high dose (400 mg/kg) treated group revealed mild vacuolar degeneration and fibrosis transition.

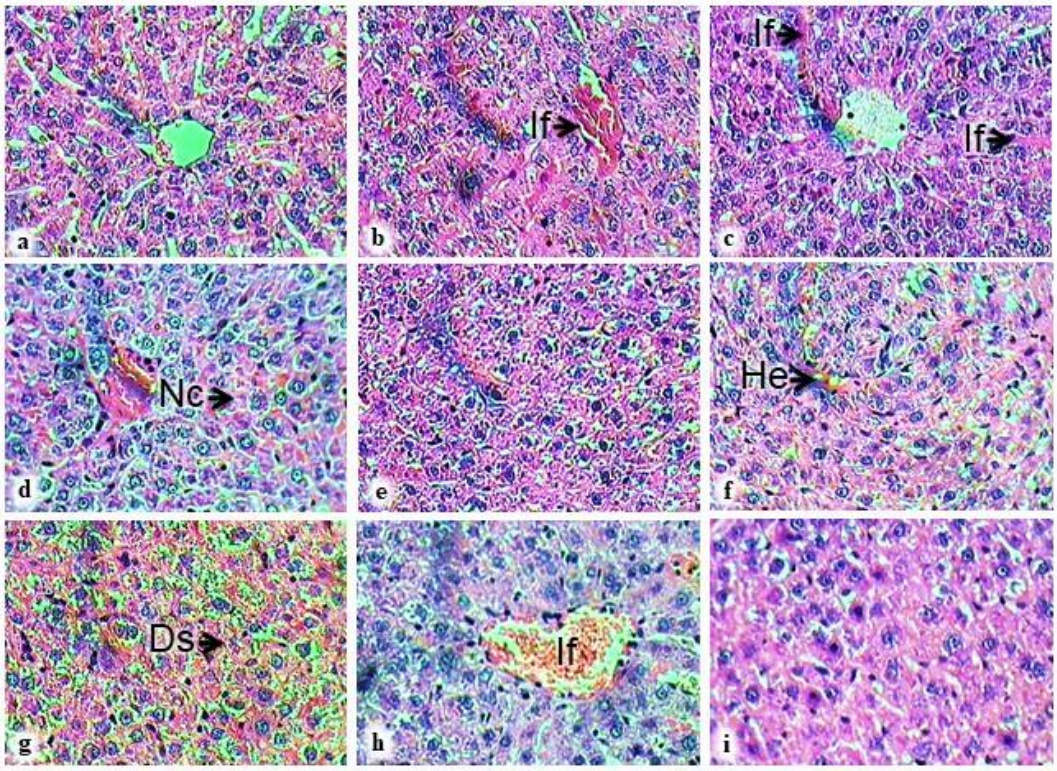

Figure 8. Representative photomicrographs of liver sections (**a**: vehicle control, 10x; **b**: carrageenan control, 10x; **c**: standard treated 10x; **d**: aqueous extract 200 mg/kg, 10x; **e**: aqueous extract 400 mg/kg, 10x; **f**: ethanol extract 200 mg/kg, 10x; **g**: ethanol extract 400 mg/kg, 10x; **h**: acetone extract 200 mg/kg, 10x; **i**: acetone extract 400 mg/kg, 10x. **If**: infiltration; **Nc**: necrosis; **He**: hemorrhage; **Ds**: dilated sinuses).

Paw tissue specimens from different groups were observed for histopathological severity of inflammatory response based on tissue alterations (**Figure 9**). Examined sections from vehicle control animals showed a typical arrangement of the epidermis, dermis with numerous sebaceous glands, and sub-epidermal and subcutaneous layers. Meanwhile, infiltrations with polymorphonuclear inflammatory cells, injured blood vessels, detachment of epidermal layer, and severe dermal inflammatory reaction were observed in paw tissue of carrageenan control animals. Rats treated with standard drugs demonstrated a conspicuous regeneration with vasculitis and hyperemia around the vessels in the dermis, besides mild epithelial hyperplasia and sub-epidermal edema. Aqueous extract 200 mg/kg treated group animals exhibited marked cellular diffused infiltration in the connective tissues. Acute edematasis in the epidermis as well as in the dermis and moderate dermal inflammatory reaction were also apparent. A reduction in inflammatory cells with evident morphological

regeneration of the dermis and epidermis is noticed in the group administered with 400 mg/kg of aqueous extract. Treatment with 200 mg/kg of ethanol extract presented a near-normal structure with less inflammation alongside distended blood vessels with inflammatory cells and vast numbers of adipocytes. In contrast, a visible reduction in the number of inflammatory cells within the dermis and an importunate regeneration of epidermal layers were evident in the tissue biopsy of rats treated with a high dose of ethanol extract (400 mg/kg). Mild epithelial hyperplasia and inflammatory reaction-related inter-muscular infiltration with many neutrophils were detected in the histopathology of 200 mg/kg of acetone extract treated rats. In contrast, an influx of inflammatory cell infiltration, considerable numbers of inflammatory cells, and subepidermal edema, accompanied by regenerative changes within the epidermis, were observed in 400 mg/kg acetone extract-treated animals.

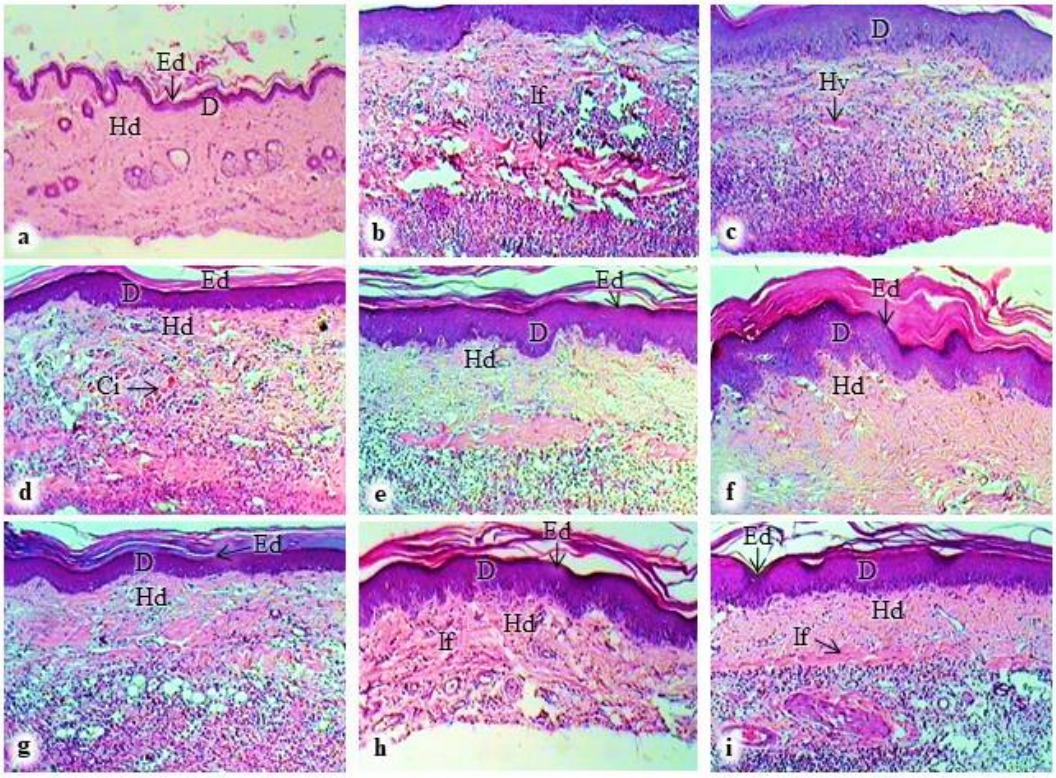

Figure 9. Representative photomicrographs of paw tissue sections (**a**: Vehicle control, 4x; **b**: carrageenan control, 10x; **c**: standard treated, 10x; **d**: aqueous extract 200 mg/kg, 10x; **e**: aqueous extract 400 mg/kg, 10x; **f**: ethanol extract 200 mg/kg, 10x; **g**: ethanol extract 400 mg/kg, 10x; **h**: acetone extract 200 mg/kg, 10x; **i**: acetone extract 400 mg/kg, 10x. **Ed**: epidermis; **D**: dermis, **Hd**: hypodermis; **If**: infiltration; **Ci**: cellular infiltration).

Paw edema was induced using carrageenan in animals administered with aqueous, acetone, and ethanol extract doses for seven days. The inflammation caused can be attributed to the release of histamine, serotonin, and kinin at the initial phase, followed by prostaglandin-like substances in the later stage[40]. Results indicated that acetone extract offered better protection than aqueous and ethanol extract. The protection offered may be ascribed to the diverse class of phytobioactives in the extract and might have been influenced by the probable antioxidant property. Free radicals are ascribed to play a role in carrageenan-induced inflammation[41]. Malondialdehyde (MDA), a metabolic product of lipid peroxidation, rises due to oxidative stress, and administration of acetone extract lowers the carrageenan-induced elevation of MDA levels, which is suggestive of the antioxidant property of the extract. The antioxidant effect was also witnessed in the liver histopathological study with mild vacuolar degeneration and fibrosis transition in the acetone extract-treated animals against the blood-filled sinusoids, cloudy swellings, and focal necrosis in the carrageenan control group. On the other hand, paw tissue histopathological study of the carrageenan control group revealed polymorphonuclear inflammatory cells, injured blood vessels, and severe dermal inflammatory reaction, while acetone extract treated groups exhibited only a considerable number of inflammatory cells[42].

CONCLUSION

The study provides insight into various pharmacognostical parameters to identify and authenticate *Z. rugosa* leaves. Phytochemical screening can be further explored to establish chemical marker compounds. Additionally, well-established pharmacognostic and phytochemical characteristics might lead to laying down Pharmacopoeial standards for the crude drug. The acetone extract may further be fractionated to determine the active fraction, followed by the isolation of active constituents responsible for the anti-inflammatory potential.

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Writing - review & editing: Kamatchi Sundara Saravanan, Judy Jays

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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Research Article

Appropriateness and Cost of Prophylaxis Stress Ulcer for Inpatient in the Internal Medicine Department in a Government Hospital: A Cross-Sectional Study

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Acid suppression therapy
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Stress ulcer
Stress ulcer prophylaxis

Abstract

Guidelines from the American Society of Health-System Pharmacists (ASHP) 1999 prohibit acid-suppressing therapy for stress ulcer prophylaxis (SUP) in patients who are not critically ill. Stress ulcer prophylaxis is not recommended in non-ICU patients with <2 risk factors. Inappropriate use of SUP can increase costs for patients. This study aims to evaluate the use and the cost of SUP. This research was a non-experimental observational study with a cross-sectional approach. Data was collected retrospectively using the consecutive sampling method with a random sampling technique on the medical records of inpatients in the internal medicine ward of Sleman Regional Public Hospital from January to December 2020, totaling 340 samples. The results showed that proton pump inhibitors were the most widely used acid-suppressing drugs, with 45.8%. Furthermore, the histamine-2 receptor antagonist was 42.6%, the sucralfate group was 7.4%, and the antacid group was 4.2%. Of 340 patients, 57 (16.8%) were in the proper indication based on the guidelines, and 283 (83.2%) were under the wrong indication for SUP. They were using SUP with the proper indication so that the therapy could save treatment costs by Rp. 19,933,582. There was a high prevalence of inappropriate SUP prescriptions among inpatients in the internal medicine department; if these drugs were given with the appropriate indications, they could save more on the prophylaxis cost. Clinician pharmacists should develop an effective intervention strategy to reduce inappropriate SUP drugs.

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INTRODUCTION

Stress ulcer prophylaxis (SUP) is generally given to critically ill patients and treated in the intensive care unit (ICU)[1]. Appropriate use of SUP is defined when proton pump inhibitors (PPIs) and histamine-2 receptor antagonists (H2RAs) are administered to patients with at least one risk factor (coagulopathy, mechanical ventilation ≥48 hours, and gastrointestinal bleeding or ulceration within a year) before hospitalization) or with some minor risk factors (sepsis, multiple organ failure, liver failure, renal insufficiency, inpatient ICU ≥7 days, hypotension or shock, organ transplant, multiple trauma, burns of more than 25-30% of body surface area, major surgery, hidden gastrointestinal bleeding ≥6 days, and use of anticoagulants, corticosteroids, or nonsteroidal anti-inflammatory drugs (NSAIDs))[2]. The American Society of Health-System (ASHP) in 1999 published guidelines for the use of SUP in medical, surgical, respiratory and pediatric patients in the ICU[3]. Research related to inappropriate prescribing of acid-suppressing therapy due to a low-risk factor for bleeding in the use of SUP based

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on the stress ulcer-related gastrointestinal bleeding (SURGIB) criteria was developed by Herzig *et al*.[4] of 88.5% and an estimated cost savings of inpatient medication hospitalization of $114,622 (approximately Rp. 1,396,095,960) in the 253 studied patients[5].

Long-term use of acid-suppressing therapy is of particular concern as complications (*Clostridium difficile*: diarrhea, osteoporosis, and pneumonia) are associated, mainly when PPIs are used for long durations at high doses[6]. Several studies[7,8] reported that *C. difficile* infection increased three times from prolonged use of SUP. If SUP is not used based on the indications of the disease in the patient, it will lead to unexpected side effects such as diarrhea due to *C. difficile*, the incidence of pneumonia, and increased unnecessary costs[9]. Therefore, evaluating SUP can be an evaluation for health workers in providing therapy to patients and obtaining optimal therapeutic effectiveness. The researcher is interested in conducting a study regarding the utilization of SUP in patients hospitalized in the internal medicine ward of Sleman Regional Public Hospital due to the completeness of medical record documentation reaching 85% out of 100% based on the patient safety and quality improvement program.

MATERIALS AND METHODS

Materials

The research instruments included medical records of patients hospitalized in the internal medicine ward of Sleman Regional Public Hospital, Special Region of Yogyakarta, Indonesia, in 2020. ASHP Therapeutic Guidelines on Stress Ulcer Prophylaxis 1999[3] and Stress Ulcer Prophylaxis Clinical Guidelines from Stanford Hospital and Clinics 2015[10] were used as therapeutic references. Sample recording was adjusted according to the inclusion criteria such as gender, patient age, length of hospitalization, drug name, drug class, drug dose, rules of use, and duration of drug use. This research has obtained research ethics approval from the Health Research Ethics Committee, Sleman Regional Public Hospital with number 180/4126.

Methods

Research design and participants

The study took medical record data of inpatients at the Sleman Regional Public Hospital and the costs of using SUP from January to December 2020. Patient characteristics and therapy data were obtained from medical records, while therapy costs were obtained from the hospital's finance department. The sample in this study was all inpatients in the internal medicine ward who used SUP and met the inclusion criteria at the Sleman Regional Public Hospital for January to December 2020. The inclusion criteria were that patients hospitalized in the internal medicine ward were given SUP during treatment with data, and the medical records were complete and legible. The exclusion criteria were patients who entered and experienced bleeding in the gastrointestinal tract, which was marked by the occurrence of hematemesis, melena, and blackish-red NG fluid; Patients with a diagnosis of gastrointestinal disorders; and a history of peptic ulcers or gastrointestinal bleeding within one year before admission.

Sample size calculation

The sample was calculated using the proportion estimation formula as shown in **Equation 1**, taking the following assumptions: the proportion of appropriate use of SUP = 0.5, a margin of error = 5%, and a 95% confidence interval[11]. The correction formula was used since the population was less than 10,000 (total patient population in a year (N) = 3000), which could represent the study sample. The corrected number of samples was then calculated, as shown in **Equation 2**. Then, 340 samples were selected with a random sampling technique.

$$n = \frac{Z_{\alpha/2}^2 P(1-P)}{d^2} = \frac{(1.96)^2\ 0.5(1-0.5)}{(0.05)^2} = 384 \quad [1]$$

$$n = \frac{N \times n}{N + n} = \frac{3000 \times 384}{3000 + 384} = 340 \quad [2]$$

Criteria establishment

Based on published evidence-based guidelines and previous literature on SUP clinical practices, we established the criteria to evaluate the appropriateness of SUP medication. Stress ulcer prophylaxis medication was considered appropriate if an inpatient in the internal medicine department had one major or at least two minor risk factors[3,10] in **Table I**.

Table I. Risk factor for stress ulcer.

The presence of of one major risk factor from the following:
1. Respiratory failure: mechanical ventilation >48 hours
2. Coagulopathy: platelet count <50,000/mm^3 (50 × 10^9/L), international normalized ratio >1.5, or partial thromboplastin time >2.0 times the control value
The presence of at least two minor risk factors of the following:
1. Head injury with a Glasgow Coma Score of ≤10 or an inability to obey simple commands
2. Thermal injury involving >35% of the body surface area
3. Partial hepatectomy
4. Hepatic or renal transplantation
5. Multiple traumas with the Injury Severity Score of ≥16
6. Acute renal failure or hepatic failure
7. Traumatic brain injury or spinal cord injury
8. Insufficiency renal
9. Sepsis
10. Occult or overt bleeding for ≥6 days
11. Length of stay >7 days
12. Corticosteroid therapy (>250 mg/day hydrocortisone or equivalent daily)
13. Using antiplatelet

Outcome measurement

Our primary outcome variable was the appropriateness evaluation of SUP prescribing patterns for inpatients in the Internal Medicine Department and the cost of using SUP, both the total cost and average cost per patient of appropriate and inappropriate indicated prophylactic use.

Data analysis

Data analysis in this study was in the form of descriptive analysis to describe the characteristics of patients based on gender, age, length of hospitalization, and risk factors to determine the profile of SUP used by inpatients in the internal medicine ward of Sleman Regional Public Hospital based on the class of drugs used, to determine the accuracy and inaccuracy of the indications for the use of SUP for inpatients in the internal medicine ward, as well as identifying the costs calculated by multiplying the total number of appropriate and inappropriate therapeutic doses given during hospitalization with the price of the drug used.

RESULTS AND DISCUSSION

Based on the data obtained from 340 samples in **Table II**, there are more male (55%) than female patients (45%). Patient characteristics by gender are dominated by males, with a higher prevalence of male smokers (62.9%). Based on Indonesian Basic Health Research 2018 (*Riset Kesehatan Dasar*, Riskesdas)[12], regularly consuming coffee could increase the risk of stress ulcers. Coffee containing caffeine can stimulate the hormone gastrin, which stimulates and accelerates the production of stomach acid, resulting in gastric ulceration[13]. In addition, regularly drinking coffee can increase the risk of 3.57 times experiencing gastritis. If left untreated, it will worsen, and the stomach acid can cause ulcers[14].

Inpatients in the internal medicine ward who receive SUP are given at >65 years old who have entered older people. The increasing age can cause a decrease in gastric mucosal function, reduced secretory function, and loss of nutritional factors in the gastric mucosa, so the stomach is prone to bleeding[15]. Age does not affect the incidence of stress ulcers as it is not included as a risk factor for gastrointestinal bleeding. However, a study revealed that older age becomes one factor in the administration of excessive gastric acid-suppressing drugs[9].

Furthermore, the maximum length of hospitalization was <7 days with a percentage of 93.24% and >7 days with a percentage of 6.76%. Farsaei *et al*.[9] explained that patients who required longer hospitalization and more medical services could unconsciously encourage doctors to provide SUP, preventing more gastrointestinal bleeding complications. Elderly

patients and longer hospitalization were shown to be significant overuse predictors of SUP. In addition, Issa *et al*.[16] have similarly identified factors contributing to the overuse of SUP. They revealed that the length of hospitalization is one of the factors in which SUP is frequently used.

In this study, the major risk factor was the incidence of coagulopathy (12.35%), in which most patients were dengue fever patients. Therefore, according to Huang *et al*.[17], it is necessary to give anti-ulcer to prevent stress ulcers. Meanwhile, the minor risk factor is the use of antiplatelets (10.59%), which can inhibit the production of prostaglandins by the gastric mucosa associated with gastric epithelial damage[18]. Our previous study[19] revealed that there were 52 patients receiving antiplatelets, where the use of antiplatelets significantly affected the incidence of bleeding.

Table II. Patients characteristics.

Parameter	Number of patients (n (%))
Gender	
Female	153 (45)
Male	187 (55)
Age (years old)	
5-11	4 (1.2)
12-16	9 (2.6)
17-25	28 (8.2)
26-35	28 (8.2)
36-45	42 (12.4)
46-55	74 (21.8)
56-65	57 (19.7)
>65	88 (25.9)
Length of Hospitalization (days)	
≤7	317 (93.24)
>7	23 (6.76)
Risk factors	
Coagulopathy	40 (12.35)
Antiplatelet use	38 (10.59)
Corticosteroid use	29 (8.53)
Congestive heart failure	26 (7.94)
Kidney insufficiency	13 (3.82)
Sepsis	12 (3.53)
Head injury	3 (0.88)

The profile of SUP in inpatients in the internal medicine ward at the Sleman Regional Public Hospital in 2020 was primarily the PPIs group of 45.8% (**Table III**). Acid suppressive therapy (AST), including PPIs and H2RAs as SUP, is one of the most common medical practices in inpatients[5]. The PPIs are more potent in increasing gastric pH than H2RAs and maintain gastric pH between 3.5 and 5.0, which can minimize the risk of gastric mucosal injury. Of the four meta-analyses comparing PPIs with H2RAs, three suggested that PPIs are superior to H2RAs[20].

Table III. Stress ulcer prophylaxis use profile.

Agent	Type	Number	%
PPIs	Lansoprazole injection	66	14
	Lansoprazole capsules	36	7.6
	Pantoprazole injection	84	17.6
	Esomeprazole injection	4	0.8
	Esomeprazole tablets	2	0.4
	Omeprazole injection	3	6
	Omeprazole tablets	21	4.4
H2RAs	Ranitidine injection	187	39.6
	Ranitidine tablets	14	3
Sucralfate	Sucralfate syrup	18	3.8
	Sucralfate tablets	17	3.6
Antacid	Antacid syrup	4	0.8
	Antacid tablets	16	3.4
Total		**472**	**100**

Evaluation of the use of SUP revealed that patients prescribed acid-suppressing drugs were 40 patients or 11.76%. One indication had a major risk factor; 17 patients, or 5%, had at least two or more indications of a minor risk factor as SUP, and 283 patients, or 83.24%, received acid-suppressing drugs without appropriate indications (**Table IV**). This is similar to several studies conducted abroad regarding the high prescription of gastric acid suppressant drugs that are not appropriate to treatment guidelines[5,21-23]. In recent years, SUP has become commonplace in patients with general treatment and little or no supporting evidence[24]. Inappropriate use of indications for SUP can increase the incidence of unexpected drug reactions, drug interactions, problems in polypharmacy, and unnecessary drug costs[25].

Table IV. The use of SUP.

Stress ulcer prophylaxis	n (%)
Correct indication	
1 major risk factor	40 (11.76)
≥2 minor risk factors	17 (5)
Incorrect indication	283 (83.24)
Total	**340**

A cost analysis was performed to assess the economic impact of SUP during therapy without incorrect indications. The cost of prophylaxis is calculated based on the total oral administration or injection of acid-suppressing drugs given during hospitalization, looking at the smallest unit of drug price from the hospital. The biggest expenditure on SUP was the inappropriate of the drug, which was Rp. 19,933,582 (**Table V**). It indicated that the hospital could save on that cost if the drug is not used Rp. 19,933,582. Moreover, there were limitations in identifying the patient's direct costs, so the cost calculation is only from the drug's price.

Table V. Drug expenses for the use of SUP.

Indication	Number of patients	Total drug cost (Rp)	Average cost (Rp)
Appropriate	57	6,240,384	109,480
Inappropriate	283	19,933,582	70,436

Researchers have not been able to explain the factors that influence the high prevalence of inappropriate prescribing, but there is a similar study that observed the factors that influence the inappropriate prescribing of prophylactic stress ulcers; a study stated that the reasons why clinicians prescribed SUP inappropriately were multifactorial. First, the fear of the development of SUP in non-ICU patients who were not on SUP therapy. Second, Due to the tense relationship between doctors and patients in China, doctors had to prescribe SUP therapy for low-risk inpatients to protect themselves from litigation. Third, the incidence of an adverse reaction related to acid suppression medicines has not been high. For this reason, doctors have believed PPIs to be safe[26]. One study[27] reported that several adverse effects (specified in *C. difficile* infections, respiratory infections, hypomagnesemia, adverse skeletal muscle effects, and psychiatric symptoms) after reducing inappropriate proton pump inhibitor use for SUP decreased significantly (35% control group versus 8% intervention group)[28]. The inappropriate use of SUP therapy can also have economic implications for patients and the healthcare system. Associated with those factors, the researcher indicated that clinicians needed to provide more information about the rationality and efficiency of their prescribing practices. Clinical pharmacists should execute effective intervention strategies to reduce improper SUP medication. The ASHP Therapeutic Guidelines on Stress Ulcer Prophylaxis 1999[3] and Stress Ulcer Prophylaxis Clinical Guidelines from Stanford Hospital and Clinics 2015[10] can be implemented in clinical practice to prevent unnecessary acid-suppressing therapy in patients due to the low risk of stress ulcer bleeding. Computerized ordering systems can reduce unnecessary use of acid suppression therapy, lower patient prescribing costs, and limit side effects[25,28].

CONCLUSION

The profile of the use of SUP drugs in patients hospitalized in the internal medicine ward at Sleman Regional Public Hospital in 2020 included PPIs of 45.8%, H2RAs of 42.6%, sucralfate of 7.4%, and antacid of 4.2%. The use of SUP in the patients described 57 patients (16.8%) with correct indications and 283 patients (83.2%) with incorrect indications. Expenditure on

the use of SUP drugs in a correct indication was Rp 6,240,384 with an average of Rp 109,480 for 57 patients and Rp 19,933,582 for an incorrect indication with an average of Rp 70,436 for 283 patients.

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Visualization: -
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DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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Research Article

Development and Validation of a Questionnaire for the Assessment of the Factors that Influence ADR Reporting by Pharmacists

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Abstract

Drug safety is a significant concern in many countries, as side effects (AE) and adverse drug reactions (ADR) have caused many deaths worldwide. One of the reasons is the low contribution of pharmacists in spontaneously reporting AE/ADR. This study aims to develop a questionnaire to assess factors that correlate with spontaneous reporting by pharmacists. A questionnaire pilot was tested on 30 pharmacist respondents who worked in type C hospitals in Surabaya and Sidoarjo, Indonesia. Respondents' responses were then evaluated for face validity, construct validity, and reliability. The results showed that the face validity of the questionnaire was ideal. Then, the results of the construct validity of the knowledge section using point biserial correlation showed that two items were invalid because the r-value was smaller than the r-table (r = 0.361). Then, construct validity uses the factor analysis method for psychological, environmental, and practical variables by paying attention to the Kaiser-Mayer-Olkin Measure (KMO) value, which must be greater than 0.5, the significance of the Bartlett test, which must be less than 0.05 and the factor loading value which conditions must be greater than 0.5. As a result, most of the psychological, environmental, and practical variables show valid and reliable results. However, further consideration should be given to eliminating some items that do not meet the requirements. In conclusion, this validated questionnaire can be used to obtain additional information regarding factors influencing spontaneous reporting by pharmacists.

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INTRODUCTION

Drug safety is a significant concern in several countries, monitored and evaluated through pharmacovigilance systems. Pharmacovigilance is an activity related to detecting, evaluating, understanding, and preventing adverse drug reactions (ADR)[1]. Pharmacovigilance activities include periodic security update reports, commonly called spontaneous reporting. Spontaneous reporting of adverse events (AE)/ADR is one element of pharmacovigilance activities that help overcome safety concerns after drug administration[2]. This reporting activity provides actual information regarding the safety profile of real-life clinical practices compared to the results of clinical trials using only a few samples and the safety of drugs studied in a limited time[3]. Spontaneous reporting is a cost-effective, flexible, and highly effective method of gathering information because health workers voluntarily submit AE/ADR case reports to the National Pharmacovigilance Center of the Food and Drug, The Indonesian Food and Drug Authority (*Badan Pengawas Obat dan Makanan Republik Indonesia*, BPOM RI) for analysis, which will later help to reduce the potential for AE/ADR in patients[4].

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Spontaneous reporting is a method that contributes significantly to the improvement of pharmacovigilance in some countries[5]. Several regulators in other countries have designed easy systems for spontaneous reporting intending to increase the participation of health workers in spontaneous reporting, but in reality, the rate of spontaneous AE/ADR reporting is still low[6,7]. Spontaneous reporting of AE/ADR in several less progressive countries is of particular concern, considering that these less developed countries contribute to 80% of the disease burden in the world. However, they only participate in AE/ADR reports in less than 1% of all global reports (11,824,804)[8].

Little participation in AE/ADR reporting in less developed countries such as Indonesia cannot be explained because the law does not require it. Spontaneous reporting of AE/ADR, reporting still needs to be improved[9]. The low level of AE/ADR reporting is caused by several factors, such as not caring about patient safety, feeling they have no responsibility to report, complicated reporting procedures, and poor knowledge and attitudes of health workers, which impacts AE/ADR reporting practices[10]. However, research on factors that correlate with spontaneous reporting practices in Indonesia still needs to be improved.

Therefore, research related to this needs to be done. Because research related to the factors that influence the practice of spontaneous reporting is still new, it requires instruments with good validity and reliability so that the data collection process is accurate and precise. Research instruments for observational research that look at the factors that influence a person generally use questionnaires because questionnaires are relatively easy to collect data in research and policy evaluation. Information containing knowledge, attitudes, opinions, behavior, and facts will be easily collected using a questionnaire[11]. Of course, a questionnaire with good validity and reliability requires a development process first from evaluating the validity and reliability of test results[12].

Validity and reliability are the two most essential and fundamental features in evaluating any measurement instrument or tool for good research, one of which is a questionnaire. Without assessing the reliability and validity of the study, it will be difficult to describe the effects of measurement errors on the theoretical relationships being measured[13]. Research with a valid questionnaire will produce data that follows the construct built by the researcher. As for reliability, it serves to minimize measurement errors from questionnaires when taking data. Reliability is an indicator of questionnaire consistency when measuring certain concepts[12].

Given the importance of validity and reliability testing for a research questionnaire, it should be a priority before taking data. However, a review of articles from 748 studies found that one-third did not attach procedures to establish validity (31%) or reliability (33%). Meanwhile, developing accurate and precise questionnaires is needed to decrease measurement errors, namely mismatches between respondent attributes and survey responses[14]. Validity and reliability tests must be carried out before data collection to reduce measurement errors and measure the questionnaire's reproducibility, and the results will be evaluated later. Therefore, this study aims to test the validity and reliability of a questionnaire developed to measure pharmacists' knowledge and management of spontaneous reporting.

MATERIALS AND METHODS

Materials

The instrument in this study was a questionnaire created by researchers based on World Health Organization (WHO) and BPOM RI pharmacovigilance guidelines[15-20]. In addition, the results of expert consultation were considered. The questionnaire consisted of five parts: pharmacist demographic characteristics, knowledge included in individual variables, psychological variables, environmental variables, and the practices that pharmacists engaged in spontaneous reporting. For the demographics section, there were 15 question items. For the part of knowledge included in individual variables, there were 13 questions. For psychological variables, there were 14 questions. Environmental variables totaled nine questions, and practice summed 19 questions. The total number of questions in the questionnaire was 55 questions (**Table I**). The questionnaire was validated in two stages: the first stage was face validation for the overall appearance of the questionnaire, and the second stage was construct validation to see whether the questionnaire could produce data following the construct developed by the researcher. Constructing validation in this questionnaire was divided into two methods. The first method was biserial point correlation for the knowledge section because the answer scale was the Guttman scale or dichotomy[21].

Because the answer scale used an ordinal scale, the factor analysis method was applied for psychological, environmental, and practice factors[22].

Table I. Question items in each instrument domain.

Variable	Questions	Number in questionnaire
Knowledge	One of the points in pharmacovigilance activities is the detection and prevention of AE/ADR so that additional reactions that are detrimental to the patient do not occur.	1
	Adverse drug reaction monitoring applies to drugs that have been around for a long time, such as captopril, simvastatin, and the like	2
	Drug reconstitution that is carried out haphazardly and without sterility has the potential to cause adverse events (AE) in patients.	3
	Adverse Drug Reactions (ADR) are part of adverse events whose causes are known from the drugs consumed by the patient.	4
	As health workers, pharmacists should report any AE/ADR encountered as part of their professional responsibility.	5
	The spontaneous reporting of AE/ADR can only be done manually via a yellow form sent by post to the pharmacovigilance centre of Balai POM.	6
	Post-Immunization Adverse Events during vaccine use are not required to be reported to BPOM.	7
	The institution that acts as the national pharmacovigilance centre in Indonesia is BPOM.	8
	Spontaneous reporting is only those that are unexpected (Unexpected Adverse Reaction).	9
	AE/ADR is one of the contributors to the highest number of deaths in various countries.	10
	Decreased absorption of omeprazole due to drug interactions with antacids does not need to be reported.	11
	Incidents of side effects due to drug overdose or medication errors need to be reported.	12
	The withdrawal of the Albothyl product in 2018 and the call to improve the drug's indications so that it is not used for mouth ulcers is an example of implementing pharmacovigilance activities so that the public can avoid serious drug side effects.	13
Psychological	Pharmacists in healthcare facilities play an essential role in pharmacovigilance activities.	14
	Pharmacists in health service facilities must regularly update their knowledge regarding pharmacovigilance.	15
	If a drug side effect occurs in their practice, the pharmacist is not obliged to report it.	16
	Pharmacists in health service facilities are the public's first reference in reporting AE/ADR.	17
	Pharmacists must receive special training regarding pharmacovigilance.	18
	Spontaneous reporting of AE/ADR must be done voluntarily or as part of professionalism.	19
	Reporting and monitoring of AE/ADR will be beneficial for patients.	20
	AE/ADR that occur due to over-the-counter drugs/limited over-the-counter and over-the-counter drugs must also be reported.	21
	Reporting AE/ADR will add more insight regarding the side effects of drugs encountered in practice.	22
	Reporting AE/ADR experienced by patients is a sign that their concerns are being taken seriously.	23
	Spontaneous reporting of AE/ADR is part of pharmaceutical care.	24
	AE/ADR must be reported even if the impact does not result in hospitalization, life-threatening conditions, disability, or death.	25
	All adverse events/ESOs that occur as a result of drugs that have just received distribution permits and medicines that have been on the market for a long time must be reported.	26
	AE/ADR reporting must be done immediately, especially for dangerous or unexpected events (Unexpected Adverse Reaction).	27
Environment	The pharmacist where I practice applies a regular shift work system.	28
	In one work shift, the pharmacist at my workplace practices according to the specified working hours.	29
	The pharmacists' working hours where I work follow the given workload.	30
	My workplace will give rewards/awards to pharmacists if they make innovations in their work or succeed in achieving specific targets.	31
	Promotions at my workplace are carried out objectively based on the achievements and contributions of a pharmacist.	32
	The income I get from my workplace is enough for me because it matches my workload.	33
	My workplace will provide additional income if there is extra work or overtime provided	34
	The portion of work at my workplace is proportional enough to do other work without needing overtime.	35
	I complete work while at work and never do work at home/outside of my working hours	36
Practice	The frequency with which I encounter reports of drug side effects or adverse events from patients at work.	37
	The frequency with which the hospital where I work reports drug side effects or adverse events to the BPOM National Pharmacovigilance Center	38

	I immediately report all drug side effects or adverse events reported by the patient to the unit head/head of pharmacy installation/pharmacist responsible for spontaneous reporting to BPOM.	39
	I document all reports of drug side effects or adverse events from patients, both unexpected and expected.	40
	I report and document all actions and interventions I provide to patients according to the patient's complaints.	41
	Suppose there is a complaint that a patient has a dry cough due to using the drug captopril. In that case, I will report it to the unit head/head of pharmacy installation/pharmacist responsible for spontaneous reporting to BPOM.	42
	Suppose there is a complaint that a patient experiences extrapyramidal syndrome due to the use of the drug metoclopramide. In that case, I will report it to the unit head/head of pharmacy installation/pharmacist responsible for spontaneous reporting to BPOM.	43
	Suppose there is an incident of decreased absorption of the drug omeprazole as a result of drug interactions with antacids. In that case, I will report it to the unit head/head of pharmacy installation/pharmacist responsible for spontaneous reporting to BPOM.	44
	I carried out a causality analysis first with the doctor who provided therapy to the patient to ensure the causality of the side effects of the medication experienced by the patient.	45
	I include information in the form of reporting data, data on patients who submit complaints, complaints felt by patients, and data on suspected drugs in every report I submit to the head of the unit/head of the pharmacy installation/pharmacist responsible for spontaneous reporting to BPOM.	46
	I discussed with the doctor who provides therapy to treat patients who experience side effects from drugs.	47
	I take my time at work to handle drug safety incident complaints from patients immediately.	48
	I take the time to do documentation and report cases of drug side effects or adverse events encountered in patients.	49
	I prioritize work related to patient safety while undergoing therapy.	50
	I apply all points of clinical pharmacy services, including monitoring drug side effects (MESO) following the Minister of Health Regulations, where I practice.	51
	I actively participate in the spontaneous reporting of drug side effects or adverse events as a form of professionalism and compliance with regulations.	52
	I have a spontaneous reporting account at e-meso.pom.go.id or the e-meso mobile smartphone application and operate it actively.	53
	I provide a yellow form for spontaneous reporting of drug side effects to BPOM manually at my practice.	54
	I participated in multilevel pharmacovigilance training held by BPOM as the national pharmacovigilance centre.	55

Methods

Design and participant

This study was conducted between September and October 2023 in pharmacists working full-time at type C hospitals in Surabaya and Sidoarjo, East Java, Indonesia. Type C hospitals were chosen because the number of type C hospitals is the largest in Indonesia, but the contribution of reports of adverse drug reactions is low[16,23]. There were 30 samples recommended by Hertzog[24]. The Health Research Ethics Commission (KEPK), Faculty of Dentistry, Universitas Airlangga, issued the certificate of ethical eligibility for this study, with number 987/HRECC. FODM/VIII/2023.

Face validity

The method used to assess face validity was to provide a suggestion and improvement column at the end of the questionnaire to comment on all parts of the questionnaire in terms of language, font size, font, and word choice. Face validity was a subjective assessment of the operation of a construct. A test was valid if its content seemed relevant to the person working on it. It evaluated the appearance of the questionnaire in terms of feasibility, readability, consistency of style and format, and clarity of the language used. In other words, face validity referred to the researcher's subjective assessment of the presentation and relevance of the measuring instrument, whether the items appeared relevant, reasonable, clear, and transparent[25].

Data analysis

Construct validity test

The construct validity test used two methods to determine the questionnaire's construct conformity. The first method used the biserial point correlation for the knowledge section, and the second used factor analysis for psychological,

environmental, and practice variables questionnaires. For biserial point correlation to check the validity of the knowledge section, the method using Microsoft Excel tools to show the difference between the r-value and the r-table, in which Rpb was biserial point correlation coefficient, xi was average total score of respondents who answered correctly, xt was average total score of all respondents, Pi was proportion of correct answers item i, Qi was 1-Pi, and St was standard deviation of the total score, as shown in **Equation 1**. **Equation 1** was used to calculate the r-value to show the validity of the questionnaire item[26]. This calculation was applied to each knowledge question item. The question item was considered valid if the computed r-value offered a value greater than the r-table, and vice versa.

$$Rpb = \frac{xi-xt}{St}\sqrt{Pi}/q \qquad [1]$$

The second method was factor analysis using several indicators using IBM SPSS Statistics 26 (https://www.ibm.com/support/pages/downloading-ibm-spss-statistics-26). The first indicator was the Kaiser-Meyer-Olkin (KMO) measurement. This parameter compared the correlation coefficient value with the partial coefficient value. The requirement for factor analysis was that the KMO value had to be higher than 0.5. Bartlett's Test of sphericity tested the dependence between the variables being tested. This parameter helped indicate the absence of correlation between variables with each other in the community. The significance value in Bartlett's Test had to be less than 0.05 so that the process could continue for factor analysis. The following indicator showed the result of the calculation of the anti-image correlation. Grades with 'A' indicated the Measure of Sampling Adequacy (MSA) value. If the MSA number for a variable was below 0.5, then the variable had to be excluded, and variable selection had to be repeated. The last indicators were the Component Matrix and Rotated Component Matrix, which helped explain the spread of variables into factors formed. The Component Matrix confirmed whether or not there was a correlation between items and components. A high correlation value showed a solid relationship between the items and the components so that the items could be used as a factor. In a complex matrix, interpreting these factors was quite rare because it was difficult. Therefore, the factor alteration used in matrix factor rotation was converted into a more friendly form to understand. The steps for factor analysis in SPSS were selecting the analysis menu, selecting dimension and factor reduction, selecting the variables to be analyzed, selecting the descriptive option, checking the initial solution, KMO, Bartlett's Test of sphericity, and anti-image, selecting OK, then selecting the rotation menu to select varimax and check rotated solution and loading plots, and finally selecting OK for the analysis process[27].

Reliability test

Reliability testing aimed to see how consistent a questionnaire was when used for data collection. The method used was to look at the value of Cronbach α, which had to be greater than 0.6 for qualified reliability[28]. However, another theory was that if the Cronbach α value was 0.5-0.7, the questionnaire could be considered moderately reliable and still be used for research data collection[29,30]. The way to carry out reliability analysis in IBM SPSS Statistics 26 tools was to select the analyze menu, click scale and select reliability analysis, then choose the variable to be measured, click the statistics menu, and check the scale if the item deleted option, then clicked ok to process the analysis, later the results would display the Cronbach α value of analyzed variables.

RESULTS AND DISCUSSION

This questionnaire pilot test found 30 pharmacist respondents who worked in type C hospitals in Surabaya and Sidoarjo. The majority of respondents were 28 (93.3%) female. Most respondents were 28 (93.3%) from type C hospitals in Surabaya and 2 (6.6%) from type C hospitals in Sidoarjo. Face validity shows that overall, the pilot test respondents said that the grammar of the questionnaire was excellent and easy to understand, and the sentence structure was not ambiguous so that respondents could understand the meaning of the questions on the questionnaire. The font size and typeface used are also ideal, according to respondents. The form validation method is similar to testing the validity of questionnaires conducted in India when testing the validity of work-related stress questionnaires (TAWS-16)[31].

The results of construct validation of the knowledge question item section that is included in individual variables can be seen in **Table II**. The calculation results in **Table II** are interpreted by comparing the calculated r-value with the r-table. The question item is arguably valid if the calculated r-value exceeds the r-table[32]. Based on the data above, two knowledge

question items show invalid results because the calculated r-value is smaller than the r-table: question items number 4 and 13. Several factors can cause the invalidity of the question item. The first possibility is that the question item contains a sentence that leads the respondent to lean toward one answer choice, or the other option is that the question item cannot describe the intention the researcher wants to ask. Hence, the respondent gives an inappropriate response[33].

Table II. Knowledge question item validation results.

Number	R table	R-value	Interpretation of results	Number in Questionnaire
1	0.361	0.559027	Item valid	1
2	0.361	0.374257	Item valid	2
3	0.361	0.455515	Item valid	3
4	0.361	-0.2747*	Item not valid	4
5	0.361	0.403602	Item valid	5
6	0.361	0.488807	Item valid	6
7	0.361	0.52928	Item valid	7
8	0.361	0.421993	Item valid	8
9	0.361	0.548176	Item valid	9
10	0.361	0.471245	Item valid	10
11	0.361	0.397606	Item valid	11
12	0.361	0.569651	Item valid	12
13	0.361	0.268317*	Item not valid	13

* invalid item

The next validity test is for psychological, environmental, and practical variables, using factor analysis methods. The first indicator shows the value of the KMO Measure and the significance values of psychological, environmental, and practice variables. The KMO and Bartlett's tests are data suitability tests that must be performed before interpreting the factor analysis results. The MSA is a statistical value that indicates the proportion of diversity in the variables on which factor analysis is based[34]. If the MSA value >0.50, It is concluded that the questionnaire can be used to measure respondents' answers precisely. If it shows a KMO value of more than 0.5 and a significance value of less than 0.05, the variable can be used for further data collection and analysis[27].

Bartlett's test examines whether the indicators used correlate and are suitable for factor analysis. If the value of Bartlett's test is less than 0.05, it is concluded that the indicators used are correlated and ideal for factor analysis. The KMO values of psychological, environmental, and practice variables are 0.535 each, 0,582, and 0.634, with each significant value below 0.05. These results show that the indicators used in this study are correlated and appropriate for factor analysis[27].

Factor analysis requires the data matrix to correlate factor analysis. The correlation value is shown in the anti-image correlation matrix. The MSA value on the diagonal anti-image correlation with the sign is expected to be above 0.5[27]. **Table III** shows that each variable has a question item whose value is less than 0.5. The first of the psychological variables shows that items 4, 8, and 13 have values less than 0.5, which are 0.229, 0.386, and 0.306. The second environmental variable is shown in item number 3; whose value is less than 0.5, which is 0.4[35]. Finally, from the practice variables, there are four items whose value is less than 0.5: items 1, 13, 18, and 19. Based on these results, question items with an MSA value of less than 0.5 cannot be continued for the data retrieval process, while other items with an MSA value of more than 0.5 can be used for the data retrieval process[22].

The results of the subsequent construct validation can be seen in **Table IV**. **Table IV** presents loading factor coefficient data explaining the connection between the origin variable and the factor. A significant correlation value denotes a solid relationship between the factor and the original variable, which means that the variable can be used as a factor. In a complex matrix, interpreting these factors is quite rare because it is difficult. Therefore, the factor alteration used in matrix factor rotation is converted into a more friendly form to understand[36].

Rotated Component Matrix is the value of the distribution of variables that have been extracted into factors that are formed based on the loading factor after the transformation process to a form that is easier to understand. The loading factor value could turn after the process rotation. Component variables with a loading factor of less than 0.5 are deemed not to contribute to the factors formed significantly, so they must be eliminated from the factors formed[27]. However, for this loading factor value, there is a theory that says if the value is more than 0.3, then the item has shown a close relationship between items on the factor formed[35]. However, in this study, all items on each variable have a loading factor value of more than 0.5, meaning that all indicator items have a close relationship with the factors formed[36].

Table III. Measurement results of MSA of psychological, environment, and practice variables.

Number of items	Standard	Anti-image correlation					
		MSA value					
		Var. psychological	Number in questionnaire	Var. environment	Number in questionnaire	Var. practice	Number in questionnaire
1	0.5	0.724	14	0.628	28	0.494*	37
2	0.5	0.513	15	0.576	29	0.705	38
3	0.5	0.560	16	0.435*	30	0.719	39
4	0.5	0.229*	17	0.623	31	0.820	40
5	0.5	0.713	18	0.518	32	0.653	41
6	0.5	0.666	19	0.596	33	0.511	42
7	0.5	0.778	20	0.630	34	0.727	43
8	0.5	0.386*	21	0.616	35	0.525	44
9	0.5	0.529	22	0.547	36	0.544	45
10	0.5	0.517	23			0.719	46
11	0.5	0.555	24			0.651	47
12	0.5	0.777	25			0.705	48
13	0.5	0.306*	26			0.443*	49
14	0.5	0.541	27			0.620	50
15	0.5					0.628	51
16	0.5					0.675	52
17	0.5					0.667	53
18	0.5					0.161*	54
19	0.5					0.492*	55

* item does not meet the requirement

Table IV. Results of loading factor measurement of psychological, environmental, and practice variables from the Rotated Component Matrix.

Item number	Rotated Component Matrix								
	Var. psychological			Var. environment			Var. practice		
	Loading factor value	Factor categories	Number in questionnaire	Loading factor value	Factor categories	Number in questionnaire	Loading factor value	Factor categories	Number in questionnaire
1	0.764	Factor 2	14	0.775	Factor 1	28	0.698	Factor 5	37
2	0.681	Factor 1	15	0.890	Factor 1	29	0.728	Factor 1	38
3	0.798	Factor 2	16	0.890	Factor 4*	30	0.849	Factor 1	39
4	0.889	Factor 4	17	0.660	Factor 2	31	0.905	Factor 1	40
5	0.641	Factor 2	18	0.829	Factor 2	32	0.572	Factor 3*	41
6	0.602	Factor 1	19	0.853	Factor 3*	33	0.821	Factor 4*	42
7	0.759	Factor 2	20	0.804	Factor 2	34	0.745	Factor 1	43
8	0.599	Factor 3*	21	0.780	Factor 3*	35	0.597	Factor 6*	44
9	0.805	Factor 1	22	0.530	Factor 1	36	0.682	Factor 5	45
10	0.757	Factor 1	23				0.867	Factor 1	46
11	0.799	Factor 1	24				0.724	Factor 2*	47
12	0.777	Factor 3*	25				0.733	Factor 2*	48
13	0.832	Factor 3*	26				0.918	Factor 3*	49
14	0.639	Factor 2	27				0.689	Factor 3*	50
15							0.687	Factor 2*	51
16							0.560	Factor 1	52
17							0.663	Factor 2*	53
18							0.907	Factor 6*	54
19							0.849	Factor 4*	55

* item does not meet the requirement

The calculated component transformation analysis results must support the results in **Table IV**. Suppose the component value of a variable shows a value that is large or more than 0.5. In that case, the relationship between the factors or components that make up a variable is getting closer[37]. Based on the component transformation matrix calculation, psychological variables are divided into four components. Still, component number three has a value of less than 0.5, so component number three is considered not to describe the construct of psychological variables. In **Table IV**, the psychological variables section results from factor loadings on psychological variables, which are included in factor or component 3 in items 8, 12, and 13, which are not included as components that make up psychological variables. Likewise, for environmental variables where the results of the transformation component matrix are only components 1 and 2, which have a solid correlation, meaning that the environmental variable question items included in components 3 and 4 are considered weak variable constituents, therefore environmental variable question items number 3, 6, and 8 are deemed

unable to represent environmental variables. In the last part, based on the results of the component transformation matrix, the training variables show that only components 1 and 5 have a strong correlation, meaning that the training questions included in components 2, 3, 4, and 6 cannot represent the training variables.

The reliability test aims to see how consistent a questionnaire item is when tested on several research samples. The reliability value is potentially high if each item has a close correlation[38]. The Cronbach α value must be greater than 0.6 to be eligible for reliability[28]. Nevertheless, another theory[39] says that if the value of Cronbach α is 0.4 to 0.6, it can be reliable, calculated, and used for data collection. The reliability test results in **Table V** show that the Cronbach α value of all variables is classified as reliable because the value is more than 0.6, meaning that question items from psychological, environmental, and practice variables are reproducible and worthy of being used as research instruments[28]. However, the corrected item's total correlation value is another parameter to see a question item's reliability.

Table V. Cronbach α value of psychological, environmental, and practice variables.

Cronbach α value		
Var. psychological	**Var. environment**	**Var. practice**
0.865	0.636	0.850

The function of the corrected item-total correlation value is to select items whose measuring function is under the test measuring function as the compiler desires. In other words, it is to choose an item that measures the same thing as what the test as a whole measure[40]. According to Azwar[41], a coefficient limit of >0.30 is commonly employed as a criterion for selecting items based on item-total correlation. As part of the test, all items with a correlation coefficient of at least 0.30 were certified psychometrically eligible. However, another theory[42] says that the item-total correlation value must be greater than the r-table to be reliable. After analysis, results show unqualified values based on the two theories above; in psychological variables, item 4 shows values less than 0.3 and more minor than the r-table. For environment variables, items 2, 3, 5, and 9 indicate low values, then practice items 6, 18, and 19, whose values do not qualify. Therefore, some of these items can be removed from the questionnaire.

The results of this validity and reliability test aim to select question items suitable for use in the data collection process because they relate to the purpose of the questionnaire, which is to get answers under the construct built into the questionnaire. Regarding the validity and reliability test results, there is a theory that states that every valid questionnaire question item must also have good reliability because if the item is accurate, then the reproducibility is also good. Unlike reliability, not all questionnaires with good reliability will result in valid question items because the accuracy of the answers has not been tested[43].

Based on the validity and reliability test results, several question items must be eliminated because they cannot provide accurate answers according to the variable construct created. The final form of the questionnaire, which has been evaluated for validity and reliability, can be seen in **Table VI**. From the 55 initial question items, the number was reduced to only 34, and this was because 21 question items in the questionnaire did not meet the validity and reliability requirements.

This research has limitations, and the sample size is only 30 respondents because few pharmacists in type C hospitals are willing to be pilot test respondents. This small number of respondents causes a lack of representation, potentially affecting the results' validity and reliability[44]. However, on the other hand, this research has strength. The strength of this research is that there are no open questions in the questionnaire developed, so the respondents' answers are common to process. Apart from that, the analysis used to test the construct validity of the questionnaire is relatively common because most questions use an ordinal answer scale, so the researcher can use the factor analysis method to construct validity.

Regardless of the strengths and limitations above, this questionnaire benefits researchers in finding out the factors that influence spontaneous reporting practices by pharmacists because this questionnaire can provide accurate, precise, and reproducible results. This questionnaire can be used for pharmacist respondents who work in type C hospitals in East Java. Suppose this questionnaire will be used for pharmacists in other types of hospitals or health services, such as community health centers or drug stores, or for pharmacists outside East Java. This questionnaire can be used but requires a verification process to adapt it to the pharmacist's workplace and location. Suggestions for the next step when developing a research questionnaire instrument: the researcher must start with making the correct conceptual framework design, compiling the questions that the researcher wants to make in the questionnaire, and determining what type of question-answer it looks like, then make a filter that suits the target respondent, then eliminate various potential biases and double questions in one

question item. Then, it also made a picture of what the analysis will be like and the last and main one that pilots must test before being used for research[45].

Table VI. Question items in each instrument domain after evaluation of validity and reliability results.

Variable	Questions	Number in questionnaire	Explanation
Knowledge	One of the points in pharmacovigilance activities is the detection and prevention of AE/ADR so that additional reactions that are detrimental to the patient do not occur.	1	Valid
	Adverse drug reaction monitoring applies to drugs that have been around for a long time, such as captopril, simvastatin, and the like	2	Valid
	Adverse events (AE) can occur in patients if medication reconstitution is done hastily and without an aseptic technique.	3	Valid
	Adverse Drug Reactions (ADR) are part of adverse events whose causes are known from the drugs consumed by the patient.	4	Invalid*
	As health workers, pharmacists should report any AE/ADR encountered as part of their professional responsibility.	5	Valid
	The spontaneous reporting of AE/ADR can only be done manually via a yellow form mailed to the Balai POM pharmacovigilance centre.	6	Valid
	Post-Immunization Adverse events occurring during vaccine administration are not required to be reported to BPOM.	7	Valid
	The institution that acts as the national pharmacovigilance centre in Indonesia is BPOM.	8	Valid
	Spontaneous reporting is only those that are unexpected (Unexpected Adverse Reaction).	9	Valid
	AE/ADR is one of the contributors to the highest number of deaths in various countries.	10	Valid
	Decreased absorption of omeprazole due to drug interactions with antacids does not need to be reported.	11	Valid
	Incidents of side effects due to drug overdose or medication errors need to be reported.	12	Valid
	The discontinuation of the Albothyl product in 2018 and the subsequent call to improve the drug's indications so that it is not used for mouth ulcers are examples of pharmacovigilance efforts being implemented to protect the public from significant adverse drug effects.	13	Invalid*
Psychological	Pharmacists in healthcare facilities play an essential role in pharmacovigilance activities.	14	Valid
	Pharmacists in health service facilities must regularly update their knowledge regarding pharmacovigilance.	15	Valid
	If a drug side effect occurs in their practice, the pharmacist is not obliged to report it.	16	Valid
	Pharmacists in health service facilities are the public's first reference in reporting AE/ADR.	17	Invalid*
	Pharmacists must receive special training regarding pharmacovigilance.	18	Valid
	Spontaneous AE/ADR reporting must be done willingly or as part of professionalism.	19	Valid
	Reporting and monitoring of AE/ADR will be beneficial for patients.	20	Valid
	AE/ADR that occur due to over-the-counter drugs/limited over-the-counter and over-the-counter drugs must also be reported.	21	Invalid*
	Reporting AE/ADR will add more insight regarding the side effects of drugs encountered in practice.	22	Valid
	Reporting AE/ADR experienced by patients is a sign that their concerns are being taken seriously.	23	Valid
	Spontaneous reporting of AE/ADR is part of pharmaceutical care.	24	Valid
	AE/ADR must be reported even if the impact does not result in hospitalization, life-threatening conditions, disability, or death.	25	Invalid*
	All adverse events/ESOs that occur as a result of drugs that have just received distribution permits and medicines that have been on the market for a long time must be reported.	26	Invalid*
	AE/ADR reporting must be done immediately, especially for dangerous or unexpected events (Unexpected Adverse Reaction).	27	Valid
Environment	The pharmacist where I practice applies a regular shift work system.	28	Valid
	In one work shift, the pharmacist at my workplace practices according to the specified working hours.	29	Valid
	The pharmacists' working hours where I work follow the given workload.	30	Invalid*
	My workplace will give rewards/awards to pharmacists if they make innovations in their work or succeed in achieving specific targets.	31	Valid

	Promotions at my workplace are carried out objectively based on the achievements and contributions of a pharmacist.	32	Valid
	The income I get from my workplace is enough for me because it matches my workload.	33	Invalid*
	My workplace will provide additional income if there is extra work or overtime provided	34	Valid
	The portion of work at my workplace is proportional enough to do other work without needing overtime.	35	Invalid*
	I complete work while at work and never do work at home/outside of my working hours	36	Valid
Practice	The frequency with which I encounter reports of drug side effects or adverse events from patients at work.	37	Invalid*
	The frequency with which the hospital where I work reports drug side effects or adverse events to the BPOM National Pharmacovigilance Center	38	Valid
	All drug side effects or adverse events reported by the patient are immediately reported to the unit head/head of pharmacy installation/pharmacist responsible for spontaneous reporting to BPOM.	39	Valid
	I document all reports of drug side effects or adverse events from patients, both unexpected and expected.	40	Valid
	I report and document all actions and interventions I provide to patients according to the patient's complaints.	41	Invalid*
	Suppose there is a complaint that a patient has a dry cough due to using the drug captopril. In that case, I will report it to the unit head/head of pharmacy installation/pharmacist responsible for spontaneous reporting to BPOM.	42	Invalid*
	Suppose there is a complaint that a patient experiences extrapyramidal syndrome due to the use of the drug metoclopramide. In that case, I will report it to the unit head/head of pharmacy installation/pharmacist responsible for spontaneous reporting to BPOM.	43	Valid
	Suppose there is an incident of decreased absorption of the drug omeprazole as a result of drug interactions with antacids. In that case, I will report it to the unit head/head of pharmacy installation/pharmacist responsible for spontaneous reporting to BPOM.	44	Invalid*
	I carried out a causality analysis first with the doctor who provided therapy to the patient to ensure the causality of the side effects of the medication experienced by the patient.	45	Valid
	I include information in the form of reporting data, data on patients who submit complaints, complaints felt by patients, and data on suspected drugs in every report I submit to the head of the unit/head of the pharmacy installation/pharmacist responsible for spontaneous reporting to BPOM.	46	Valid
	I discussed with the doctor who provides therapy to treat patients who experience side effects from drugs.	47	Invalid*
	I take my time at work to handle drug safety incident complaints from patients immediately.	48	Invalid*
	I take the time to do documentation and report cases of drug side effects or adverse events encountered in patients.	49	Invalid*
	I prioritize work related to patient safety while undergoing therapy.	50	Invalid*
	I apply all points of clinical pharmacy services, including monitoring drug side effects (MESO) following the Minister of Health Regulations, where I practice.	51	Invalid*
	I actively participate in the spontaneous reporting of drug side effects or adverse events as a form of professionalism and compliance with regulations.	52	Valid
	I have a spontaneous reporting account at e-meso.pom.go.id or the e-meso mobile smartphone application and operate it actively.	53	Invalid*
	I provide a yellow form for spontaneous reporting of drug side effects to BPOM manually at my practice.	54	Invalid*
	I participated in multilevel pharmacovigilance training held by BPOM as the national pharmacovigilance centre.	55	Invalid*

* invalid item

CONCLUSION

Evaluation of validation results on knowledge question items found two invalid items and four question items could not represent psychological variables. There are three invalid items for environmental variables, and then for practice variables, there are twelve invalid items. Thirty-four question items can still be used to acquire further data.

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DATA AVAILABILITY

None.

CONFLICT OF INTEREST

There are no conflicts of interest.

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