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RESEARCH ARTICLE

Activity of rambutan *(Nephelium lappaceum l.)* leaves ethanolic extract in inhibiting Methicillin-Resistant *Staphylococcus Aureus* (MRSA) and *Streptococcus Pyogenes'* growth

ARTICLE INFO

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ABSTRACT

Rambutan (Nephelium lappaecum l.) is one of Indonesia's ethnomedicinal plants with large spectrum of benefits for health, such as antimicrobe. Many studies have explored the role of rambutan's leaves for medicinal purpose as they are rich of secondary metabolites such as alkaloid, flavonoid, tannin, saponin, polyphenol, and glycoside. Due to increase rate of antibiotic resistance, more research is open to discover more alternatives and back to nature is one of the strategies. Thus, the purpose of the study is to evaluate the antibacterial activity from rambutan's leaves extract to Methicillin-Resistant Staphylococcus aureus (MRSA) and Streptococcus pyogenes by measure the clear zone on petri discs and determine the minimal inhibitory concentration (MIC). Priorly, the rambutan's leaves extract is prepared by maceration technique with ethanol 96% as diluent, continued by phytochemistry screening. The thick extract is divided into several different concentrations, following by disk diffusion and visual turbidity test. Phytochemical screening gives blackish blue color after FeCl3 droplets and red color after HCl droplets in the extract. It indicates flavonoid and tannin compound. The widest clear zone of Methicillin-Resistant Staphylococcus aureus (MRSA) and Streptococcus pyogenes are obtained from concentration 100% (14,6 mm and 24 mm). The turbidimetry test showed the apparent clear tube appears in concentration 25%.

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1. Introduction

Rambutan (*Nephelium lappaceum l.*) is one of the millions exotic and tropical fruits that can be found across Indonesia. The popularity of the fruit comes from the appearance and the taste. For many decades, rambutan has been part of main commodity especially in traditional trade market in several big islands in Indonesia, for example Java, Sumatra and Borneo island. The latest production report of rambutan is at 2023 where the fruit is cultivated approximately 845.107 tons in 2023 and Java island distributes nearly more than half of the national production—West Java (154.037 tons), Central Java (146.428 tons), and East Java (129.998 tons) (Badan Pusat Statistik ~~(BPS)~~, 2023; Rozana & Sunardi, 2021). It has been proven in various empirical and experimental studies that all parts of rambutan have health benefits. The fruit of rambutan can be eaten directly or processed into various foods such as pickles or other things. Meanwhile, other parts of the body have been widely used as traditional medicine to cure various health problems such as diabetes, hypertension and infectious diseases. Rambutan skin can be used to treat mouth ulcers, the roots can be used to treat fever, seed fiber to treat diabetes mellitus, and leaves to treat diarrhea and blacken hair (Rumaolat & Husada, 2020). Rambutan leaves, especially old leaves with dark green characteristics, are also often used as antibacterial because they contain tannins and saponins which are effective in preventing bacterial growth (Anggresani et al., 2019; Indrayati & Sugiarto, 2020).

Compounds and extracts found from various parts of rambutan show antidiabetic, antimicrobial, antibacterial, anti-inflammatory, and anti-aging properties, as suggested by many previous studies (Peixoto Araujo *et al.,* 2021). A study that tested the effect of rambutan leaf extract as an antibacterial for the cause of dental caries, *Staphylococcus mutans*, showed strong inhibition of bacterial growth at a concentration of 10% with an inhibition zone reaching 12.5

mm. In addition, another study conducted by Putri *et al.* which tested the inhibition of rambutan leaf extract on *Propionibacterium acnes* bacteria that causes acne vulgaris reported an inhibition zone reaching 11 mm at a concentration of 20% (Febrina Karim *et al.,* 2023; Putri *et al.,* 2021). In addition to being antibacterial, rambutan leaf extract also shows protective ability against ultraviolet light exposure with an ultra-protection category (A. Lestari et al., 2023). Using a similar method, the diameter of the inhibition zone of *Staphylococcus aureus* also appeared positive at 7.96 mm at the smallest concentration of 3.125%. The antibacterial ability of rambutan leaf extract is suspected to come from the effects of secondary metabolites contained therein, namely flavonoids, tannins and saponins (Irmayanti *et al.,* 2022; ~~W. D.~~ Lestari *et al.,* 2023). From various reported studies, in general flavonoids have the ability as anti-inflammatory caused by cell hyperglycemic environment and prevent the senescence process. Thus, flavonoids can function as anti-diabetic, anti-aging to antimicrobial. Saponins are known to have the potential to inhibit pathogenic activity from microbes by suppressing related signaling pathways. While tannins have been widely studied for their antioxidant effects, stopping bleeding, and the ability to precipitate proteins (Wang *et al.,* 2018; Zahra *et al.,* 2023; Zaynab *et al.,* 2021).

One of the components of the body's natural defense that inhabits human skin and mucosa is normal flora. In a balanced immune condition, these normal flora bacteria can provide protection rather than pathogenic effects and trigger infections. Some normal flora that often cause infections of the skin and mucosa include *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes* to *Cutibacterium acnes* (Byrd *et al.,* 2018; Flowers & Grice, 2020). Streptococcus pyogenes is a pathogenic Gram-positive coccus-shaped bacteria that looks like a long chain under a microscope and is a normal flora in the respiratory tract. When the host's defenses are disrupted, these bacteria can cause infections, especially in the esophagus and skin. Clinical manifestations due to infection from these bacteria can range from pain when swallowing to reddish vesicles that easily break on the skin (Agustin *et al.,* 2019; Hasanah *et al.,* 2021). Methicillin-Resistant *Staphylococcus aureus* (MRSA) is a strain of *Staphylococcus aureus* that is resistant to isoxazolyl penicillin antibiotics such as methicillin, oxacillin and flucloxacillin which spreads through two main mechanisms, namely the spread of clones that exist between humans and animals, either from animals to humans or vice versa, and through the acquisition of Staphylococcal Cassette Chromosome mec (SCCmec) elements through horizontal gene transfer (Brown *et al.*, 2005; Kemalaputri *et al.,* 2017; Lee *et al.,* 2018). Until now, antibiotics are still the main treatment for treating bacterial infections. However, the impact of extensive and irrational use increases the risk of resistance in the future (Hossain *et al.,* 2023). Therefore, a strategy is needed to strengthen the ability of antibiotic and recued the further potential risk of resistant, one of which is by exploring natural materials. Most natural materials contain secondary metabolites that are rich in antibacterial activity. Flavonoids can inhibit the growth activity of MRSA especially in ring A chemical structure 5,7-dihydroxylation and ring B chemical structure 4-hydroxylation while tannin and saponin are known to inhibit bacterial growth by damaging the integrity of bacterial cell membranes through the activity of chemical groups on their surfaces (Chigurupati *et al.,* 2019; Dong *et al.,* 2020; Shamsudin *et al.,* 2022). Among all parts of the rambutan plant, the leaves are known to have total phenolic content, total flavonoids and are expressed in gallic acid (19.6 ± 0.04 mg GAE/g) and rutin equivalents (16.7 ± 0.01 mg RUE/g) respectively (Aini *et al.,* 2023).

Due to the great benefits reported in previous studies related to rambutan leaves as an antibacterial agent, a study was conducted to experimentally examine the bactericidal ability of rambutan leaf extract in 96% ethanol on several normal flora bacteria such as MRSA (Methicillin-Resistant *Staphylococcus aureus*) and *Streptococcus pyogenes*. This study assessed the ability of rambutan leaf extract on antibacterial effects on gram-positive bacteria. Assessing the inhibitory ability of rambutan extract against *Streptococcus pyogenes* and Methicillin-Resistant *Staphylococcus aureus*

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(MRSA) bacteria to determine the effective concentration in inhibiting these bacteria.

2. Materials and Methods

The study design was quasi experimental with post-test only analysis. It compared a few ethanolic extract of rambutan leaves (*Nephelium lappaceum l.)* concentrations to MRSA (Methicillin-Resistant *Staphylococcus aureus*) and *Streptococcus pyogenes'* growth inhibition through disk diffusion and turbidimetry method. The research was conducted at Laboratory of Organic Chemistry, Faculty of Pharmacy and Laboratory of Microbiology, Faculty of Medicine, Universitas Ahmad Dahlan. The main material in this study was rambutan leaves (*Naphelium lappceum l.),* which came from the Special Region of Yogyakarta, the leaves used were old leaves (Felicia *et al.,* 2017). The rambutan leaf determination process was carried out in the Biology Laboratory, Universitas Ahmad Dahlan. The bacterial samples used Methicillin-Resistant *Staphylococcus aureus* (MRSA) RES 22-2020 and *Streptococcus pyogenes* ATCC 19615. Other materials used included 96% ethanol, NaCl, distilled water, DMSO, flannel filter cloth, filter paper (Whatmann), aluminum foil, $FeCl_3$, microtube (Labselect), microtip (Biologix), sterile cotton swabs, H2SO4, BaCl2, Mueller Hinton Agar (MHA) media (Himedia), Mueller Hinton Broth (MBH) (Himedia), 70% alcohol, Streptomycin vial, Novobiocin antibiotic disc (Oxoid), and blank disc (Oxoid). The tools used in this study were: laboratory blender (Waring), a set of maceration extraction tools and rotary evaporator (Heidolph), water bath (Memmert), oven (Memmert), autoclave (GEA), jars, electric analytical balance (Ohaus), sieve/mesh, water bath, porcelain cup, flacon, ruler, 3 cc syringe, measuring cup, micropipette (Dlab scientific) (size 1000 µl, 200 µl and 100 µl), test tube, petri dish, inoculation loop, Bunsen burner, laminar air flow (Thermo Scientific), and incubator (Memmert).

To prepare the ethanolic extract of rambutan (*Naphelium lappceum l.)* leaves, the rambutan leaves are washed and oven-dried at 50° Celcius for 24 hours, then the leaf veins are separated (Yuvakkumar *et al.,* 2015). Rambutan simplisia is made into powder and filtered with a mesh size of 100. The powder that is ready is macerated by pouring 250 grams into a glass jar containing 1000 mL of 96% ethanol as a solvent for up to 24 hours (Tingting *et al.,* 2022). The macerate are filtered using filter cloth and filter paper. The filtered macerate is separated between the solvent and residue to obtain a liquid extract with a rotary evaporator at a temperature of 50° Celcius at rotation of 80 rpm. The thick extract is obtained by placing the liquid extract on a water bath at a temperature of 40° Celcius for 24 hours. After the thick extract is ready, phytochemical screening is necessary to evaluate any secondary metabolites of the leaves. Testing for the presence of flavonoids is done by dissolving the extract with ethanol. The solution then is added with 0,1 grams magnesium (Mg) and homogenized then add a few drops of HCl through the tube wall (Setyawaty *et al.,* 2020). Red color change in the extract layer indicates flavonoid compound inside the extract. The presence of tannins is done by dissolving the extract with ethanol. The solution is then added a few drops of 1% $FeCl_3$ through the wall (Retno Priamsari *et al.,* 2023). A change in the color of the extract to blackish blue indicates a positive result for the presence of tannins.

According to Irmayanti *et al.*, using the same method to evaluate *Staphylococcus aureus* dan *Escherichia coli*'s growth inhibition by ethanolic extract of rambutan (*Naphelium lappceum l.)* leaves, at least it requires three replications to strengthen the result validation (Irmayanti *et al.,* 2022). Prior to antibacterial activity test, confirmation test of bacteria sample used in this research was done by applying Gram staining. The sample then continued to have antibacterial test by disk diffusion method. The first step is to prepare the series of the thick extract concentrations by diluted them with 5% DMSO. The concentrations used were 100%, 50%, 25%, 10%, and 5%, each concentration was dropped on a sterile empty disk as much as 20 µL. Both bacteria were made into a

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suspension with NaCl solution in a sterile tube and then homogenized. The resulting suspension was then compared for its hardness using Mc.Farland solution. The bacterial suspension that had been inoculated in accordance with the standard was dipped using a inoculation loop and then spread on the surface of the agar media. The media that had been spread with bacteria was then planted with a disk containing a concentration of rambutan leaf extract and Novobiocin used as an antibiotic standard. Observations were made after the media was incubated in an incubator at a temperature of 27° Celcius for 18-24 hours (Retno Priamsari *et al.,* 2023).

Turbidimetric test is used to observe the MIC (minimum inhibitory concentration) value of extract on bacterial growth. The principle of turbidimetric test is visual observation of turbidity. The concentrations of rambutan leaf extract used are 100%, 50%, 25%, 12.5%, 6.25%, and 3.125%. The positive control (K+) is a bacterial suspension equivalent to the McFarland 0.5 standard while the negative control (K-) contains the antibiotic Streptomycin. Each tube is labeled according to the concentration series and positive/negative controls needed. Tube 1 is filled with 2 ml of 100% concentration of rambutan leaf extract, tubes 2-6 are filled with 1 ml of Mueller Hinton Broth (MHB) media. 1 ml of solution is taken from tube 1 using a micropipette, inserted into tube 2, mixed until homogeneous to obtain a concentration of 50%. The same thing is done up to tube 6 and all extract concentrations are obtained with a ratio of 1:2. At the end, each tube is given 0.5 ml of bacterial suspension equivalent to McFarland 0.5. Then each tube was incubated at 37°C for 1x24 hours. After 24 hours, the MIC was determined by visual observation of turbidity (Sekeon *et al.,* 2018; Warokka *et al.,* 2016). The absorbance value of each tube was then measured using a UV-vis Spectrophotometer by comparing before and after 1x24-hour incubation at a wavelength of 625. If the final absorbance value (after incubation) of each tube is higher than the initial absorbance value (before incubation), then bacterial growth is considered to continue. However, if the final absorbance value does not change or the absorbance value is lower than before incubation, then bacterial growth stops or is inhibited (Warokka *et al.,* 2016).

To obtain the concentration with the best inhibitory activity, statistical analysis will be carried out with SPSS 29.0. The variables in this study are numeric interval data with more than one group test so that if the data is normally distributed, the test used is One way-ANOVA, while if the data is not normally distributed, the test used is the non-parametric Kruskall-Wallis test. The results of the antibacterial clear zone measurements were then presented using the GraphPad Prism 9 program. The official number of ethical approval of this study was 012402029 and was approved by Universitas Ahmad Dahlan's Research Ethic Committee.

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3. Results

To obtain a thick extract from rambutan leaves, several extraction methods can be applied. In a study conducted by Tambunan *et al.* (2024), the maceration method was the best method of choice for making thick extract of rambutan leaves (Mistryanto Tambunan *et al.,* 2024). From 1.5 liters of a mixture of simplicia powder that had been obtained that was soaked with 96% ethanol, approximately 400 ml of liquid extract was produced. Then continued by soaking the liquid extract in a water bath at a temperature of 45° Celcius for 1x24 hours until at the end of the extraction process using the maceration method, 10 grams of thick extract was produced (Figure 1).

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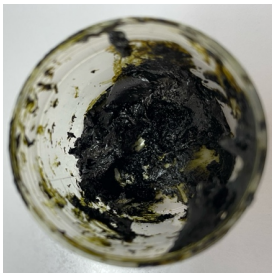

Figure 1. Results of thick extract of rambutan leaves using the maceration method

The organoleptic results of the extract produced a thick consistency. Blackish brown in color with a distinctive matcha odor is the characteristic of the final thick extract. Phytochemical screening of rambutan leaf extract showed positive results for flavonoids with a sign of a red color change in the extract after Mg and HCl drops. In addition, there was a blue-black color change after $FeCl_3$ drops which indicated the presence of tannin in the extract.

In bacterial identification, Gram staining is considered as the standard and easily conducted. The first Gram staining step is to fix the bacteria on the object glass. After that, Gentian violet immersion was carried out for 1 minute, Lugol for 1 minute, alcohol drops, and safranin immersion for 30-45 seconds. The staining results were read using a light microscope with a magnification of 100 x with immersion oil given to the preparation. In the identification of bacteria, it was obtained in accordance with the theory, namely Gram (+) and coccus and colonies that could grow on Nutrient Agar media for 24 hours at a temperature of 37° Celcius (Figure 2) (Tripathi & Sapra, 2023).

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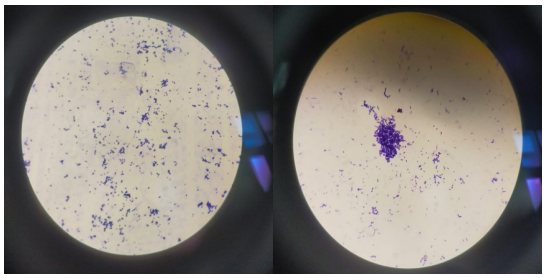

Figure 2. The appearances of colonies and Gram Staining of MRSA RES 22-2020 (left) and *Streptococcus pyogenes* (right) bacteria performed

The results of the antibacterial test of rambutan leaves against MRSA showed good activity visually (Figure 3). The results of each bacterial inhibition zone are shown in Table 1. A concentration of 5% rambutan leaf extract did not produce a clear zone after 3 repetitions of the test. The best concentration results were shown at a concentration of 100% with an average inhibition zone of 14.6 mm. There was an increase in antibacterial activity at a concentration of 25% when compared to a concentration of 50%. The results of the One Way ANOVA quantitative analysis showed that there was a significant value in the standard (novobiocin) for all concentrations, with a p value <0.0001. The 5% concentration had no inhibition zone at all so that there was a significant value for the 100%, 50%,

and 25% concentrations. The 100% concentration was significant when compared to the 10% concentration (p value 0.0402) and 5% (p value 0.0004).

Table 1. Results of the Inhibition Zone Test on Methicillin Resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pyogenes* Bacteria

Concentration (%)	MRSA				*Streptococcus pyogenes*			
	I (mm)	II (mm)	III (mm)	Average (mm)	I (mm)	II (mm)	III (mm)	Average (mm)
Standard	45	45	40	43,3	35	36	35	35,3
100%	15	10	19	14,7	23	23	26	24
50%	14	11	14	13	22	22	23	22,33
25%	14	10	14	11,3	23	20	20	21
10%	3	8	9	6,7	20	17	18	18,3
5%	0	0	0	0	17	14	10	13,7

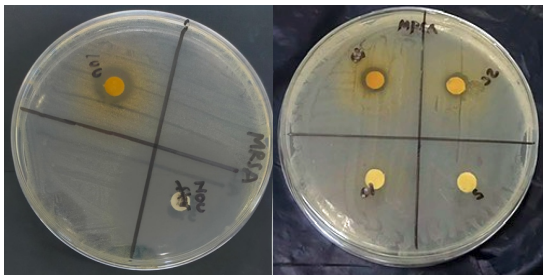

Figure 3. Clear zone of MRSA bacteria on petri dish after incubation for 1x24 hours.

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The results of the antibacterial test of rambutan leaves against *Streptococcus pyogenes* are also shown in Table 1. From the observation results, the inhibition activity is evenly distributed visually. The concentration of 100% has the largest zone with an average inhibition zone of 24 mm (Figure 4). The results of the One Way ANOVA quantitative analysis show that the standard has a significant value with all extract concentrations. The concentration of 100% has a significant value with a concentration of 10% (p 0.0485) and a concentration of 5% (p 0.0005). A significant difference is also shown in the comparison of the concentration of 50% with 5% (p 0.0035) and the concentration of 25% with 5% (p value 0.0091). Thus, it can be concluded that increasing the concentration has a significant ability to inhibit bacterial growth. The average diameter of the inhibition zone formed by the ethanol extract of rambutan leaves against MRSA bacteria and *Streptococcus pyogenes* bacteria will increase longitudinally as the concentration rise (Diagram 1). The diameter of the inhibition zone indicates the presence of bacterial growth inhibition activity originating from compounds contained in the extract.

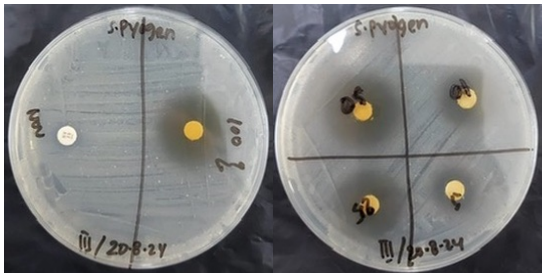

Figure 4. Clear zone of *Streptococcus pyogenes* bacteria on petri dish after incubation for 1x24 hours.

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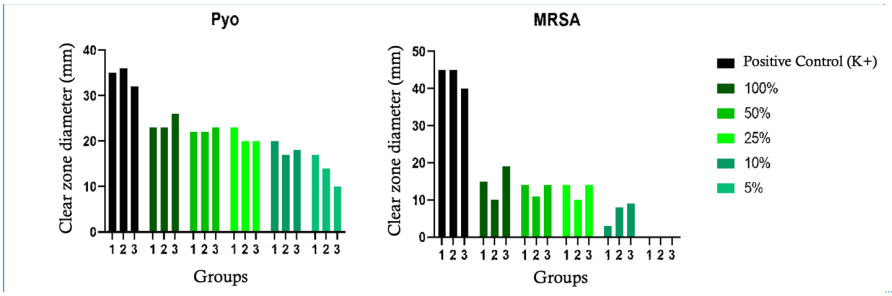

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Diagram 1. Results of inhibition of the growth of MRSA bacteria (right) and *Streptococcus pyogenes* (left) using the disk diffusion method.

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To support the results obtained from the disk diffusion method, the assessment of the MIC (minimum inhibitory concentration value) of the ethanol extract of rambutan leaves was carried out using the turbidimetry method and confirmation using UV-vis Spectrophotometer. The results obtained showed that visually in the third tube which was a concentration of 25%, both for MRSA and *Streptococcus pyogenes* bacteria, still showed a clear appearance after being incubated at 37° Celcius for 1x24 hours (Figure 5).

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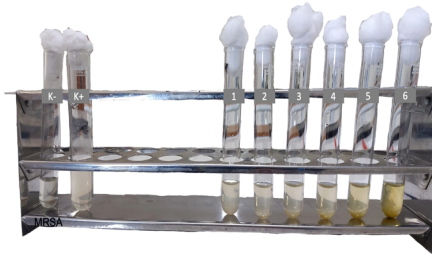

Figure 5. Results of the MIC test using the turbidimetric method of MRSA bacteria (top) and Streptococcus pyogenes (bottom). K(-) is a negative control, K(+) is a positive control, respectively from 1-6 contains extract concentrations of 100%, 50%, 25%, 12.5%, 6.25%, and 3.125%

According to the observation, the clarity of the tubes became increasingly apparent starting from the 3rd tube (25% concentration) to 100% concentration. In contrast, the tubes began to become cloudy in the 4th tube (12.5% concentration) although they were faintly visible in both MRSA and *Streptococcus pyogenes* bacteria up to the 6th tube (3.125% concentration). The turbidity that appeared indicated an increase in the density level of the suspension originating from the bacteria. The bacterial inhibition power was assessed based on the clarity of the suspension in the tube compared to the positive control containing McFarland 0.5. Turbidimetric observations were carried out using three replications followed by using a UV-vis Spectrophotometer with a wavelength of 625. The results of the MIC of MRSA and *Streptococcus pyogenes* bacteria are shown in tables 2 and 3. From the results of absorbance observations using a UV-vis Spectrophotometer, it is known that the decrease in absorbance is only seen at concentrations of 100% and 50% in MRSA bacteria with a respectively decrease of 0.224 and 0.049. However, in *Streptococcus pyogenes* bacteria, it only shows a decrease in absorbance at a concentration of 100%. Concentrations of 50% -100% show clarity because the molecules of the material or compound read by the device are only slightly absorbed at the specified wavelength. The increase in absorbance begins to be seen at concentrations of 12.5 to 3.125% in MRSA bacteria, while in *Streptococcus pyogenes* bacteria, the increase in absorbance begins to be seen from concentrations of 25% to 3.125%.

Table 2. Results of the MIC value using the turbidimetric method of ethanol extract of rambutan leaves (*Nephelium lappaceum l.)* on the growth of MRSA and *Streptococcus pyogenes* bacteria in three replications.

Tube(s)	Concentration of ethanolic extract of rambutan (*Nephelium lappaceum L.*) leaves	MRSA			*Streptococcus pyogenes*		
		I	II	III	I	II	III
1	100%	-	-	-	-	-	-
2	50%	-	-	-	-	-	-
3	25%	-	-	-	-	-	-
4	12,5%	+	-	+	+	-	+
5	6,25%	+	+	+	+	+	+
6	3,125%	+	+	+	+	+	+
K+	*McFarland* 0,5	+	+	+	+	+	+
K-	Antibiotik Streptomycin	-	-	-	-	-	-

Description: The (+) sign indicates the presence of bacterial growth, which is shown by the presence of turbidity, while the (-) sign indicates the presence of inhibited bacterial growth, which is shown by the clarity.

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Table 3. Absorbance results of the UV-vis Spectrophotometer of ethanol extract of rambutan leaves (*Nephelium lappaceum l.)* on the growth of MRSA and *Streptococcus pyogenes* bacteria.

Concentration of ethanolic extract of rambutan (*Nephelium lappaceum L.*) leaves	MRSA			*Streptococcus pyogenes*		
	Pre-incubation	Post-incubation	Interpretation	Pre-incubation	Post-incubation	Interpretation
100%	0,633	0,409	Decrease	0,644	0,523	Decrease
50%	0,354	0,305	Decrease	0,362	0,584	Increase
25%	0,202	0,428	Increase	0,216	0,169	Increase
12,5%	0,123	0,568	Increase	0,141	0,416	Increase
6,25%	0,081	1,18	Increase	0,078	1,024	Increase
3,125%	0,072	1,23	Increase	0,07	0,947	Increase
K(+)	0,03	0,45	Increase	0,03	0,45	Increase
K(-)	0,104	0,085	Decrease	0,104	0,085	Decrease

Description: "Increase" indicates an increase in bacterial growth as indicated by an increase in absorbance after incubation, while "Decrease" indicates that bacterial growth is inhibited or decreases after incubation.

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4. Disscussion

The bacteria used in this study were MRSA (Methicillin Resistant *Staphylococcus aureus*) and *Streptococcus pyogenes*. MRSA is a type of *Staphylococcus aureus* bacteria that experiences resistance mainly due to the expression of penicillin-binding protein (PBP2a) which tends to reduce the sensitivity of beta lactam antibiotics to the bacterial receptors to carry out the bactericidal process. This bacteria is often found on human mucous membranes and is the most common cause of skin infections, especially if there is a wound (Kemalaputri *et al.,* 2017). Like MRSA, Streptococcus pyogenes is a bacteria that is largely responsible for skin and respiratory infections, one of which is impetigo to pneumonia. Both bacteria have similarities such as possess thicker cell walls as well as the peptidoglycan layer. In addition, their proteins, teichoic acids and lipoteichoic acids are distinctively prominent compared to Gram-negative bacteria (Fischetti, 2017). From the Gram examination, a purplish image will appear due to the binding ability of crystal violet as a primary dye from one of the Gram reagents to bacterial peptidoglycan. Thus, both bacteria are included in Gram-positive (Tripathi & Sapra, 2023). From the tests conducted, it was proven that MRSA and *Streptococcus pyogenes* bacteria showed a bright purple color, thus both bacteria were Gram-positive bacteria.

Phytochemical screening of ethanolic extract of rambutan leaves was carried out using two different methods to assess each secondary metabolite. From the results of the experiment, it was concluded that ethanolic extract of rambutan leaves contains flavonoids and tannins. Flavonoid testing was carried out by observing the reactions between the extract with Mg followed by dripping HCl. Because Mg and HCl are metals by nature, they can reduce the benzopyrone core in flavonoids, thus the red flavylium salt is formed (A. Lestari *et al.,* 2023). The blackish blue color that appears after dropping $FeCl_3$ indicates the formation of a complex compound between Fe metal and tannin. The coordination covalent bond between metal ions or atoms with non-metal atoms causes the formation of this complex compound. $FeCl_3$ reagent is usually used to identify phenolic compounds, including tannins, when this solution is added. It is estimated that this solution will react with one of the hydroxyl groups in the tannin compound. Because tannins are phenolic compounds that tend to dissolve in water and polar solvents, the addition of $FeCl_3$ is a way to find out if they contain phenol groups; the blackish blue color after adding $FeCl_3$ indicates the presence of phenol groups (Febrina Karim *et al.,* 2023; Rumaolat & Husada, 2020). This is in accordance with the results obtained in this study. Both compounds have activity as natural antibacterial found in plants. Flavonoids are important

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In litertature

In the literature written in this paper there are other compounds such as saponins which are described in the introduction which also have antibacterial activity

components and are most often found in the phenolic compound class (Panche *et al.,* 2016). The structure of flavonoids shows an important role as antibacterial that help damage the integrity of bacterial membranes (Shamsudin *et al.,* 2022). The antibacterial activity of flavonoids is suspected of ability to disrupt cell membrane permeability and adhesion to biofilm formation which ultimately triggers bacterial death (Xie *et al.,* 2014). In addition, flavonoids also inhibit bacterial DNA gyrase enzymes. The DNA gyrase enzyme functions to maintain the protein synthesis process in the formation of prokaryotic cell membranes such as bacteria. Compared to Gram-negative bacteria, flavonoids have faster activity in Gram-positive bacteria. This may be due to differences in cell envelope structure and higher quinone composition. The types of quinones most commonly found in Gram-positive are menaquinone and ubiquinone. Quinones are known to play a role in the cellular respiration process of Gram-positive bacteria (Yan *et al.,* 2024). Other secondary metabolite, tannins, also have antibacterial activity by disrupting the formation of peptidoglycan and bacterial cellular membranes, chelating iron, inhibiting antibiotic efflux pumps, and disrupting bacterial fatty acid synthesis (Villanueva *et al.,* 2023).

The results of this study indicate that both MRSA and *Streptococcus pyogenes* bacteria showed significant inhibition after administration of ethanolic extract of rambutan (*Nephelium lappaecum l.)* leaves. The antibacterial ability of a certain chemical component has been determined based on the classification made by Davis and Stout (1971) according of clear zone diameter. The diameter of the clear zone <5 mm is classified as weak, 5-10 mm is classified as moderate, 10-20 mm is classified as strong, and >20 mm is classified as very strong (G.S. *et al.,* 2021). This study showed the antibacterial effect of ethanolic extract of rambutan (*Nephelium lappaecum l.)* leaves at a concentration of 100% given to MRSA bacteria and *Streptococcus pyogenes* bacteria is included in the strong category with an average inhibition zone diameter in the range of 10-20 mm with the highest mean obtained from *Streptococcus pyogenes* group (24 mm). The inhibitory ability of rambutan (*Nephelium lappaecum l.)* leaves extract in ethanol was also shown in a study conducted by Irmayanti *et al.* (2022) on *Staphylococcus aureus* bacteria. The widest inhibition zone was shown when the extract concentration was 100%, which was 12.9 mm, and the smallest was at a concentration of 3.125%, which was 7.96 mm (Irmayanti *et al.,* 2022). Using the same bacterial strain, in a study conducted by Rumaolat (2020), methanol extract of rambutan leaves provided an inhibition zone of up to 2 times than Irmayanti *et al.* study where the obtained clear zone at concentration of 75% was 26 mm (Rumaolat & Husada, 2020). Another study reported by Ratna et al. (2018) showed that at a concentration of 10% ethanol extract of rambutan leaves, the average inhibition zone was 19.64 mm (Ratna *et al.,* 2018). Purba *et al.* (2023) also reported from their study that rambutan leaves extract has the widest mean of inhibition diameter of *Streptococcus mutans'* growth at concentration 100% which is 17,40±0,10 mm (Purba *et al.,* 2023).

Based on observations on both bacteria using the disk diffusion method, it was seen that the ethanol extract of rambutan leaves had better effectiveness on *Streptococcus pyogenes* bacteria when compared to MRSA. At a concentration of 5%, rambutan leaves extract did not have any effect on bacterial growth in a petri dish, in contrast to *Streptococcus pyogenes* bacteria which had a strong inhibition zone. Several things can cause an inhibition zone not to form from specific extract at a concentration of 5%, including weak diffusion power of the extract, inappropriate use of solvents, and low concentrations of active ingredients, causing the extract to be unable to effectively inhibit bacterial growth (Rumaolat & Husada, 2020). There have been no studies that specifically compare the effectiveness of ethanolic extract of rambutan leaves on MRSA and *Streptococcus pyogenes* bacteria. However, the opposite result was shown in a study that tested the skin of rambutan fruit against MRSA and *Streptococcus pyogenes* bacteria. In their study testing the effect of rambutan fruit skin extract on MRSA, Rostinawati *et al.* (2018) reported that the inhibition zone at 100% extract concentration reached 23.4 mm, while a study conducted by Sekar *et al.* (2014) showed that the

inhibition zone of red and yellow rambutan skin extract at the highest concentration could only inhibit 10 mm of *Streptococcus pyogenes* bacterial growth (Rostinawati *et al.,* 2018; Sekar *et al.,* 2014).

The main principle of the turbidimetry method is to test the concentration of the solution in the tube from various concentrations of the test material. The higher the turbidity that appears visually, the bacterial activity also increases, thus the tube clarity becomes a standard in assessing the inhibition of the test material against bacterial growth. However, the turbidity that arises has a dual interpretation, namely the number of bacteria that have died cannot be known so that both living and dead bacteria will both give a cloudy appearance. Although visual observation is considered sufficient to obtain the MIC value, the researcher's eye ability also has limitations. It will lead to the variation in observation results that can appear because the brownish color between one tube and another looks the same (Warokka *et al.,* 2016). The results shown in this study on both MRSA and *Streptococcus pyogenes* bacteria showed the best clarity in tubes with concentrations of 100%, 50%, and 25%. However, in one study conducted by Mukaromah *et al.,* (2023) reported that extracts of rambutan's peel and seed have MIC 15,625 mg/mL in inhibiting the growth of MDR (Multi Drug Resistant)-*Klebsiella pneumoniae* bacteria through microdilution test. To confirm the bacterial MIC, post-incubation absorbance calculations can be carried out, one of which is by utilizing a UV-vis Spectrophotometer. In this study, it was found that the significant decrease of absorbance for both bacteria were concentration 100%. It is also relevant to other study reported by Salsabila *et al.* (2019) in one experiment to evaluate the best concentration for *Aggregatibacter actinomycetemcomitans* and *Treponema denticola* biofilm inhibition after given the rambutan (*Nephelium lappaecum l.)* leaves extract. According to the findings, rambutan leaves extract was most successful in preventing *Aggregatibacter actinomycetemcomitans* biofilm adherence at 100% concentration, with an average optical density of 0.028, and Treponema denticola at 50% concentration, with an average optical density of 0.120 (Salsabila *et al.,* 2022). In general, the working principle of a Spectrophotometer is to assess the absorption of light on the molecules of a particular material. However, Spectrophotometers also have several weaknesses that are often related to the operation of the tool or limitations in distinguishing between living and dead bacteria. Spectrophotometers can still read contaminants or other particles that are absorbed by the same wavelength so that to determine specifically whether bacterial death occurs or not, it can be continued by growing bacteria on Plate Count Agar. In addition, it is recommended to use a high-performance liquid chromatography (HPLC) tool to analyze the amount per compound more accurately (Gloria Rambet *et al.*, 2017; J Soelama *et al.*, 2015).

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5. Conclusions

Ethanolic extract of rambutan (*Nephelium lappaceum l.)* leaves contains secondary metabolites of flavonoids and tannins which are shown from the color change after being dripped with the appropriate reagent. Ethanol extract of rambutan leaves also has the activity of inhibiting the growth of Methicillin-Resistant *Staphylococcus aureus* (MRSA) bacteria with a strong category inhibition ability (14.9 mm) at a concentration of 100% while the concentration with the highest inhibition power in *Streptococcus pyogenes* bacteria is 100% (24 mm). There was a significant value for the 100%, 50%, and 25% concentrations in inhibiting the MRSA growth. Meanwhile, all concentrations of ethanolic extract of rambutan (*Nephelium lappaceum l.)* leaves have significant ability in decreasing the *Streptococcus pyogenes* growth. Based on the visual turbidimetry method, the minimum inhibition ability of both bacteria began to appear at a concentration of 25%. Confirmation using a UV-vis Spectrophotometer showed a decrease in absorbance in MRSA bacteria seen at a concentration of 100%-50% while in *Streptococcus pyogenes* bacteria only at a concentration of 100%.

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Conflict of interest

All authors have no conflict of interest in this article.

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RESEARCH ARTICLE

Activity of rambutan *(Nephelium lappaceum l.)* leaves ethanolic extract in inhibiting Methicillin-Resistant *Staphylococcus Aureus* (MRSA) and *Streptococcus Pyogenes'* growth

ARTICLE INFO

Keywords:
Growth inhibition
MRSA
Rambutan's leaves
Streptococcus pyogenes

ABSTRACT

Rambutan (Nephelium lappaecum l.) is one of Indonesia's ethnomedicinal plants with large spectrum of benefits for health, such as antimicrobe. Many studies have explored the role of rambutan's leaves for medicinal purpose as they are rich of secondary metabolites such as alkaloid, flavonoid, tannin, saponin, polyphenol, and glycoside. Due to increase rate of antibiotic resistance, more research is open to discover more alternatives and back to nature is one of the strategies. Thus, the purpose of the study is to evaluate the antibacterial activity from rambutan's leaves extract to Methicillin-Resistant Staphylococcus aureus (MRSA) and Streptococcus pyogenes by measure the clear zone on petri discs and determine the minimal inhibitory concentration (MIC). Priorly, the rambutan's leaves extract is prepared by maceration technique with ethanol 96% as diluent, continued by phytochemistry screening. The thick extract is divided into several different concentrations, following by disk diffusion and visual turbidity test. Phytochemical screening gives blackish blue color after FeCl3 droplets and red color after HCl droplets in the extract. It indicates flavonoid and tannin compound. The widest clear zone of Methicillin-Resistant Staphylococcus aureus (MRSA) and Streptococcus pyogenes are obtained from concentration 100% (14,6 mm and 24 mm). The turbidimetry test showed the apparent clear tube appears in concentration 25%.

1. Introduction

Rambutan (*Nephelium lappaceum l.*) is one of the millions exotic and tropical fruits found across Indonesia. The popularity of the fruit comes from the appearance and the taste. For many decades, rambutan has been a main commodity, especially in the traditional trade market in several big islands in Indonesia, such as Java, Sumatra, and Borneo. The latest production report of rambutan is at 2023 where the fruit is cultivated approximately 845.107 tons in 2023 and Java island distributes nearly more than half of the national production—West Java (154.037 tons), Central Java (146.428 tons), and East Java (129.998 tons) (Badan Pusat Statistik (BPS), 2023; Rozana & Sunardi, 2021). It has been proven in various empirical and experimental studies that all parts of rambutan have health benefits. The fruit of rambutan can be eaten directly or processed into various foods such as pickles or other things. Meanwhile, other parts of the body have been widely used as traditional medicine to cure various health problems such as diabetes, hypertension and infectious diseases. Rambutan skin can be used to treat mouth ulcers, the roots can be used to treat fever, seed fiber to treat diabetes mellitus, and leaves to treat diarrhea and blacken hair (Rumaolat & Husada, 2020). Rambutan leaves, especially old leaves with dark green characteristics, are also often used as antibacterial because they contain tannins and saponins which are effective in preventing bacterial growth (Anggresani et al., 2019; Indrayati & Sugiarto, 2020).

Compounds and extracts found from various parts of rambutan show antidiabetic, antimicrobial, antibacterial, anti-inflammatory, and anti-aging properties, as suggested by many previous studies (Peixoto Araujo *et al.,* 2021). A study that tested the effect of rambutan leaf extract as an antibacterial for the cause of dental caries, *Staphylococcus mutans*, showed strong inhibition of bacterial growth at a concentration of 10% with an inhibition zone reaching 12.5

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Edit for grammatical precision and academic tone.
Avoid redundancy (e.g., repetitive descriptions of results in the text and tables).

Commented [A24]: Clarify the study's novelty and significance in the global context.
Ensure the abstract concisely encapsulates the methodology and key findings.

Commented [A25]: Emphasize the global burden of antibiotic resistance and the relevance of the study in addressing this challenge.
Include a succinct comparison of similar ethnomedicinal studies.

mm. In addition, another study conducted by Putri *et al*. which tested the inhibition of rambutan leaf extract on *Propionibacterium acnes* bacteria that causes acne vulgaris reported an inhibition zone reaching 11 mm at a concentration of 20% (Febrina Karim *et al.,* 2023; Putri *et al.,* 2021). In addition to being antibacterial, rambutan leaf extract also shows protective ability against ultraviolet light exposure with an ultra-protection category (A. Lestari et al., 2023). Using a similar method, the diameter of the inhibition zone of *Staphylococcus aureus* also appeared positive at 7.96 mm at the smallest concentration of 3.125%. The antibacterial ability of rambutan leaf extract is suspected to come from the effects of secondary metabolites contained therein, namely flavonoids, tannins and saponins (Irmayanti *et al.,* 2022; W. D. Lestari *et al.,* 2023). From various reported studies, in general flavonoids can be anti-inflammatory caused by cell hyperglycemic environment and prevent the senescence process. Thus, flavonoids can function as anti-diabetic, anti-aging to antimicrobial. Saponins are known to have the potential to inhibit pathogenic activity from microbes by suppressing related signaling pathways. While tannins have been widely studied for their antioxidant effects, stopping bleeding, and the ability to precipitate proteins (Wang *et al.,* 2018; Zahra *et al.,* 2023; Zaynab *et al.,* 2021).

One of the components of the body's natural defense that inhabits human skin and mucosa is normal flora. In a balanced immune condition, these normal flora bacteria can provide protection rather than pathogenic effects and trigger infections. Some normal flora that often cause infections of the skin and mucosa include *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes* to *Cutibacterium acnes* (Byrd *et al.,* 2018; Flowers & Grice, 2020). Streptococcus pyogenes is a pathogenic Gram-positive coccus-shaped bacteria that looks like a long chain under a microscope and is a normal flora in the respiratory tract. When the host's defenses are disrupted, these bacteria can cause infections, especially in the esophagus and skin. Clinical manifestations from infection from these bacteria can range from pain when swallowing to reddish vesicles that easily break on the skin (Agustin *et al.,* 2019; Hasanah *et al.,* 2021). Methicillin-Resistant *Staphylococcus aureus* (MRSA) is a strain of *Staphylococcus aureus* that is resistant to isoxazolyl penicillin antibiotics such as methicillin, oxacillin and flucloxacillin which spreads through two main mechanisms, namely the spread of clones that exist between humans and animals, either from animals to humans or vice versa, and through the acquisition of Staphylococcal Cassette Chromosome mec (SCCmec) elements through horizontal gene transfer (Brown *et al*., 2005; Kemalaputri *et al.,* 2017; Lee *et al.,* 2018). Until now, antibiotics are still the main treatment for treating bacterial infections. However, the impact of extensive and irrational use increases the risk of resistance in the future (Hossain *et al.,* 2023). Therefore, a strategy is needed to strengthen the ability of antibiotic and recued the further potential risk of resistant, one of which is by exploring natural materials. Most natural materials contain secondary metabolites that are rich in antibacterial activity. Flavonoids can inhibit the growth activity of MRSA especially in ring A chemical structure 5,7-dihydroxylation and ring B chemical structure 4-hydroxylation while tannin and saponin are known to inhibit bacterial growth by damaging the integrity of bacterial cell membranes through the activity of chemical groups on their surfaces (Chigurupati *et al.,* 2019; Dong *et al.,* 2020; Shamsudin *et al.,* 2022). Among all parts of the rambutan plant, the leaves are known to have total phenolic content, total flavonoids and are expressed in gallic acid (19.6 ± 0.04 mg GAE/g) and rutin equivalents (16.7 ± 0.01 mg RUE/g) respectively (Aini *et al.,* 2023).

Due to the great benefits reported in previous studies related to rambutan leaves as an antibacterial agent, a study was conducted to experimentally examine the bactericidal ability of rambutan leaf extract in 96% ethanol on several normal flora bacteria such as MRSA (Methicillin-Resistant *Staphylococcus aureus*) and *Streptococcus pyogenes*. This study assessed the ability of rambutan leaf extract on antibacterial effects on gram-positive bacteria. Assessing the inhibitory ability of rambutan extract against *Streptococcus pyogenes* and Methicillin-Resistant *Staphylococcus aureus*

(MRSA) bacteria to determine the effective concentration in inhibiting these bacteria.

2. Materials and Methods

The study design was quasi experimental with post-test only analysis. It compared a few ethanolic extract of rambutan leaves (*Nephelium lappaceum l.)* concentrations to MRSA (Methicillin-Resistant *Staphylococcus aureus*) and *Streptococcus pyogenes'* growth inhibition through disk diffusion and turbidimetry method. The research was conducted at Laboratory of Organic Chemistry, Faculty of Pharmacy and Laboratory of Microbiology, Faculty of Medicine, Universitas Ahmad Dahlan. The main material in this study was rambutan leaves (*Naphelium lappceum l.),* which came from the Special Region of Yogyakarta, the leaves used were old leaves (Felicia *et al.,* 2017). The rambutan leaf determination process was carried out in the Biology Laboratory, Universitas Ahmad Dahlan. The bacterial samples used Methicillin-Resistant *Staphylococcus aureus* (MRSA) RES 22-2020 and *Streptococcus pyogenes* ATCC 19615. Other materials used included 96% ethanol, NaCl, distilled water, DMSO, flannel filter cloth, filter paper (Whatmann), aluminum foil, $FeCl_3$, microtube (Labselect), microtip (Biologix), sterile cotton swabs, H2SO4, BaCl2, Mueller Hinton Agar (MHA) media (Himedia), Mueller Hinton Broth (MBH) (Himedia), 70% alcohol, Streptomycin vial, Novobiocin antibiotic disc (Oxoid), and blank disc (Oxoid). The tools used in this study were: laboratory blender (Waring), a set of maceration extraction tools and rotary evaporator (Heidolph), water bath (Memmert), oven (Memmert), autoclave (GEA), jars, electric analytical balance (Ohaus), sieve/mesh, water bath, porcelain cup, flacon, ruler, 3 cc syringe, measuring cup, micropipette (Dlab scientific) (size 1000 µl, 200 µl and 100 µl), test tube, petri dish, inoculation loop, Bunsen burner, laminar air flow (Thermo Scientific), and incubator (Memmert).

To prepare the ethanolic extract of rambutan (*Naphelium lappceum l.)* leaves, the rambutan leaves are washed and oven-dried at 50° Celcius for 24 hours, then the leaf veins are separated (Yuvakkumar *et al.,* 2015). Rambutan simplisia is made into powder and filtered with a mesh size of 100. The ready powder is macerated by pouring 250 grams into a glass jar containing 1000 mL of 96% ethanol as a solvent for up to 24 hours (Tingting *et al.,* 2022). The macerate are filtered using filter cloth and filter paper. The filtered macerate is separated between the solvent and residue to obtain a liquid extract with a rotary evaporator at a temperature of 50° Celcius at rotation of 80 rpm. The thick extract is obtained by placing the liquid extract on a water bath at a temperature of 40° Celcius for 24 hours. After the thick extract is ready, phytochemical screening is necessary to evaluate any secondary metabolites of the leaves. Testing for the presence of flavonoids is done by dissolving the extract with ethanol. The solution then is added with 0,1 grams magnesium (Mg) and homogenized then add a few drops of HCl through the tube wall (Setyawaty *et al.,* 2020). Red color change in the extract layer indicates flavonoid compound inside the extract. The presence of tannins is done by dissolving the extract with ethanol. The solution is then added a few drops of 1% $FeCl_3$ through the wall (Retno Priamsari *et al.,* 2023). A change in the color of the extract to blackish blue indicates a positive result for the presence of tannins.

According to Irmayanti *et al.*, using the same method to evaluate *Staphylococcus aureus* dan *Escherichia coli*'s growth inhibition by ethanolic extract of rambutan (*Naphelium lappceum l.)* leaves, at least it requires three replications to strengthen the result validation (Irmayanti *et al.,* 2022). Prior to antibacterial activity test, confirmation test of bacteria sample used in this research was done by applying Gram staining. The sample then continued to have antibacterial test by disk diffusion method. The first step is to prepare the series of the thick extract concentrations by diluted them with 5% DMSO. The concentrations used were 100%, 50%, 25%, 10%, and 5%, each concentration was dropped on a sterile empty disk as much as 20 µL. Both bacteria were made into a suspension with NaCl solution in a sterile tube and then homogenized. The resulting suspension was then compared for its hardness using Mc.Farland solution.

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The bacterial suspension that had been inoculated in accordance with the standard was dipped using a inoculation loop and then spread on the surface of the agar media. The media that had been spread with bacteria was then planted with a disk containing a concentration of rambutan leaf extract and Novobiocin used as an antibiotic standard. Observations were made after the media was incubated in an incubator at a temperature of 27° Celcius for 18-24 hours (Retno Priamsari *et al.,* 2023).

Turbidimetric test is used to observe the MIC (minimum inhibitory concentration) value of extract on bacterial growth. The principle of turbidimetric test is visual observation of turbidity. The concentrations of rambutan leaf extract used are 100%, 50%, 25%, 12.5%, 6.25%, and 3.125%. The positive control (K+) is a bacterial suspension equivalent to the McFarland 0.5 standard while the negative control (K-) contains the antibiotic Streptomycin. Each tube is labeled according to the concentration series and positive/negative controls needed. Tube 1 is filled with 2 ml of 100% concentration of rambutan leaf extract, tubes 2-6 are filled with 1 ml of Mueller Hinton Broth (MHB) media. 1 ml of solution is taken from tube 1 using a micropipette, inserted into tube 2, mixed until homogeneous to obtain a concentration of 50%. The same thing is done up to tube 6 and all extract concentrations are obtained with a ratio of 1:2. At the end, each tube is given 0.5 ml of bacterial suspension equivalent to McFarland 0.5. Then each tube was incubated at 37°C for 1x24 hours. After 24 hours, the MIC was determined by visual observation of turbidity (Sekeon *et al.,* 2018; Warokka *et al.,* 2016). The absorbance value of each tube was then measured using a UV-vis Spectrophotometer by comparing before and after 1x24-hour incubation at a wavelength of 625. If the final absorbance value (after incubation) of each tube is higher than the initial absorbance value (before incubation), then bacterial growth is considered to continue. However, if the final absorbance value does not change or the absorbance value is lower than before incubation, then bacterial growth stops or is inhibited (Warokka *et al.,* 2016).

To obtain the concentration with the best inhibitory activity, statistical analysis will be carried out with SPSS 29.0. The variables in this study are numeric interval data with more than one group test so that if the data is normally distributed, the test used is One way-ANOVA, while if the data is not normally distributed, the test used is the non-parametric Kruskall-Wallis test. The results of the antibacterial clear zone measurements were then presented using the GraphPad Prism 9 program. The official number of ethical approval of this study was 012402029 and was approved by Universitas Ahmad Dahlan's Research Ethic Committee.

3. Results

To obtain a thick extract from rambutan leaves, several extraction methods can be applied. In a study conducted by Tambunan *et al.* (2024), the maceration method was the best method of choice for making thick extract of rambutan leaves (Mistryanto Tambunan *et al.,* 2024). From 1.5 liters of a mixture of simplicia powder that had been obtained that was soaked with 96% ethanol, approximately 400 ml of liquid extract was produced. Then continued by soaking the liquid extract in a water bath at a temperature of 45° Celcius for 1x24 hours until at the end of the extraction process using the maceration method, 10 grams of thick extract was produced (Figure 1).

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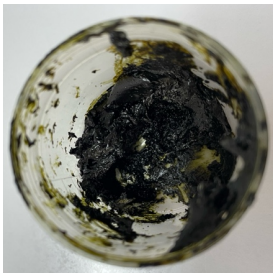

Figure 1. Results of thick extract of rambutan leaves using the maceration method

The organoleptic results of the extract produced a thick consistency. Blackish brown in color with a distinctive matcha odor is the characteristic of the final thick extract. Phytochemical screening of rambutan leaf extract showed positive results for flavonoids with a sign of a red color change in the extract after Mg and HCl drops. In addition, there was a blue-black color change after $FeCl_3$ drops which indicated the presence of tannin in the extract.

In bacterial identification, Gram staining is considered as the standard and easily conducted. The first Gram staining step is to fix the bacteria on the object glass. After that, Gentian violet immersion was carried out for 1 minute, Lugol for 1 minute, alcohol drops, and safranin immersion for 30-45 seconds. The staining results were read using a light microscope with a magnification of 100 x with immersion oil given to the preparation. In the identification of bacteria, it was obtained in accordance with the theory, namely Gram (+) and coccus and colonies that could grow on Nutrient Agar media for 24 hours at a temperature of 37° Celcius (Figure 2) (Tripathi & Sapra, 2023).

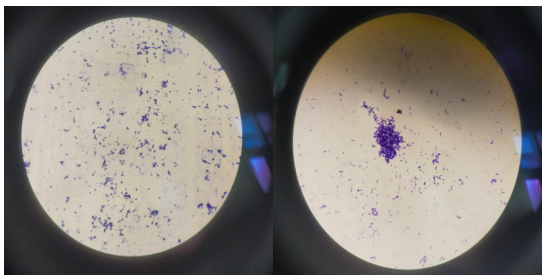

Figure 2. The appearances of colonies and Gram Staining of MRSA RES 22-2020 (left) and *Streptococcus pyogenes* (right) bacteria performed

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The results of the antibacterial test of rambutan leaves against MRSA showed good activity visually (Figure 3). The results of each bacterial inhibition zone are shown in Table 1. A concentration of 5% rambutan leaf extract did not produce a clear zone after 3 repetitions of the test. The best concentration results were shown at a concentration of 100% with an average inhibition zone of 14.6 mm. There was an increase in antibacterial activity at a concentration of 25% when compared to a concentration of 50%. The results of the One Way ANOVA quantitative analysis showed that there was a significant value in the standard (novobiocin) for all concentrations, with a p value <0.0001. The 5% concentration had no inhibition zone at all so that there was a significant value for the 100%, 50%, and 25% concentrations. The 100% concentration was significant when compared to the 10% concentration (p value 0.0402) and

5% (p value 0.0004).

Table 1. Results of the Inhibition Zone Test on Methicillin Resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pyogenes* Bacteria

Concentration (%)	MRSA				*Streptococcus pyogenes*			
	I (mm)	II (mm)	III (mm)	Average (mm)	I (mm)	II (mm)	III (mm)	Average (mm)
Standard	45	45	40	43,3	35	36	35	35,3
100%	15	10	19	14,7	23	23	26	24
50%	14	11	14	13	22	22	23	22,33
25%	14	10	14	11,3	23	20	20	21
10%	3	8	9	6,7	20	17	18	18,3
5%	0	0	0	0	17	14	10	13,7

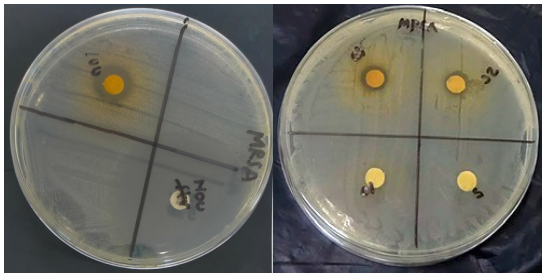

Figure 3. Clear zone of MRSA bacteria on petri dish after incubation for 1x24 hours.

The results of the antibacterial test of rambutan leaves against *Streptococcus pyogenes* are also shown in Table 1. From the observation results, the inhibition activity is evenly distributed visually. The concentration of 100% has the largest zone with an average inhibition zone of 24 mm (Figure 4). The results of the One Way ANOVA quantitative analysis show that the standard has a significant value with all extract concentrations. The concentration of 100% has a significant value with a concentration of 10% (p 0.0485) and a concentration of 5% (p 0.0005). A significant difference is also shown in the comparison of the concentration of 50% with 5% (p 0.0035) and the concentration of 25% with 5% (p value 0.0091). Thus, it can be concluded that increasing the concentration has a significant ability to inhibit bacterial growth. The average diameter of the inhibition zone formed by the ethanol extract of rambutan leaves against MRSA bacteria and *Streptococcus pyogenes* bacteria will increase longitudinally as the concentration rise (Diagram 1). The diameter of the inhibition zone indicates the presence of bacterial growth inhibition activity originating from compounds contained in the extract.

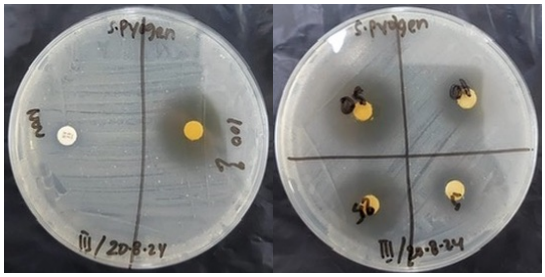

Figure 4. Clear zone of *Streptococcus pyogenes* bacteria on petri dish after incubation for 1x24 hours.

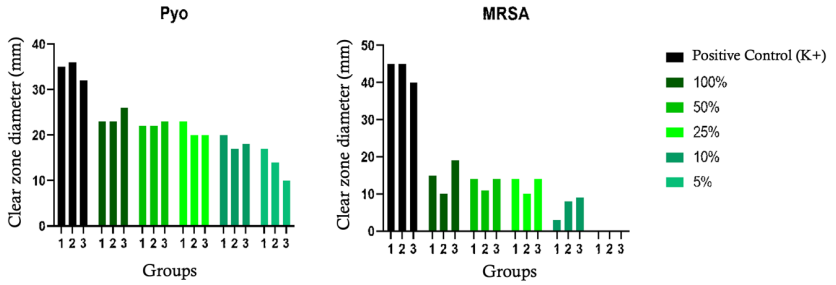

Diagram 1. Results of inhibition of the growth of MRSA bacteria (right) and *Streptococcus pyogenes* (left) using the disk diffusion method.

To support the results obtained from the disk diffusion method, the assessment of the MIC (minimum inhibitory concentration value) of the ethanol extract of rambutan leaves was carried out using the turbidimetry method and confirmation using UV-vis Spectrophotometer. The results obtained showed that visually in the third tube which was a concentration of 25%, both for MRSA and *Streptococcus pyogenes* bacteria, still showed a clear appearance after being incubated at 37° Celcius for 1x24 hours (Figure 5).

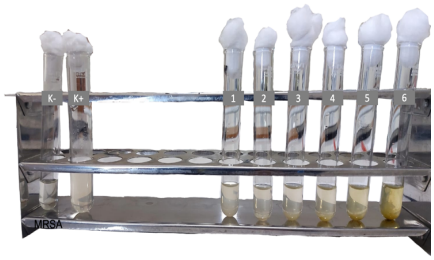

Figure 5. Results of the MIC test using the turbidimetric method of MRSA bacteria (top) and Streptococcus pyogenes (bottom). K(-) is a negative control, K(+) is a positive control, respectively from 1-6 contains extract concentrations of 100%, 50%, 25%, 12.5%, 6.25%, and 3.125%

According to the observation, the clarity of the tubes became increasingly apparent starting from the 3rd tube (25% concentration) to 100% concentration. In contrast, the tubes began to become cloudy in the 4th tube (12.5% concentration) although they were faintly visible in both MRSA and *Streptococcus pyogenes* bacteria up to the 6th tube (3.125% concentration). The turbidity that appeared indicated an increase in the density level of the suspension originating from the bacteria. The bacterial inhibition power was assessed based on the clarity of the suspension in the tube compared to the positive control containing McFarland 0.5. Turbidimetric observations were carried out using three replications followed by using a UV-vis Spectrophotometer with a wavelength of 625. The results of the MIC of MRSA and *Streptococcus pyogenes* bacteria are shown in tables 2 and 3. From the results of absorbance observations using a UV-vis Spectrophotometer, it is known that the decrease in absorbance is only seen at concentrations of 100% and 50% in MRSA bacteria with a respectively decrease of 0.224 and 0.049. However, in *Streptococcus pyogenes* bacteria, it only shows a decrease in absorbance at a concentration of 100%. Concentrations of 50% -100% show clarity because the molecules of the material or compound read by the device are only slightly absorbed at the specified wavelength. The increase in absorbance begins to be seen at concentrations of 12.5 to 3.125% in MRSA bacteria, while in *Streptococcus pyogenes* bacteria, the increase in absorbance begins to be seen from concentrations of 25% to 3.125%.

Table 2. Results of the MIC value using the turbidimetric method of ethanol extract of rambutan leaves (*Nephelium lappaceum l.)* on the growth of MRSA and *Streptococcus pyogenes* bacteria in three replications.

Tube(s)	Concentration of ethanolic extract of rambutan (*Nephelium lappaceum L.*) leaves	MRSA			*Streptococcus pyogenes*		
		I	II	III	I	II	III
1	100%	-	-	-	-	-	-
2	50%	-	-	-	-	-	-
3	25%	-	-	-	-	-	-
4	12,5%	+	-	+	+	-	+
5	6,25%	+	+	+	+	+	+
6	3,125%	+	+	+	+	+	+
K+	*McFarland* 0,5	+	+	+	+	+	+
K-	Antibiotik Streptomycin	-	-	-	-	-	-

Description: The (+) sign indicates the presence of bacterial growth, which is shown by the presence of turbidity, while the (-) sign indicates the presence of inhibited bacterial growth, which is shown by the clarity.

Table 3. Absorbance results of the UV-vis Spectrophotometer of ethanol extract of rambutan leaves (*Nephelium*

lappaceum l.) on the growth of MRSA and *Streptococcus pyogenes* bacteria.

Concentration of ethanolic extract of rambutan (*Nephelium lappaceum L.*) leaves	MRSA			*Streptococcus pyogenes*		
	Pre-incubation	Post-incubation	Interpretation	Pre-incubation	Post-incubation	Interpretation
100%	0,633	0,409	Decrease	0,644	0,523	Decrease
50%	0,354	0,305	Decrease	0,362	0,584	Increase
25%	0,202	0,428	Increase	0,216	0,169	Increase
12,5%	0,123	0,568	Increase	0,141	0,416	Increase
6,25%	0,081	1,18	Increase	0,078	1,024	Increase
3,125%	0,072	1,23	Increase	0,07	0,947	Increase
K(+)	0,03	0,45	Increase	0,03	0,45	Increase
K(-)	0,104	0,085	Decrease	0,104	0,085	Decrease

Description: "Increase" indicates an increase in bacterial growth as indicated by an increase in absorbance after incubation, while "Decrease" indicates that bacterial growth is inhibited or decreases after incubation.

4. Disscussion

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The bacteria used in this study were MRSA (Methicillin Resistant *Staphylococcus aureus*) and *Streptococcus pyogenes*. MRSA is a type of *Staphylococcus aureus* bacteria that experiences resistance mainly due to the expression of penicillin-binding protein (PBP2a) which tends to reduce the sensitivity of beta lactam antibiotics to the bacterial receptors to carry out the bactericidal process. This bacteria is often found on human mucous membranes and is the most common cause of skin infections, especially if there is a wound (Kemalaputri *et al.,* 2017). Like MRSA, Streptococcus pyogenes is a bacteria that is largely responsible for skin and respiratory infections, one of which is impetigo to pneumonia. Both bacteria have similarities such as possess thicker cell walls as well as the peptidoglycan layer. In addition, their proteins, teichoic acids and lipoteichoic acids are distinctively prominent compared to Gram-negative bacteria (Fischetti, 2017). From the Gram examination, a purplish image will appear due to the binding ability of crystal violet as a primary dye from one of the Gram reagents to bacterial peptidoglycan. Thus, both bacteria are included in Gram-positive (Tripathi & Sapra, 2023). From the tests conducted, it was proven that MRSA and *Streptococcus pyogenes* bacteria showed a bright purple color, thus both bacteria were Gram-positive bacteria.

Phytochemical screening of ethanolic extract of rambutan leaves was carried out using two different methods to assess each secondary metabolite. From the results of the experiment, it was concluded that ethanolic extract of rambutan leaves contains flavonoids and tannins. Flavonoid testing was carried out by observing the reactions between the extract with Mg followed by dripping HCl. Because Mg and HCl are metals by nature, they can reduce the benzopyrone core in flavonoids, thus the red flavylium salt is formed (A. Lestari *et al.,* 2023). The blackish blue color that appears after dropping $FeCl_3$ indicates the formation of a complex compound between Fe metal and tannin. The coordination covalent bond between metal ions or atoms with non-metal atoms causes the formation of this complex compound. $FeCl_3$ reagent is usually used to identify phenolic compounds, including tannins, when this solution is added. It is estimated that this solution will react with one of the hydroxyl groups in the tannin compound. Because tannins are phenolic compounds that tend to dissolve in water and polar solvents, the addition of $FeCl_3$ is a way to find out if they contain phenol groups; the blackish blue color after adding $FeCl_3$ indicates the presence of phenol groups (Febrina Karim *et al.,* 2023; Rumaolat & Husada, 2020). This is in accordance with the results obtained in this study. Both compounds have activity as natural antibacterial found in plants. Flavonoids are important components and are most often found in the phenolic compound class (Panche *et al.,* 2016). The structure of flavonoids shows an important

role as antibacterial that help damage the integrity of bacterial membranes (Shamsudin *et al.,* 2022). The antibacterial activity of flavonoids is suspected of ability to disrupt cell membrane permeability and adhesion to biofilm formation which ultimately triggers bacterial death (Xie *et al.,* 2014). In addition, flavonoids also inhibit bacterial DNA gyrase enzymes. The DNA gyrase enzyme functions to maintain the protein synthesis process in the formation of prokaryotic cell membranes such as bacteria. Compared to Gram-negative bacteria, flavonoids have faster activity in Gram-positive bacteria. This may be due to differences in cell envelope structure and higher quinone composition. The types of quinones most commonly found in Gram-positive are menaquinone and ubiquinone. Quinones are known to play a role in the cellular respiration process of Gram-positive bacteria (Yan *et al.,* 2024). Other secondary metabolite, tannins, also have antibacterial activity by disrupting the formation of peptidoglycan and bacterial cellular membranes, chelating iron, inhibiting antibiotic efflux pumps, and disrupting bacterial fatty acid synthesis (Villanueva *et al.,* 2023).

The results of this study indicate that both MRSA and *Streptococcus pyogenes* bacteria showed significant inhibition after administration of ethanolic extract of rambutan (*Nephelium lappaecum l.)* leaves. The antibacterial ability of a certain chemical component has been determined based on the classification made by Davis and Stout (1971) according of clear zone diameter. The diameter of the clear zone <5 mm is classified as weak, 5-10 mm is classified as moderate, 10-20 mm is classified as strong, and >20 mm is classified as very strong (G.S. *et al.,* 2021). This study showed the antibacterial effect of ethanolic extract of rambutan (*Nephelium lappaecum l.)* leaves at a concentration of 100% given to MRSA bacteria and *Streptococcus pyogenes* bacteria is included in the strong category with an average inhibition zone diameter in the range of 10-20 mm with the highest mean obtained from *Streptococcus pyogenes* group (24 mm). The inhibitory ability of rambutan (*Nephelium lappaecum l.)* leaves extract in ethanol was also shown in a study conducted by Irmayanti *et al.* (2022) on *Staphylococcus aureus* bacteria. The widest inhibition zone was shown when the extract concentration was 100%, which was 12.9 mm, and the smallest was at a concentration of 3.125%, which was 7.96 mm (Irmayanti *et al.,* 2022). Using the same bacterial strain, in a study conducted by Rumaolat (2020), methanol extract of rambutan leaves provided an inhibition zone of up to 2 times than Irmayanti *et al.* study where the obtained clear zone at concentration of 75% was 26 mm (Rumaolat & Husada, 2020). Another study reported by Ratna et al. (2018) showed that at a concentration of 10% ethanol extract of rambutan leaves, the average inhibition zone was 19.64 mm (Ratna *et al.,* 2018). Purba *et al.* (2023) also reported from their study that rambutan leaves extract has the widest mean of inhibition diameter of *Streptococcus mutans'* growth at concentration 100% which is 17,40±0,10 mm (Purba *et al.,* 2023).

Based on observations on both bacteria using the disk diffusion method, it was seen that the ethanol extract of rambutan leaves had better effectiveness on *Streptococcus pyogenes* bacteria when compared to MRSA. At a concentration of 5%, rambutan leaves extract did not have any effect on bacterial growth in a petri dish, in contrast to *Streptococcus pyogenes* bacteria which had a strong inhibition zone. Several things can cause an inhibition zone not to form from specific extract at a concentration of 5%, including weak diffusion power of the extract, inappropriate use of solvents, and low concentrations of active ingredients, causing the extract to be unable to effectively inhibit bacterial growth (Rumaolat & Husada, 2020). There have been no studies that specifically compare the effectiveness of ethanolic extract of rambutan leaves on MRSA and *Streptococcus pyogenes* bacteria. However, the opposite result was shown in a study that tested the skin of rambutan fruit against MRSA and *Streptococcus pyogenes* bacteria. In their study testing the effect of rambutan fruit skin extract on MRSA, Rostinawati *et al.* (2018) reported that the inhibition zone at 100% extract concentration reached 23.4 mm, while a study conducted by Sekar *et al.* (2014) showed that the inhibition zone of red and yellow rambutan skin extract at the highest concentration could only inhibit 10 mm of *Streptococcus pyogenes* bacterial growth (Rostinawati *et al.,* 2018; Sekar *et al.,* 2014).

The main principle of the turbidimetry method is to test the concentration of the solution in the tube from various

concentrations of the test material. The higher the turbidity that appears visually, the bacterial activity also increases, thus the tube clarity becomes a standard in assessing the inhibition of the test material against bacterial growth. However, the turbidity that arises has a dual interpretation, namely the number of bacteria that have died cannot be known so that both living and dead bacteria will both give a cloudy appearance. Although visual observation is considered sufficient to obtain the MIC value, the researcher's eye ability also has limitations. It will lead to the variation in observation results that can appear because the brownish color between one tube and another looks the same (Warokka *et al.,* 2016). The results shown in this study on both MRSA and *Streptococcus pyogenes* bacteria showed the best clarity in tubes with concentrations of 100%, 50%, and 25%. However, in one study conducted by Mukaromah *et al.,* (2023) reported that extracts of rambutan's peel and seed have MIC 15,625 mg/mL in inhibiting the growth of MDR (Multi Drug Resistant)-*Klebsiella pneumoniae* bacteria through microdilution test. To confirm the bacterial MIC, post-incubation absorbance calculations can be carried out, one of which is by utilizing a UV-vis Spectrophotometer. In this study, it was found that the significant decrease of absorbance for both bacteria were concentration 100%. It is also relevant to other study reported by Salsabila *et al.* (2019) in one experiment to evaluate the best concentration for *Aggregatibacter actinomycetemcomitans* and *Treponema denticola* biofilm inhibition after given the rambutan (*Nephelium lappaecum l.)* leaves extract. According to the findings, rambutan leaves extract was most successful in preventing *Aggregatibacter actinomycetemcomitans* biofilm adherence at 100% concentration, with an average optical density of 0.028, and Treponema denticola at 50% concentration, with an average optical density of 0.120 (Salsabila *et al.,* 2022). In general, the working principle of a Spectrophotometer is to assess the absorption of light on the molecules of a particular material. However, Spectrophotometers also have several weaknesses that are often related to the operation of the tool or limitations in distinguishing between living and dead bacteria. Spectrophotometers can still read contaminants or other particles that are absorbed by the same wavelength so that to determine specifically whether bacterial death occurs or not, it can be continued by growing bacteria on Plate Count Agar. In addition, it is recommended to use a high-performance liquid chromatography (HPLC) tool to analyze the amount per compound more accurately (Gloria Rambet *et al*., 2017; J Soelama *et al*., 2015).

5. Conclusions

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Suggest directions for future research explicitly.

Ethanolic extract of rambutan (*Nephelium lappaceum l.)* leaves contains secondary metabolites of flavonoids and tannins which are shown from the color change after being dripped with the appropriate reagent. Ethanol extract of rambutan leaves also has the activity of inhibiting the growth of Methicillin-Resistant *Staphylococcus aureus* (MRSA) bacteria with a strong category inhibition ability (14.9 mm) at a concentration of 100% while the concentration with the highest inhibition power in *Streptococcus pyogenes* bacteria is 100% (24 mm). There was a significant value for the 100%, 50%, and 25% concentrations in inhibiting the MRSA growth. Meanwhile, all concentrations of ethanolic extract of rambutan (*Nephelium lappaceum l.)* leaves have significant ability in decreasing the *Streptococcus pyogenes* growth. Based on the visual turbidimetry method, the minimum inhibition ability of both bacteria began to appear at a concentration of 25%. Confirmation using a UV-vis Spectrophotometer showed a decrease in absorbance in MRSA bacteria seen at a concentration of 100% -50% while in *Streptococcus pyogenes* bacteria only at a concentration of 100%.

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Conflict of interest

All authors have no conflict of interest in this article.

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