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RESEARCH ARTICLE

Inhibitory effects of rambutan (*Nephelium lappaceum* l.) leaves ethanolic extract on Methicillin-Resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pyogenes*

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ABSTRACT

Antimicrobial resistance poses a global health threat, with Methicillin-Resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pyogenes* contributing to the highest mortality rates among Gram-positive pathogens over the last three decades. The search for alternative antimicrobial agents has increasingly concentrated on natural products. Rambutan (*Nephelium lappaceum* L.), a widely used ethnomedicinal plant in Indonesia, is recognized for its numerous health benefits, including its antimicrobial properties. Despite extensive phytochemical studies on various parts of rambutan, the specific inhibitory effects of its leaf extract against MRSA and *S. pyogenes* have yet to be explored. This study aimed to evaluate the antimicrobial activity of ethanolic rambutan leaf extract against MRSA and *S. pyogenes*. The extract was prepared using maceration with 96% ethanol, followed by qualitative phytochemical screening, which confirmed the presence of flavonoids (blackish-blue reaction with $FeCl_3$) and tannins (reddish hue with HCl). Antibacterial activity was assessed by measuring inhibition zones on agar plates and determining the minimum inhibitory concentration (MIC) through UV-vis spectrophotometry. The 100% ethanolic extract demonstrated significant inhibitory effects, with a mean inhibition zone of 14.6 mm against MRSA, outperforming the 5% ($p = 0.0004$) and 10% ($p = 0.0402$) concentrations. For *S. pyogenes*, the 100% extract produced the largest inhibition zone (24 mm), showing superior activity compared to the 5% ($p = 0.0005$) and 10% ($p = 0.0485$) concentrations. MIC analysis indicated optimal antimicrobial activity at both 50% and 100% concentrations for the two pathogens. These findings underscore the potential of rambutan leaf extract as a natural antimicrobial agent against resistant Gram-positive bacteria, warranting further investigation into its bioactive compounds and mechanisms of action.

1. Introduction

The global rise of antimicrobial resistance (AMR) is primarily driven by the irrational use of antibiotics and the remarkable adaptability of bacteria. While AMR is a universal challenge, Southeast Asia, sub-Saharan Africa, and tropical Latin America bear a disproportionately high burden. By 2050, AMR will cause approximately 8.22 million deaths annually (Naghavi *et al.*, 2024). Indonesia, a member of the Global Antimicrobial Resistance and Use Surveillance System (GLASS) since 2019, reports the highest prevalence of AMR among Southeast Asian nations. Data from 20 sentinel hospitals attribute

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over-the-counter antibiotic purchases as a key factor in AMR proliferation. Priority pathogens identified by the World Health Organization (WHO)—including Klebsiella pneumoniae, Escherichia coli, Acinetobacter baumannii, Pseudomonas aeruginosa, and Methicillin-Resistant *Staphylococcus aureus* (MRSA)—underscore the urgency of developing effective antimicrobial strategies (Siahaan *et al.*, 2022).

The 2023 Surveillance of Indonesia Network on Antimicrobial Resistance (SINAR) study analyzed 54,688 clinical samples from 70 hospitals across 15 provinces, revealing resistance to third-generation cephalosporins in 34% of MRSA cases and 25% of Pseudomonas aeruginosa cases (Anggraini *et al.*, 2024). These findings highlight the critical need for innovative solutions to combat AMR. Natural products, mainly from biodiverse regions like Indonesia, offer promising prospects for drug development (Putra *et al.*, 2023). Historically, 80% of the global population has relied on ethnomedicine to treat various conditions, with plants as a significant resource (Bandibas & Roxas, 2017).

One notable example is rambutan (*Nephelium lappaceum* L.), a tropical fruit widely cultivated in Indonesia. In 2023, Indonesia produced approximately 845,107 tons of rambutan, with Java Island contributing over half of the national yield (BPS, 2023; Rozana & Sunardi, 2021). Rambutan has long been utilized in traditional medicine, with its various parts—such as fruit peels, leaves, seeds, and roots—demonstrating therapeutic properties. Rambutan leaves, in particular, are known for their antibacterial activity due to their rich content of tannins, flavonoids, and saponins (Anggresani *et al.*, 2019; Indrayati & Sugiarto, 2020).

Previous studies have demonstrated the antibacterial efficacy of rambutan leaf extracts against pathogens like Streptococcus mutans, Propionibacterium acnes, and *Staphylococcus aureus*. Flavonoids in rambutan leaves exhibit anti-inflammatory, antimicrobial, and antioxidant properties, while tannins contribute to bacterial membrane disruption and protein precipitation (Mistriyani *et al.*, 2018; Wang *et al.*, 2018; Zahra *et al.*, 2023). Additionally, ethanolic extracts of rambutan peels have shown activity against multidrug-resistant bacteria such as ampicillin-resistant Escherichia coli and Salmonella typhi (Joy *et al.*, 2022).

Normal flora bacteria, including *Streptococcus pyogenes* and MRSA, can become opportunistic pathogens under compromised immune conditions, causing infections ranging from skin lesions to systemic illness (Byrd *et al.*, 2018; Flowers & Grice, 2020). MRSA, a notorious multidrug-resistant strain, spreads via horizontal gene transfer and clonal dissemination, posing significant challenges to antibiotic therapy (Kemalaputri *et al.*, 2017; Lee *et al.*, 2018).

Given the promising antibacterial properties of rambutan leaves, this study aims to evaluate the inhibitory potential of 96% ethanolic rambutan leaf extract against Gram-positive bacteria, specifically *Streptococcus pyogenes* and MRSA. The findings will contribute to developing alternative antimicrobial agents to address the global AMR crisis.

2. Materials and Methods

2.1 Study Design

This study employed a quasi-experimental design with post-test-only analysis. The growth inhibition effects of various concentrations of ethanolic extract of rambutan leaves (*Nephelium lappaceum* L.) on Methicillin-Resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pyogenes* were assessed using the disk diffusion and turbidimetry methods. The research was conducted at the Laboratory of Organic Chemistry, Faculty of Pharmacy, and the Laboratory of Microbiology, Faculty of Medicine, Universitas Ahmad Dahlan. Ethical approval for this research was granted by Universitas Ahmad Dahlan's Research Ethics Committee (Approval No. 012402029, issued on February 27, 2024).

2.2. Materials

The primary material in this study was old rambutan leaves (*Nephelium lappaceum* L.) collected from the Special Region of Yogyakarta (Felicia *et al.*, 2017). The botanical determination was performed in the Biology Laboratory, Universitas Ahmad Dahlan. The bacterial strains used were MRSA RES 22-2020 and *Streptococcus pyogenes* ATCC 19615. Other materials include: (1) Solvents and chemicals: 96% pure ethanol, NaCl, distilled water, DMSO, $FeCl_3$, H_2SO_2, $BaCl_2$, (2) Media and reagents: Mueller Hinton Agar (MHA), Mueller Hinton Broth (MHB) (Himedia), Novobiocin antibiotic disc (Oxoid), blank discs (Oxoid), and (3) Equipment: Laboratory blender (Waring), maceration apparatus, rotary evaporator (Heidolph), water bath (Memmert), oven (Memmert), autoclave (GEA), UV-vis spectrophotometer (Genesys 150, ThermoFisher Scientific), micropipettes (Dlab Scientific), laminar airflow (Thermo Scientific), incubator (Memmert), and various laboratory glassware and consumables.

2.3. Preparation of Rambutan Leaf Extract

The rambutan leaves were washed and oven-dried at 50°C for 24 hours. Leaf veins were removed, dried leaves were ground into a fine powder using a laboratory blender, then sieved through a 100-mesh sieve (Yuvakkumar *et al.*, 2015). 200 g of the powdered leaves was macerated in 1000 mL of 96% ethanol for 24 h. The mixture was filtered using flannel cloth and Whatman filter paper, and the solvent was separated

from the residue using a rotary evaporator at 50°C and 80 rpm. The liquid extract was concentrated further in a water bath at 40°C to produce a thick extract.

2.4. Phytochemical Screening

Phytochemical screening was performed to identify secondary metabolites, including flavonoids and tannins.

1. Flavonoid detection: The extract was dissolved in ethanol, mixed with 0.1 g magnesium, and a few drops of HCl were added. A red color change indicated the presence of flavonoids (Setyawaty *et al.*, 2020).
2. Tannin detection: The extract was dissolved in ethanol, and a few drops of 1% $FeCl_3$ were added. A blackish-blue color change confirmed the presence of tannins (Priamsari *et al.*, 2023).

2.5. Bacterial Identification with Gram Staining

Gram staining was performed as a standard method for bacterial identification. Smears were fixed on glass slides and stained sequentially with gentian violet, Lugol solution, alcohol, and safranin. The results were observed under a light microscope at 100x magnification with immersion oil.

2.6. Disk Diffusion Test

The disk diffusion test, following the method described by Irmayanti *et al.* (2022), measured the antibacterial activity of the ethanolic extract of rambutan leaves. The extract was diluted with 5% DMSO to prepare concentrations of 100%, 50%, 25%, 10%, and 5%. Each concentration was applied to sterile blank disks (20 µL per disk). Bacterial suspension of MRSA and *Streptococcus pyogenes* were prepared in NaCl solution, adjusted to the 0.5 McFarland standard, and evenly spread on MHA plates. Disks impregnated with the extract and a Novobiocin antibiotic disk (positive control) were placed on the inoculated plates. The plates were incubated at 27°C for 18–24 hours. The diameter of the inhibition zones was measured to evaluate antibacterial activity (Indrayati & Sugiarto, 2020; Rumaolat & Husada, 2020).

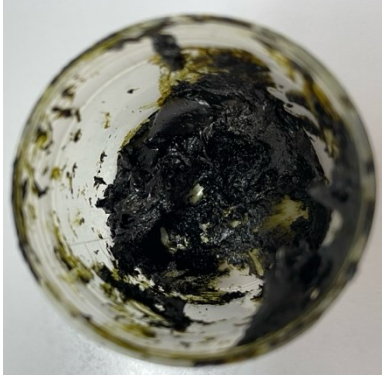

Figure 1. Thick extract of rambutan leaves prepared via the maceration method

2.7. Turbidimetry Test

The turbidimetry test determined the rambutan leaf extract's minimum inhibitory concentration (MIC) (Warokka *et al.*, 2016). A series of extract concentrations (100%, 50%, 25%, 12.5%, 6.25%, and 3.125%) were prepared in MHB using a two-fold dilution method. A positive control (bacterial suspension only) and a negative control (Streptomycin antibiotic) were included. Each tube was inoculated with 0.5 mL of bacterial suspension equivalent to 0.5 McFarland standard. Tubes were incubated at 37°C for 24 h. Absorbance values before and after incubation were measured at 625 nm using a UV-vis spectrophotometer. MIC was defined as the lowest concentration with no turbidity (absorbance) increase. "Increase" signifies an observed rise in bacterial growth, as evidenced by a higher absorbance value after incubation. Conversely, "Decrease" indicates inhibition or reduction of bacterial growth, demonstrated by a lower absorbance value following incubation.

2.8. Statistical Analysis

Data were analyzed using SPSS 29.0. A one-way ANOVA test was performed for normally distributed data, while the Kruskal-Wallis test was used for non-normally distributed data. Results were presented as mean values with standard deviations, and inhibition zone measurements were visualized using GraphPad Prism 9.

3. Results

3.1. Extraction of Rambutan Leaf Ethanolic Extract

The maceration method was employed to extract rambutan leaves (*Nephelium lappaceum* L.), consistent with findings by Tambunan *et al.* (2024), who identified this method as optimal for producing thick extracts from rambutan leaves. A yield of 10 grams of thick extract was obtained from 200 grams of dried simplicia, resulting in a rendement of 5% (Figure 1). The resulting extract exhibited a thick consistency, a characteristic blackish-brown color, and a distinctive matcha-like odor. Phytochemical screening confirmed the presence of flavonoids and tannins, indicated by red color development upon adding Mg and HCl and blue-black color change upon $FeCl_3$ addition, respectively.

3.2. Bacterial Identification

The bacterial isolates Methicillin-Resistant *Staphylococcus aureus* (MRSA) RES 22-2020 and *Streptococcus pyogenes* ATCC 19615 were confirmed as

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Gram-positive cocci through Gram staining, which revealed bright purple staining (Figure 2). Colonies were successfully cultivated on Nutrient Agar medium at 37°C for 24 hours, consistent with previous findings (Tripathi & Sapra, 2023).

3.3. Antibacterial Activity via Disk Diffusion Method

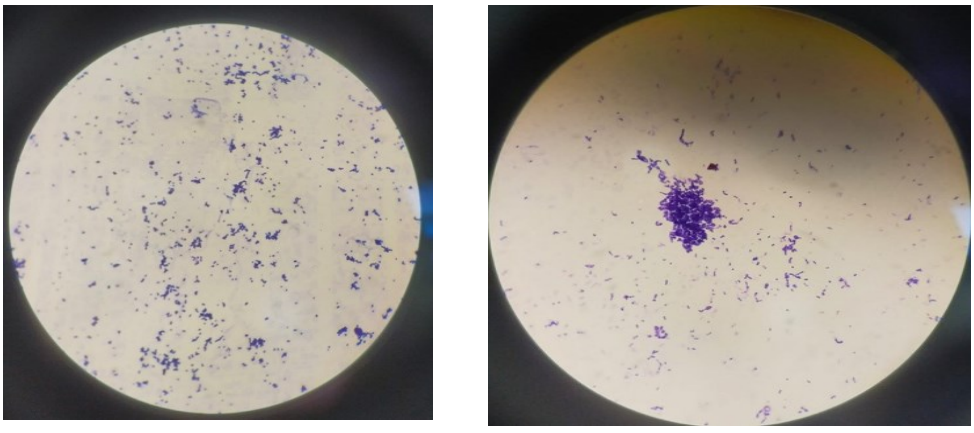
The ethanolic extract of rambutan leaves showed concentration-dependent antibacterial activity against MRSA and *Streptococcus pyogenes*. The inhibition zones produced by different extract concentrations are summarized in Figure 3 and Figure 4. No inhibition zone was observed at a 5% concentration after three trials. The 100% concentration demonstrated the highest activity, with an average inhibition zone of 14.7 mm for MRSA and 24 mm for *Streptococcus pyogenes*. Statistical analysis using one-way ANOVA revealed significant differences ($p < 0.0001$) between the standard antibiotic (novobiocin) and all extract concentrations. The 100% extract concentration showed significant differences compared to 10% ($p = 0.0402$) and 5% ($p = 0.0004$) concentrations.

3.4. Minimum Inhibitory Concentration (MIC) Assessment

The MIC of the rambutan leaf extract was determined using turbidimetry and UV-vis spectrophotometry. The third tube (25% concentration) was clear (Figure 5), indicating bacterial growth inhibition, while turbidity increased in tubes with lower concentrations (Table 1). The absorbance values measured pre- and post-incubation revealed a decrease in bacterial growth for 100% and 50% concentrations for MRSA and only the 100% concentration for *Streptococcus pyogenes* (Table 2).

4. Discussion

The World Health Organization (WHO) has identified six bacterial pathogens as the primary



(a) (b)

Figure 2. Gram staining results: (a) MRSA RES 22-2020, (b) *Streptococcus pyogenes* ATCC 19615.

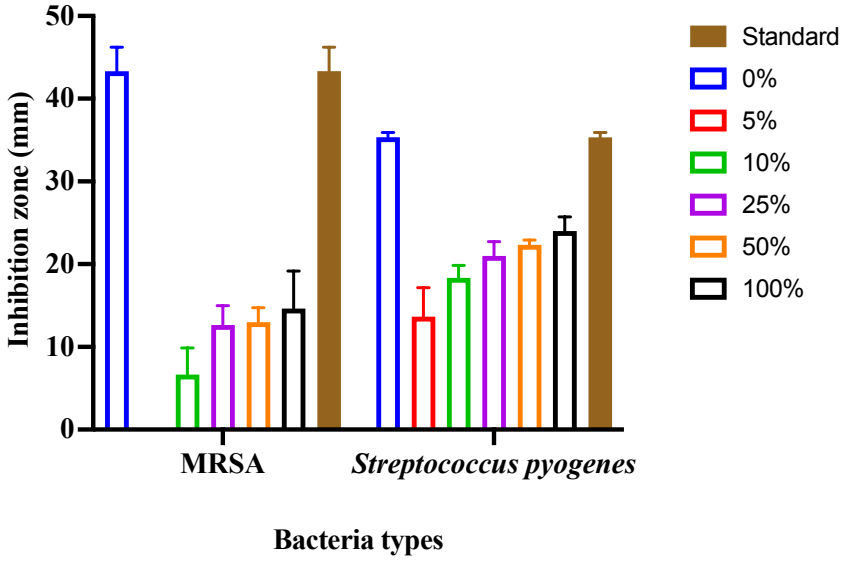
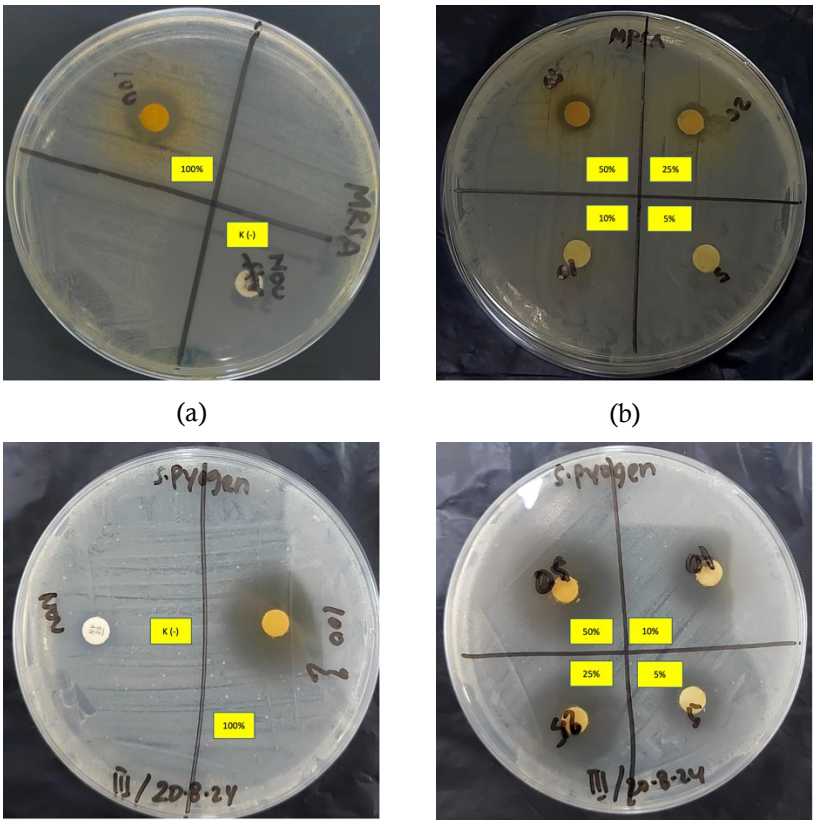


Figure 3. Inhibition Zone Test on Methicillin Resistant Staphylococcus aureus (MRSA) and Streptococcus pyogenes Bacteria



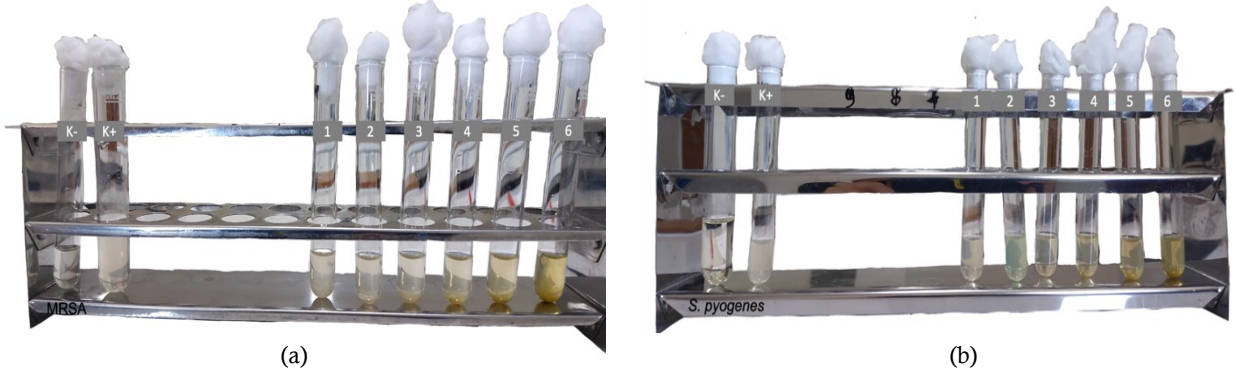
(a) (b)

(c) (d)

Figure 4. Clear zones of MRSA (a, b) and *Streptococcus pyogenes* (c, d) after incubation (1×24 hours) for various extract concentrations.

Table 1. MIC values for MRSA and *Streptococcus pyogenes* using the turbidimetry method.

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-	Antibiotic: Streptomycin	-	-	-	-	-	-



(a) (b)

Figure 4. Results of the MIC test using the turbidimetry method for MRSA (a) and *Streptococcus pyogenes* (b). Tubes 1–6 correspond to extract concentrations of 100%, 50%, 25%, 12.5%, 6.25%, and 3.125%, respectively. K(-) represents the negative control, and K(+) represents the positive control.

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Table 2. Absorbance values measured by UV-vis spectrophotometry for MRSA and *Streptococcus pyogenes*.

Tube(s)	Concentration of ethanolic extract of rambutan (*Nephelium lappaceum L.*) leaves	MRSA			*Streptococcus pyogenes*		
		Pre-incubation	Post-incubation	Interpretation	Pre-incubation	Post-incubation	Interpretation
1	100%	0,633	0,409	Decrease	0,644	0,523	Decrease
2	50%	0,354	0,305	Decrease	0,362	0,584	Increase
3	25%	0,202	0,428	Increase	0,216	0,169	Increase
4	12,5%	0,123	0,568	Increase	0,141	0,416	Increase
5	6,25%	0,081	1,18	Increase	0,078	1,024	Increase
6	3,125%	0,072	1,23	Increase	0,07	0,947	Increase
K+	K(+)	0,03	0,45	Increase	0,03	0,45	Increase
K-	K(-)	0,104	0,085	Decrease	0,104	0,085	Decrease

contributors to antimicrobial resistance (AMR)-associated mortality, accounting for 73% of AMR-related deaths in 2019. Among these, Methicillin-Resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pyogenes* are notable contributors (Global Burden of Disease, 2022). AMR-related infections were directly responsible for approximately 1.27 million deaths and indirectly implicated in 4.95 million deaths globally in 2019 out of an estimated 13.7 million infection-related fatalities (Ho *et al.*, 2024). Notably, MRSA represented the most lethal pathogen-drug combination, contributing to 121,000 AMR-associated deaths in 2019 (Naghavi *et al.*, 2024). In parallel, surveillance data from the United Kingdom in 2020–2021 revealed an increase in case fatality and death rates from Pseudomonas aeruginosa bacteremia compared to the preceding year (UK Health Security Agency, 2023). These findings underscore the urgent need for sustained action against AMR, which remains a significant global health challenge.

In response to AMR, natural antimicrobial products have gained substantial attention due to their chemical diversity and bioactivity. The ethanolic extract of rambutan (*Nephelium lappaceum* L.) leaves has shown potential as a natural antimicrobial agent, attributed to its bioactive secondary metabolites, including flavonoids and tannins.

MRSA and *Streptococcus pyogenes*, the bacterial models used in this study, are Gram-positive pathogens known for their significant resistance mechanisms and pathogenicity. MRSA exhibits resistance primarily through the expression of penicillin-binding protein 2a (PBP2a), which diminishes the efficacy of β-lactam antibiotics by reducing receptor sensitivity, thereby compromising bactericidal activity (Kemalaputri *et al.*, 2017). *Streptococcus pyogenes* is a causative agent of skin and respiratory infections, including impetigo and pneumonia. Both pathogens share structural features such as a thick peptidoglycan layer and prominent teichoic acids, contributing to their Gram-positive classification (Tripathi & Sapra, 2023). In this study, Gram staining confirmed the Gram-positive nature of these bacteria, showing a characteristic bright purple color.

Previous studies have demonstrated that ethanolic extracts of rambutan exhibit more potent antibacterial activity than extracts derived from other solvents, such as n-hexane and chloroform (Mukaromah *et al.*, 2023). For example, Yunusa *et al.* (2018) reported that ethanolic rambutan peel extracts demonstrated potent free radical scavenging activity, with inhibitory concentration (IC50) values of 24.99 ± 2.82 and 144.59 ± 1.36 µg/mL.

Phytochemical screening confirmed the presence of flavonoids and tannins in ethanolic rambutan leaf extract, compounds with known antibacterial properties. Flavonoids exert their antibacterial effects by disrupting bacterial membrane integrity, inhibiting biofilm formation, and interfering with bacterial DNA gyrase activity (Shamsudin *et al.*, 2022; Xie *et al.*, 2014). Conversely, tannins disrupt peptidoglycan synthesis, chelate iron, and inhibit antibiotic efflux pumps, thus impairing bacterial cell wall and membrane integrity (Jantapaso & Mittraparp-Arthorn, 2022).

The current study demonstrated that the ethanolic rambutan leaf extract exhibited significant antibacterial activity against MRSA and *Streptococcus pyogenes*, with a concentration of 100% achieving the most potent inhibition. According to the classification by Davis and Stout (1971), the inhibition zone diameters observed in this study for *Streptococcus pyogenes* at 100% concentration (24 mm) fall within the "strong" category. Statistical one-way ANOVA analysis confirmed that the 100% extract concentration was the most effective against MRSA and *Streptococcus pyogenes*.

Compared to MRSA, *Streptococcus pyogenes* exhibited greater susceptibility to the rambutan leaf

extract, as evidenced by larger inhibition zones. This observation aligns with previous findings that variations in extract diffusion, solvent compatibility, and active compound concentration can influence antibacterial efficacy (Rumaolat & Husada, 2020).

Turbidimetric assays confirmed that higher extract concentrations (100%, 50%, and 25%) reduced bacterial growth, as evidenced by lower absorbance values. These findings are consistent with earlier studies that demonstrated effective bacterial inhibition at high extract concentrations, including the work of Mukaromah *et al.* (2023), which reported a minimum inhibitory concentration (MIC) of 15.625 mg/mL for rambutan peel and seed extracts against multidrug-resistant Klebsiella pneumoniae.

While turbidimetry provides valuable insights, its inability to differentiate between live and dead bacteria necessitates complementary methods, such as Plate Count Agar, for more accurate assessments of bacterial viability. Additionally, advanced analytical techniques like high-performance liquid chromatography (HPLC) are recommended for precise quantification of active compounds (Gloria Rambet *et al.*, 2017).

In conclusion, this study highlights the significant antibacterial potential of ethanolic rambutan leaf extract against Gram-positive pathogens, particularly *Streptococcus pyogenes* and MRSA. These findings support the exploration of rambutan leaf extract as a natural antimicrobial agent, offering a promising alternative in the fight against AMR. Further research is needed to elucidate the specific mechanisms of action and optimize the application of this extract in clinical settings.

5. Conclusions

The ethanolic extract of rambutan (*Nephelium lappaceum* L.) leaves contains secondary metabolites, specifically flavonoids and tannins, as indicated by characteristic color changes when reacted with appropriate reagents. The extract demonstrated significant antibacterial activity against Methicillin-Resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pyogenes*. At a concentration of 100%, the extract exhibited a strong inhibition zone for MRSA (14.9 mm) and a substantial inhibition zone for *Streptococcus pyogenes* (24 mm). Statistical analysis revealed that concentrations of 100%, 50%, and 25% significantly inhibited MRSA growth, while all tested concentrations displayed significant inhibitory effects on *Streptococcus pyogenes*.The minimum inhibitory concentration (MIC), as assessed through visual turbidimetry, was 25% for both bacteria. Further confirmation using UV-Vis spectrophotometry indicated a decrease in absorbance for MRSA at concentrations of 100% and 50%, whereas for *Streptococcus pyogenes*, the reduction was observed only at 100%.

Further exploration of natural products is crucial to address the ongoing global challenge of antibiotic resistance. Future studies should focus on the detailed quantification and analysis of flavonoid and tannin content in rambutan leaves, as these compounds demonstrate significant bioactivity, particularly against Gram-positive bacteria.

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Conflict of interest

All authors have no conflict of interest in this article.

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