In vitro Antibacterial Activity of *Seribu kuman* Leaf (*Rhinacanthus nasutus* (l.) Kurz) Extracts Against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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Abstract

Seribu Kuman leaves (Rhinacanthus nasutus (L.) Kurz) are the herb most commonly used in traditional medicine to treat various diseases. The presence of secondary metabolites, including flavonoids, alkaloids, and phenolics, has led to its known antibacterial activity. This study aims to verify the antibacterial activity of *Seribu Kuman* leaf extract against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. This study involved experimental design using the Kirby-Bauer method, inoculating Mueller-Hilton Agar (MHA) test media containing *S. aureus* and *P. aeroginosa* bacteria with *Seribu Kuman leaves* ethanol extracts at concentrations of 15%, 30%, and 60%. Penicillin and ciprofloxacin were used as positive controls, whereas sterile aquabidest was used as a negative control for the study. The findings of this study showed that *Seribu Kuman* (*R. nasutus*) ethanol extract, at a concentration of 60%, presented the most significant inhibition zone against *S. aureus*, measuring 13.6 mm. In contrast, no inhibition zone was observed for *P. aeroginosa*. It can be concluded that *Seribu Kuman* (*R. nasutus*) ethanol extract can inhibit the growth of *S. aureus* but not *P. aeruginosa*. So, *Seribu Kuman leaves* (*R. nasutus*) ethanol extract could be an alternative for treating infections caused by *S. aureus*.

Keywords: Antibacterial, *Seribu Kuman* leaves, *Rhinacanthus nasutus* (L.) Kurz, *Staphylococcus aureus*, *Pseudomonas aeruginosa*

Significance | *Rhinacanthus nasutus* extract inhibits the growth of *Staphylococcus aureus* and could serve as an alternative in treating infections caused by *S. aureus*.

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1. Introduction

Antibacterials, also known as antibiotics in pharmacology, are substances that can inhibit the growth of bacteria (bacteriostatic) and eliminate pathogenic bacteria (bactericidal) (Magani et al., 2020; Sadikin et al., 2021)

Antibiotics exert their effects in several ways, including impairing bacterial cell walls, altering bacterial membrane permeability, interrupting bacterial protein synthesis, and decreasing enzyme activity (Magani et al., 2020; Septiani et al., 2017). Antibiotics significantly contribute to the global reduction of infectious diseases due to their utilization in treating diseases caused by bacterial infections. Nevertheless, the misuse of antibiotics can diminish their efficacy, leading to a global escalation in the prevalence of bacterial resistance (Rani et al., 2017).

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The inability of bacteria to suppress or eliminate harmful bacteria is known as bacterial resistance (Pratiwi, 2017; Sukertiasih et al., 2021). Bacterial resistance will increase the number of individuals as carriers in the community, lengthen hospital stays, and prolong healing time (Purnomo and Azzahra, 2021). As a result, antibiotic sensitivity and effectiveness will decrease, rendering treatment ineffective and ultimately increasing patient morbidity and mortality (Pratiwi, 2017; Purnomo ans Azzahra, 2021; Sukertiasih et al., 2021).

Staphylococcus aureus is a Gram-positive bacteria responsible for many infections globally (Septiani et al., 2017; Sukertiasih et al., 2021). Multiple studies have confirmed that *S. aureus* has developed resistance to several antibiotics (Jamilatun, 2019). *S. aureus* exhibits antibiotic resistance due to its ability to produce the penicillin beta-lactam enzyme, making penicillin-class drugs ineffective (Sukertiasih et al., 2021). Meanwhile, *Pseudomonas aeruginosa* is a Gram-negative bacteria known for causing infections. It has also shown antibiotic resistance (Purnomo and Azzahra, 2021).

The increasing incidence of antibiotic resistance has led to studying antibacterial agents derived from chemical compounds in rich biodiversity (Purnomo and Azzahra, 2021). Herbal plants have been used as a therapeutic choice since ancient times. Despite the significant improvements in access to medical services, several individuals keep choosing herbal therapy as an option for treatment (Johari and Khong, 2019). Phytochemical substances, such as phenols, flavonoids, and alkaloids, may work as antibacterial agents by damaging bacterial cell walls. These compounds are antibacterial against *S. aureus* and *E. coli* (Septiani et al., 2017). As such, developing new antibiotics by utilizing active compounds from the rich biodiversity could provide an alternative. Furthermore, they are safer and do not cause any significant side effects (Singh et al., 2017).

Indonesia offers many natural biological resources that can be used for medical purposes (Andry et al., 2023). *Rhinacanthus nasutus* is among these plants. *R. nasutus* (Figure 1) is a flowering plant belonging to the *Acanthaceae* family that grows in the tropics and subtropics, such as in Southeast Asia, India, and China. *R. nasutus* is known as Snake Jasmine, *Bai He Ling Zhi* in China, and *Manukan* in Indonesia. In Padang, West Sumatera, *R. nasutus* is known as *Daun Seribu Kuman*. The roots, stems, and leaves are used in traditional medicinal practices for various diseases (Irawan et al., 2021).

Flavonoids, alkaloids, phenolics, benzenoids, anthraquinones, coumarins, naphthoquinones, saponins, and tannins are some active compounds in *R. naustus* leaves. Rinacanthin is the major compound in this leaf (Sunarti and Paninsari, 2020). Rinacanthin and alkaloids extracted from *R. nasutus* exhibit antibacterial activity against several bacteria, including *S. epididermidis, S. mutans, P. acnes,* and *S. aureus* (Brimson et al., 2020; Sunarti and Paninsari, 2020).

Although rinachantin and the alkaloids found in *R. nasutus* have demonstrated antibacterial activity against various microorganisms, there is still limited report on the antibacterial activity of *R. nasutus* leaf extract against *S. aureus* and *P. aeruginosa.* Therefore, this study aims to prove the *in vitro* antibacterial activity of *Seribu Kuman* leaves (*Rhinacanthus nasutus*) ethanol extracts against *S. aureus* and *P. aeruginosa* at 15%, 30%, and 60% extract concentrations carried out by the disc diffusion method.

Material and Methods

Plant material

Seribu Kuman leaves (*Rhinacanthus nasutus* (L.) Kurz) were collected from Lubuk Minturun village in West Sumatera. It is authenticated and registered from the national herbarium at the ANDA Herbarium with the registration number ANDA00015732. This research was conducted from July to October 2022 at the UPTD Health Laboratory in West Sumatera and the Laboratory of the University of Baiturrahmah. This type of research is a proper experimental design. In this study, there were five groups:

i). **Group 1:** Positive control (Nutrient Agar + bacterial culture + penicillin and ciprofloxacin)

ii). **Group 2:** Treatment 1 (Nutrient Agar + bacterial culture + ethanol extract of *Rhinacanthus nasutus* (L.) Kurz 15%)

iii). **Group 3:** Treatment 2 (Nutrient Agar + bacterial culture + ethanol extract of *Rhinacanthus nasutus* (L.) Kurz 30%)

iv). **Group 4:** Treatment 3 (Nutrient Agar + bacterial culture + ethanol extract of *Rhinacanthus nasutus* (L.) Kurz 60%)

v). **Group 5:** Negative control (Nutrient Agar + bacterial culture + aquabidest sterile)

and all groups were repeated three times. The type of data obtained is primary data in the form of growth inhibition zones of *S. aureus* and *P. aeruginosa* using the Kirby-Bauer method by administering an ethanol extract of *Seribu Kuman* leaves (*R. nasutus*) on Mueller-Hilton Agar (MHA) test media that has been incubated with *S. aureus* and *P. aeruginosa* bacteria in the control group and treatment group.

Equipment Sterilization

The glass apparatus was washed and dried before use. They were wrapped in paper and autoclaved at 121°C at 2 atm pressure for 15 minutes, then dried in an oven at 150°C for 3 hours.

Preparation of Seribu Kuman Leaves Extract

The collected *Seribu Kuman* leaves were washed and aerated in the open air at room temperature for 3-5 days until they turned dry

brownish. They were then ground into a fine powder. Ethanol (96%) as a solvent was distilled to purify it so that the ethanol had a good quality. 100 grams of the leaves were macerated in 1000 mL of 96% ethanol (1:10). The extract was allowed to stand in a dark place with minimal light for 72 hours at 24-hour intervals and stirred or shaken for 60 minutes; the filtrate was filtered using filter paper. The filtrate was evaporated using a vacuum rotary evaporator at 50-60°C until a thick extract was obtained, then diluted with DMSO to obtain concentrations of 15%, 30%, and 60%.

Preparation of S. aureus and P. aeruginosa Bacteria and Experimental Procedures

Isolates of *S. aureus* and *P. aeruginosa* were obtained from the UPTD health laboratory in West Sumatra. The bacterial suspensions were taken using pure colonies of *S. aureus* and *P. aeruginosa* bacteria. Colonies were made into a suspension in a test tube containing 0.9% physiological NaCl using an eye dropper, then vortexed to make it homogeneous. The turbidity of the suspension was equated with McFarland's 0.5 standard solution to obtain 1.5 x 108 cells/mL of bacteria. MHA (Mueller-Hilton Agar) media was prepared, and bacterial cultures of *S. aureus* and *P. aeruginosa* were applied with sterile cotton sticks. The 20 µL of *Seribu kuman* leaves ethanol extract concentrations of 15%, 30%, and 60% each were dripped on the disc paper for ±15 minutes. 20 µL of penicillin antibiotic, ciprofloxacin, which acts as a positive and negative control, and 20 µL of sterile distilled water were dropped on the disc paper for ± 15 minutes. The disc distance was set at about ± 15 mm between other discs. The samples were incubated at 37°C for 24 hours. The diameter of the clear zone was measured using a caliper. The largest clear zone diameter has the most significant antimicrobial activity.

Results

This study was conducted in 3 repetitions using Mueller-Hilton Agar (MHA) media with paper discs containing 15%, 30%, and 60% extract concentrations. Penicillin and ciprofloxacin were the positive control (K+), and aquabidest sterile was the negative control (K-). Table 1 shows the average zone of inhibition of the *Seribu Kuman* leaves (*Rhinacanthus nasutus* L. Kurz) ethanol extract against *S. aureus* bacteria. The strong inhibition zone (13.6 mm) was indicated in the extract of *Seribu Kuman* (*R. nasutus* L. Kurz) at 60% concentration. The second more potent inhibition was shown by 30% concentration with an inhibition zone of 12.3 mm. Meanwhile, a moderate inhibition zone (8.3 mm) was demonstrated by the extract of 15% concentration. The positive control obtained an average inhibition zone of 7.3 mm with moderate inhibition criteria; in the negative control, no inhibition zone formed.

Figure 1 shows the results of the antibacterial activity test of *Seribu Kuman* (*R. nasutus* L. Kurz) against *P. aeruginosa*, namely that the clear zone is not visible around the paper disc that has been given *Seribu Kuman* leaves extracts with a concentration of 15%, 30%, 60%, and negative control. Only the positive control in the center of the petri dish showed a clear zone around the paper disc.

The average value of the inhibition zone in the antibacterial activity of *Seribu Kuman* (*R. nasutus* L. Kurz) leaves extract against *P. aeruginosa* with a concentration of 15%, which is 0.00 mm, 30%, which is 0.00 mm and 60% which is 0.00 mm, while the positive control (K+) which is 27.00 mm and the negative control (K-) is 0.00 mm (Table 2). The strength of bacterial inhibition is known through the average diameter of the inhibition zone, namely the extract concentrations of 15%, 30%, and 60%, and negative control (K-) (0.00 mm) has no bacterial inhibition. In contrast, the positive control (K+) (27.00 mm) has very strong antibacterial strength (clear zone >20 mm).

Based on the analysis test that has been performed, it is known that the data obtained do not show a significant difference in the average rank of the inhibition zone on *P. aeruginosa* between each test concentration treatment because Asymp. Sig. shows p=1.00. The group of inhibition zone measurement data on *S. aureus* obtained the p-value of 0.011, meaning that H_a is accepted or there is *in vitro* antibacterial activity of *Rhinacanthus nasutus* (L.) Kurz ethanol extract against *S. aureus.*

Discussion

The ethanol extract of *Seribu Kuman* leaves (*R. nasutus* (L.) Kurz) at concentrations of 15%, 30%, and 60% did not inhibit the growth of *P. aeruginosa* in this study. These concentrations were deemed insufficient to provide growth activity for Gram-negative bacteria. This finding is in agreement with research by Nanthakumar et al. (2014), which showed that an ethanol extract of *Seribu Kuman* leaves had no activity on the Gram-negative bacteria *Salmonella paratyphi* at a concentration of 50 mg/mL but only showed an inhibitory effect at a concentration of 100 mg/mL with weak inhibition criteria (Nanthakumar et al., 2014).

Another study by Febria et al. (2021), an antibacterial activity test of *Seribu Kuman* (*R. nasutus*) leaves extracts at concentrations of 20%, 40%, 60%, 80%, and 100% found the best antibacterial activity at a concentration of 100% with an inhibition zone diameter of 26 mm against Gram-positive *S. aureus* and 17 mm against *Methicillin-Resistant Staphylococcus aureus* (MRSA) (Febria et al., 2021). In addition, Sunarti and Paninsari (2020) tested the activity of *Seribu Kuman* (*R. nasutus*) leaves extracts against *S. aureus* at concentrations of 15%, 30%, and 60%, where antibacterial activity was only found at concentrations of 30% and 60% (Sukertiasih et al., 2021; Sunarti and Paninsari, 2020).

Table 1. Results of Antimicrobial activity test of *Seribu Kuman* leaf extracts against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Concentrate	Mean Zone of Inhibition ± SD* (mm)		Inhibition Criteria	
	S. aureus	*P. aeruginosa*	*S. aureus*	*P. aeruginosa*
15%	8.3 ± 1.5	0.0 ± 0.0	Moderate	Resistant
30%	12.3 ± 0.6	0.0 ± 0.0	Strong	Resistant
60%	13.7 ± 0.6	0.0 ± 0.0	Strong	Resistant
Ciprofloxacin (K^+)	-	27.0 ± 1.0	-	Very Strong/ Sensitive
Penicillin (K^+)	7.3 ± 1.5	-	Very Strong	-
Sterile distilled water (K^-)	0.0 ± 0.0	0.0 ± 0.0	Resistant	Resistant

Note: Experiments performed in triplicates; K^+= Positive control; K^-= Negative control * SD = standard deviation

Figure 1. Leaves of *Rhinacanthus nasutus*

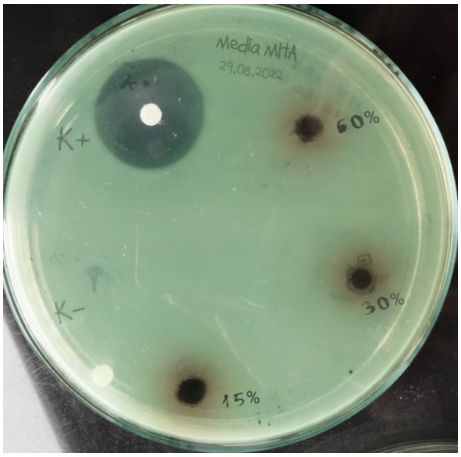

Figure 2. Results of Antimicrobial activity test of *Seribu Kuman* leaf extracts against *Pseudomonas aeruginosa*

From the data of previous studies, it was found that the inhibition zone produced by the ethanol extract of *Seribu Kuman* (*R. nasutus*) leaves against the growth of Gram-positive bacteria (*Bacillus subtilis*) was more significant than the inhibition zone of the *Seribu Kuman* leaves ethanol extract against the growth of Gram-negative bacteria (*Salmonella paratyphi*) (Nanthakumar et al., 2014). The difference in bacterial sensitivity to antibacterial can be caused by differences in cell wall structure. Gram-positive bacteria tend to be more sensitive to antibacterial because Gram-positive bacteria's cell wall structure is simpler than Gram-negative bacteria's cell wall structure, making it easier for antibacterial compounds to enter Gram-positive bacterial cells (Breijyeh et al., 2020). In addition, the presence of flavonoids, alkaloids, phenolics, benzenoids, anthraquinones, coumarins, naphthoquinones, saponins, and tannins enable it to work synergistically to inhibit the growth of Gram-positive and Gram-negative bacteria as reported by previous studies (Nanthakumar et al., 2014).

Based on this study on the relationship between the increase in concentration and the inhibition of *R. nasutus* (L.) Kurz ethanol extract against *S. aureus* has R=0.939, R2=0.883, and p=0.000 values. These results indicate a relationship between the increase in concentration and the inhibition of an ethanol extract of *R. nasutus* (L.) Kurz against *S. aureus*, which has a very high correlation strength, or that there is a meaningful correlation with a positive correlation direction. So, the higher the concentration of *R. nasutus* (L.) ethanol extract, the more significant the increase in inhibition against *S. aureus* bacteria. This is in line with research conducted by Sunarti & Paninsari (2020), which showed that there was a significant increase in the inhibition zone of an ethanol extract of *Manukan* leaves (*R. nasutus* L. Kurz) against Gram-positive bacteria *S. aureus* at the concentration of *R. nasutus* (L.) Kurz extracts 15%, 30%, and 60% of each inhibition zone, namely, 9.97 mm, 10.95 mm, and 13.18 mm (Sunarti & Paninsari, 2020).

The bacteria used in this study is *P. aeruginosa*. *P. aeruginosa* is a Gram-negative bacterium whose cell wall is covered by an outer membrane containing proteins, phospholipids, and lipopolysaccharides. The outer wall of *P. aeruginosa* bacteria is highly permeable, contains hydrophilic porins, and contains a nonpolar lipid layer (Singh et al., 2017). As a result, the active ingredients of *Seribu Kuman* (*R. nasutus*) leaf extract cannot penetrate bacterial cells optimally, so the extract cannot inhibit the growth of *P. aeruginosa* bacteria.

P. aeruginosa is a Gram-negative bacterium that can develop resistance to various antibiotics, and the widespread existence of resistant strains creates an urgency to find new antibacterials against this bacterium (Singh et al., 2017). Based on WHO data in 2017, *P. aeruginosa* and *Acinetobacter baumannii* ranked first and second on the list of multidrug-resistant (MDR) bacteria due to their high resistance to most antibiotics (Deni & Pangalila, 2019). Many researchers are researching by testing various extracts for alternative antibiotics with less chemical risk.

Research by Noviyanto et al. (2020) examined that bangle leaves (*Zingiber purpureum roxb*) at a concentration of 100% showed the most significant activity against the growth of *P. aeruginosa* bacteria with an inhibition zone of 10 mm (Noviyanto et al., 2020). The results of Purnomo and Azzahra (2021) research on the antibacterial activity test of avocado (*Persea americana Mill.*) leaves ethanol extract against *P. aeruginosa*, which has the most significant zone diameter of 7 mm (Purnomo and Azzahra, 2021). Extracts from other plants can also inhibit the growth of *P. aeruginosa*, as shown in the research of Anggita et al. (2018) on *Putri Malu* (*Mimosa pudica*) leaves extract at a concentration of 10% against *P. aeruginosa* bacteria, which is 1.01 mm (Anggita et al., 2018).

The negative control group in this study used DMSO, the extract solvent, and showed no antibacterial activity. The positive control in this study used ciprofloxacin, an antibiotic with an average inhibition zone diameter of 27 mm, so it is classified as being in the strong inhibition category. The solvent in this study was 96% ethanol. The extracted material influences the type of solvent used in the extraction process. The solubility of a compound in a solvent depends on the groups bound to the solvent. Solvents with hydroxyl (alcohol) and carbonyl (ketone) groups are included in polar solvents, while hydrocarbons are included in non-polar solvents. Solvent selection should be based on the nature of polarity and stability (Rarassari and Maftuch, 2016). The choice of solvent is by the solubility principle of like dissolves like, namely that polar solvents will dissolve polar compounds. In contrast, non-polar solvents will dissolve non-polar compounds as well.

There are two primary considerations in selecting the type of solvent: the solvent must have a high solubility and be a solvent that is not harmful or toxic. Polar and semi-polar solvents have been commonly used to extract polyphenolic compounds from plants such as fruits and vegetables. Solvents often used are distilled water, ethanol, methanol, acetone, and ethyl acetate (Trimanto et al., 2018). Research by Abdulsalami et al. (2016), using the maceration method with aqueous and ethanol, produced a percentage of extracts of 14.95% (aqueous) and 17.63% (ethanol). This shows that ethanol solvents produce more extracts than aqueous solvents because of the ability of ethanol to dissolve more active components in the extract (Abdulsalami et al., 2016).

The extraction method used in this research is maceration. Maceration is the process of soaking samples in organic solvents at room temperature. This method has advantages because the equipment is simple and easy to use, and it avoids damage to chemical components due to the heating process. Factors that affect extraction include time, temperature, type of solvent, ratio

of material and solvent, and particle size. Maceration time is another factor that must be considered in the extraction process. The longer the maceration time, the longer the contact between the solvent and the material will increase the number of broken cells and dissolved active ingredients. This condition will continue until an equilibrium is reached between the concentration of compounds in the material and the concentration of compounds in the solvent (Chairunnisa et al., 2019).

Several factors can affect the results of antibacterial tests, such as the turbidity of bacterial suspensions, the incubation temperature, and the thickness of agar media. In addition, dilution is also a crucial factor. In this study, it is known that the ethanol extract of *Seribu Kuman* leaves with the highest dilution (60% concentration) has the largest inhibition zone because the higher the concentration of the extract, the lower the solubility (it thickens like a gel), so this can slow down the diffusion of the active ingredients of the extract into the media and can ultimately reduce the ability of extracts with high concentrations to inhibit the growth of *P. aeruginosa* bacteria (Widyananda et al., 2021).

Flavonoids act as antibacterial by damaging the permeability of bacterial cell walls, microsomes, lysosomes, and bacterial cells due to their interaction with bacterial cell DNA. The antibacterial effect of phenol compounds depends on the number of hydroxyl groups and the concentration. *Flavonoids* are able to penetrate the polar peptidoglycan layer in the cell wall of gram-positive bacteria better than Gram-negative bacteria, which have membranes with high lipid content so that they are nonpolar. The concentration of phenol in the *Seribu Kuman* leaves extract in this study may not be able to damage the cell wall and inhibit *P. aeruginosa* bacteria (Sunarti and Paninsari, 2020). In addition, differences in the content of metabolite compounds can also occur due to differences in the environment where plants grow, types of varieties, physiological conditions (old and young), and post-harvest processing (Nanthakumar et al., 2014).

Conclusion

This study concludes that the average zone of inhibition of ethanol extract of *Rhinacanthus nasutus* (L.) Kurz against *S. aureus* bacteria is highest at a concentration of 60%, 13.6 mm, with strong inhibition bacteria. At the same time, ethanol extract from *Seribu Kuman* leaves (*R. nasutus*) has no inhibition zone on *P. aeruginosa* bacteria *in vitro*. There is a relationship between the concentration of ethanol extract of *Seribu Kuman* leaves (*R. nasutus*) and the inhibition zone in inhibiting the growth of *S. aureus* bacteria. However, there is no relationship between the concentration of ethanol extract of *Seribu Kuman* leaves (*R. nasutus*) and the inhibition zone in inhibiting the growth of *P. aeruginosa* bacteria.

Author contribution

B.F. conceptualisation, prepared methodology, managed project, S.F. Investigated, collected data, visualized, drafted, W.S. wrote, reviewed, M.R.S. Investigated, collected data, analysed data, K.H.Y. wrote, validated, reviewed, and edited.

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Competing financial interests

The authors have no conflict of interest.

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