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Antibacterial Potential of Rhinacanthus Nasutus Leaf Extract Against Escherichia Coli and Streptococcus Pneumoniae in Vitro

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Abstract**:** *Antibiotic resistance is a growing global health problem that impacts the effectiveness of infection therapy (World Health Organization [WHO], 2024). Therefore, safe and effective antibacterial alternatives are needed, including those derived from natural ingredients such as Rhinacanthus nasutus (L.) Kurz, which is known to contain bioactive compounds. This study aimed to evaluate the antibacterial activity of ethanolic extract of Rhinacanthus nasutus leaves against Escherichia coli and Streptococcus pneumoniae in vitro. The method used was well diffusion on Mueller–Hinton Agar media with varying extract concentrations of 15%, 30%, and 60%. Ciprofloxacin was used as a positive control and distilled water as a negative control. Antibacterial activity was measured based on the diameter of the inhibition zone after 24 hours of incubation at 37°C and analyzed using one-way ANOVA. The results showed that the extract was able to inhibit the growth of both test bacteria, with an increase in the diameter of the inhibition zone with increasing concentration. Statistical analysis showed a significant difference between treatment groups (p < 0.05). However, the inhibitory power of the extract was still lower than that of the positive control. These findings indicate that Rhinacanthus nasutus has the potential to be developed as a natural antibacterial, but further research is needed to optimize its effectiveness and safety.*

Keywords: Rhinacanthus nasutus (L.) Kurz, antibacterial activity, Escherichia coli, Streptococcus pneumoniae, antibiotic resistance, well diffusion

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INTRODUCTION

This phenomenon is closely related to irrational antibiotic use, such as unintended use, premature discontinuation of therapy, and use without medical supervision. This situation contributes to the emergence of bacteria that are resistant to various antimicrobial agents, thus decreasing the effectiveness of therapy and increasing the complexity of infection management.

The impact of antibiotic resistance is not limited to reduced therapeutic efficacy but also has implications for increased morbidity and mortality rates, as well as increased healthcare costs.

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Infections previously treatable with first-line antibiotics now require more aggressive therapy, longer treatment durations, and the use of broader-spectrum drugs. Therefore, innovative efforts are needed to develop alternative antibacterial agents that are effective, safe, and sustainable.

One widely developed approach is the use of natural ingredients, particularly medicinal plants, as sources of bioactive compounds with antibacterial potential. Herbal plants are known to contain various secondary metabolites such as flavonoids, alkaloids, tannins, and other phenolic compounds that play a role in inhibiting the growth of microorganisms. These compounds can work through various mechanisms, including damaging the structure of bacterial cell walls, disrupting membrane function, and inhibiting metabolic processes and protein synthesis in microbial cells.

Infections caused by pathogenic bacteria such as Escherichia coli and Streptococcus pneumoniae remain a significant health problem, particularly in developing countries. Escherichia coli is a gram-negative bacterium often associated with gastrointestinal infections, while Streptococcus pneumoniae is a gram-positive bacterium that plays a role in various serious diseases such as pneumonia, meningitis, and bacteremia. Both bacteria are known to exhibit increasing resistance to various classes of antibiotics, complicating clinical management.

One plant with potential for development as a natural antibacterial agent is Rhinacanthus nasutus (L.) Kurz. This plant is reported to contain active compounds such as rhinacanthin, flavonoids, and alkaloids, which have biological activities, including antibacterial properties. Several previous studies have shown that extracts of this plant can inhibit the growth of various pathogenic bacteria, both gram-positive and gram-negative, thus offering potential as an alternative natural-based therapy.

Based on this description, further research is needed to evaluate the antibacterial activity of ethanol extract of Rhinacanthus nasutus (L.) Kurz leaves against the growth of pathogenic bacteria in vitro. This research is expected to provide scientific contributions to the development of natural antibacterial agents as an alternative effort to address the increasing problem of antibiotic resistance

METHODOLOGY

This study used a post-test only control group design and was conducted in vitro. The aim of the study was to evaluate the antibacterial activity of ethanol extract of Rhinacanthus nasutus (L.) Kurz leaves against the growth of Streptococcus pneumoniae and Escherichia coli. All research procedures were conducted in a microbiology laboratory, adhering to research ethics principles and applicable occupational safety standards.

The leaves of the thousand-buman plant, extracted using ethanol as a solvent, were the main component used in this study. Using the maceration method, the leaves of the thousand-buman plant were soaked in 96% ethanol for a specified period of time to extract the active chemicals as effectively as possible. After filtering and evaporation, a thick extract was produced, which was then diluted to concentrations of 15%, 30%, and 60%.

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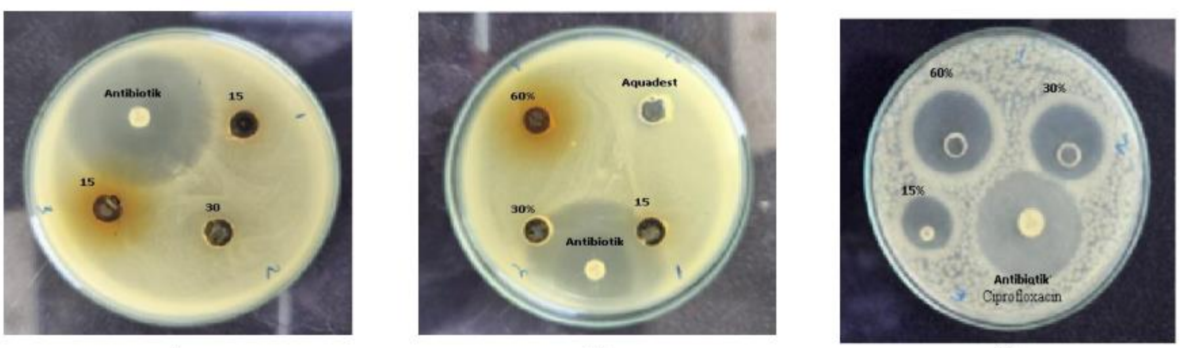
The McFarland standard was used to adjust the turbidity of the test bacterial suspensions, namely Streptococcus pneumoniae and Escherichia coli. The antibacterial activity test was carried out using Mueller–Hinton Agar (MHA) media with the well diffusion method. The bacterial suspension was inoculated evenly on the surface of the MHA media, then wells were made in the media. Each well was filled with ethanol extract of Rhinacanthus nasutus (L.) Kurz leaves at various concentrations. Distilled water was used as a negative control, while ciprofloxacin was used as a positive control. Next, the petri dishes were incubated for 24 hours at 37°C. Antibacterial activity was determined based on the measurement of the diameter of the inhibition zone formed around the well, which is expressed in millimeters.

The diameter of the inhibition zone formed was measured in millimeters, then systematically recorded and statistically analyzed to evaluate differences in antibacterial effectiveness between treatment groups. A normality test was performed first as a prerequisite for analysis, followed by a one-way ANOVA test to identify significant differences at the 95% confidence level. Furthermore, the antibacterial effectiveness of the ethanol extract of Rhinacanthus nasutus (L.) Kurz leaves at various concentrations was compared with the control group based on the results of the statistical analysis obtained

RESULTS AND DISCUSSION

A. Inhibition Zone Against Escherichia coli Bacteria

Antibacterial Activity of Rhinacanthus nasutus (L.) Kurz Leaf Extract against Escherichia coli Based on Inhibition Zone Diameter.



A B C

Figure 1. Inhibition Zone of Escherichia coli Bacteria. A. (A) Concentration of 15% with an average inhibition zone of 9.68 mm. (B) Concentration of 30% with an average inhibition zone of 11.6 mm. (C) Concentration of 60% with an average inhibition zone of 12.93 mm.

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Table 1.Average Diameter of Inhibition Zone of Ethanol Extract of Rhinacanthus nasutus (L.) Kurz Leaves on Escherichia coli

Treatment Group	Concentration	Inhibition Zone Diameter (mm)
Ethanol extract	15%	9.68
Ethanol extract	30%	11.16
Ethanol extract	60%	12.92
Positive control	Ciprofloxacin	> extract*
Negative control	Aquadest	0

Description: Compared with all extract concentrations, the positive control showed higher inhibitory power.

Ethanol extract of Rhinacanthus nasutus (L.) Kurz leaves also showed the ability to inhibit the growth of Gram-negative bacteria, as demonstrated by testing against Escherichia coli (Table 1). The diameter of the inhibition zone increased gradually with increasing extract concentration, from 9.68 mm at 15% concentration to 12.92 mm at 60% concentration. This pattern indicates a positive relationship between extract concentration and the resulting antibacterial activity. In other words, increasing extract concentration correlates with the increasing ability of the active compound to inhibit bacterial growth.

However, the inhibitory effectiveness of the extract was still lower than that of the positive control, which showed the highest antibacterial activity. Conversely, the negative control showed no inhibition zone formation, confirming that the observed antibacterial effect stems from the bioactive compounds in the leaf extract.

B. Inhibition Zone Against Streptococcus pneumoniae Bacteria

The diameter of the inhibition zone formed around the wells on Mueller–Hinton Agar (MHA) media after administration of ethanol extract of Rhinacanthus nasutus (L.) Kurz leaves is used as a parameter to assess antibacterial activity against Streptococcus pneumoniae. The clear zone formed reflects the area of inhibition of bacterial growth due to the presence of antibacterial compounds in the extract. The diameter of the inhibition zone is directly proportional to the strength of the antibacterial activity produced, so that the measurement of this parameter is the main indicator in evaluating the effectiveness of the leaf extract against the test bacteria.

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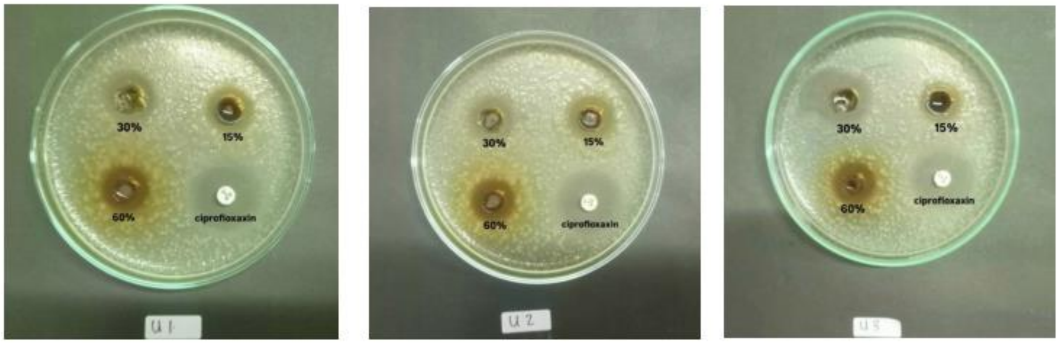

Figure 2. Inhibition Zone of Streptococcus pneumoniae Bacteria

The following table shows the information gathered from the inhibition zone measurements:

Table 2. Average Diameter of Inhibition Zone of Ethanol Extract of Seribu Kuman Leaves against Streptococcus pneumonia

Treatment Group	Concentration	Inhibition Zone Diameter (mm)
Ethanol extract	15%	18.65
Ethanol extract	30%	19.22
Ethanol extract	60%	19.38
Positive control	Ciprofloxacin	25.37
Negative control	Aquadest	0

Based on Table 2, the results of the study indicate that the ethanol extract of Rhinacanthus nasutus (L.) Kurz leaves has antibacterial activity against Streptococcus pneumoniae at all concentrations tested. The diameter of the inhibition zone formed tends to increase with increasing extract concentration, with the highest value reaching 19.38 mm at a concentration of 60%. This pattern indicates a concentration-dependent relationship, where increasing extract levels contribute to increased inhibitory power against the test bacteria.

However, the antibacterial activity of the extract was still below that of the positive control, ciprofloxacin, which produced an inhibition zone diameter of 25.37 mm. On the other hand, the absence of an inhibition zone in the negative control indicated that the observed antibacterial effect originated from the active compounds in the leaf extract, not from the solvent or other factors used in the study.

ANOVA Test Results on Escherichia coli

Table 3. ANOVA Test Results on Escherichia coli

Statistical Parameters	Mark
Statistical Test	One-Way ANOVA

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p-value	$p < 0.05$
Significance Level	$p < 0.05$
Information	The resulting inhibitory power showed significant differences between treatment groups based on the results of statistical analysis.

Based on Table 3, the results of the one-way ANOVA test show that the antibacterial activity of the ethanol extract of Rhinacanthus nasutus (L.) Kurz leaves against Escherichia coli differed significantly between treatment groups. The significance value obtained ($p < 0.05$) confirmed the existence of a significant difference in inhibitory power, so that variations in extract concentration were proven to affect the diameter of the resulting inhibition zone. This finding indicates that increasing extract concentration contributes to increasing antibacterial activity, indicating a concentration-response relationship. Thus, the null hypothesis (H_0) stating that there is no difference between treatment groups is rejected, while the alternative hypothesis (H_1) is accepted.

ANOVA Test Results on Streptococcus pneumonia

Table 4. ANOVA Test Results on Streptococcus pneumoniae

Statistical Parameters	Mark
Statistical Test	One-Way ANOVA
p-value	0.004
Significance Level	$p < 0.05$
Information	The resulting inhibitory power differed significantly between treatment groups based on the results of statistical analysis.

Based on Table 4, the results of the one-way ANOVA test show that the antibacterial activity of the ethanol extract of Rhinacanthus nasutus (L.) Kurz leaves against Streptococcus pneumoniae differed significantly between treatment groups. The significance value obtained ($p = 0.004$; $p < 0.05$) indicates that variations in extract concentration significantly affected the diameter of the inhibition zone formed. These results indicate a positive relationship between increasing extract concentration and the inhibitory power against the growth of Streptococcus pneumoniae. Thus, the null hypothesis (H_0) which states that there is no difference between treatment groups is rejected.

In addition, statistical analysis using one-way ANOVA test also showed that variations in the concentration of ethanol extract of Rhinacanthus nasutus (L.) Kurz leaves had a significant effect on the diameter of the inhibition zone in both test bacteria, namely Streptococcus pneumoniae and Escherichia coli ($p < 0.05$). This finding confirms that differences in extract concentrations result in significant variations in inhibitory power between treatment groups.

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DISCUSSION

Based on the synthesis of both studies, the ethanol extract of Rhinacanthus nasutus (L.) Kurz leaves was proven to have antibacterial activity against Gram-positive (Streptococcus pneumoniae) and Gram-negative (Escherichia coli) bacteria in vitro. This activity was demonstrated by the formation of inhibition zones at all tested concentrations (15%, 30%, and 60%), which reflects the extract's ability to inhibit bacterial growth. The increase in the diameter of the inhibition zone along with increasing extract concentration indicated a concentration-response relationship pattern, thus indicating that antibacterial effectiveness is influenced by the amount of active compounds available. These findings indicate that Rhinacanthus nasutus leaves have the potential as a source of natural antibacterials with a relatively broad spectrum.

Both studies consistently showed that increasing extract concentration was followed by increased inhibition. In Escherichia coli, the average inhibition zone diameter increased from 9.68 mm at a concentration of 15% to 12.92 mm at a concentration of 60%. A similar pattern was also found in Streptococcus pneumoniae, with inhibition zone diameters ranging from 18.65 to 19.38 mm at the same concentration range. This consistency strengthens the hypothesis that the higher the extract concentration, the greater the number of bioactive compounds that interact with bacterial cells, resulting in a more optimal inhibitory effect.

The results of the one-way ANOVA test showed a statistically significant difference between the treatment groups ($p < 0.05$) in both test bacteria. This confirms that variations in extract concentration significantly affect the level of bacterial growth inhibition. However, the effectiveness of the extract was still lower than that of the positive control, ciprofloxacin, which produced the largest inhibition zone diameter. Therefore, despite its potential as a natural antibacterial, this extract still requires further development, including concentration optimization, isolation of active compounds, and further in vivo testing.

The antibacterial activity of Rhinacanthus nasutus leaf extract is thought to be related to its secondary metabolite content, such as flavonoids, alkaloids, tannins, and the unique compound rhinacanthin. These compounds are known to work through various mechanisms, including disrupting cell wall integrity, increasing membrane permeability, inhibiting protein synthesis, and disrupting bacterial cell metabolism. This damage to cell structure and function ultimately inhibits growth and can even lead to bacterial cell death. Despite differences in cell wall structure between Gram-positive and Gram-negative bacteria, these mechanisms of action are generally effective in both groups, thus supporting the extract's potential as a broad-spectrum antibacterial.

However, both studies had several limitations. Antibacterial activity testing used only the well diffusion method, without determining the Minimum Inhibitory Concentration (MIC) or Minimum Bactericidal Concentration (MBC). Furthermore, the testing scope was limited to a single bacterial species, so generalizing the antibacterial spectrum of the extracts requires further study.

In line with these findings, several previous studies have also reported consistent results. A study by Fitriyasti et al. (2024) showed that Rhinacanthus nasutus leaf extract had in vitro antibacterial activity that increased at higher concentrations. Similar results were reported by Potti et al. (2023) and Sofyana et al. (2024), who found a dose-response relationship between various plant extracts against pathogenic bacteria. This consistent pattern strengthens the evidence that increasing extract concentration plays a significant role in enhancing antibacterial effectiveness.

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Furthermore, research by Akilandeswari et al. (2020) explains that the antibacterial activity of herbal plants is closely related to their secondary metabolite content. Alkaloids are reported to disrupt nucleic acid synthesis and cell wall structure, tannins play a role in precipitating bacterial cell proteins, while flavonoids can damage cell membranes and inhibit the activity of important enzymes. This mechanism aligns with the findings of this study, where increasing extract concentration is followed by increased inhibitory power.

Further developments have also been carried out, as reported by Solecha (2022), who synthesized Rhinacanthus nasutus extract in the form of silver nanoparticles (AgNPs) to enhance its antibacterial effectiveness against Escherichia coli. This approach suggests that the potential of this plant extract can still be enhanced through formulation innovation. From a global health perspective, these findings are relevant to the growing problem of antibiotic resistance. The World Health Organization (WHO, 2022; 2024) reports that antibiotic resistance is a serious threat, especially in bacterial infections such as pneumonia and diarrhea. Furthermore, increasing resistance of Streptococcus pneumoniae to β-lactam and fluoroquinolone antibiotics has also been reported (Zahari et al., 2023).

Therefore, the development of natural antibacterial sources, such as Rhinacanthus nasutus leaf extract, is crucial as an alternative or complementary therapy to address the challenge of antibiotic resistance. Overall, these synthesis results demonstrate that the ethanolic extract of Rhinacanthus nasutus leaves has potential as a promising natural antibacterial agent, although further research is needed to optimize its use in clinical applications.

CONCLUSIONS

Based on the research results and discussion, the formation of inhibition zones at all tested extract concentrations (15%, 30%, and 60%), and the absence of inhibition zones in the negative control, indicate that the ethanol extract of Rhinacanthus nasutus (L.) Kurz leaves has antibacterial activity against Escherichia coli and Streptococcus pneumoniae in vitro. The results of the one-way ANOVA test showed a statistically significant effect ($p < 0.05$), which indicates a concentration-response relationship, where an increase in extract concentration was followed by an increase in the diameter of the inhibition zone in both test bacteria. However, the inhibitory power of the extract was still lower than that of the positive control, ciprofloxacin. These findings indicate that the ethanol extract of Rhinacanthus nasutus leaves has the potential to be developed as an alternative natural antibacterial source. However, further research is needed to determine the Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), and to evaluate its safety and effectiveness aspects in vivo. The observed antibacterial activity is thought to be related to the content of bioactive compounds, such as flavonoids, alkaloids, tannins, and rhinacanthin, which play a role in inhibiting bacterial growth through various mechanisms.

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