

Antibacterial Activity of Ceremai Leaf (*Phyllanthus acidus* (L.) Skeels) Extract Against the Growth of *Staphylococcus aureus* ATCC 25923

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ABSTRACT

Staphylococcus aureus is a major bacterial pathogen responsible for various infectious diseases. The widespread and inappropriate use of antibiotics has accelerated the emergence of antimicrobial resistance, highlighting the need for alternative antibacterial agents derived from medicinal plants. Ceremai (*Phyllanthus acidus* (L.) Skeels) leaves contain several bioactive phytochemicals, including flavonoids, tannins, saponins, alkaloids, and steroids, which are reported to possess antibacterial properties. This study aimed to evaluate the antibacterial activity of ceremai leaf extract against the growth of *Staphylococcus aureus* ATCC 25923. A true experimental study employing a post-test-only control group design was conducted. Seven extract concentrations (100%, 50%, 25%, 12.5%, 6.25%, 3.125%, and 1.56%) were tested using the Kirby–Bauer disk diffusion method. Erythromycin (15 µg) and sterile distilled water served as the positive and negative controls, respectively. Each treatment was performed in triplicate, and the inhibition zone diameters were analyzed using one-way ANOVA. Serological test results showed that the prevalence of toxoplasmosis infection was 58%. Chronic toxoplasmosis infection occurred in 62% of owners with poor hygiene of drums, and 91% occurred in owners with poor personal hygiene. As a preventive measure against the risk of infection, breeders of cats need to always maintain the sanitation of the cage, such as frequent cleaning of the cage, cat feces, and cleaning the floor around the cage so that infective oocysts do not multiply around the cage area. Also, must use personal protective equipment and wash your hands after contact with cats. **Conclusion:** Ceremai (*Phyllanthus acidus* (L.) Skeels) leaf extract exhibits concentration-dependent antibacterial activity against *Staphylococcus aureus* ATCC 25923, with the strongest inhibitory effect observed at the 100% concentration. These findings support the potential use of ceremai leaves as a natural source of antibacterial compounds and warrant further investigation to isolate and characterize their active constituents.

INTRODUCTION

Infectious diseases remain one of the major health problems in many developing countries, including Indonesia. Infections caused by bacteria, viruses, fungi, and protozoa contribute to high rates of morbidity and mortality, and therefore require effective management. One of the most common pathogenic bacteria causing infections in humans is *Staphylococcus aureus*. This Gram-positive bacterium is part of the normal flora of the skin and nasal cavity, but can become pathogenic when certain predisposing factors are present. The infections it causes vary, ranging from mild skin infections to pneumonia, osteomyelitis, meningitis, endocarditis, urinary tract infections and sepsis. The ability of *S. aureus* to cause disease is closely linked to various virulence factors that trigger inflammation, tissue necrosis and the formation of abscesses (Anand et al., 2019)(Bustanussalam et al., 2016)(Khairunnisa et al., 2023)(Suyasa & Mastra, 2020)(Ambarsari et al., 2020).

To date, the treatment of bacterial infections still relies on the use of antibiotics. However, the irrational use of antibiotics—such as administration not in accordance with indications, incorrect dosing, and overuse—has led to an increase in the incidence of antimicrobial resistance. Antibiotic resistance results in higher rates of morbidity, mortality, treatment failure, and the financial burden on the healthcare system. Furthermore, the discovery of new classes of antibiotics has declined in recent decades, making it necessary to seek alternative antibacterial agents derived from natural sources (Anand et al., 2019)(Maulana, 2022)(Ambarsari et al., 2020)(Sukanda & Kusuma, 2023).

Indonesia is a country with high biodiversity and a wealth of medicinal plants. The use of plants as traditional medicines has been practised for generations because they are relatively easy to obtain, more economical, and have fewer side effects than synthetic medicines when used correctly. According to data from the Ministry of Health, thousands of species of medicinal plants have been successfully identified and utilised as ingredients in traditional medicine, and thus have the potential to be developed as sources of natural antibacterial compounds(Maulana, 2022)(Sukanda & Kusuma, 2023).

One plant with potential antibacterial properties is the ceremai (*Phyllanthus acidus* (L.) Skeels). This plant, belonging to the Euphorbiaceae family, is widely found in tropical regions and has long been used in traditional medicine. Ceremai leaves are known to contain various secondary metabolites, such as flavonoids, tannins, saponins, alkaloids and steroids, which possess antioxidant,

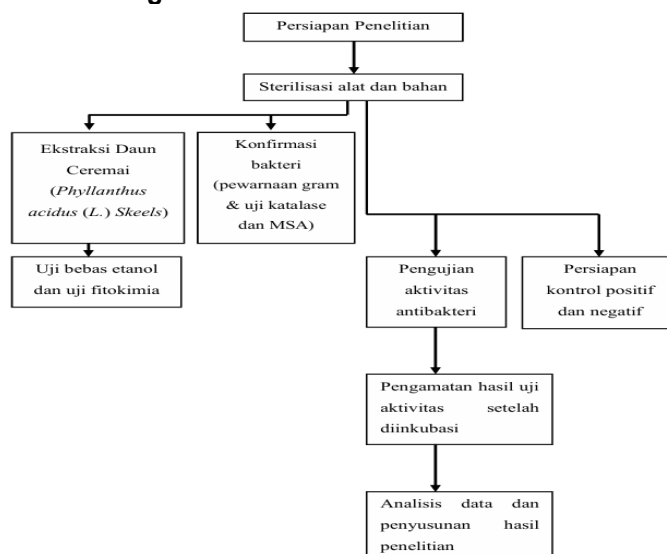
anti-inflammatory, analgesic and antibacterial properties. These compounds act through various mechanisms, including damaging bacterial cell membranes, inhibiting the synthesis of proteins and nucleic acids, and increasing cell membrane permeability, thereby inhibiting bacterial growth (Andriani et al., 2017) (Marpaung et al., 2022) (Nisa et al., 2023) (Wahyudi, 2021).

Several studies have reported the antibacterial activity of ceremai leaf extract against various pathogenic bacteria. (Hasibuan, 2020) demonstrated that ethanol extract of ceremai leaves was capable of inhibiting the growth of *Staphylococcus aureus*, with the diameter of the inhibition zone increasing as the extract concentration rose. Another study by (Susanti & Safitri, 2019) also demonstrated antibacterial activity against *Streptococcus mutans*, whilst (Nafis et al., 2023) reported that both ethanol and methanol extracts of ceremai leaves were capable of inhibiting the growth of *S. aureus*, although the inhibition zone diameters obtained were still relatively low. These differing results indicate that the antibacterial efficacy of ceremai leaves is influenced by the type of solvent, the extraction method, and the concentration of the extract used (Hasibuan, 2020) (Nafis et al., 2023) (Susanti & Safitri, 2019).

Although the antibacterial activity of ceremai leaves has been widely reported, studies evaluating the effectiveness of ceremai leaf extract against *Staphylococcus aureus* ATCC 25923 as a laboratory standard strain remain limited. The use of a standard strain is necessary to produce more valid data that can be compared with other studies. Therefore, this study aims to analyse the antibacterial activity of ceremai leaf extract (*Phyllanthus acidus* (L.) Skeels) against the growth of *Staphylococcus aureus* ATCC 25923 using the Kirby–Bauer disc diffusion method. The results of this study are expected to provide scientific evidence regarding the potential of ceremai leaves as a source of natural antibacterial agents and to support the development of phytopharmaceuticals to address the rising incidence of antibiotic resistance.

RESEARCH METHODS

Figure 1. Research Workflow



This study is a purely experimental study (true experimental design) using a post-test only control group design. The study was conducted at the Laboratory of the Medical Education Programme, Faculty of Medicine and Veterinary Medicine, Nusa Cendana University. The independent variable in this study was ceremai leaf extract (*Phyllanthus acidus* (L.) Skeels) at seven concentration levels, whilst the dependent variable was the growth of *Staphylococcus aureus* ATCC 25923, measured based on the diameter of the inhibition zone. Control variables included incubation temperature, sterilisation, incubation time and medium composition, which were kept constant throughout the study.

Error! Reference source not found. shows the research workflow, starting from research preparation, sterilisation of equipment and materials, the ceremai leaf extraction process, confirmation of bacterial identity, preparation of controls, testing of antibacterial activity, observation of results following incubation, through to data analysis. This flowchart illustrates the stages of the research carried out systematically to evaluate the antibacterial activity of ceremai leaf extract against *Staphylococcus aureus* ATCC 25923.

Ceremai leaves were extracted using the maceration method with 70% ethanol as the solvent. Subsequently, an ethanol-free test and phytochemical screening were carried out to identify the secondary metabolites present, including flavonoids, alkaloids, tannins, saponins, and steroids. The extraction process and identification of metabolites followed the procedures used in previous studies (Marpaung et al., 2022)(Nisa et al., 2023).

The antibacterial activity of ceremai leaf extract was tested using the Kirby–Bauer disc diffusion method in accordance with the standards of the Clinical and Laboratory Standards Institute (CLSI, 2023). The extract was tested at seven concentrations, namely 100%, 50%, 25%, 12.5%, 6.25%, 3.125% and 1.56%. Erythromycin 15 µg was used as the positive control, whilst sterile distilled water was used as the negative control. Each treatment group was repeated three times. A suspension of *Staphylococcus aureus* ATCC 25923 was inoculated onto Mueller-Hinton Agar (MHA); discs soaked in the extract were then placed on the surface of the medium and incubated for 24 hours at 37°C. The diameter of the inhibition zones formed was measured using a vernier calliper in millimetres as an indicator of antibacterial activity (CLSI, 2023).

Data on inhibition zone diameters are presented as mean ± standard deviation. Statistical analysis began with a normality test using the Shapiro–Wilk test and a homogeneity test using Levene’s test. A one-way analysis of variance (ANOVA) was then conducted to determine differences in inhibition zone diameters between treatment groups. Where significant differences were found ($p < 0.05$), the analysis was continued using the Dunnett T3 test as a post hoc test. All statistical analyses were carried out at a significance level of 95% ($\alpha = 0.05$) (Dahlan, 2015).

RESULTS

A total of 5 kg of fresh ceremai leaves (*Phyllanthus acidus* (L.) Skeels) were collected from Kupang City, East Nusa Tenggara, then cleaned, dried and ground to yield 1 kg of crude drug. The crude drug was subsequently extracted using the maceration method with 70% ethanol as the solvent for three days. Following filtration and evaporation using a vacuum rotary evaporator, 164 grams of concentrated extract were obtained. An ethanol-free test showed that the extract no longer contained any ethanol residue and was therefore suitable for use in antibacterial activity testing.

The results of the phytochemical screening indicate that ceremai leaf extract contains five classes of secondary metabolites, namely alkaloids, saponins, tannins, steroids and flavonoids. All of these compounds tested positive in the qualitative assays, as shown in **Table 1**.

Table 1. Results of the phytochemical screening of ceremai leaf extract (*Phyllanthus acidus* (L.) Skeels)

Metabolite Seconds	Result
Alkaloid	+
Saponin	+
Tanin	+
Terpenoid	+ Steroid
Flavonoid	+

The identity of the test bacteria was confirmed using Gram staining, the catalase test and the Mannitol Salt Agar (MSA) test. The Gram staining results showed purple, coccus-shaped bacteria, indicating Gram-positive bacteria. The catalase test produced bubbles following the addition of hydrogen peroxide, whilst the MSA test showed a colour change in the medium to yellow. These three results confirmed that the bacterium used in the study was *Staphylococcus aureus* ATCC 25923.

The antibacterial activity of ceremai leaf extract against *Staphylococcus aureus* ATCC 25923 was evaluated using the Kirby–Bauer disc diffusion method. The test was carried out using seven concentrations of the extract (100%; 50%; 25%; 12.5%; 6.25%; 3.125%; and 1.56%), with 15 µg of erythromycin as the positive control and sterile distilled water as the negative control. The diameter of the inhibition zone was measured after incubation for 24 hours at 37°C.

The results of the measurements showed that ceremai leaf extract was able to inhibit the growth of *Staphylococcus aureus* at concentrations ranging from 6.25% to 100%. The largest inhibition zone diameter was obtained at a concentration of 100%, namely 11.3 mm, whilst concentrations of 50%, 25%, 12.5% and 6.25% produced inhibition zone diameters of 9.4 mm, 9.1 mm, 7.5 mm and 7.5 mm, respectively. No inhibition zone formed at concentrations of 3.125% and 1.56%. The positive control produced an inhibition zone diameter of 22.4 mm, whilst the negative control showed no formation of an inhibition zone.

Table 2. Diameter of the inhibition zone produced by ceremai leaf extract against the growth of *Staphylococcus aureus* ATCC 25923

Concentration	Average Inhibition Zone (mm)
Extract 100%	11,3
Extract 50%	9,4
Extract 25%	9,1
Extract 12,5%	7,5
Extract 6,25%	7,5
Extract 3,125%	0
Extract 1,56%	0
Control (-)	0
Control (+)	22,4

As presented in Table 2, the diameter of the inhibition zone increased with increasing concentrations of *Phyllanthus acidus* leaf extract. The highest antibacterial activity was observed at the 100% concentration, which produced an inhibition zone of 11.3 mm, followed by the 50% (9.4 mm), 25% (9.1 mm), 12.5% (7.5 mm), and 6.25% (7.5 mm) concentrations. In contrast, no inhibition zone was observed at the 3.125% and 1.56% concentrations or in the negative control. The positive control (erythromycin 15 µg) produced the largest inhibition zone (22.4 mm), indicating greater antibacterial efficacy than the plant extract. Statistical analysis showed that the data on the diameter of the inhibition zone were normally distributed, as indicated by the Shapiro–Wilk test ($p > 0.05$). However, the results of the homogeneity test using Levene's test indicated that the data were not homogeneous ($p = 0.001$). Therefore, the analysis was continued using a one-way analysis of variance (ANOVA), which revealed a significant difference in the mean inhibition zone diameters between the treatment groups ($p < 0.001$). Furthermore, the Dunnett T3 post hoc test showed that the 100 % and 50 % concentrations differed significantly from the 3.125 %, 1.56 %, negative control and positive control concentrations ($p < 0.05$), whilst there were no statistically significant differences between the higher concentration groups.

DISCUSSION

The results of the study indicate that ceremai leaf extract (*Phyllanthus acidus* (L.) Skeels) exhibits antibacterial activity against *Staphylococcus aureus* ATCC 25923. This activity was demonstrated by the formation of inhibition zones at concentrations of 6.25%, 12.5%, 25%, 50% and 100%, whilst no inhibition zones were formed at concentrations of 3.125% and 1.56%. The largest inhibition zone diameter was obtained at a concentration of 100% (11.3 mm), whilst the positive control, erythromycin, produced an inhibition zone diameter of 22.4 mm. These results indicate that ceremai leaf extract has the ability to inhibit the growth of *S. aureus*, although its effectiveness is still lower than that of standard antibiotics. The antibacterial activity exhibited by ceremai leaf extract is thought to be related to its secondary metabolite content. Phytochemical screening results indicate the presence of flavonoids, alkaloids, tannins, saponins and steroids in the ceremai leaf extract. The presence of these compounds suggests that the extract contains bioactive components that may act synergistically to inhibit bacterial growth. These findings are consistent with studies by (Marpaung et al., 2022) and (Nisa et al., 2023), which reported that ceremai leaf extract contains various secondary metabolites that contribute to its antibacterial activity.

Flavonoids are a class of phenolic compounds widely reported to possess antibacterial activity. These compounds act through several mechanisms, including disrupting the permeability of bacterial cell membranes, inhibiting nucleic acid synthesis, and inhibiting the action of enzymes involved in cellular metabolism. Membrane damage leads to the leakage of intracellular components, thereby disrupting the physiological processes of bacteria and ultimately causing cell death. Furthermore, flavonoids are also known to form complexes with cell wall proteins, thereby inhibiting the growth of Gram-positive bacteria such as *Staphylococcus aureus*. This mechanism explains why ceremai leaf extract is still capable of producing an inhibition zone despite being a crude extract containing a mixture of various active compounds. In addition to flavonoids, tannins are also thought to play an important role in the antibacterial activity of ceremai leaf extract. Tannins have the ability to precipitate proteins, thereby damaging the structure of bacterial cell walls and membranes. The interaction of tannins with proteins disrupts enzyme function and the transport of nutrients into bacterial cells. The saponins identified in ceremai leaf extract also contribute to its antibacterial activity. Saponins possess detergent-like properties as they are able to interact with cell membrane lipids. This interaction increases membrane permeability, causing the cell contents to leak out and leading to lysis. Meanwhile, alkaloids are known to inhibit the formation of peptidoglycan in bacterial

cell walls, thereby destabilising the cell structure. Steroids are thought to act by disrupting the integrity of the cytoplasmic membrane, thereby impairing the membrane's function in regulating substance transport. Consequently, the antibacterial activity of ceremai leaf extract is likely the result of the synergistic action of various secondary metabolites present within it, rather than stemming from a single compound. The results of the study indicate a tendency for the diameter of the inhibition zone to increase as the concentration of the extract rises. A 100 % concentration produced the largest inhibition zone diameter, whilst lower concentrations showed progressively lower activity. This phenomenon suggests a relationship between the concentration of the extract and the amount of active compounds capable of diffusing into the agar medium. The higher the extract concentration, the greater the number of bioactive compounds available to diffuse and interact with bacterial cells, resulting in a greater inhibitory effect. Conversely, at low concentrations, the number of active compounds diffusing is insufficient to effectively inhibit bacterial growth. This is evident at concentrations of 3.125 per cent and 1.56 per cent, which produced no inhibition zones at all. The absence of an inhibition zone at concentrations of 3.125% and 1.56% indicates that the amount of active compound present in the extract has not yet reached a concentration effective for inhibiting the growth of *S. aureus*. As well as being influenced by concentration, the size of the inhibition zone is also affected by the ability of the active compounds to diffuse through the agar medium, the molecular size of the compounds, the solubility of the extract, and the sensitivity of the bacteria to the antibacterial compounds. Therefore, the diameter of the inhibition zone not only reflects antibacterial potential but is also influenced by the physical and chemical characteristics of the compounds contained in the extract. According to the classification by Davis and Stout, the diameter of the inhibition zone for the ceremai leaf extract at a concentration of 100% falls into the 'strong' category, whilst concentrations of 50%, 25%, 12.5% and 6.25% fall into the 'moderate' category. Nevertheless, all antibacterial activity of the extract remains below that of the positive control, erythromycin. These results are understandable as erythromycin is a pure antibiotic with a specific mechanism of action, namely inhibiting bacterial protein synthesis by binding to the 50S ribosomal subunit. In contrast, the ceremai leaf extract is still a crude extract containing various active compounds in unpurified concentrations, meaning its effectiveness is relatively lower than that of standard antibiotics.

The findings of this study are consistent with several previous studies reporting that ceremai leaf extract exhibits antibacterial activity against Gram-positive bacteria. (Marpaung et al., 2022) reported that ethanol extract of ceremai leaves was able to inhibit the growth of *Staphylococcus aureus*, with the diameter of the inhibition zone increasing as the extract concentration rose. Similar results were also reported by (Hasibuan, 2020) and (Nafis et al., 2023), who demonstrated that the flavonoid, tannin, saponin, and alkaloid content in ceremai leaves plays a significant role in their antibacterial activity. The consistency of these results reinforces the hypothesis that the secondary metabolites contained in ceremai leaves have potential as a source of natural antibacterial agents. Conversely, differences in the diameter of the inhibition zones between studies are likely influenced by variations in extraction methods, the type of solvent, the origin of the plants, the age of the leaves, the environmental growing conditions, and the testing methods employed. Statistical analysis supported the laboratory test results. The Shapiro–Wilk test indicated that the data were normally distributed, whilst Levene's test showed that the variances between groups were not homogeneous. Therefore, the analysis continued using a one-way ANOVA, which revealed a significant difference in the diameter of the inhibition zone between the treatment groups ($p < 0.001$). The Dunnett T3 post-hoc test confirmed that there were significant differences between some of the concentration groups. These results indicate that variations in the concentration of ceremai leaf extract have a significant effect on its ability to inhibit the growth of *Staphylococcus aureus*. Consequently, the research hypothesis stating that ceremai leaf extract possesses antibacterial activity against *Staphylococcus aureus* can be accepted. This study indicates that ceremai leaves have the potential to be developed as a source of natural antibacterial agents. This potential is significant given the rising incidence of antibiotic resistance, which is driving the search for new antibacterial compounds from natural sources. Nevertheless, this study has several limitations. Testing was conducted solely using the disc diffusion method on a single bacterial species; consequently, it cannot yet provide a comprehensive picture of the antibacterial activity. Furthermore, this study has not determined the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values, nor has it identified the dominant active compound responsible for the antibacterial activity. Therefore, further research is required using more comprehensive methods, including the isolation of active compounds, testing against other bacteria, and in vivo evaluation to support the development of ceremai leaf extract as a candidate phytopharmaceutical.

CONCLUSIONS AND RECOMMENDATIONS

Ceremai leaf extract (*Phyllanthus acidus* (L.) Skeels) has been shown to possess antibacterial activity against *Staphylococcus aureus* ATCC 25923. Antibacterial activity was demonstrated by the formation of inhibition zones at concentrations ranging from 6.25% to 100%, with the highest inhibitory effect observed at a concentration of 100% (11.3 mm), which falls within the 'strong' category. Phytochemical screening results indicate that ceremai leaf extract contains flavonoids, alkaloids, tannins, saponins and steroids, which are thought to act synergistically to inhibit bacterial growth. Statistical analysis using One-Way ANOVA showed that variations in extract concentration had a significant effect on the diameter of the inhibition zone ($p < 0.05$); thus, the higher the extract concentration, the greater the antibacterial activity produced. Consequently, ceremai leaf extract has the potential to be developed as a natural source of antibacterial agents against *Staphylococcus*

Further research is recommended to conduct a quantitative analysis of the secondary metabolite content of ceremai leaf extract in order to identify the active compounds that play the most significant role in its antibacterial activity. In addition, testing should be carried out on other Gram-positive and Gram-negative bacteria, the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values should be determined, and ceremai leaf extract should be developed into a pharmaceutical formulation so that its potential as a natural antibacterial agent can be evaluated more comprehensively.

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