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Antibacterial Effectiveness Test of Sandalwood Leaf Extract (*Santalum album*) Against Pathogenic Bacteria *Escherichia coli* and *Staphylococcus aureus*

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Abstract. Infectious diseases are diseases caused by organisms such as bacteria, viruses, fungi or parasites. One of the causes of most infectious diseases comes from bacterial infections, including *Escherichia coli* and *Staphylococcus aureus*. Treatment for bacterial infections is generally by administering antibiotics. Treatment using plants containing antibacterials is an alternative that is being widely studied as an effort to kill bacteria other than the use of antibiotics. One of the plants cultivated and used as a medicinal plant is sandalwood (*Santalum album*). This research was carried out to evaluate the antibacterial activity of sandalwood leaf extract (*Santalum album*) against *Escherichia coli* and *Staphylococcus aureus*. The research utilized a true experimental framework with a posttest-only control group design, featuring treatment groups consisting of a Ciprofloxacin positive control, a sterile aquadest negative control, and varying concentrations of sandalwood leaf extract (ranging from 3.12% to 100%), each replicated three times against *Escherichia coli* and *Staphylococcus aureus* strains. Data analysis used the Kruskal-Wallis statistical test with a 95% degree of confidence. Based on the results of testing the antibacterial potential of sandalwood leaf extract on the growth of *Escherichia coli* and *Staphylococcus aureus* bacteria, it was shown that the extract had antibacterial potential. Analysis of the Kruskal-Wallis test showed that $p = 0.002$ was smaller than $\alpha = 0.05$, showing there was a significant variation in the mean diameter of the inhibition zones between the different treatment groups. There is an antibacterial effectiveness of sandalwood leaf extract (*Santalum album*) against *Escherichia coli* and *Staphylococcus aureus* bacteria.

Keywords: Antibacterial; *Escherichia coli*; *Santalum Album*; *Staphylococcus aureus*

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INTRODUCTION

Infectious diseases are diseases caused by organisms such as bacteria, viruses, fungi, or parasites (J. Larry Jameson et al., 2018) Currently, infectious diseases remain a health issue in both developing and developed countries. One of the most common causes of infectious diseases is bacterial infections. Antibiotics are used to treat bacterial infections (Calhoun et all., 2022). Antibiotics are natural substances generated by microbes, especially fungi, which inhibit or eliminate the growth of other microbial organisms. Antibiotics can also be synthetically producedan (Andarsari et al., 2024). *Escherichia coli* and *Staphylococcus aureus* are commensal bacteria in the human body. *Escherichia coli* is generally found in the human intestines, while *Staphylococcus aureus* is found on the skin, respiratory tract, and digestive tract. Diseases caused by *Escherichia coli* include diarrhea, urinary tract infections, and respiratory tract infections. On the other hand, *Staphylococcus aureus* can cause cellulitis, wound infections, abscesses, osteomyelitis, and pneumonia (Maharani et al., 2017).

Indonesia has an area of about 9 million km², around 17,500 islands, and a very high level of biodiversity, including about 25% of the plant species in the world. Therefore, the potential for medicinal plants in Indonesia is very high due to its rich biodiversity (Fadilah, 2018). Plants with active ingredients are one of the sources of natural antibacterial compounds (Abebe et al.,2015).

Sandalwood *(Santalum album)* is a medicinal plant that grows in tropical regions and is endemic to the island of Sumba, East Nusa Tenggara (NTT). The phytochemical content of the methanol extract includes alkaloids, terpenoids, flavonoids, phenols, and steroids. All compounds in sandalwood leaves have potential as antibacterial compounds (Puspawati et al., 2018).

Based on the above description, to consider the antibacterial effectiveness of the sandalwood leaf extract, research is needed to test the antibacterial effectiveness of this extract on pathogenic bacteria, specifically *Escherichia coli* and *Staphylococcus aureus*, as used in this study.

MATERIALS AND METHODS

This is a true experimental design study with a posttest-only control group design. Bivariate analysis was performed using the Kruskal Wallis test, followed by Post Hoc testing using the Dunnett T3 test. The research was conducted at the Laboratory of the Faculty of Medicine and Health, Bioscience, and the Faculty of Agriculture, Nusa Cendana University, from June to August.

Sandalwood leaves (*Santalum album*) were collected from the Kupang City area. Samples of *Escherichia coli* and *Staphylococcus aureus* bacteria were obtained from the Surabaya Central Laboratory. The samples consisted of 9 groups, including 6 groups of sandalwood leaf extract at concentrations of 100%, 50%, 25%, 12.5%, 6.25%, and 3.12%, with a negative control (sterile distilled water) and positive control (Ciprofloxacin). The experiment was repeated 3 times.

Two kilograms of sandalwood leaves were washed thoroughly and air-dried, then ground into a powder. This powder was macerated with 70% ethanol for 3 days, with daily agitation. The filtrate was evaporated using a Rotary Evaporator to obtain a concentrated extract (Riswana, 2022).

Ethanol-free testing was carried out by reacting potassium dichromate ($K_2Cr_2O_7$) with ethanol in an acidic environment. Phytochemical screening of the sandalwood leaf extract included tests for polyphenols, terpenoids, and alkaloids.

Glassware and media were wrapped in paper and aluminum foil and sterilized in an autoclave at 121°C for 15-20 minutes. Needles and forceps were sterilized by flaming. Plastic equipment was sterilized with 70% alcohol (Riswana, 2022).

Bacterial confirmation testing was performed using Gram staining. Nutrient agar media was prepared by cooking and sterilizing, then poured into Petri dishes. Bacterial suspensions were made using 0.9% NaCl and 1-2 bacterial inocula until reaching the 0.5 McFarland standard. (Misra & Dey, 2021)

Antibacterial testing was conducted using a sterile cotton swab dipped in the bacterial suspension and spread on the nutrient agar. A 6 mm paper disc soaked in the extract and controls was placed on the agar and incubated for 30 minutes. All media were incubated at 37°C for 24 hours. The diameter of the inhibition zones was measured with a caliper. The inhibition zone measurement data were recorded as the research results.

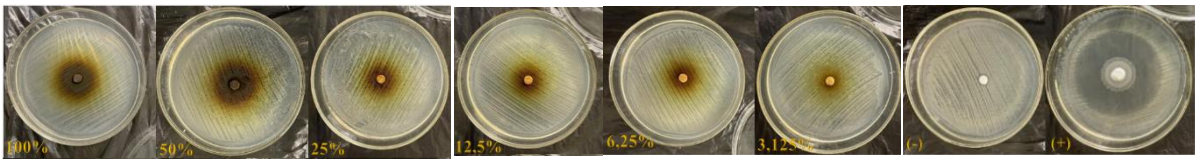
RESULTS AND DISCUSSION

Extraction of Sandalwood Leaves Two kilograms of sandalwood leaves (*Santalum album*) were used to obtain 100 ml of concentrated sandalwood leaf extract, Ethanol-Free Test The reaction showed an orange color or a mixture of the extract, potassium dichromate ($K_2Cr_2O_7$), and sulfuric acid (H_2SO_4), indicating that the extract did not contain ethanol, Phytochemical Test The test results showed that the extract contained active compounds, namely polyphenols, terpenoids, and alkaloids.

Bacterial Confirmation Test

The gram staining results showed a reddish color with rod-shaped bacteria, indicating Gram-negative bacteria (*Escherichia coli*). The second bacterial sample showed a purple color and coccus shape, indicating Gram-positive bacteria (*Staphylococcus aureus*). The results of antibacterial testing of sandalwood leaf extract on the growth of *Escherichia coli* bacteria and *Staphylococcus aureus* can be seen in Figure 1 and Figure 2.

Antibacterial Test



(a) (b) (c) (d) (e) (f) (g) (h)

Figure 1. The results of the antibacterial test of sandalwood leaf extract on the growth of *Escherichia coli* bacteria (a) 100% concentration, (b) 50% concentration, (c) 25% concentration, (d) 12.5% concentration, (e) 6.25% concentration, (f) 3.12% concentration, (g) negative control and (h) positive control.

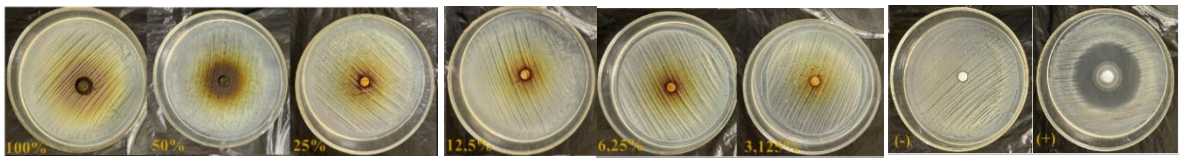
Figure 1 depicts the clear inhibition zones around the paper discs for Concentrations from 100% to 6.25%, with progressively smaller zones as concentration decreases. No visible zone is observed at 3.12% or in the negative control, while the positive control (Ciprofloxacin) shows a large zone, confirming the extract's dose-dependent activity against *Escherichia coli*, a Gram-negative bacterium. This pattern aligns with studies showing ethanol extracts inhibit Gram-negative bacteria through membrane disruption, though less effectively than against Gram-positive ones due to the outer lipopolysaccharide layer.

The results of measuring the average diameter of the inhibition zone of sandalwood leaf extract on the growth of *Escherichia coli* bacteria can be seen in Table 1.

Table 1. Average measurement results of the inhibition zone diameter of sandalwood leaf extract against the growth of *Escherichia coli* bacteria

Extract Concentration	Average Inhibition Zone Diameter (mm)	Potential
Control (+)	51.3	Very Strong
100%	10.1	Strong
50%	9.3	Moderate
25%	7.26	Moderate
12.5%	7.13	Moderate
6.25%	7.13	Moderate
3.12%	0	Weak
Control (-)	0	Weak

Table 1 summarizes the dose-response relationship for *Escherichia coli*, with strong inhibition at 100% (10.1 mm) tapering to moderate levels down to 6.25% (7.13 mm), and no activity at 3.12%. Criteria, this indicates effective antibacterial potential at higher concentrations, comparable to aqueous extracts in other studies that showed 80–87% inhibition at similar doses. The lack of activity at low concentrations highlights a minimum inhibitory concentration (MIC) threshold around 6.25–12.5%.



(a) (b) (c) (d) (e) (f) (g) (h)

Figure 2. The results of the antibacterial test of sandalwood leaf extract on the growth of *Staphylococcus aureus* bacteria (a) 100% concentration, (b) 50% concentration, (c) 25% concentration, (d) 12.5% concentration, (e) 6.25% concentration, (f) 3.12% concentration, (g) negative control and (h) positive control.

Figure 2 illustrates similar inhibition zones for *Staphylococcus aureus*, with slightly larger diameters at equivalent concentrations compared to *Escherichia coli*, indicating higher susceptibility. Zones are evident up to 6.25%, absent at 3.12%, and maximal for the positive control. This suggests the extract's phytochemicals,

such as terpenoids, more readily penetrate the thicker peptidoglycan layer of Gram-positive bacteria like *Staphylococcus aureus*.

The results of measuring the average diameter of the inhibition zone of sandalwood leaf extract on the growth of *Escherichia coli* bacteria can be seen in Table 2.

Table 2. Average measurement results of the inhibition zone diameter of sandalwood leaf extract against the growth of *Staphylococcus aureus* bacteria

Extract Concentration	Average Inhibition Zone Diameter (mm)	Potential
Control (+)	42.03	Very Strong
100%	10.26	Strong
50%	9.5	Moderate
25%	7.8	Moderate
12.5%	7.26	Moderate
6.25%	7.06	Moderate
3.12%	0	Weak
Control (-)	0	Weak

Table 2 reveals slightly larger zones for *Staphylococcus aureus* (e.g., 10.26 mm at 100%) than for *Escherichia coli*, supporting greater efficacy against Gram-positive bacteria. Moderate activity persists to 6.25% (7.06 mm), with no zone at 3.12%, suggesting an MIC similar to but potentially lower than reported in methanol extract studies (around 17%) (Puspawati et al., 2018). This difference may stem from the 70% ethanol solvent enhancing extraction of active compounds.

Confirms statistically significant differences in inhibition zones across treatment groups ($p = 0.002 < 0.05$), rejecting the null hypothesis and indicating the extract's concentration-dependent effects on both bacteria. This non-parametric test was appropriate given the small sample size and non-homogeneous data, aligning with similar experimental designs in phytochemical antibacterial research.

The results of the Post Hoc analysis using the Dunnett T3 test on the growth of *Escherichia coli* bacteria are presented in Table 3.

Table 3. Post Hoc analysis results using Dunnett T3 test on the growth of *Escherichia coli* bacteria

Test Group 1	Test Group 2							
	100%	50%	25%	12.5%	6.25%	3.12%	K(+)	K(-)
100%		0.933	0.059	0.048*	0.048*	0.005*	0.002*	0.005*
50%	0.933		0.203	0.178	0.178	0.012*	0.012*	0.001*
25%	0.059	0.203		0.787	0.787	0.000*	0.004*	0.000*
12.5%	0.048*	0.178	0.787		1.000	0.000*	0.000*	0.004*
6.25%	0.048*	0.178	0.787	1.000		0.000*	0.004*	0.000*
3.12%	0.005*	0.012*	0.000*	0.000*	0.000*		0.003*	-
K(+)	0.002*	0.001*	0.004*	0.004*	0.004*	0.003*		0.003*
K(-)	0.005*	0.012*	0.000*	0.000*	0.000*		0.003*	

Note: denotes a significant difference ($p < 0.05$)

Table 3 highlights pairwise differences for *Escherichia coli*, with significant variations between higher concentrations (100–6.25%) and lower ones (3.12%, controls). For instance, 100% differs significantly from 12.5% and below ($p < 0.05$), underscoring a threshold effect where efficacy drops sharply below 12.5%. This supports the dose-dependency observed in comparable studies on plant extracts.The results of the Post Hoc analysis using the Dunnett T3 test on the growth of *Staphylococcus aureus* bacteria are presented in Table 4.

Table 4. Post Hoc analysis results using Dunnett T3 test on the growth of *Staphylococcus aureus* bacteria

Test Group 1	Test Group 2						
	100%	50%	25%	12.5%	6.25%	3.12%	K(+)
100%		0.899	0.008*	0.025*	0.001*	0.001*	0.000*
50%	0.899		0.381	0.223	0.199	0.016*	0.016*	0.001*
25%	0.008*	0.381		0.790	0.132	0.000*	0.000*	0.000*
12.5%	0.025*	0.223	0.790		1.000	0.009*	0.019*	0.000*
6.25%	0.001*	0.199	0.132	1.000		0.002*	0.002*	0.000*
3.12%	0.001*	0.016*	0.000*	0.009*	0.002*		-	0.000*
K(+)	0.001*	0.016*	0.000*	0.009*	0.002*	-		0.000*
K(-)	0.000*	0.000*	0.001*	0.000*	0.000*	-	0.000*	

Note: denotes a significant difference ($p < 0.05$)

Table 4 shows similar patterns for *Staphylococcus aureus*, with more pronounced significances at mid-concentrations (e.g., 100% vs. 25%: p = 0.008*). Differences are evident between active groups and controls/low concentrations, reinforcing the extract's stronger impact on Gram-positive bacteria, as seen in external research where *Staphylococcus aureus* inhibition reached 87% with *Santalum album* extracts.

The study aims to test the antibacterial effectiveness of sandalwood leaf extract (*Santalum album*) against *Escherichia coli* and *Staphylococcus aureus* by observing the inhibition zones. The average inhibition zone differences produced by the sandalwood leaf extract against both pathogenic bacteria were larger for *Staphylococcus aureus* compared to *Escherichia coli*. At a concentration of 3.12% sandalwood leaf extract, the inhibition zone could no longer be measured, which contrasts with the study by Ni Made Puspawati in 2018, who found the minimum inhibition zone at a concentration of 17% sandalwood leaf extract (Puspawati et al., 2018).

The extraction process using the 70% ethanol maceration technique allows the necessary compounds in the plant to dissolve in the solvent used (Elma Pebryna Putri, 2021) This mixture is then filtered and the solvent is evaporated to obtain a concentrated extract with a high concentration (Elma Pebryna Putri, 2021).

The average inhibition zone diameter measurements for *Escherichia coli* growth at a 100% concentration (10.1 mm) are categorized as strong, 50% concentration (9.3 mm) as moderate, 25% concentration (7.26 mm) as moderate, 12.5% concentration (7.13 mm) as moderate, and 6.25% concentration (7.13 mm) as moderate. The 3.12% concentration and negative control (-) both had a value of 0, while the positive control (+) had a diameter of 51.3 mm.

For *Staphylococcus aureus*, the 100% concentration (10.26 mm) is categorized as strong, 50% concentration (9.5 mm) as moderate, 25% concentration (7.8 mm) as moderate, 12.5% concentration (7.26 mm) as moderate, and 6.25% concentration (7.06 mm) as moderate. The 3.12% concentration and negative control (-) are categorized as weak, as the inhibition zone had a value of 0, while the positive control (+) had a diameter of 42.03 mm.

Data analysis of the research results for both bacteria was conducted using the Kruskal Wallis test, followed by Post Hoc analysis using the Dunnett T3 test. The Kruskal Wallis test is performed under the condition that the data distribution is not normally distributed. Normality testing was done using the Shapiro-Wilk test since this study used a sample of fewer than 50. The normality test results indicated that the data distribution was normal, as the p-value was > 0.05. The Kruskal Wallis test yielded a p-value of 0.002, which is less than $\alpha = 0.05$, so H0 is rejected and H1 is accepted, indicating that there is a significant difference in the mean inhibition zone diameter between the treatment groups. To determine which treatment groups had significant differences in the mean inhibition zone diameter, a Post Hoc test using the Dunnett T3 test was performed because the data were non-homogeneous.

The inhibition zones produced by the sandalwood leaf extract increased with the concentration of the extract. This is in line with the statement Jawetz et al.,(2019) who stated that the effectiveness of an antimicrobial substance is influenced by the concentration of the substance used. An increase in the extract concentration is proportional to an increase in its ability to inhibit microbial growth (Jawetz et al,.2019). The inhibition zone testing of sandalwood leaf extract against the growth of *Escherichia coli* and *Staphylococcus aureus* showed different values. The largest inhibition zone diameter based on the test bacteria was for *Staphylococcus aureus*, followed by *Escherichia coli*. This study used Ciprofloxacin as a positive control and sterile distilled water as a negative control. Ciprofloxacin was used as the positive control (comparator) because it is a second-generation fluoroquinolone with a broad spectrum, active against both Gram-positive and Gram-negative bacteria (Thai et al., 2022).

Based on the discussion above, sandalwood leaf extract (*Santalum album*) has been proven to have antibacterial effectiveness, capable of killing *Escherichia coli* and *Staphylococcus aureus*. The sandalwood leaf extract is expected to be further researched to provide a positive impact on the development of natural antibacterial substances. This proof was conducted through phytochemical examination to identify the chemical compounds in the extract. The phytochemical screening results showed that the extract contains polyphenols, terpenoids, and alkaloids.

Polyphenols work by interfering with the components of peptidoglycan in bacterial cells, preventing the proper formation of the cell wall, leading to cell death (Bae, 2022). Terpenoids can react with porins (transmembrane proteins) in the outer membrane of bacterial cell walls, forming strong polymer bonds and damaging the porins, reducing the permeability of the bacterial cell wall, causing the bacteria to lack nutrients, thus inhibiting growth or causing cell death. (Raza, 2023). The antimicrobial effect of alkaloids is due to their ability to interfere with the components of peptidoglycan in bacterial cells, which prevents the proper formation of the cell wall, resulting in cell death (Tenea, 2022).

CONCLUSION

There is antibacterial effectiveness produced by the sandalwood leaf extract against *Escherichia coli* and *Staphylococcus aureus* bacteria. The inhibition zone diameter produced by the sandalwood leaf extract against *Escherichia coli* bacteria is 10.1 mm at a 100% concentration, 9.3 mm at a 50% concentration, 7.26 mm at a 25% concentration, 7.13 mm at both 12.5% and 6.25% concentrations, and could not be measured at a 3.12% concentration. The inhibition zone diameter produced by the sandalwood leaf extract against *Staphylococcus aureus* bacteria is 10.26 mm at a 100% concentration, 9.5 mm at a 50% concentration, 7.8 mm at a 25% concentration, 7.26 mm at a 12.5% concentration, 7.06 mm at a 6.25% concentration, and could not be measured at a 3.12% concentration.

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CONFLICT OF INTEREST

The authors declare no conflict of interest and take full responsibility for the content of the article, including the implications of AI-generated content.

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