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Comparison of Antibacterial Activity Tests of 70% and 96% Ethanol Extract on Siam Sambas Orange Leaves (Citrus nobilis var. microcarpa)

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Abstract

Background: Antibiotic resistance against bacterial infections such as Escherichia coli and Staphylococcus aureus, prevalent in tropical regions, necessitates alternative treatments. One potential solution is utilizing secondary metabolites found in Sambas Siamese orange leaves. These compounds may serve as antibacterial agents. **Objective:** This study aims to compare the effectiveness of 70% and 96% ethanol as solvents for extracting secondary metabolites from Sambas Siamese orange leaves. **Methods:** Extraction was performed using two types of solvents (70% and 96% ethanol) to obtain secondary metabolites. Antibacterial activity was tested using the disc diffusion method (Kirby-Bauer), and data were analyzed with One-Way Analysis of Variance (ANOVA). **Results:** Extraction with 96% ethanol yielded flavonoids (+++), terpenoids (++), steroids (++), and phenols (++). Meanwhile, extraction with 70% ethanol produced Mayer alkaloids (++), flavonoids (++), saponins (+), steroids (+++), and phenols (+++). The 96% ethanol solvent was more effective in extracting limonene compounds from the leaves. **Conclusion:** The 96% ethanol solvent demonstrated higher effectiveness compared to 70% ethanol in extracting secondary metabolites, particularly limonene, which has potential antibacterial properties, from Sambas Siamese orange leaves.

Keywords: *70% ethanol, 96% ethanol, antibacterial activity, comparison, Sambas Siamese.*

Introduction

Tropical regions are among the areas with high potential for disease spread. Diseases in tropical regions generally relate to individual hygiene in maintaining health[1]. West Kalimantan, included in tropical regions, has a relatively high prevalence of tropical diseases such as diarrhea and pneumonia[2,3]. These diseases are most commonly caused by Escherichia coli and Staphylococcus aureus bacteria.

The potential spread of Escherichia coli and Staphylococcus aureus bacteria is very easy and widespread. Besides being supported by West Kalimantan's geographical conditions as a tropical region, both bacteria have also developed resistance to several antibiotic classes [4,5]. The main trigger for this condition can be inappropriate antibiotic consumption in the community [6,7]. Therefore, treatment using traditional natural ingredients containing compounds that can inhibit the growth of Escherichia coli and Staphylococcus aureus bacteria is needed, one of which is using Sambas Siamese orange leaves (Citrus nobilis var. macrocarpa) [8].

Phytochemical compounds contained in Sambas Siamese orange leaves (Citrus nobilis var. macrocarpa) contain secondary metabolite compounds that can be beneficial, including

flavonoids, terpenoids, alkaloids, steroids, saponins, and phenolics. Among all these compounds, the most dominant is limonene, which belongs to the terpenoid category [9,10]. The limonene compound has many benefits, including functioning as an antioxidant for the body, having the ability to capture free radicals, and containing antibacterial properties [11]. To extract the limonene content in Sambas Siamese orange leaves (Citrus nobilis var. macrocarpa), an extraction process requiring ethanol solvent is needed.

This research was conducted in response to increasing antibiotic resistance against Escherichia coli and Staphylococcus aureus bacteria, which is spreading widely due to inappropriate antibiotic use in the community[12]. This resistance becomes a serious problem in the health world, so effective and safe alternative treatments are needed. One potential solution is utilizing natural ingredients like Sambas Siamese orange leaf extract (Citrus nobilis var. macrocarpa), known to contain secondary metabolite compounds, especially limonene, with potential as antibacterial agents. However, the effectiveness of limonene extraction from these leaves depends on the method and solvent used. This research aims to answer the research question: Is 70% or 96% ethanol solvent more effective in extracting secondary metabolite compounds (limonene) from Sambas Siamese orange leaves? To answer this question, maceration was conducted using both solvents to compare their effectiveness in extracting limonene as the primary bioactive compound.

Materials and Methods

Study Design

This study is an experimental laboratory research with a post-test only group design to evaluate the antibacterial activity of Sambas Siamese orange leaf ethanol extract against Escherichia coli ATCC 25922 and Staphylococcus aureus ATCC 25923 [13].

Sample

The Sambas Siamese orange leaves used as the main research material were obtained from Sungai Kunyit Hamlet, Sambas Regency, West Kalimantan. Leaves were picked in the morning between 08:00–10:00 to maintain quality, with criteria of being fresh, not too old, and undamaged. Sample preparation included washing with running water, cutting into small pieces, and natural drying under sunlight with cloth cover. Dried simplicia were produced by grinding the leaves using a blender until becoming dry powder. This simplicia was extracted using the maceration method with 70% and 96% ethanol solvents in a 3:1 ratio for 72 hours [14]. The maceration filtrate was then concentrated using a rotary evaporator at 50°C to produce concentrated ethanol extract with 100% concentration [15].

Data Collection Techniques

Data collection was conducted through antibacterial activity testing using the disc diffusion method. This test involved six treatment concentrations of extract against Escherichia coli (25%, 50%, 75%, 100%, positive control ciprofloxacin, and negative control sterile distilled water) and six treatments against Staphylococcus aureus (25%, 40%, 55%, 70%, positive control clindamycin, and negative control sterile distilled water) [16–18]. Each treatment was repeated four times. The surface of Mueller Hinton Agar (MHA) media was inoculated with test bacteria using the swab method, then discs soaked in extract were placed on the media [16–18]. After incubation at 35°C for 24 hours, the inhibition zones formed were measured using calipers. Inhibition zone diameters were categorized as weak (≤5 mm), moderate (6-10 mm), and strong (≥10 mm) [19].

Data Analysis Techniques

Antibacterial test result data were analyzed using One-Way Analysis of Variance

(ANOVA) statistical test with 95% confidence level ($p < 0.05$). Before ANOVA, normality tests were conducted using the Shapiro-Wilk method and homogeneity tests using Levene's Test. If significant differences were found, analysis continued with Post Hoc Least Significant Difference (LSD) to determine significant differences between treatment groups. Data analysis was performed using SPSS Statistics version 25.0 software [20].

Ethical Consideration

This research has received ethical approval from the Health Research Ethics Committee of the Faculty of Medicine, Universitas Tanjungpura Pontianak through Ethical Clearance letter No: 6765/UN22.9/PG/2023.

Result

Phytochemical screening was conducted qualitatively by submitting Sambas Siamese orange leaf (Citrus nobilis var. macrocarpa) extract samples to the Chemistry Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Tanjungpura (Table 1).

Table 1. Phytochemical Results

Compound	70% Ethanol	96% Ethanol
Alkaloid Mayer	++	-
Alkaloid Wagner	-	-
Alkaloid Dragendroff	-	-
Flavonoids	++	+++
Terpenoids	-	++
Saponins	+	-
Steroids	+++	++
Phenols	+++	++

Qualitative phytochemical screening results showed that Sambas Siamese orange leaf extract using 70% ethanol solvent contained Mayer alkaloid compounds (++), flavonoids (++), saponins (+), steroids (+++), and phenols (+++). However, the target secondary metabolite compound in Sambas Siamese orange leaf (Citrus nobilis var. macrocarpa) components, namely limonene (terpenoid), could not be extracted using 70% ethanol solvent. This condition contradicts research by Riwanti et al. which showed that 70% ethanol solvent effectiveness in extracting secondary metabolite compounds was better compared to 50% and 96% ethanol solvents [21].

Table 2. Antibacterial Activity Test Results

Concentration	Average (mm)	Description
Escherichia coli 70% ethanol		
25%	0	No inhibition zone formed
50%	0	No inhibition zone formed
70%	0	No inhibition zone formed
100%	0	No inhibition zone formed
Control (-)	0	No inhibition zone formed
Control (+)	33.49	Sensitive
Staphylococcus aureus 96% ethanol		
25%	7.15	Moderate
40%	8.43	Moderate
55%	10.88	Moderate
70%	9.23	Moderate
Control (-)	0	No inhibition zone formed
Control (+)	29.86	Sensitive

Qualitative phytochemical screening results also showed that Sambas Siamese orange leaf extract using 96% ethanol solvent contained flavonoid compounds (+++), terpenoids (++), steroids (++), and phenols (++). The use of 96% ethanol solvent could extract the target secondary metabolite compound in Sambas Siamese orange leaf components, namely limonene (terpenoid). This condition aligns with the theory that 96% ethanol has a polarity level very similar to bioactive compounds, one of which is found in Sambas Siamese orange leaves, namely limonene (terpenoid) [22].

In antibacterial activity testing on Sambas Siamese orange leaf extract using 96% solvent, moderate category inhibition zones were found at all test concentrations. Meanwhile, in antibacterial testing on leaf extract using 70% solvent, no inhibition zones were found at all test concentrations. Positive control using ciprofloxacin for Escherichia coli testing showed sensitive category results with an average value of 33.49 mm, while positive control using clindamycin for Staphylococcus aureus testing showed sensitive category results with an average value of 29.86 mm (Table 2).

Discussion

Based on antibacterial activity test results on Sambas Siamese orange leaf extract, significant test result differences were found. Antibacterial testing of leaf extract using 96% ethanol solvent showed moderate category inhibition zone activity at concentrations of 25%, 40%, 55%, and 70%. Meanwhile, in antibacterial testing of leaf extract using 70% ethanol solvent, no inhibition zones were found at concentrations of 25%, 50%, 75%, and 100%. The difference in test results is greatly influenced by several differences in test variables such as solvent type differences, metabolite compound content, and bacterial cell wall structure.

Solvent Type Differences

The ethanol solvent used in the research was chosen because ethanol has advantages of being inexpensive, relatively environmentally friendly, and non-toxic compared to acetone and methanol [23]. Additionally, extracts using ethanol solvent tend to contain more dominant phytochemical compounds compared to using other solvents like diethyl ether and chloroform[24]. This is because ethanol has good ability in extracting compounds with polar, non-polar, and semi-polar structures [25]. The choice of 96% ethanol solvent was proven to extract phytochemical compounds, especially terpenoids, from leaves compared to 70% ethanol solvent. Terpenoid compound content is the main target compound in Sambas Siamese orange leaf extract. This condition will greatly affect antibacterial activity against Escherichia coli and Staphylococcus aureus because terpenoid compounds have mechanisms to damage bacterial cell walls by performing reactions on transmembrane proteins [26].

Metabolite Compound Content

Differences in metabolite compound content in Sambas Siamese orange leaf extract results using 70% and 96% ethanol solvents greatly affect the antibacterial activity occurring in Escherichia coli and Staphylococcus aureus. In leaf extract with 70% solvent, low saponin content (+), moderate alkaloid content (++), moderate flavonoid content (++), high phenolic content (+++), and high steroid content (+++) were found. Meanwhile, in Sambas Siamese orange leaf (Citrus nobilis var. macrocarpa) extract with 96% solvent, moderate phenolic content (++), moderate terpenoid content (++), moderate steroid content (++), and high flavonoid content (+++) were found. The difference in content between the two solvents lies in terpenoid and alkaloid compounds. Terpenoid compounds found in 96% ethanol solvent were proven to provide antibacterial effects against Staphylococcus aureus with inhibition zones in the moderate category. The working mechanism of terpenoid compounds damaging cell walls by making bond reactions on transmembrane proteins causes damaged cell walls, allowing terpenoid compounds to enter bacteria and inhibit Staphylococcus aureus bacterial growth [26]. Staphylococcus aureus bacteria have simpler cell wall structures compared to gram-negative bacteria. Gram-positive bacteria have cell walls composed of thick peptidoglycan without lipopolysaccharides[27]. In gram-negative bacteria, lipopolysaccharides are located on the outer membrane, making phytochemical compounds more difficult to enter bacterial cytoplasm [28]. Additionally, terpenoids also have lipophilic properties that function to penetrate lipid layers of gram-negative bacterial cell walls [29]. The absence of terpenoid compound content in 70% ethanol solvent against antibacterial activity testing in Escherichia coli resulted in bacteria still growing at all test concentrations. Alkaloid compound content in 70% ethanol solvent could not provide antibacterial effects against Escherichia coli. Alkaloid compounds tend to have working mechanisms by damaging

bacterial cell walls [30]. However, in this study, alkaloid derivatives could not be identified because phytochemical screening was conducted qualitatively.

Steroid, saponin, and phenolic compound content in 70% ethanol solvent could not provide antibacterial effects against Escherichia coli. High phenolic content tends to have more effective working mechanisms against gram-positive bacteria compared to gram-negative. This is based on phenolic working mechanisms damaging cell membrane permeability by utilizing hydrogen bonds to create organelle leaks in bacterial cytoplasm [31]. This mechanism certainly depends on different bacterial growth in each variant [32]. Gram-negative bacteria tend to be more resistant to phenolics because they have more complex cell wall structures than gram-positive bacteria.

In gram-positive bacteria, steroid, saponin, and phenolic content have effectiveness in creating inhibition zones against bacteria. Phenolic compounds, which are polyphenol compounds, work by disrupting bacterial cell wall integrity and altering bacterial membrane permeability[31]. Moderate phenolic concentration prevents the formation of N-acetylmuramic acid and murepeptide acid bonds [27]. Additionally, steroids and saponins have activity to disrupt cell permeability, thus affecting permeability control of bacterial cell walls against substances entering and exiting the cytoplasm [33]. In Kim et al.'s research, it was found that antibacterial activity against Staphylococcus aureus could be disrupted in biofilm formation with diosgenin content [34].

Bacterial Cell Wall Structure

Differences in gram-positive and gram-negative bacterial cell wall structures also influence the effectiveness of phytochemical compounds present. In gram-positive bacteria, cell walls consist of 2 layers: peptidoglycan and plasma membrane. The peptidoglycan structure in gram-positive bacteria is much thicker than in gram-negative bacteria [35]. In gram staining, gram-positive bacteria appear purple because crystal violet dye is trapped in peptidoglycan [36]. This peptidoglycan layer contains sugars (N-acetylglucosamine and N-acetylmuramic acid) and short amino acid chains. With this structure, gram-positive bacterial cell walls have rigid and quite strong structures [37].

Gram-negative bacteria have more complex cell wall arrangements consisting of 3 layers: outer layer, lipopolysaccharide, and inner membrane. The outer membrane of gram-negative bacteria contains phospholipids bound to bacterial membranes and lipopolysaccharides. Gram-negative bacterial cell wall ability can also perform selection against micronutrients entering cells[38]. The complexity of gram-negative bacterial structures makes it difficult for phytochemical compounds to enter the cytoplasm, and this is also supported by the content of target phytochemical compounds having effective working mechanisms against gram-negative bacteria, namely terpenoid compounds. The working mechanisms of phytochemical compounds in 70% ethanol solvent tend not to be very effective against gram-negative bacteria.

Conclusion

Sambas Siamese orange leaf ethanol extract with 96% ethanol solvent was more effective for attracting secondary metabolite compounds compared to Sambas Siamese orange leaf ethanol extract with 70% ethanol solvent, resulting in better antibacterial activity test results. For further research, it is recommended to use the same test bacteria for both solvents so results can be more optimal.

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