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POTENTIAL OF SAMBAS SIAM ORANGE (*Citrus nobilis var. microcarpa*) LEAVES ETANOL EXTRACT AGAINST *Escherichia coli*

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ABSTRAK

Escherichia coli memiliki faktor virulensi dan mampu untuk menghindari pertahanan inang serta resisten terhadap antibiotik. Peningkatan resistensi memerlukan terapi alternatif, diantaranya dari bahan alam. Potensi yang berasal dari Kalimantan Barat ialah jeruk siam sambas (Citrus nobilis var. microcarpa). Semua bagian tanaman ini mengandung metabolit sekunder yang dapat dimanfaatkan, misalnya bagian daun. Senyawa-senyawa yang terkandung dalam metabolit sekunder memiliki sifat sebagai antibakteri. Penelitian ini untuk mengetahui potensi antibakteri dari ekstrak etanol daun jeruk siam sambas terhadap pertumbuhan bakteri Escherichia coli. Metode Daun jeruk diekstrak menggunakan etanol 70% kemudian dilanjutkan analisis fitokimia dan uji aktivitas antibakteri menggunakan metode difusi cakram. Hasil Analisis fitokimia menunjukkan ekstrak etanol daun jeruk siam sambas mengandung fenolik, alkaloid, saponin, flavonoid, dan steroid. Pengujian aktivitas antibakteri pada semua konsentrasi menunjukkan tidak adanya zona hambat. Kesimpulan Ekstrak etanol daun jeruk siam sambas tidak memiliki aktivitas antibakteri terhadap pertumbuhan bakteri Escherichia coli.

ABSTRACT

Potential Of Sambas Siam Orange (Citrus Nobilis Var. Microcarpa) Leaves Etanol Extract Against Escherichia coli. Escherichia coli has virulence factors and is able to avoid host defenses, and is resistant to antibiotics. Increasing resistance requires alternative therapies, including natural ingredients. The potential that comes from West Kalimantan is the Siamese orange (Citrus nobilis var. microcarpa). All parts of this plant contain secondary metabolites that can be utilized, for example the leaves. The compounds contained in secondary metabolites have antibacterial properties. This study was to determine the antibacterial potential of the ethanol extract of Siamese orange leaves on the growth of Escherichia coli bacteria. Method The orange leaves were extracted using 70% ethanol then continued with phytochemical analysis and antibacterial activity testing using the disc diffusion method. The results of the phytochemical analysis showed that the ethanol extract of Siamese orange leaves contained phenolics, alkaloids, saponins, flavonoids, and steroids. Antibacterial activity testing at all concentrations showed no inhibition zone. Conclusion The ethanol extract of Siamese orange leaves did not have antibacterial activity on the growth of Escherichia coli bacteria.

INTRODUCTION

The use of antibiotics is one way that is often used in cases of infection.[1] However, the World Health Organization (WHO) states that antibiotic resistance in *Escherichia coli* has become a worrying problem worldwide.[2] *Escherichia coli* isolates taken from several specimens in Pontianak City, Indonesia, are reported to be resistant to several classes of antibiotics such as aminopenicillin, cephalosporin, monobactam, beta-lactam, fluoroquinolone, and sulfonamide.[3] Without the effective use of antibiotics, many medical procedures are at risk of causing resistance. Therefore, it is necessary to find alternative antibiotics from herbs easily found around, especially in West Kalimantan, namely sambas siam orange (*Citrus nobilis var. microcarpa*).

There is potential genetic diversity in citrus fruits found in Indonesia.[4] One type of citrus that grows in Indonesia is Siam oranges from the Sambas district (*Citrus nobilis var. microcarpa*), with a total yield 2019 of 2,296,297 tonnes.[5,6] Sambas Siam oranges (*Citrus nobilis var. microcarpa*) are from the Rutaceae family and the genus Citrus. In this plant, both the fruit and other plant parts, have content that can be used for medical purposes, especially the leaves of the plant, which are still minimally utilized. The most common compound in the essential oil of citrus plant leaves is limonene, one of the terpenoid groups. This compound has antimicrobial properties that can be utilized as an antibacterial.[7]

Essential oils from citrus leaves have strong antioxidants and antimicrobial activities. Previous research shows that limonene compound is 62% in most orange leaf essential oils.[7] Limonene is an antioxidant that fights free radicals, prevents and treats cancer, and reduces the risk of degenerative diseases.[8] Another potential property of limonene is as an antimicrobial. Based on research conducted by *Gupta et al*.[9], limonene preparations in pure form, nano emulsion, and essential oil limonene can inhibit Gram-positive and Gram-negative bacteria growth. The high content of limonene in citrus leaf essential oil can be utilized as an antibacterial alternative. Limonene is also suspected to be present in the ethanol extract of sambas siam orange leaves (*Citrus nobilis var. microcarpa*).

The utilization of other plant parts besides the fruit of the citrus plant is still very minimal, especially the use of leaves. The leaves of citrus plants can still be extracted and utilized for the phytochemical content contained there.[10] Previous research showed that the use of ethanol extract of Siam orange leaves (*Citrus nobilis*) with a concentration of 50% was able to inhibit the growth of *Pseudomonas aeruginosa* bacteria by 8.5 mm and *Staphylococcus aureus* by 7.5 mm.[10] Another study showed that Siam orange juice (*Citrus nobilis*) with a concentration of 50% inhibited the growth of *Escherichia coli* bacteria by 10.5 mm.[11,12] In addition, the potential originating from Pontianak in the form of sambal orange juice (*Citrus microcarpa* Bunge) with a concentration of 50% was able to inhibit *Staphylococcus aureus* bacteria by 16.04 mm and Escherichia coli by 7.93 mm.[13,14]

The high cases of bacterial resistance to antibiotics in Pontianak, natural resources in the form of large sambas siam orange fruit plantations, and the lack of utilization of the leaves of plants that contain much limonene are the background of this study. Supported by the lack of similar research using sambas siam orange, this study was conducted to find the antibacterial potential of ethanol extract of sambas siam orange leaves (*Citrus nobilis var. microcarpa*) against the growth of *Escherichia coli* bacteria.

METHOD

The study was divided into preparation and making extracts to test antibacterial activity. The extract used came from sambas siam orange leaves (*Citrus nobilis var. microcarpa*) obtained from a plantation in Sungai Pinang hamlet RT.001/RW.001, Sungai Rambah village, Sambas district, Sambas regency, West Kalimantan, Indonesia. Then extracted using 70% ethanol solvent. While the test bacteria used were *Escherichia coli* ATCC 25922.

1. Extract preparation

In the morning, Sambas siam orange leaves were collected in Sungai Rambah village, Sambas district, West Kalimantan. The leaves were then sorted between leaves in good condition and those that were not. The leaves were washed and cleaned and then dried in the sun. After drying, the leaves are made into powdered simplisia and then stored in a dry place without sunlight.

The simplisia powder was then macerated with 70% ethanol. The ratio between powder and extract was 1:3 for 1x24 hours and repeated 3 times. The results of maceration (macerate) were then filtered using Whatman paper no. 1. The macerate was then concentrated using a rotary evaporator and oven at 50°C to obtain a condensed extract.

The condensed extract was then stored in a glass bottle, covered using aluminum foil, and stored in a refrigerator at 4°C. The phytochemical test process on the extract to see the content of secondary metabolites using qualitative methods. The following compounds were tested in this study:

1.1. Phenolic

As much as 50 mg of extract was dissolved in 5 mL of distilled water. Then, a few drops of neutral 5% $FeCl_3$ were added to the extract solution. A solid green colour indicates the presence of phenolic compounds in the tested extract.

1.2. Alkaloid

The alkaloid test was carried out using the Mayer method. The extract was dissolved in HCl and filtered. Then, 1-2 drops of Mayer's reagent were added to a small filtrate. The reagent was applied to the side of the test tube. A cloudy white or yellow colour indicates a positive result for the presence of alkaloids.

1.3. Saponin

As many as 0.2 g of extract sample was put into a test tube. Add 2 mL of distilled water, then shake vigorously for 30 seconds. The formation of a stable froth characterizes positive results for the presence of saponins.

1.4. Flavonoid

A sample of 0.2 g was added with 0.1 g of Mg powder and a few drops of 2N HCl. The formation of orange to red colour indicates the presence of flavonoid compounds.

1.5. Steroid and Terpenoid

A sample of 0.2 g was put into a test tube and added with 1-2 drops of glacial acetic acid and 1-2 drops of concentrated sulfuric acid solution (H_2SO_4). A greenish colour will indicate positive results for the presence of steroids, while changes in colour to red or orange indicate the presence of terpenoid compounds.

2. Antibacterial activity tests

The antibacterial activity test of ethanol extract of sambas siam orange leaves against Escherichia coli bacteria was carried out using the disc diffusion method (Kirby-Bauer) on MHA media. There were six groups: disc 1 with 25% extract concentration, disc 2 with 50% concentration, disc 3 with 75% concentration, disc 4 with 100% concentration, positive control disc (ciprofloxacin), and negative control disc (DMSO 5%). Concentration was made by mixing the extract with 5% DMSO according to the desired concentration with the formula $V_1 \times M_1 = V_2 \times M_2$. Each test was repeated 4 times. The media was then incubated, and the diameter results were measured and interpreted to evaluate the antibacterial activity using a vernier caliper.

RESULT

The results of phytochemical screening on ethanol extract of sambas siam orange leaves obtained have high levels (+++) of phenolics and steroids, moderate levels (++) of alkaloids and flavonoids, low levels (+) of saponins, and none (-) of terpenoids. The following phytochemical screening results are attached in **Table 1.** following with the interpretation of (+) low, (++) moderate, (+++) high, and (-) none.

Table 1. Phytochemical screening of ethanol extract of sambas siam orange leaves

Uji	Pereaksi	Results	Interpretation
Phenolic	$FeCl_3$ 5%	It forms an intense green colour	+++ (high)
Alkaloid	Mayer	It forms a cloudy green colour	++ (moderate)
Saponin	H_2O	It forms a small amount of froth	+ (low)
Flavonoid	Mg + HCl	Formed red colour	++ (moderate)
Steroid	$CH_3COOH + H_2SO_4$	Formed a greenish colour	+++ (high)
Terpenoid	$CH_3COOH + H_2SO_4$	Formed a greenish colour	- (none)

The results of the antibacterial activity test in **Figure 1.** showed no inhibition zone at concentrations of 25%, 50%, 75%, and 100%. The absence of an inhibition zone at each concentration can be interpreted that the extract used on MHA media has no antibacterial activity against the growth of *Escherichia coli* bacteria. The control group was also used in this study to compare the presence or absence of antibacterial activity. The negative control of 5% DMSO in this study did not form an inhibition zone on MHA media, meaning there is no antibacterial activity against *Escherichia coli* bacteria in the diluent. In contrast, **Figure 2.** shows that the positive control of ciprofloxacin 5 µg/disk produced an inhibition zone of 33.5 mm with a standard deviation of 0.46 mm, which is included in the sensitive category based on the interpretation of the diameter of the inhibition zone of ciprofloxacin antibiotic according to CLSI.[15]

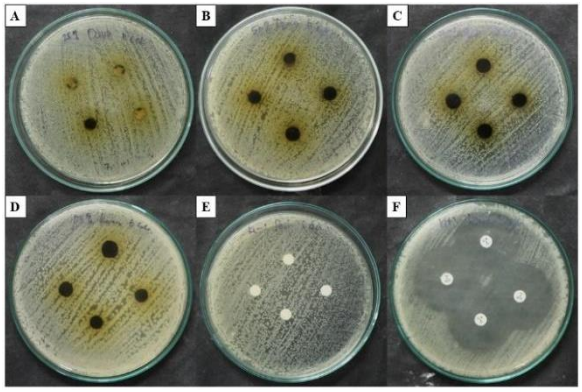

Figure 1. Antibacterial activity test of ethanolic extract of sambas siam orange leaves against *Escherichia coli*. (A) 25% extract concentration. (B) 50% extract concentration. (C) 75% extract concentration. (D) 100% extract concentration. (E) Negative control. (F) Ciprofloxacin as positive control.

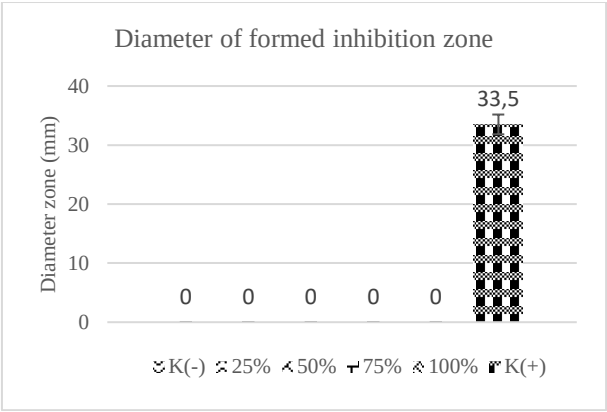

Figure 2. Interpretation of antibacterial activity test

DISCUSSION

The extraction process starts from collecting leaves in the plantation in Sungai Pinang subvillage RT.001/RW.001, Sungai Rambah village, Sambas sub-district, Sambas district, West Kalimantan, Indonesia. Based on data from the Penjabat Pengelola Informasi dan Dokumentasi (PPID) of Sambas Regency, alluvial soil types dominate the area of Sambas Regency by 36.06% (230,630 ha) of the total area, making it suitable for the agricultural sector.[16] The leaves used were shiny green old leaves that were clean from impurities and picked before 10 am. Excessive UV light affects the stability of secondary metabolite content contained in the leaves because metabolite compounds are generally thermolabile.[17]

The compounds contained in *Citrus medica*, such as alkaloids, glycosides, steroids, flavonoids, carbohydrates, phenolics, terpenoids, and phytosterols, were found to be contained in methanol extracts made from the leaves of the plant.[18] In another study using *Citrus limon* leaves, the content of secondary metabolites obtained in flavonoids, saponins, phenols, and alkaloids was found in high levels.[19] The juice of *Citrus tangerine*, *Citrus paradisi*, *Citrus limon*, and *Citrus aurantifiola*, contains alkaloid compounds, phenols, flavonoids, steroids, terpenoids, and saponins.[20] So, the compounds found align with previous studies that compounds such as phenolics, alkaloids, flavonoids, and saponins are common compounds found in the leaves of citrus plants.

The secondary metabolite level of a plant extract is strongly influenced by the type of solvent used. The use of ethanol solvents can attract more compounds than methanol solvents.[11] This is also supported by previous research that 70% ethanol can attract more diverse compounds than 50% and 96% concentrations.[21] Increasing ethanol concentration can increase the diffusion rate and extraction process. However, when the ethanol concentration is greater than 70%, the level of target components decreases slightly due to denaturation at higher ethanol concentrations. [22] This is supported by another study conducted by *Riwanti et al*.[21], which found that 70% ethanol can extract more flavonoids dissolved in 70% ethanol solvent than 50% and 96% ethanol. However, terpenoid compounds, the target compounds, are less effectively withdrawn using 70% ethanol solvent due to differences in polarity.

Secondary metabolites are also strongly influenced by the environment. The content of secondary metabolites of a plant is strongly influenced by the climatic conditions in which the plant grows. Research conducted by *Saini et al*.[23] showed that the phenolic and limonoid contents contained in known orange fruit were higher in climates with high humidity. On the other hand, in dry climates, the limonoid content tends to be lower.

The test of ethanol extract of sambas siam orange leaves at all concentrations showed that ethanol extract of sambas siam orange leaves on MHA media did not have antibacterial activity against the growth of *Escherichia coli*. The absence of an inhibition zone can be due to several factors, such as the absence of target compounds in the extract, the structure of Gram-negative bacteria, and the mechanism of action of secondary metabolites. The following factors cause the absence of inhibition zone on *Escherichia coli* bacteria in this study:

1. Absence of the target compound in the extract.

   The absence of the initial target compound, limonene, a terpenoid-derived compound, was the main factor in not forming an inhibition zone around the disc. The terpenoid group is a non-polar compound, so it can be better extracted using solvents with the same properties. Non-polar solvents such as n-hexane or 96% ethanol can be used to extract non-polar compounds.[21,24] All terpenoid derivatives have antibacterial properties against Gram-positive and Gram-negative bacteria with diverse mechanisms of action.[25] This is the same as previous research that terpenoids and terpenes in processed natural ingredients can inhibit the growth of *Escherichia coli* bacteria.[26]

   In general, the mechanism of action of terpenoids is to damage the bacterial cell wall and inhibit the synthesis of proteins and nucleic acids.[27] Limonene, the target compound in this study, also has antibacterial properties. The mechanism of the antibacterial action of limonene is inhibiting the synthesis of proteins and nucleic acids to prevent bacteria from replicating.[28] Limonene compounds can inhibit *Escherichia coli, Staphylococcus aureus*, and *Bacillus subtuilis* bacteria with a significant minimum inhibitory concentration value of 1 µg/mL.[29] In addition, the lipophilic nature of the terpenoid class makes it easy for terpenoid compounds to penetrate the lipid layer owned by bacteria so that they can attack from within the cell.[25]

   According to research from *Chueca et al.*[30], using pure limonene on *Escherichia coli* bacteria can kill bacteria. The essential oil dosage form containing limonene showed inhibition against *Escherichia coli* bacteria. Because limonene is a common content found in oranges, other citrus varieties such as *Citrus sinensis* Linn, *Citrus paradisi*, and *Citrus grandis*, which are then extracted into essential oils, have antibacterial activity against *Escherichia coli* bacteria.[31–33] This is also

supported by research conducted by *Gupta et al*.[9], who found 60 similar articles discussing the effects of limonene on Gram-positive and Gram-negative bacteria.

2. The structure of Gram-negative bacteria.

   A possible factor in the absence of antibacterial activity is that *Escherichia coli* has a Gram-negative bacterial structure, with three layers of wrapping consisting of an inner membrane, peptidoglycan, and outer membrane.[34–36] The outermost layer, called the outer membrane, is a protective envelope and has the unique ability to distinguish Gram-negative bacteria from Gram-positive ones. In addition, the outer membrane also has proteins in the form of porins that can filter small molecules such as amino acids to enter the cell.

   The outer membrane structure in Gram-negative bacteria is the main reason these bacteria can form antibiotic resistance in many spectrums. Porins in the outer membrane function as an entrance and filter what substances can enter and not into bacterial cells.[37] So, the antibacterial effect of phenolics is more effective against Gram-positive bacteria than Gram-negative bacteria because there are differences in the cell membrane structure of each bacteria.[36] In other plants, the antibacterial effect extracted from green tea seeds is greater on *Staphylococcus aureus* (Gram-positive) bacteria compared to *Salmonella enteridis* and *Escherichia coli* (Gram-negative) bacteria.[38]

3. Mechanism of action of secondary metabolites

   Phenolic is a class of antioxidants that have antibacterial properties. The mechanism of action of phenolics inhibits bacterial growth by damaging the permeability of cell membranes using hydrogen bonds; changes in intracellular functions will occur along with the loss of cell membranes that make organelles leak from the cell. Because of this mechanism of action, the impact produced by phenolics on growth in each bacterium is different. Based on research conducted by *Bouarab-Chibane, et al*.[36] the antibacterial effect of phenolics is more effective against Gram-positive bacteria than Gram-negative because there are differences in the cell membrane structure of each bacterium. This makes Gram-negative bacteria more resistant to plant secondary metabolites, including phenolics, because the complexity of the bacterial cell membrane makes phenolic compounds enter slowly.[36]

The absence of antibacterial activity of ethanol extract on *Escherichia coli* can be due to two main factors: the absence of target compounds and the structure of *Escherichia coli* bacteria. The absence of target compounds used as the initial target, namely terpenoid-derived compounds, limonene, was the first factor causing the formation of no inhibition zone. Based on the results of a study conducted by *Eloff*,[39] plant secondary metabolite compounds that can inhibit bacterial growth generally have non-polar properties. Non-polar terpenoid groups are more effectively extracted using solvents with the same polarity level. Non-polar solvents such as n-hexane or 96% ethanol can be used to draw compounds that have non-polar properties.[21,24]

The second factor that can cause the absence of antibacterial activity is the structure of Gram-negative bacteria such as *Escherichia coli*, which has a membrane consisting of three layers.[34,35,40] The structure of the outer membrane, which can adapt to antibacterial compounds by changing porin proteins or becoming hydrophobic, is the reason for the absence of antibacterial

effects.[37] Then, the structure of the bacterial cell membrane, consisting of three layers, makes it slow for secondary metabolites to enter and carry out their mechanism of action. This structure makes Gram-negative bacteria more resistant to plant secondary metabolites than Gram-positive bacteria.[36] Besides, phytochemicals contained in extracts such as phenolics, alkaloids, saponins, flavonoids, and steroids are lipophilic.[40,41]

CONCLUSION

This study concludes that ethanol extract of sambas siam orange leaves (*Citrus nobilis var. microcarpa*) contains secondary metabolites in the form of phenolics, alkaloids, saponins, flavonoids, and steroids. It does not have antibacterial activity against *Escherichia coli* bacteria. Based on the results of this study, further studies are needed regarding the potential of siam sambas orange leaves against Gram-positive bacteria. In addition, it is necessary to conduct further studies on differences in the use of solvents for the extraction process of sambas siam orange leaves (*Citrus nobilis var. microcarpa*).

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