

Comparison of the Antibacterial Activity of *Jeruk Sambal* (*Citrus microcarpa* Bunge) Peel Extract Against Gram-Positive and Gram-Negative Bacteria

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Abstract

Antimicrobial resistance has increased the need to explore plant-based sources of antibacterial agents. This study evaluated the phytochemical constituents and compared the antibacterial activity of ethanol extract of *jeruk sambal* (*Citrus microcarpa* Bunge) peels against gram negative bacteria (*Escherichia coli*) and gram positive bacteria (*Staphylococcus aureus*). A laboratory experimental study was performed using a completely randomized design with a post-test-only control group. Phytochemical compounds were identified through qualitative screening, while antibacterial activity was measured using the disc diffusion method. The extract contained phenolics (+++), flavonoids (+++), alkaloids (++), saponins (+), and tannins (+). Inhibition of gram negative bacteria was observed at 60% and 80%, with inhibition zones of 7.80 and 7.83 mm, respectively. For *gram positive bacteria*, inhibition occurred at 40%, 60%, and 80%, measuring 6.93, 8.88, and 10.25 mm, respectively. The extract showed moderate antibacterial activity, with stronger inhibition against gram positive bacteria 60% concentration demonstrated the most effective activity against both bacteria.

Keywords: Antibacterial activity; *Citrus microcarpa* Bunge Peel; Gram-negative bacteria; Gram-positive bacteria; Phytochemical screening.

Abbreviations: CRD: Completely Randomized Design; MHA: Mueller-Hinton Agar; DMSO: Dimethyl Sulfoxide; EMB: Eosin Methylene Blue; BAP: Blood Agar Plate; MSA: Mannitol Salt Agar; ATCC: American Type Culture Collection; CFU/mL: Colony Forming Units per milliliter; MRSA: Methicillin-Resistant *Staphylococcus aureus*; UTI: Urinary Tract Infection; CLSI: Clinical and Laboratory Standards Institute; ANOVA: Analysis of Variance; GH test: Games-Howell test.

INTRODUCTION

Bacterial infection is one of the most crucial health problems that has been a burden for worldwide health (Long et al., 2018). The two most common Gram-negative and Gram-positive bacteria that become the pathogen and are considered to pose the greatest threat to human health are *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) (WHO, 2017). Several infectious diseases caused by *E. coli* are urinary tract infection (UTI), diarrhea, abdominal and pelvic infections, pneumonia, bacteremia, and meningitis (Rahayu et al., 2018; Mueller & Tainter, 2022). Meanwhile, various diseases caused by *S. aureus* including skin and soft tissue infections, endocarditis, osteomyelitis, bacteremia, septic arthritis, prosthetic device infections, lung infections, gastroenteritis,

meningitis, toxic shock syndrome, and UTI (Tong et al., 2015; Siddiqui & KJ, 2022).

Common empiric therapies used to treat bacterial infections are antibiotics. However, the irrational use of antibiotics leads to the increases in resistance problems resulting the treatment to become more difficult (Guo et al., 2020; Murray et al., 2016). The research showed that *E. coli* is resistant to many of the most commonly used antibiotics (WHO, 2017; Mortazavi-Tabatabaei et al., 2019). Meanwhile, the emergence of Methicillin-Resistant *Staphylococcus aureus* (MRSA) has become a serious worldwide clinical threat (Siddiqui & KJ, 2022; Taylor & Unakal, 2022; Turner et al., 2019). Furthermore, *E. coli* and *S. aureus* has been included in WHO global priority pathogens list of antibiotic-resistant bacteria as the critical priority and high priority. The discovery of resistant *E. coli* and *S. aureus* is a serious

threat that must be resolved immediately (WHO, 2017). An effort that can be made is to look for an alternative natural resource that can be used as a drug candidate (Perdani & Hasibuan, 2021).

Indonesia is very rich in potential biological natural resources to be developed into traditional medicine. The government also continues encouraging the development of traditional medicine from natural ingredients (Kemenkes RI, 2019). *Jeruk sambal* (*Citrus microcarpa* Bunge) is a plant that is abundant found in various Asian countries including Indonesia, especially in the region of West Kalimantan (Nguyen et al., 2018; Widyasari et al., 2018). There are many fruits and drinks made from jeruk sambal, while the peel is only used as complementary certain dishes (Widyasari et al., 2018).

According to the previous research by Asseggaf et al.; (2021) it is known that jeruk sambal juice containing secondary metabolite compounds such as phenolics, flavonoids, alkaloids, saponins, and tannins. Another research by Wulandari et al. (2013) also found that jeruk sambal peel containing several similar compounds such as flavonoids, polyphenols, alkaloids, and steroids. It is known that these compounds have antibacterial activity against both negative and positive bacteria such as *E. coli* and *S. aureus* (Amiliah et al., 2021). Regarding to the potential of jeruk sambal peel as an antibacterial agent, researchers are interested in analyzing secondary metabolite compounds and in vitro antibacterial activity of ethanol extract of *jeruk sambal* (*Citrus microcarpa* Bunge) peels against the growth of *E. coli* and *S. aureus* bacteria.

MATERIALS AND METHODS

Samples Preparation

Jeruk sambal (*Citrus microcarpa* Bunge) fruits were obtained by direct picking from the trees planted in Kalimas Village, Sungai Kakap District, Kubu Raya Regency, Indonesia. The fruits then washed thoroughly with running water. Once clean, the chopping process was carried out, the fruits were cut and sliced into small pieces, leaving the peel for later air-drying process. The dry simplicia of *jeruk sambal* peels were then smoothed with a blender until it becomes a powder.

Extraction Process

500 grams of *jeruk sambal* fruits peel powder was collected and weighed then extracted using the maceration method with 70% ethanol solvent in a ratio of 3:1 for 3 x 24 hours and occasionally stirred. The macerate is then concentrated using a rotary evaporator at a temperature of 50°C so that the solvent and extract can be separated. The ethanol extract produced from this treatment is a condensed extract with a concentration of 100%.

Phytochemical Screening

Phytochemical screening of the ethanol extract of *jeruk sambal* peels was determined qualitatively using various types of reagents to identify the presence of secondary metabolite compounds such as phenolics, alkaloids, flavonoids, saponins and tannins. The results of current research's phytochemical screening were interpreted as a little (+), moderate (++), lots (+++), and none (-).

Instrument and Materials Sterilization

All instruments that will be used in the antibacterial activity testing process were washed with running water and then dried. Mueller-Hinton Agar (MHA) media, distilled water, 0.9% NaCl, and glassware were sterilized using an autoclave at a temperature of 121°C for 2 hours. Inoculating loops and tweezers were sterilized by exposing them to direct heat.

Bacterial Identification

The bacteria used in current research were Gram-negative bacteria, represented by *Escherichia coli* (ATCC 25922) and Gram-positive bacteria which represented by *Staphylococcus aureus* (ATCC 25923). *E. coli* was sub-cultured on MacConkey agar and Eosin-Methylene Blue (EMB) agar, while *S. aureus* was sub-cultured on Blood Agar Plate (BAP) and Mannitol-Salt Agar (MSA) media. The media were incubated for 24 hours at the temperature of 37°C then the colonies were identified morphologically. Furthermore, both *E. coli* and *S. aureus* were identified microscopically by Gram-staining method. Specifically for *S. aureus*, biochemical tests were determined with catalase and coagulase tests.

Variations in Extract Concentrations Making

The condensed extract with a concentration of 100% diluted by adding 1% DMSO solution so the varying concentrations of *jeruk sambal* peels ethanol extract could be made at 20%, 40%, 60% and 80%. The formula used for making extract variations was $M1 \times V1 = M2 \times V2$.

Bacterial Suspensions Making

Both *E. coli* and *S. aureus* colonies were taken with each inoculation loops and then suspended in test tubes containing 5 mL of sterile 0.9% NaCl solution. The densities of the suspensions were equalized with the McFarland 0.5 standard solution (1.5×10^8 CFU/mL) (Vandepitte et al., 2011).

Antibacterial Activity Testing

The antibacterial activity testing in this research was determined using the disc diffusion method. There were on a total of six treatment groups consisting of two control groups and four tested groups that were treated by placing the paper discs on both *E. coli* and *S. aureus* colonies cultured on MHA media. The tested groups were ethanol extract of *jeruk sambal* peels with varying

concentrations of 20%, 40%, 60%, and 80%, while the control groups were ciprofloxacin 5 µg/disc (*E. coli*) and clindamycin 2 µg/disc (*S. aureus*) as positive control, and 1% DMSO as negative control.

The MHA media were incubated at 37°C for 18 hours and then observed whether an inhibition zone in the form of a clear zone was formed (Nurhayati et al., 2020). Measurement of the inhibition zone on MHA media was carried out by calculating the diameter of the clear zone formed around the paper disc using a caliper from three different sides by adding them then dividing by three, so the means were obtained. The mean diameter of the inhibition zones formed during 18 hours of tested groups were categorized as either weak (<6 mm), moderate (6-10 mm), strong (11-20 mm) or very strong (>20 mm) (Morales et al., 2003). Whereas, the inhibition zones of each antibiotics used as positive controls were categorized according to CLSI standardizations (CLSI 2022; 2023).

Statistical Analysis

Data obtained from the antibacterial activity test of the ethanol extract of *jeruk sambal* peels were statistically analyzed using the SPSS version 24 application, and were analyzed using the one-way ANOVA (analysis of variances) method, then continued with a follow-up Post Hoc test that is Games-Howell (GH) test to find out which groups were significantly different.

RESULTS AND DISCUSSION

Result

Phytochemical Screening

Current research on phytochemical screening showed that the ethanol extract of jeruk sambal peels positively contains all of the secondary metabolites tested with phenolics and flavonoids as the most dominant compounds (+++), while tannins and saponins were the least of all (+). The results can be seen in the Table 1 below.

Table 1. Results of Phytochemical Screening.

Types	Reagent	Results	Interpretation
Phenolics	FeCl ₃ 5%	Dark green color formed	+++
Flavonoids	Mg powder + HCl 2N	Orange color formed	+++
Tanins	FeCl ₃ 1%	Blackbluish color formed	+
Saponins	H ₂ O + HCl 2N	Stable foam formed (> 25 minutes)	+
Alkaloids	Mayer	White precipitate formed	++

Antibacterial Activity Testing

The observation results of the antibacterial activity test of the ethanol extract of *jeruk sambal* peels against both *E. coli* and *S. aureus* bacteria with the incubation period of 18 hours and the temperature of 37°C can be seen in Table 2 below.

Table 2. The Results of Antibacterial Activity Testing of *E. coli* and *S. aureus*.

Treatment Group	Inhibition Zones ± Std. (mm)		Interpretations (<i>E. coli</i> / <i>S. aureus</i>)
	<i>E. coli</i>	<i>S. aureus</i>	
Negative Control	0.00 ± 0.00	0.00 ± 0.00	-/-
Ethanol Extract of Jeruk Sambal Peels	20%	0.00 ± 0.00	-/-
	40%	0.00 ± 0.00	-/M
	60%	7.80 ± 0.96	M/M
	80%	7.83 ± 1.54	10.25 ± 1.04 M/M
Positive Control	33.24 ± 1.27	28.14 ± 0.14	S/S

Notes: M = moderate; S = sensitive; (-) = no activity.

The antibacterial activity testing of the ethanol extract of *jeruk sambal* peels against *E. coli* and *S. aureus* were determined in four various extract concentrations, those are 20%, 40%, 60% and 80%. The results of antibacterial activity testing showed that *S. aureus* had the greater inhibition zone than *E. coli*. There were inhibition zones at extract concentrations of 60% and 80% with diameters of 7.80 mm and 7.83 mm respectively against *E. coli*, whereas there were antibacterial activities at 40%, 60%, and 80% extract concentrations with respectively 6.93 mm, 8.88 mm, and 10.25 mm diameters inhibition zones against *S. aureus*. Meanwhile, concentrations of extract

that did not show any inhibition zone were at 20% and 40% for *E. coli*, yet only at 20% for *S. aureus*. The positive controls used in this study were ciprofloxacin 5 µg/disk (*E. coli*) and clindamycin 2 µg/disk (*S. aureus*) which showed the formation of inhibition zone of 33.24 mm and 28.14 mm respectively, thus both were included in the sensitive category. Meanwhile, the 1% DMSO that was used as negative control did not formed any inhibition zone against the growth of both *E. coli* and *S. aureus*.

The data of inhibition zones of the ethanol extract of *jeruk sambal* peels were statistically analyzed using the

SPSS version 24 application. The results of the one-way ANOVA test in this study showed a value of $p = 0.000$ ($p < 0.05$) for both *E. coli* and *S. aureus*. Therefore, there were a significant difference between the groups. To find out which groups were significantly different, the Games-Howell test as a further test was determined and the results can be shown on the Figure 1 below.

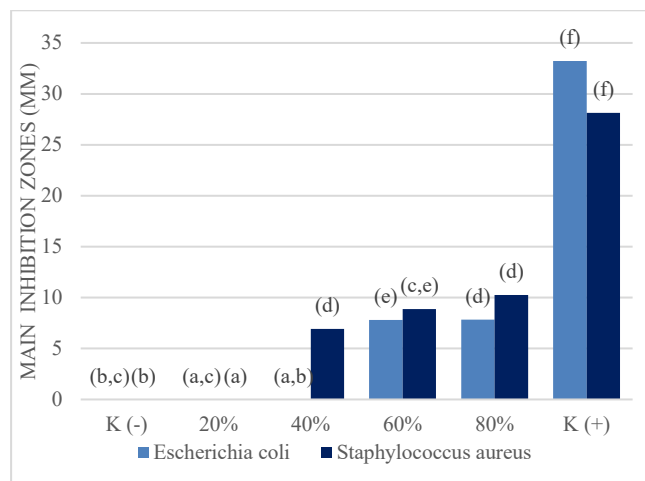


Figure 1. Graphic of extract concentration against the zone of inhibitions: (a) not significantly different against the negative control; (b) not significantly different against 20% concentration; (c) not significantly different against 40% concentration; (d) not significantly different against 60% concentration; (e) not significantly different against 80% concentration; (f) significantly different against all treatment groups.

According to the data analyzed, it is known that the ethanol extract of *jeruk sambal* peels against both *E. coli* and *S. aureus* at concentrations of 60% and 80% were significantly different ($p < 0.05$) from the negative control, 20% extract, 40% extract, and positive control, but did not have significant differences between each other ($p > 0.05$). Additionally, both positive controls used in the current research were significantly different from all of the groups tested ($p < 0.05$).

Discussion

The results of qualitative phytochemical screening showed that the ethanol extract of *jeruk sambal* peels contained all the secondary metabolite compounds tested, those are alkaloids (++), flavonoids (+++), phenolics (+++), saponins (+), and tannins (+). This is in accordance with the results of previous research by Asseggaf et al., 2021 who studied the juice of *jeruk sambal* obtained from the same plantation. This research also showed that saponin is the most dominant secondary metabolite compound (Asseggaf et al., 2021). Another previous research by Amiliah et al., 2021 on kalamansi citrus peel from Bengkulu area using 96% ethanol extract as a solvent obtained results containing similar secondary metabolite compounds such as alkaloids, flavonoids, tannins, saponins and terpenoids. Meanwhile, another research by Wulandari et al., 2013 which used different solvents such as n-hexane, ethyl acetate, and methanol, obtained the results that *jeruk sambal* peel contains

secondary metabolite compounds such as steroids, flavonoids, polyphenols, and alkaloids.

Solvent selection plays an important role in the extraction process so that secondary metabolite compounds contained in plants can be extracted perfectly. The use of different solvents can result in variations or differences of the secondary metabolite compounds that will be extracted. Secondary metabolite compounds such as alkaloids tend to be more soluble in non-polar solvents such as alcohol and difficult to dissolve in water, while phenolic compounds (simple phenols and phenolic acids, flavonoids and tannins) are more soluble in polar solvents. The environment, such as cultivation location, altitude, temperature, time of exposure to sunlight, rainfall, climate and soil, can influence the secondary metabolites contained in a plant both qualitatively and quantitatively, so that their bioactivity can also vary (Julianto, 2019).

Antibacterial activity tests were determined on a total of 6 treatment groups consisting of 4 tested groups and 2 control groups. The tested group was ethanol extract of *jeruk sambal* peels with varying concentrations of 20%, 40%, 60% and 80%. The results of the antibacterial activity test of the ethanol extract of *jeruk sambal* peels at concentrations of 20% and 40% against *E. coli* and at concentration of 20% against *S. aureus* did not show any inhibition zone formed. Meanwhile, there were inhibition zones which were classified as moderate antibacterial activities against both bacteria, *E. coli* at concentrations of 60% and 80%, whereas *S. aureus* at concentrations of 40%, 60%, and 80% (Morales et al., 2003).

Previous research by Amiliah et al., 2021 who investigated *jeruk kalamansi* peels extract with concentrations of 5%, 10%, 20% and 40% showed antibacterial activity against both *E. coli* and *S. aureus* in the weak category except at a concentration of 40% which was classified as moderate with the inhibition zone diameters of 7.2 mm and 10 mm respectively. The difference in results between the current research and previous research can be influenced by several factors, one of which is the use of different solvents resulting in secondary metabolite compounds that differ both in types or quantity. Previous research is known to have used ethanol solvent with a concentration of 96%, whereas in this current research the solvent used was ethanol with a concentration of 70%. The solvent in current research was chosen based on its polarity which is able to dissolve various secondary metabolite compounds (both polar and non-polar) (Julianto, 2019). Increasing ethanol concentration can increase the dissolution and extraction rate. When the ethanol concentration is greater than 70%, the extraction rate of the target component decreases slightly due to protein denaturation increasing diffusion resistance (Fan et al., 2020; Chen et al., 2020). Furthermore, high viscosity liquids are also more difficult to penetrate plant tissue due to their high density and low diffusivity (Chen et al., 2020).

The inhibition zone formed in this study could be caused by the synergy of various secondary metabolite compounds contained in the ethanol extract of *jeruk sambal* peels. Based on the results of the phytochemical screening that has been determined, it is known that the extract used contains alkaloids (++), phenolics (+++), flavonoids (+++), tannins (+), and saponins (+). Alkaloids are secondary metabolite compounds that contain nitrogen and have pharmacological effects on humans (Endarini, 2016). Alkaloids have various mechanisms as antibacterials, such as inhibiting nucleic acid synthesis, inhibiting protein synthesis, damaging cell membranes, damaging cell walls, inhibiting efflux pumps, inhibiting bacterial metabolism, inhibiting bacterial functional protease activity, and affecting DNA topoisomerase and respiration (Yan et al., 2021).

Phenolic compounds which are divided into several groups, such as simple phenols and phenolic acids, phenyl propanoids, flavonoids and tannins, can inhibit the growth and activity of many microorganisms including *E. coli* and *S. aureus*. Phenolics have varied molecular structures, giving them a wide range of antibacterial effects. These molecules can suppress several microbial virulence factors, such as inhibiting biofilm formation, reducing bacterial ligand adhesion, and neutralizing bacterial toxins. Furthermore, phenolics can also reduce membrane fluidity, inhibit nucleic acid synthesis, inhibit cell wall synthesis, and inhibit bacterial energy metabolism (Takó et al., 2020).

Flavonoids are compounds from the phenolics group that was most dominant in current research, which are known to have various antibacterial mechanisms such as inhibition of nucleic acid synthesis, inhibition of cytoplasmic membrane function, inhibition of energy metabolism, inhibition of attachment and biofilm formation, inhibition of porins in cell membranes, changes in membrane permeability and reduced pathogenicity (Xie et al., 2015). Meanwhile, tannins, which are also the members of phenolic compounds, can inhibit bacteria by iron chelation, inhibiting cell wall synthesis, disrupting cell membranes, and inhibiting the fatty acid biosynthesis pathway (Farha et al., 2020).

Saponins are secondary metabolite compounds with distinctive foaming characteristics when it is diluted in aqueous solutions and are included in steroid or triterpenoid aglycones attached to one or more sugar units. Saponins can damage the permeability of bacterial extracellular membranes. Approximately 90% of the surface of the extracellular membrane of Gram-negative bacterial cell walls that do not contain natural cholesterol is covered by lipopolysaccharide (LPS). Saponin can interact with the lipid A part of proteus LPSs, thus increasing the permeability of bacterial cell walls and causing cell leakage (Tatli Cankaya & Somuncuoglu, 2021). However, based on the results of current research, only a small amount of saponin was contained in the *jeruk sambal* peels extract tested, resulting in minimal damage extracellular membrane of *E. coli* which leads to

less than optimally antibacterial activity produced by the extract.

The type of bacteria used greatly influences the size of the inhibition zone formed. In the current study, the samples of *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were used, with the results showed that *S. aureus* had a greater inhibition zone than *E. coli*. As it is known, *E. coli* is a Gram-negative bacterium which has an extracellular membrane layer in their cell wall in the form of protein, phospholipids and lipopolysaccharides, which makes its permeability relatively high (Widyasanti et al., 2015). Moreover, the high lipid content in the cell walls of *E. coli* makes it easier for non-polar secondary metabolite compounds to penetrate, whereas most of the secondary metabolite compounds contained in *jeruk sambal* peels extract in this research were polar (Julianto, 2019; Widyasanti et al., 2015). The high permeability properties and non-polar cell walls of *E. coli* were preventing the bioactive compounds in the ethanol extract of *jeruk sambal* peels from diffusing optimally, which ultimately results in the extract being less than optimal in inhibiting the growth of *E. coli* (Widyasanti et al., 2015).

On the other hand, *S. aureus* is a Gram-positive bacterium that does not have an extracellular membrane but it has thick peptidoglycan with teichoic acid of its cell wall. Teichoic acid is a polymer that dissolves in water and act as a transport for positive ions (Liswandari, 2018). The polarity of its cell walls makes *S. aureus* more easily penetrated and damaged by secondary metabolite compounds contained in *jeruk sambal* peels that are equally polar such as phenolics, flavonoids, tannins, and saponins (Julianto, 2019; Liswandari, 2018). This is supported by the research of Amiliah et al., 2021 which found that the ethanol extract of *jeruk kalamansi* peel was more effective in inhibiting *S. aureus* as Gram-positive bacteria compared to Gram-negative bacteria such as *E. coli*.

The results of the statistical analysis of the current research showed that there was no significant difference in the treated groups of ethanol extract of *jeruk sambal* peels at concentrations of 60% and 80% against both *E. coli* and *S. aureus*. This can be caused by a lack of diffusion power of the extract into the medium which is influenced by the viscosity level of the extract. The higher the concentration, the thicker the extract, so the solubility will be lower. This can slow down the diffusion of the bioactive compounds of the extract into the media which can ultimately reduce the ability of extracts with high concentrations to inhibit the growth of *E. coli* and *S. aureus* (Admi et al., 2022). This is supported by Zeniusa et al., (2019) which shows that greater concentrations do not provide a greater inhibitory effect.

According to the results of statistical analysis of current research which showed that there was no significant difference between the 60% and 80% extract treated groups, it can be concluded that the 60%

concentration of jeruk sambal peels ethanol extract with moderate inhibitory power is an effective concentration in inhibiting the growth of both *E. coli* and *S. aureus*. The effective concentration referred to in this research was the concentration that best inhibits the growth of *E. coli* and *S. aureus* among all the treated groups for the ethanol extract of jeruk sambal peels tested. However, these effective concentrations were still not strong enough to compete with the effectiveness of ciprofloxacin and clindamycin as the positive controls in inhibiting the growth of *E. coli* and *S. aureus* bacteria.

CONCLUSIONS

Our research confirmed that the ethanol extract of jeruk sambal (*Citrus microcarpa* Bunge) peels had various secondary metabolites compounds which provides antibacterial activities against the growth of gram negative bacteria (*E. Coli*) and gram positive bacteria (*S. Aureus*) with moderate strength category, while gram positive bacteria had the greater inhibition zone than gram negative bacteria, and the most effective extract concentration in inhibiting the growth of both was at 60%.

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