

Research Article

Antibacterial Activity of Extract Combination of Leaves and Peels Kaffir Lime (*Citrus Hystrix DC.*) Against Some Test Bacteria

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ABSTRACT

Leaf and peel of the kaffir lime plant (*Citrus hystrix DC.*) can be used as herbal preparations to treat infections by opportunistic bacteria. Kaffir lime plant extract was prepared using maceration method with ethanol 96 % and then dissolved using aquadest. This study aimed to analyze the antibacterial activity of the combination of leaf and peel extract against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, and *Pseudomonas aeruginosa* bacteria isolates. This study used a posttest-only control group design, including phytochemical and in vitro antibacterial activity tests. The combination of leaf and peel extract of kaffir lime tested was 12.5%, 25%, 50%, and 75% (w/v). The results of phytochemical tests on the leaf extract and rind of limes were found to have antibacterial compounds, namely flavonoids, tannins, phenols, and alkaloids. The results of the antibacterial activity test used the paper disc diffusion method showed that there were differences in the size of the inhibition zone for the four test bacteria; The combination treatment of 75% (1:1) leaf-peel extract gave the greatest inhibitory zone effect on the four test bacteria and was equivalent to the positive control. The conclusion of the study, the combination of leaf and peel extract had different antibacterial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, and *Pseudomonas aeruginosa*.

Keywords: extract combination, kaffir lime, antibacterial, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa*.

Introduction

Infectious diseases caused by *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, and *Pseudomonas aeruginosa* are still health problems in the community. These bacteria are opportunistic bacteria that act as

normal flora in the human body and in the environment, but due to certain predisposing factors that can cause some abnormalities. Generally the transmission of bacteria through direct contact or equipment in some communities,

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there is a tendency to use herbal preparations to overcome infectious diseases by bacteria.

Among the plants that have been used as herbs are orange limes (*Citrus hystrix DC.*). Kaffir lime is a member of the genus *Citrus*, the family Rutaceae. The scientific name is *Citrus hystrix DC.* Common names of *Citrus hystrix DC.* are jeruk purut, limau kuit, limau purut, kaffir lime, leech lime, and makrut (Thai) [1], [2], [3]. Kaffir lime leaves have been used in Southeast Asian recipes since they provide a unique and strong aroma [3]. In Kalimantan Indonesian, lime leaves and peels are aromatic and used as spices for various flavoring purposes, such as seasoning or preparing savory soup, but they can also use in traditional medicine. This lime fruit is used for headaches, inflammation, flu, fever, sore throat, bad breath, and digestive disorders, hypertension, stomach pain, diarrhea in infants, flavoring, eliminating body odor; the leaves are used to maintain healthy teeth, and gums and cure scurvy [4].

Many studies have demonstrated various biological activities of kaffir lime. The peel and leaf are sources of phenolic compounds and antioxidative substances [5]. These phytochemical compounds exhibited many advantages, such as antioxidant, antibacterial, antifungal, anticholinesterase, anticancer, cardioprotective, and antidiabetic activities [4], [6], [7]. The previous phytochemical report showed that this plant contains various phytoconstituents such as high phenolic, flavonoid, alkaloid, tannins, glycerolglycolipids, tocopherols, and furanocoumarins in several parts of CH, such as a leaf, peel, and juice [6], [8]. Results of phytochemical tests, known kaffir peel essential oil contains the main components of β -pine (21.44%), citronellal (20.91%), limonene (12.59%), and terpinene-4-ol (11.93%). While the essential oil of kaffir lime leaves has the main component of citronellal (85.07%), linalool (3.46%) and sabinene (2.79%) [9].

Most of the studies on kaffir lime bioactivities are associated with its antimicrobial effect. The extract of kaffir lime leaves showed antibacterial activity against *S.aureus* and *E.coli* [1], [2]. Waikedre et al. (2010) tested the leaf's essential oil against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*,

Klebsiella pneumonia, and *Escherichia coli* [10]. The test result essential oils of leaf and peel have antimicrobial activity on *Shigella*, *S.thypi*, *E.coli*, *S.mutants*, *S.aureus*, *S.cerevisiae*, and *C.albicans* [11]. Kaffir antibacterial activity lime leaf extracts in *Salmonella typhi*, *Staphylococcus aureus*, *Bacillus cereus*, and *Escherichia* form a diameter of inhibition zone $6.83 \pm 0.17 - 8.67 \pm 0.67$ mm, which is smaller than gentamicin control of 16-21mm [12]. The test antibacterial activity of different citrus fruits' peel ethanolic extracts and blending extracts against *S.aureus*, *E.coli*, and *P.aeruginosa* showed two ethanolic extracts and three blending extracts of peels' powder had a good activity antibacterial [13].

Several test results of ethanol extract of leaves and lime peel on a single preparation showed antibacterial effects under positive control. A combination of two extract preparations is expected to provide a better antibacterial effect. However, no studies regarding the antibacterial activity of combination extract of lime leaf and peels have been conducted to date.

The study aimed to analyze the antibacterial activity of a combination of leaf extract and skin of the fruit against the bacteria *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Antibacterial activity is observed in vitro, i.e., through measurement of the inhibition zones formed around the growth of test bacteria. This study has passed the ethical test based on the letter statement of approval of the ethics committee of the Faculty of Medicine, University of Lambung Mangkurat Number 834/KEPK-FK ULM/EC/IX/2021.

Materials and Method

The method used in this study is the proper experimental laboratories method, with a posttest-only control group design.

Plant material. The fresh fruits of *Citrus hystrix* (kaffir lime) were collected from Banjarbaru City, located in South Kalimantan Province, in October 2021. The plant sample was identified and the determination certificate specimen (No.167/LB.LABDASAR/X/2021) was Laboratory Biology of the Faculty of

Science Lambung Mangkurat University, Banjarbaru.

Process of making extract. The extraction method used for this study was maceration [14] Total of 100-gram samples of leaf powder and peel lime (*C. hystrix*) were put into a maceration device, then ethanol 96 % solution was poured slowly into the maceration device [15]. The maceration process is carried out within 3 x 24 hours by stirring until distributed, every 1 x 24 hours the filtrate is filtered and the solvent is replaced with a new one. Then the extract was put into a *rotatory evaporator* at a temperature of 600 C until a concentrated ethanol extract was obtained, then evaporated in a water bath [16]. The extraction results can be stored in a refrigerator at 40 C [17]. Furthermore, we do phytochemical screening on the extracts.

The concentrations of lime leaf extract and peels tested were 12.5%, 25%, 50%, and 75% (w/v). The stew is filtered through filter paper while hot. Then combine the lime extract of the leaf and peel in a 1:1 ratio.

Phytochemical Screening. Flavonoid Test, **Alkaline Reagent Test:** 100 mg of sample is dissolved in 50 ml of the solvent, then 1 ml of sample is taken and a few drops of NaOH solution are added. It contains flavonoids if they form faded yellow after adding aqueous acid [18]. **Pb Acetate Test:** 100 mg sample is dissolved in 50 ml of the solvent, then 1 ml of sample is taken, and 1 ml of **Pb Acetate** 10 % is added to the test tube and shaken. If there is a change in color of the solution to a yellowish-brown, it means that it positively contains flavonoids [19]. **Alkaloid Test; Dragendroff Test:** 100 mg of sample is dissolved in 50 ml of the solvent, 1 ml of sample is taken, and 1 ml of Dragendroff reagent is added. If a red precipitate is formed, it means that it positively contains an alkaloid. **Mayer Test:** 100 mg of sample is dissolved in 50 ml of the solvent, then 1 ml of sample is taken, and 1 ml of Mayer reagent is added. If a yellow precipitate is formed, it means that it positively contains alkaloid [19]. **Tannin Test, Gelatin Test:** 100 mg of sample is dissolved in 50 ml of the solvent, then 2 ml of sample is taken and 2 ml of gelatin solution 1% containing NaCl. If a white precipitate is formed, it means that it positively contains

tannin [19]. **Phenol Test:** Iron (III) Chloride Test: 100 mg of sample is dissolved in 50 ml of the solvent, then 1 ml of sample is taken and 1 ml of FeCl₃ 3% is added. If a blackish-green precipitate is formed, it means that it positively contains phenol [20]. **Saponin Test, Foam Method:** 100 mg of sample is dissolved in 50 ml of the solvent, then 2 ml of sample is taken and shaken with 2 ml of water. If the foam appears for 10 minutes, it indicates positively contains saponin [19]. **Steroid Test, Libermann Burchard's Test:** 50 mg of sample were dissolved with chloroform and filtered. The filtrate obtained was added with acetic acid hydrate, then heated and cooled. Concentrated sulfuric acid is added to the tube wall slowly if a brown ring is formed, indicating the presence of steroids [19].

Bacterial organisms. The bacterial organisms determined in this study contained 4 American Type Culture Collection (ATCC) bacterial. The ATCC bacterial strains were *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853. These bacteria were obtained from the Faculty of Medical, Lambung Mangkurat University. The bacteria were cultivated on blood agar at 37° C for 18–24 hrs.

Agar disk diffusion method. Agar disk diffusion was performed to screen the in vitro antibacterial activity as previously described (Aryani et al., 2018) with some modifications. The bacterial suspension was spread on either Mueller Hinton Agar (MHA), depending on the type of bacteria. The 24 hr bacterial cultures were placed inside culture bottles containing saline and compared with 0.5 McFarland standard ($\pm 1.0 \times 10^8$ CFU), and 0.5 mL bacteria suspensions were spread on MHA (Mueller Hinton agar, Oxoid). Next, 6 mm blank paper discs (Oxoid antimicrobial susceptibility test disc) were dipped into the kaffir lime leaves extract, placed on the agar, and incubated overnight at 37° C. Then, the agar plates were observed for clear inhibition zones, and the diameter of inhibition zones was measured [12]. Ciprofloxacin antibiotic disc (Oxoid) was used as a positive control and distilled water was used as a negative control. The plates were incubated at 37° C

for 18–24 hrs. The diameter of the zone of inhibition in mm was recorded after incubation.

The experiment was performed in five replicates, and the average diameter of the zone of inhibition was obtained. The inhibition zone diameter (ID) of extract combination to lime leaf and peel was measured and interpreted using the following criteria: no activity, IZD = 6 mm; weak activity, 6 mm < IZD < 12 mm; moderate activity, 12 mm < IZD < 20 mm; and strong activity, IZD > 20 mm [21].

Results and Discussion

Results of the phytochemical test (Table 1) showed that the lime *C.histryx* extract employed contains such as compounds active such as flavonoids, phenols, tannins, alkaloids, and saponins. The bioactive compounds in the lime leaf and peel parts have inhibitory potency as an antibacterial. Proof that the inhibiting compounds were extractable by the employed solvents against the tested bacteria.

Table 1. Analysis Content Phytochemical From Leaves and Peels Extract Lime *Citrus hystrix*

Number	Com- pounds	Lime <i>C.hystrix</i> parts		Descriptions
		Leave	Peel	
1	Flavonoids	+	+	Formed yellowish chocolate precipitate
2	Alkaloid	+	+	Formed color yellow precipitate
3	Tanin	+	+	Formed color white precipitate
4	Phenol	+	+	Formed blackish-green precipitate

Table 2. Average Diameter of Inhibitory Zone and Standard Deviation In Some Bacteria Tested with Treatment of Leaf Extract and Peel *Citrus hystrix*

Samples extract leaf + peel of lime <i>C.hystrix</i> and CIX	Zones of inhibition (mm)			
	<i>S.aureus</i>	<i>S.epidermidis</i>	<i>E.coli</i>	<i>P.aeruginosa</i>
12,5% leaf + 12,5% peel	13,9±0,2 ^l	14,6±0,9 ^j	13,4±0,4 ⁿ	11,8±0,3 ^k
12,5% leaf + 25% peel	15,7±0,4 ^k	16,4±0,1 ⁱ	14,0±0,2 ^m	13,1±0,3 ^j
12,5% leaf +50% peel	16,1±0,2 ^k	16,6±0,2 ⁱ	16,3±0,3 ^j	15,5±0,3 ^h
12,5% leaf +75% peel	18,1±0,1 ^h	18,3±0,2 ^h	16,9±0,3 ⁱ	16,8±0,5 ^{fg}
25% leaf +12,5% peel	16,6±0,3 ^j	16,9±0,4 ⁱ	14,7±0,2 ^j	13,5±0,8 ^j
25% leaf +25% peel	17,4±0,3 ⁱ	18,1±0,7 ^h	15,5±0,4 ^k	14,3±0,4 ⁱ
25% leaf +50% peel	18,6±0,3 ^g	19,1±0,4 ^g	17,0±0,1 ⁱ	16,3±0,4 ^{gh}
25% leaf +75% peel	19,4±0,5 ^f	21,0±0,2 ^{ef}	17,8±0,2 ^{gh}	17,1±0,1 ^f
50% leaf +12,5% peel	19,0±0,0 ^{fg}	19,6±0,4 ^g	17,5±0,4 ^h	15,8±0,5 ^h
50% leaf +25% peel	20,1±0,1 ^e	20,6±0,7 ^f	18,1±0,3 ^g	17,3±1,0 ^{ef}
50% leaf +50% peel	21,3±0,6 ^d	21,9±0,3 ^{de}	18,9±0,3 ^f	18,0±0,5 ^{de}
50% leaf +75% peel	23,2±0,2 ^c	23,7±0,4 ^c	20,3±0,5 ^d	19,6±0,2 ^c
75% leaf +12,5% peel	21,3±0,3 ^d	21,9±0,4 ^d	19,4±0,4 ^e	18,4±0,1 ^d
75% leaf +25% peel	23,1±0,1 ^c	23,6±0,4 ^c	20,9±0,1 ^c	19,7±0,4 ^c
75% leaf +50% peel	25,3±0,3 ^b	25,9±0,4 ^b	23,1±0,2 ^b	21,5±0,9 ^b
75% leaf +75% peel	28,1±0,2 ^a	28,3±0,3 ^a	24,6±0,4 ^a	23,8±0,6 ^a
Ciprofloxacin 5 ug	27,9±0,1 ^a	28,2±0,2 ^a	24,5±0,2 ^a	23,7±0,0 ^a

a, b, c, d, e, f, g, h, i, j, k, l, m, n superscript in the same column shows no significant difference (P>0.05)

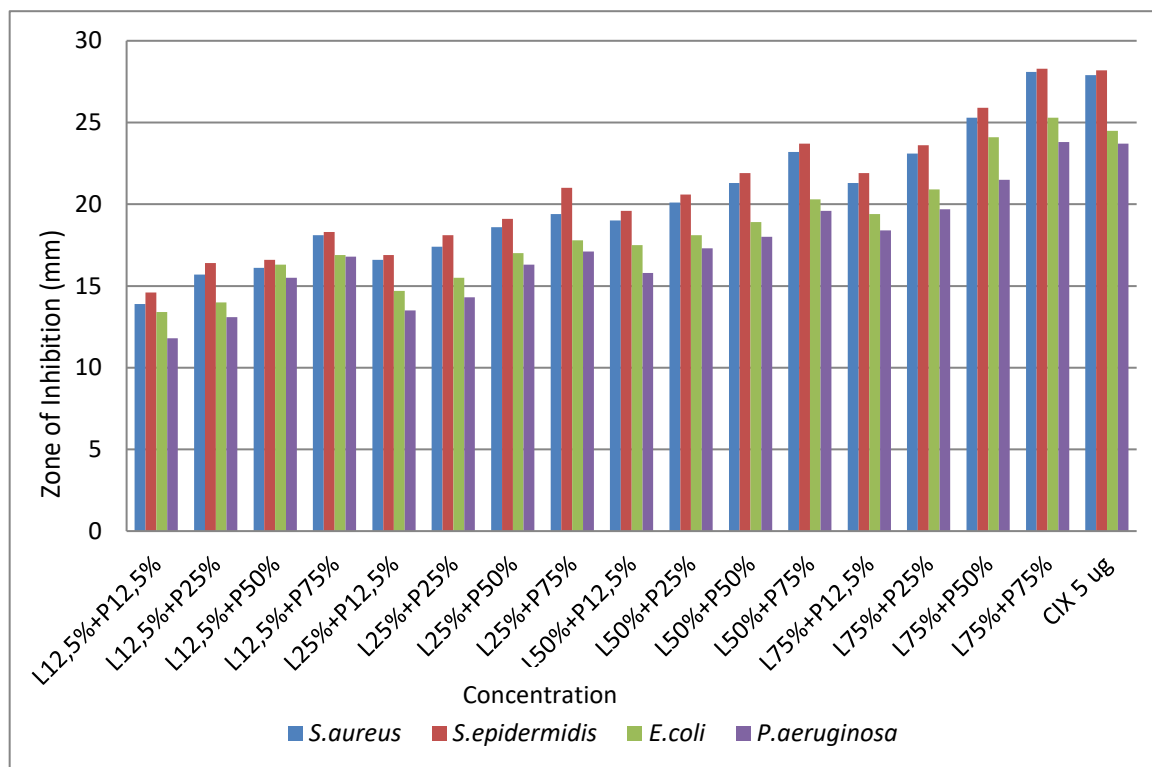


Figure 1. Average Diameter of Inhibition Zone of Leaf (L) and Peel (P) Extract of Lime *C. hystrix* Against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, and *Pseudomonas aeruginosa*.

Disk diffusion methods for antibacterial activity show significant activity against bacterial growth in inhibitory zones around the disk. Among the bacteria tested, *S. aureus* and *S. epidermidis* were more sensitive to leaf extracts and lime peels than *E. coli* and *P. aeruginosa*. The inhibitory zone increases with the increased concentration of extracts in the disk. Inhibition zone of leaf and peel extract of lime *C. hystrix* showed the concentration-dependent activity (Table 2 and Figure 1)

The inhibitory effect on bacteria thought to be due to the role of the active compounds of the more dominant leaf extract contributed to the combination preparations tested. Kaffir lime peel has weaker antibacterial activity than kaffir lime leaves since the compound's main chemicals have hydrocarbon groups. Kaffir lime leaves have antibacterial activity twice as strong as kaffir lime leaves because the main chemical

compounds have aldehyde and alcohol, functional groups with high antibacterial activity. In addition, antibacterial activity can also be influenced by the synergy between the chemical compounds it contains [22].

In Table 2, it appears that showed the smallest inhibitory zone in all test bacteria was obtained from the 12.5% combination of leaf and orange peel extract treatment, and the largest inhibitory zone was obtained from the 75% combination treatment. The results of this study are not much different from studies that use a combination of orange peel powder of different types. Results testing antibacterial activity of different citrus fruits' peel ethanolic extracts and blending extracts against *S. aureus*, *E. coli*, and *P. aeruginosa* showed two ethanolic extracts and three blending extracts of peels' powder from some lime types had a good activity antibacterial [13].

Secondary compounds in lime kaffir, such as flavonoid, phenol, alkaloid, and tannin, can work as antibacterials. Flavonoids inhibit the enzyme topoisomerase II (DNA gyrase), which is an essential enzyme in the process of replication and transcription of bacterial DNA so that bacterial growth is disrupted. In flavonoids also, phenolic compounds can interfere with bacterial growth. Phenol is acidic alcohol that can denature proteins and damage bacterial cell membranes [23], [24]. Alkaloid interferes with the peptidoglycan constituent components of bacterial cells so that the cell wall layers are not fully formed and cause the death of the cell. Alkaloids also have a nitrogen-containing base group that will inhibit the topoisomerase enzyme that plays a role in replication, transcription, and recombination of bacterial DNA [25]. Tannins can inhibit reverse transcriptase and DNA topoisomerase enzymes so that bacterial cells cannot replicate, and the toxicity of tannins can act on bacterial cell membranes [24], [25].

Phytochemical compounds in *C.hystrix*, also including citronella, phenolic, and saponins. Citronella is effective against several pathogenic bacteria and fungi.[4] Wattanastcha et al. (2012) observed that citronellal was effective in the growth inhibition of *S. aureus*; however, for *E. coli*, no antimicrobial. Additionally, phenolic compounds with hydrophobic character have the bactericidal potential to interact with the outer membrane (OM). [26]. Saponins are active substances whose surface is similar to detergent, which lowers the surface tension of bacterial cell walls and impairs membrane permeability, thereby interfering with the survival of bacteria. When the surface tension is disturbed, saponins will easily enter cells and interfere with metabolism, causing denaturation of membrane proteins, where it can make cell membranes become damaged and lysis [27].

The largest inhibition zone from the combination treatment of 75% lime leaf extract and 75% lime peel extract against *S.aureus* was 28.1 mm, *S.epidermidis* was 28.3 mm, and *E.coli* was 24.6 mm, and against *P.aeruginosa* of 23.8 mm. The strength of antibacterial activity resulting from the combination treatment of leaf and

peel extract against *S.aureus*, *S.epidermidis*, *E.coli*, and *P.aeruginosa* was categorized as strong and very strong at several concentrations. The positive control (Ciprofloxacin) had a very strong effect. Based on the average value of the inhibitory zone and standard deviation, the combination of leaves and lime peel gives a greater inhibitory zone effect to *S.aureus* and *S.epidermidis* than *E.coli* and *P.aeruginosa*. The present study was similar to that obtained by Lopez-Romero et al. (2015) and other authors, and the Gram-positive bacterium was more resistant than the Gram-negative one[26].

The Anova test analysis results showed a significant difference ($p < 0.05$) between the combined treatment of leaf and peel extract of *C.hystrix* and the positive control tested on all the tested bacteria. The results of Duncan's Post Hoc test showed that there were treatments that produced different and no different effects (inhibition zone size) on the two test bacteria ($p > 0.05$). The effect of the combination treatment of *C.hystrix* lime leaf-peel 75% (1:1) gave an effect that was not significantly different (equivalent) to the positive control (ciprofloxacin); The effect of the combination treatment of lime leaf and peel 75% (1:1) gave a significantly different effect with the combination treatment of *C.hystrix* lime leaf and peel at a concentration combination below 75%.

Based on the results of this study, it was found that the combination treatment of leaf and peel extract (75% combination) had the same effect as the positive control. This result is influenced by the active compounds contained in the combination preparations that can work to inhibit the growth of the test bacteria optimally and are equivalent to the positive control activity.

The action of secondary compounds in *C.hystrix* could diffuse and penetrate the bacterial outer membrane causing loss of membrane permeability and cell death. Synergistic interaction of leaf and peels *C.hystrix* in the combination extract can increase the membrane permeability and precipitation of cytoplasmic molecules. The synergistic activity may be due to their actions on the same target at the bacterial cell membrane[28][29]

This study showed that the combination treatment of lime leaf-peel had different effects on the test bacteria. There are different zones of inhibition in gram-positive bacteria (*Staphylococcus aureus* and *Staphylococcus epidermidis*) and gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). In general, the size of the inhibition zone for *S.aureus* and *S.epidermidis* was greater than the inhibition zone for *E.coli* and *P.aeruginosa*.

This research is not different from previous studies using different types of bacteria. The antibacterial activity of kaffir lime leaf essential oils to *E.coli* was lower than *S.aureus* [1]. In this sense, some studies suggest that the susceptibility to antimicrobials between Gram-positive and Gram-negative bacteria could be attributed to the cell envelope (cytoplasmic membrane and/or outer membrane and cell wall) structure and composition. In Gram-negative bacteria, the cell wall is more complex. It is constituted by a thin peptidoglycan layer adjacent to the cytoplasmic membrane and an outer membrane (OM) composed of phospholipids and lipopolysaccharides (LPS) [26][28].

The difference in bacterial sensitivity is related to differences in the structure and bacterial cell wall composition. *S.aureus* is a Gram-positive bacteria with a cell wall with an outer layer of peptidoglycan, making it easier for antibacterial compounds to pass. In opposite, *E.coli* is a Gram-negative bacteria that have a cell wall with an outer layer of lipopolysaccharide that does not easily pass antibacterial compounds [1][26]. This difference could be explained by the fact that the cell wall of Gram-negative bacteria contains high contents of phospholipids and lipopolysaccharides, which is more complex than Gram-positive bacteria [26][30]. The lipopolysaccharide layer could limit the permeability of extra through the outer membrane of Gram-negative bacteria. On the other hand, Gram-positive bacteria contain high peptidoglycan content in their cell wall, which allows hydrophobic molecules to penetrate the bacterial cell [30] [31].

The cell walls of *S.aureus* and *S.epidermidis* bacteria have simpler structure that make it easier for antibacterial compounds to enter the cells. These bacteria have polysaccharide

compounds and amino acids in their peptidoglycan layer. *P.aeruginosa* and *E. coli* bacteria have a more complex cell wall structure and contain more lipid components than Gram-positive bacteria. The lipid layer in gram-negative bacteria protects the cytoplasm from the environment and has a selective system against foreign substances in the lipopolysaccharide layer. The selective nature of gram-negative bacteria provides benefits in its defense system [6] [26][30].

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