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Antibacterial Activity Test of Ethanol Extract Citrus Leaf Against *Staphylococcus epidermidis*

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Abstract

One of the causes of acne is an increase in colonies of *Staphylococcus epidermidis* bacteria. One of the acne treatments that trigger bacterial resistance is the irrational use of antibiotics. Therefore, it is necessary to discover natural materials with antibacterial potential, namely orange plants. This study aims to determine the effect of single and combination of lime, kaff, lemon, and sweet leaves extract on of *S. epidermidis.* The treatment of this study was the concentration of orange leaf ethanol extract of 2%, 4%, and 8% with a bacteria sample of *S. epidermidis.* Phytochemical screening test results contain alkaloids, flavonoids, saponins, tannins, phenols, and essential oils. The results of the One-Way ANNOVA test showed a significant difference in the average diameter of the inhibition zone of orange leaf ethanol extract against the growth of *S. epidermidis* (Sig.<0.05). Post Hoc test showed that the effective concentration of lime leaves, kaffles, lemons, sweet oranges, and combination was 8%, while sweet oranges are the most significant treatment group in inhibiting the growth of *S. epidermidis*. The conclusion is that 8% sweet lime leaf ethanol extract can be used as a pharmaceutical raw material in inhibiting the growth of *S. epidermidis* with a strong category.

Keywords: acne; citrus; Phytochemicals; kirby bauer; *S. epidermidis*.

INTRODUCTION

Acne is one of the diseases in the skin area. According to data from the Indonesian Cosmetic Dermatology Study Group (ICDSG), the incidence of acne in Indonesia until 2024 is 87.5% (Asbullah et al., 2021). The impact of acne on psychology is embarrassment, loss of confidence, anxiety, and depression. An increase in psychological problems will affect the quality of life of acne sufferers (Duru & Örsal, 2021).

According to Sibero et al. (2019), one of the causes of acne is infection with the bacteria *Propionibacterium acnes*, *Staphylococcus aureus,* and *Staphylococcus epidermidis*. Therefore, acne treatment is often using antibiotics. However, irrational use of antibiotics often causes bacterial resistance to antibiotics (Rahman, 2019), thus increasing the number of morbidity, deaths, and treatment time which has implications for increasing medical costs (Yunita & Sukmawati, 2021). The percentage of acne bacteria resistance to antibiotics in Indonesia is 32.6%-52.2% (Marsudi et al., 2021).

Referring to the problems and impacts of acne and bacterial resistance to antibiotics, efforts to prevent acne by bacteria are needed. One of the prevention efforts is testing plant raw materials that have the potential to inhibit the growth of acne-causing bacteria. The selection of plants as antibacterial agents is because it is safer from the risk of side effects than antibiotics with high costs and risk of bacterial resistance. The natural materials from the plants to be tested in this study are orange leaves, including lime (*Citrus x aurantiifolia* (Christm.) Swingle), kaffir lime (*Citrus hystrix* DC.), lemon (*Citrus x limon* (L. Osbeck), and sweet orange (*Citrus x aurantium* L.).

Citrus simplicia tests against bacteria have been researched using an infusion of lime leaves and kaffir lime 100% can inhibit *E. coli* (11.7 mm and 14.3 mm) (Siregar et al.,2020), Ethyl acetate extract of lemon leaves 25% and 50% inhibits *S. aureus* (16.16 mm) and *E. coli* (10.57 mm) (Sormin et al., 2023), lime peel ethanol extract inhibits *P. acnes* with a Minimum Inhibition Concentration (MIC) value of 1%, Minimum Bactericidal Concentration (MBC) of 40% and *S. epidermidis* with MIC 1% and MBC 50% (Rachmawati et al., 2021), Sweet orange peel essential oil 50 µg/mL is able to inhibit *E. coli*, *S. Aureus,* and *S. Agalactia* (Anwar *et al*., 2023). Research Nurjannah et al. (2022) concluded that the combination of kaffir lime and moringa leaf extracts with 20%, 40%, 60%, 80%, and 100% could inhibit *S. aureus* (7.20-10.68 mm).

Based on previous research, was more focused on the use of a single extract, so this study wanted to test the

effect of citrus leaf extract with novelty on the use of low concentrations single and combination on *Staphylococcus epidermidis*. This study is expected to provide information on the single and combined effects of ethanol extracts of lime leaves, kaffir lime, lemon orange, and sweet orange on *Staphylococcus epidermidis.*

MATERIALS AND METHODS

Materials

Materials and tools include lime leaves (*Citrus x aurantiifolia* (Christm.) Swingle), kaffir lime leaves (*Citrus hystrix* DC.), lemon leaves (*Citrus x limon* (L.) Osbeck), sweet lime leaf (*Citrus x aurantium* L.), pure culture of *S. epidermidis* ATCC: 12228 (microbiology laboratory, University of Indonesia), Mueller Hinton Agar (MHA) (Himedia), Agar Nutrient (NA) (Himedia), Agar Powder (Himedia), Ethanol 96% (technical), Dimethyl sulfoxide (DMSO) (Merck), *blank disc* (Oxoid), Chloramphenicol disc (Oxoid), disc paper (Oxoid), sterile cotton swab (Onemed).

Sample preparation

Sample preparation includes plant determination at the Faculty of Mathematics and Natural Sciences Indonesia University, 1.5 kg orange leaf samples, wet sorting, washing, slicing, drying, dry sorting, and making powder.

Extraction, evaporation, and screening of phytochemicals

The simplicia powder weighed 200 grams each, dissolved with 96% ethanol solvent 1 Litre. The solution is soaked for 3 days, occasionally stirring until completely mixed. The filtrate was filtered using Whatman No 1 paper, then remaceration in the same way for 5 days. The liquid extract from the maceration process is evaporated by a rotary evaporator at a temperature of 40°C, a pressure of 200 Mbar and a speed of 60 rpm for 3 days. The viscous extract obtained is then heated on a waterbath at a temperature of 60°C. The results of the viscous extract were carried out phytochemical screening tests, including alkaloids, flavonoids, saponins, tannins, phenols, and essential oils.

Antibacterial Activity Test

The preparation of bacteria begins by taking pure culture *S.epidermidis* 3 ose, suspended in a test tube containing 10 ml of 0.9% NaCl solution, divortex until homogeneous (Hindun et al., 2021), compared to the turbidity with Mc Farland 0.5 solution (equivalent to a bacterial suspension of 1.5 x 10^8 CFU/ml). The suspension of bacteria is inoculated on MHA media in a petri dish using the streak plate method and incubated for 15 minutes. Blank disks (6 mm) dripped with ethanol extracts of lime leaves, kaffles, lemons, and sweets using micropipettes 100 µL, awaited 30 minutes, and placed on MHA media on a petri dish containing streak cultures of *S. epidermidis* using sterile tweezers. Incubation 18-24 hours at 37°C. Observations were made by measuring the diameter of the inhibition zone (clear zone) using a ruler (Karim et al., 2023).

Data Analysis

Data analysis uses IBM SPSS 25 statistics. Parametric data (ratio) was tested for normal distribution, homogeneity, then *One Way ANNOVA* (Sig<0.05). If significant, continued *Post-Hoc* test to find groups with significant influence.

RESULTS AND DISCUSSION

Plant Determination

The plant determination test aims to find out the truth of the species name and plant family (Purwanti, 2022). The results of the determination can be seen in Table 1.

Table 1. Plant determination results.

Sampel	Species	Family
Lime	*Citrus x aurantiIfolia* (Christm.) Swingle	*Rutaceae*
Kaffir Limer	*Citrus hystrix DC.*	*Rutaceae*
Lemon	*Citrus x limon* (L.) Osbeck	*Rutaceae*
Sweet Orange	*Citrus x aurantium* L.	*Rutaceae*

Results in Table 1 show that all samples include the Rutaceae family with the Latin names lime (*Citrus x aurantiIfolia* (Christm.) Swingle), kaffir lime (*Citrus hystrix* DC), lemon (*Citrus x limon* (L.) Osbeck), and sweet orange (*Citrus x aurantium* L.)

Macroscopic Powder

The organoleptic test of medicinal plant raw materials aims to determine their shape, color, odor, and taste. The results are shown Tables 2 and 3.

Table 2. Organoleptic orange leaf powder.

Sample	Organoleptic			
	Shape	Colour	Odor	Taste
Lime	Powder	Brownish Green	Aromatic	bitter
Kaffir Lime	Powder	Light Green	Aromatic	bitter
Lemon	Powder	Dark Green	Strong aromatic	bitter
Sweet Orange	Powder	Dark Brown Green	Aromatik	bitter

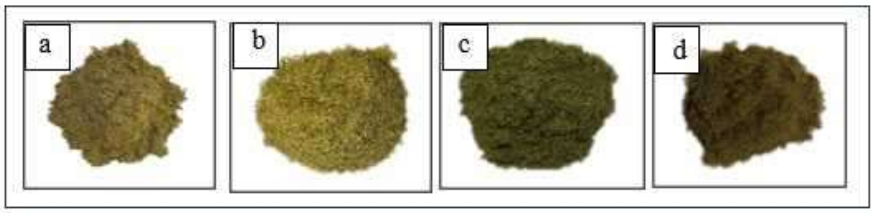

Figure 1. a. Lime. B. Kaffir lime. c. Lemon. d. Sweet orange.

Based on Table 2 and Figure 1, lime leaf powder has a brownish-green color, kaffir lime leaves light green, lemon dark green color, and sweet lime brownish dark green. Lime and sweet leaf samples have a distinctive aromatic odor while kaffir lime and lemon leaves have a strong aromatic distinctive odor. All samples have a bitter taste.

Organoleptic of viscous extracts

Table 3. Organoleptic of ethanol extract of orange leaf.

Sampel	Organoleptic		
	Shape	**Colour**	**Odor**
Lime	Viscous exstract	Blackish Brown	Aromatic
Kaffir Lime	Viscous exstract	Blackish Brown	Strong aromatic
Lemon	Viscous exstract	Blackish Brown	Strong aromatic
Sweet Orange	Viscous exstract	Dark Brown Blackish	Aromatic

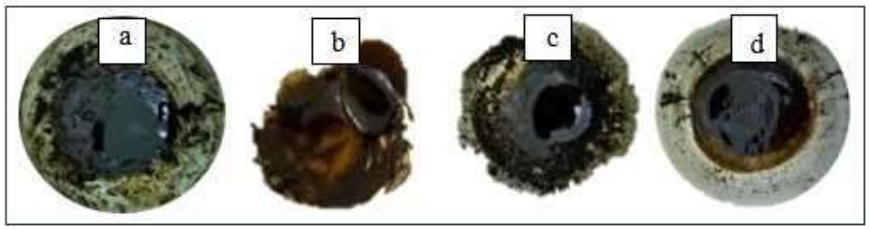

Figure 2. a. Lime. b. Kaffir lime. c. Lemon. d. Sweet orange.

Based on Table 3, ethanol extracts of orange leaves are blackish-brown to blackish-dark brown, have a weak to strong aromatic aroma, and are bitter aromatic.

Yield of extract

The extraction in this study uses the maceration and maceration method with 96% ethanol solvent. The selection of the maceration method is simple and is carried out at room temperature without involving heating to minimize the damage to secondary metabolite compounds that are thermostabile (Ulfa et al., 2023), meanwhile 96% ethanol solvent was chosen because it has polar properties that are able to bind to the dominance of secondary metabolite compounds that are polar (Wendersteyt et al., 2021). Remaceration is carried out to make the extraction of secondary metabolite compounds more optimal (Nurjannah et al., 2022). Remaceration with solvent replacement aims to extend the duration of contact between the solvent and simplicia to increase the content of secondary metabolite compounds in the viscous extract (Wahyudi & Minarsih, 2023). The results of the viscous extract were calculated by comparing the weight of the viscous extract with the weight of simplicia powder (Wijaya et al., 2022). The yield value of ethanol extract of orange leaves can be seen in Table 4.

Table 4. Yield value of local orange leaf ethanol extract.

Sample	Powder weight (g)	Extract weight (g)	Extract yield (%)
Lime	200 g	16,87 g	8.43 %
Kaffir Lime	200 g	20,06 g	10.03 %
Lemon	200 g	25,05 g	12.52 %
Sweet Orange	200 g	22,56 g	11.28 %

Table 4 shows the yield of ethanol extracts of lime, kaffir lime, lemon, and sweet orange are 8.43%, 10.03%, 12.52%, and 11.28%. The yield results correlate with the secondary metabolite compounds contained in the viscous extract. According to research by Subaryanti et al., (2022), the yield value is high, the content of secondary metabolite compounds is high. The yield results in this study were greater than the previous study: 3.037%, 9.945%, 8.5%, 5.16% (Magfirah et al., 2022; Maimunah et al., 2020; Rachmawati et al., 2021; Sriarumtias et al., 2020). The results of this study are by the criteria of the Indonesian Herbal Pharmacopoeia (not less than 7.2%). Several factors cause the difference in the percentage value of the extract yield, namely the extraction method, the length of extraction, the type of solvent, the particle size of the extraction sample, and the comparison of the number of samples with the solvent (Putra & Surahmaida, 2023).

Phytochemical screening

Phytochemical screening is a qualitatively preliminary test stage to provide an overview of the secondary metabolite compound class contained in a sample to be studied. Testing is carried out by observing the color testing reaction using a color reagent (Saragih & Arsita, 2019). This study was carried out to test the existence of secondary metabolites contained in lime, kaffir lime, lemon, and sweet orange leaves using a color reagent. The compounds to be tested include alkaloids, flavonoids, saponins, tannins, phenols, and essential oils because these compounds are able to inhibit microbial growth (Nafisa et al., 2021; Astriani et al., 2021; Rachmawati et al., 2021; Sriarumtias et al., 2020).

Table 5. Phytochemical screening orange leaf ethanol extract.

Sample	Compound	Reagents	Results	Indicator
Lime Kaffir Lime Lemon Orange	Alkaloid	Mayer	+	white precipitate
		Dragendorf	+	Orange solution
		Wagner	+	Brown solution
	Flavonoid	HCl pekat + Mg powder	+	Brownish-red solution
	Saponin	Aquadest + Heat	+	Foam formed
	Tannin	Aquadest + Heat + $FeCl_3$ 1%	+	Greenish-brown solution
	Phenol	$FeCl_3$	+	Deep black solution
	Essential oils	96% Ethanol	+	distinctive odor

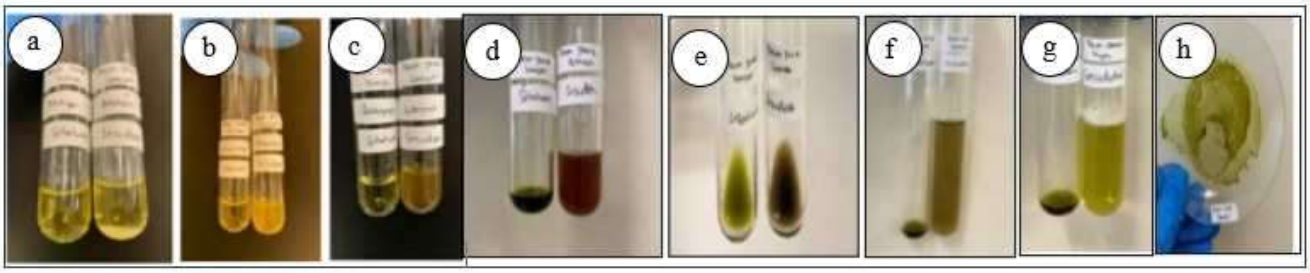

Figure 3. a. meyer. b. dragendorf. c. wagner. d.flavonoid.e. phenol. f. tannin. g. saponin.h. essential oils.

Phytochemical screening tests showed positive results for alkaloids with mayer (white precipitate), dragendoff (orange), wagner (brown), flavonoids (brownish-red), saponins (foam-formed), tannins (greenish-brown), phenols (deep black), and essential oils (special odors). The white precipitation results in the meyer test are due to the interaction of the alkaloid nitrogen group with the tetraiodomercury (II) ($K_2[HgI_2]$) metal ion (Syafitri et al., 2023) (Harahap & Situmorang, 2021). (Reiza et al., 2019) (Sulistyarini et al., 2020), orange color with dragendorff reagent due to potassium-alkaloid (KI) complex with tetraiodobismutate ion, brown color with Wagner reagent due to iodine (I_2) reaction with I- ion from potassium iodide (KI) and form I_3 (Yanti & Vera, 2019). Brownish-red color in the flavonoid test due to the addition of concentrated HCl and Mg powder (Ni'ma & Lindawati, 2022). A positive result of saponins indicates a positive result of foam formation. The formation of foam because saponins have hydrophilic and hydrophobic groups so that when the solution is shaken, the hydrophilic group will bind to water while the hydrophobic group will bind to air so that the reaction will form foam (Suleman et al., 2022). Greenish-brown discoloration due to the addition of $FeCl_3$ (Khafid et al., 2023). The positive result of phenol compounds is due to the reaction between $FeCl_3$ and the OH group (Anindita et al., 2022). The essential oil compound test gave positive results, marked by the aromatic odor of residue after being dissolved with 96% ethanol and evaporated on a hot plate using the watch glass. These results are in

accordance with the research Rahmasiahi et al. (2023), The positive results of the essential oil test are characterized by aromatic odor.

Antibacterial Activity Test of Kirby Bauer Method

The activity test of antibacterial compounds in this study aims to determine the effect of a single extract and a combination of lime leaves, kaff, lemon, and sweet on the growth of *S. epidermidis.* The antibacterial test carried out in this study used the *Kirby bauer* method with concentrations of 2%, 4%, and 8% single and a combination of comparisons (1:1:1:1) with replication three times.

The concentration variation single or combination purpose is to find out which concentration has good antibacterial activity. The principle of *the Kirby bauer* method is to use disc paper as an antimicrobial agent to determine microbial sensitivity by placing disc paper containing antibacterial compounds into agar media that has been inoculated with bacteria. The inhibition of the growth of bacteria produced can be determined by measuring the clear zone around the disc paper (Sari et al., 2022). The results of the test on the effect of ethanol extract of orange leaves on *S. epidermidis* bacteria can be seen in Table 6

Table 6. Test results of ethanol extract of orange leaves.

Sample	Treatment	x̄ ± SD (mm)	Sig.
Lime	2 %	1.67 ± 0.57	0.002
	4 %	3 ± 1.00	
	8 %	5.67 ± 0.57	
	Control (+)	24	
	Control (-)	0	
Kaffir Lime	2 %	2 ± 0.00	0.004
	4 %	5 ± 1.00	
	8 %	8 ± 2.00	
	Control (+)	24	
	Control (-)	0	
Lemon	2 %	3.33 ± 1.15	0.032
	4 %	6.33 ± 2.51	
	8 %	9.67 ± 2.51	
	Control (+)	24	
	Control (-)	0	
Sweet Orange	2 %	3.67 ± 1.15	0.000
	4 %	9.33 ± 0.57	
	8 %	14.33 ± 1.15	
	Control (+)	24	
	Control (-)	0	
Combination leaves orange	2 %	5.33 ± 0.57	0.008
	4 %	9 ± 2.00	
	8 %	10.67 ± 1.15	
	Control (+)	24	
	Control (-)	0	

Control (+) = chloramfenikol
Control (-) = DMSO
*Significance(Sig<0,05)

Based on Table 6, the results of the One Way ANNOVA obtained Sig<0.05, or there was a significant effect of single extract and combination of orange leaves on the diameter of the growth inhibition zone of *S. epidermidis*. Visualization of the inhibition zone between treatment groups can be seen in Figure 4

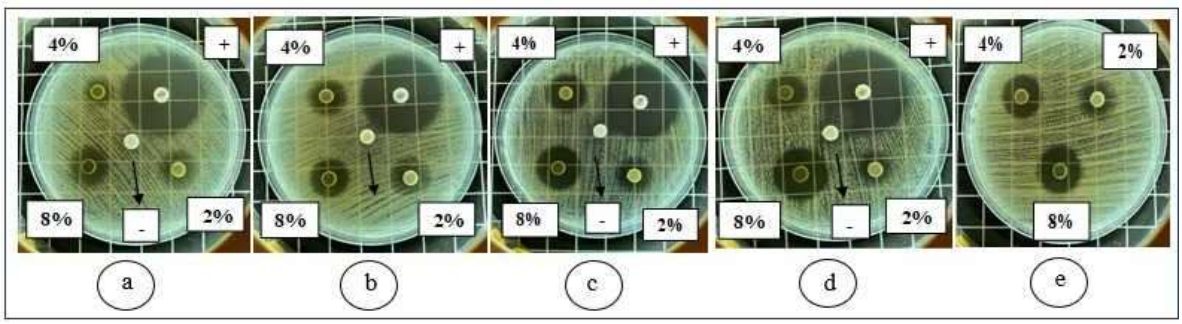

Figure 4. a. lime. b. kaffir lime. c. lemon. d. sweet orange. e. Orange combination.

The results of the Post Hoc test to determine the group with significant influence can be seen in table 7.

Table 7. Results of post hoc tests between groups of orange leaf extract treatment.

Group		Sig.
Lime	Kaffir Lime	1,000
	Lemon	0,395
	Sweet Orange	0,006*
Kaffir Lime	Lime	1,000
	Lemon	1,000
	Sweet Orange	0,082
Lemon	Lime	0,395
	Kaffir Lime	1,000
	Sweet Orange	0,601
Sweet Orange	Lime	0,006*
	Kaffir Lime	0,082
	Sweet Orange	0,601

*Significance(Sig<0,05)

Based on table 7. Post Hoc test results of *ethanol* extract samples of sweet orange leaves most significantly inhibited the growth of *S. epidermidis* (Sig0.006<0.05). The graphic comparison of the effect of single test and combination of ethanol extract of orange leaves on the average diameter of the growth inhibition zone of *S. epidermidis* can be seen in Figure 5.

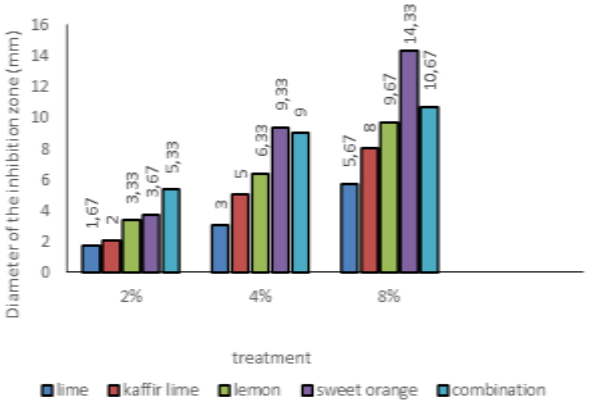

Figure 5. Comparison of the average diameter of the inhibition zone between treatment groups

The treatment of lime leaf ethanol, kaffir lime, lemon, and combination (Table 6) with concentrations of 2%, 4%, and 8% was able to inhibit the growth of *S. epidermidis* with the average diameter of the inhibition zone in lime leaf ethanol extract of 1.67 mm, 3 mm, and 5.67 mm, kaffir lime : 2 mm, 5 mm, and 8 mm, lemon is 3.33 mm, 6.33 mm, and 9.67 mm, sweet orange was 3.67 mm, 9.33 mm, and 14.33 mm, and a combination orange leaf is 5.33 mm, 9 mm, and 10.67 mm. Significant treatment was found at a concentration of 8% with the inhibition category for lime (medium), kaffir lime (medium), lemon (medium), sweet orange (strong), and combination (medium). The sweet orange group is the significant influence of treatment in inhibiting the growth of *S. epidermidis* (table 7). The classification of *S. epidermidis* sensitivity response to Citrus leaf ethanol extract in this study refers to Davis and Stout (1971) in Sakul et al. (2020). It is classified into 4 categories: ≤ 5 mm (weak), 6-10 mm (medium), 11-20 mm (strong), and ≥ 21 mm (very strong).

The antibacterial activity of *S. epidermidis* orange leaf is due to the content of alkaloid, flavonoids, saponins, tannins, phenols, and essential oils. The synergy of secondary metabolite compounds in inhibiting bacterial growth by disrupting peptidoglycan, cell wall surface tension, cell membrane permeability, protein synthesis, and cell leakage results in metabolic disorders, growth, lysis, loss of cytoplasmic substance, and bacterial cell death (Saputera et al., 2019; Nurjannah et al., 2022; Hidayatullah & Mourisa, 2023; Yunilawati et al., 2021). The factors that affect the diameter of the inhibition zone in this study include the turbidity of the bacterial suspension, the thickness of the agar medium, the incubation time, the diffusion of the extract in the media, the type of bacteria, and the concentration of the extract (Rahman et al., 2022; Fransisca et al., 2020).

Related to the type of bacteria factor is proven in research by Wardani et al (2019); Sari & Asri (2022), which used 25%, 50%, and 75% lime peel ethanol extracts were able to inhibit *S. epidermidis* (11.66 mm, 15.66 mm, and 18.33 mm), concentrations of 12.5%, 25%, 37.5%, and 50% against *Shigella dysenteriae* (1.15 mm, 2.69 mm, 2.96 mm, and 3.35 mm). S. *epidermidis* belongs to Gram positive whereas *Shigella dysenteriae* is Gram negative. Based on research by Hermawati et al. (2023) Gram-positive bacteria have a layer of the hydrophobic cell wall that is easily penetrated by secondary metabolite compounds compared to Gram-negative bacteria.

The incubation factor is proven in the research by Maimunah et al. (2020) which used 5%, 10%, 15%, and 20% kaffir lime leaf samples after 48 hours of incubation was able to inhibit *S. aureus* (6.6 mm, 6.8 mm, 7.3 mm, and 8.2 mm), while in this study the concentration of 8% with an incubation time of 18-24 hours was 8 mm

Dewi et al. (2020) using 5%, 12.5%, 25%, 50%, 75%, 100% fruit juice and lemon peel as much as 20 µL was able to inhibit *P. acne* bacteria with (7.15 mm, 9.20 mm, 11.25 mm, 12.50 mm, 14.10 mm, and 16.90 mm). Results Anindita et al. (2022), ethanol extracts of 20%, 40%, 60%, 80%, and 100% lemon peels showed negative alkaloid results so only able to inhibit *S. aureus* bacteria (1.5-3.5 mm). The factor of the length of the soaking time of the disc paper is proven in the study Niken et al. (2023), sweet orange peel extract 20%, 40%, 60%, 80%, and 100% with 15 minutes of soaking was able to inhibit the growth of *S. aureus* (12.3 mm – 21.4 mm), while in this study, soaking for 30 minutes was able to produce an inhibition zone of 14.33 mm.

CONCLUSIONS

The results of the phytochemical screening test of ethanol extract of orange leaf were positive for alkaloids, flavonoids, saponins, tannins, phenols and essential oils. The results of the One Way ANNOVA showed that there was a significant influence on the average diameter of the growth inhibition zone of *S. epidermidis*. The Post Hoc test showed that the effective concentration of lime leaf, kaffir lime, lemon, sweet orange, and the combination was 8%, while for the most significant group category was sweet orange leaf with strong categories. Therefore, it can be concluded that 8% sweet orange leaf ethanol extract can be recommended as a pharmaceutical raw material in inhibiting the growth of *S. epidermidis*.

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Competing Interests: The authors declare that there are no competing interests.

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