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Evaluation of the Bioactivity of MeOH:DMSO (1:1, v/v) Lime Peel Extract on Methicillin-Resistant *Staphylococcus aureus*

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Abstract: This laboratory study aimed to evaluate the bioactivity of MeOH:DMSO (1:1, v/v) lime peel extract on the methicillin-resistant bacteria *Staphylococcus aureus* (MRSA) isolated from infected skin wounds. A screening test was performed on the lime peel extract, and the extract's total flavonoid level (mgHSP/g) was determined. The bioactivity test was based on the results of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests using extract concentrations of 6.25, 12.5, 25, 50, and 800 ppm as treatments, penicillin G as a positive control, and DMSO 10% as a negative control. The extract was found to contain a total flavonoid level of 6.7371 mgHSP/g. The MIC was found to be 50 ppm with an ΔOD value of −0.151. An MBC simple non-linear regression test showed that the increase in extract concentration from 6.25 to 800 ppm decreased the MRSA bacterial count by 87.1%. The eight variations in extract concentration could not determine MBC because all concentrations could only reduce the number of MRSA colonies, and none could kill MRSA.

Keywords: lime peels, flavonoids, methicillin-resistant Staphylococcus aureus, skin infections.

评估甲醇：二甲基亚砜（1：1，v/v）青柠皮提取物对耐甲氧西林的金黄色葡萄球菌的生物活性

摘要：本实验室研究旨在评估甲醇:二甲基亚砜(1:1,体积/体积)石灰皮提取物对从感染的皮肤伤口中分离出的耐甲氧西林金黄色葡萄球菌(MRSA)的生物活性。对酸橙皮提取物进行了筛选测试，并确定了提取物的总黄酮含量 (毫克热电偶/克)。生物活性测试基于最小抑菌浓度(麦克风)和最小杀菌浓度(MBC)测试结果，提取物浓度为6.25、12.5、25、50和800百万分之一作为处理，青霉素G作为阳性对照，DMSO10%作为阴性对照。发现提取物含有6.7371毫克热电偶/克的总黄酮类化合物。发现麦克风为 50百万分之一，Δ外径值为-0.151。MBC 简单非线性回归测试表明，提取物浓度从 6.25百万分之一增加到 800百万分之一会使 MRSA 细菌计数降低 87.1%。提取物浓度的八种变化无法确定 MBC，因为所有浓度都只能减少 MRSA 菌落的数量，而没有一种能杀死 MRSA。

关键词：石灰皮，类黄酮，耐甲氧西林的金黄色葡萄球菌，皮肤感染。

1. Introduction

In recent decades, the pharmacology industry has produced a number of new antibiotics to overcome the increasing resistance of pathogenic microorganisms to many antibiotics [1]. Increasing bacterial resistance to antibiotics has led researchers to focus on finding new drugs derived from medicinal plants. Natural herbal products, both in pure compound form and as standardized plant extracts, provide endless opportunities for developing new drugs because plants

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contain secondary metabolites that have many health benefits [2]. There is, therefore, an urgent need to find new antimicrobial compounds with chemical structures and mechanisms of action that can be used to overcome the problem of pathogen resistance to antibiotics for both recurring and new diseases [3].

The main causes of skin wounds are complications arising from surgery, trauma, and insect bites to the mucosa or skin surface [4]. Skin infections often occur around the hair follicles, subcutaneous tissue, and muscles, and damage or loss of skin integrity due to trauma or disease may cause significant morbidity, even death [5]. Aerobic culture results show that such infections are caused by various types of bacteria, including *Staphylococcus aureus* [6, 31].

S. aureus produces the beta-lactamase enzyme that beta-lactam antibiotics cannot bind to proteins in the bacterial wall, especially those in the penicillin class, such as methicillin, oxacillin, penicillin G, and ampicillin. The presence of this enzyme will damage the β-lactam ring, rendering the antibiotic inactive. The *S. aureus* strain that has developed resistance to the antibiotic methicillin is known as methicillin-resistant *S. aureus* (MRSA) [6, 7].

Phytotherapy represents an alternative and safe form of treatment for infection problems and has minimal side effects. Lime is one phytotherapy option [8]. Lime peel contains the active flavonoid compounds eriocitrin, narirutin, hesperidin, neohesperidin, neoponcirin, poncirin, isorhoifolin, diosmin, neodiosmin, sinensetin, nobiletin, tangeretin, and heptametoxyflavone [9]. Lime peel has higher flavonoid content than the juice of the pulp. Besides containing flavonoid compounds, lime peel also contains saponin compounds [10]. The substances contained in lime peel are able to act as anti-inflammatory, antibacterial, antimicrobial, antiviral, antiulcerogenic, anticancer, cholesterol-lowering, antineoplastic, antitumor, antiplatelet, antihepatotoxic, and antihypertensive substances [8, 11].

2. Methods/Material

Data were collected in this laboratory experimental study between November 2019 and March 2020.

2.1. Lime Peel Collection

The lime fruit used in this study was obtained from a plantation in Bumiaji, Batu, East Java, Indonesia. To reduce the possibility of sample identity errors, plant samples were first identified at the Biology Service Unit, Faculty of Science and Technology, Universitas Airlangga, Surabaya, Indonesia.

Lime fruits were collected from the previously identified plants. The fruit was washed several times with clean running water. The peel was separated from the pulp, cut into small pieces using a stainless-steel knife, and dried in the shade.

2.2. Preparation of Lime Peel Extract

A 2.5-kg sample of lime peel was immersed in a fresh 6 L n-hexane solution for 48 hours. This was then filtered to separate the residue from the filtrate. The filtrate was evaporated, producing a thick extract of n-hexane (non-polar). The residue was immersed in 6 L of ethyl acetate (EtOAc) for 48 hours, then filtered. The resulting filtrate was evaporated, producing a thick (semi-polar) ethyl acetate extract, and the resulting residue was immersed in 6 L of methanol (MeOH) for 48 hours and then filtered. The newly resulting filtrate was evaporated, producing a thick (polar) methanol extract, and the newly resulting residue was immersed in a mixture of methanol and DMSO with a ratio 1:1 for 48 hours and then filtered. The newly resulting filtrate was evaporated, producing an MeOH:DMSO extract (highly polar) [12, 13]. Each of the extracts produced in the previous steps was weighed. This study used the MeOH:DMSO (1:1, v/v) extract, which was freeze-dried after weighing.

2.3. Flavonoid Screening Test

A total of 2 g of MeOH:DMSO (1:1, v/v) lime peel extract was placed in a test tube, to which 3 drops of concentrated HCl solution and 2 mg of Mg powder were added. The sample was shaken, and the resulting changes in color were observed. Flavonoid presence was indicated by a red, yellow, or orange color, depending on the structure of the flavonoids contained in the sample [14].

2.4. Determination of the Extract's Total Flavonoid Levels (mgHSP/g)

Standard solutions of 10, 20, 30, 40, and 50 ppm were prepared from 100 ppm of stock solution by weighing 1 mg of standard hesperidin. 0.5 mL of each standard solution was placed in a 5 mL volumetric flask, to which 1.5 mL of methanol, 0.1 mL of 10% $AlCl_3$, and then 0.1 mL of 1 M potassium acetate were added. The flask was then left to stand for 6 minutes. The solution was diluted to 5 mL and then left to stand for 30 minutes, and the absorption of the solution was measured at gamma = 415 nm using a spectrophotometer.

For the sample, the extract weighed as much as 0.1074 g and 0.1037 g, respectively, and dissolved with 10 mL of methanol in a 10 mL volumetric flask. From each sample solution, as much as 0.5 mL was taken and put into a 5 mL volumetric flask and then added with 1.5 mL methanol and 0.1 mL of 10% $AlCl_3$. It was added with 0.1 mL of 1 M potassium acetate and then left to stand for 6 minutes. The solution was diluted to 5 mL and then left to stand for 30 minutes, and the absorption of the solution was measured at wavelength = 415 nm using a spectrophotometer [15], [16].

2.5. Bioactivity Test of MeOH:DMSO (1:1,v/v) Extract Lime Peels

The bioactivity test of lime peel MeOH:DMSO (1:1, v/v) extract by turbidity measurement method was carried out using a spectrophotometer to determine the MIC value, while the MBC value was tested using the pour plate method. The test bacteria used in this study were methicillin-resistant staphylococcus aureus (MRSA), which was isolated from pus samples and obtained from the Clinical Microbiology Laboratory, Dr. Soetomo Hospital, Surabaya. MRSA bacterial suspensions were prepared, and its turbidity levels were standardized to the McFarland standard of 0.5, where the turbidity was equivalent to a bacterial density of 10^8 CFU/mL. The produced suspension was then diluted by pipetting 0.1 mL of bacterial suspension (10^8 CFU/mL), inserted into a sterile tube, and added with 9.9 mL of 0.9% NaCl solution so that the bacterial density of the test became 10^6 CFU/mL.

The MIC test was carried out by preparing seven tubes, each containing 8.8 mL of Nutrient Broth media. Tube 1 was added with 1 mL of penicillin-G 10 units as a positive control treatment. Tube 2 was added with 10% DMSO as a negative control treatment. Tubes 3, 4, 5, 6, 7, 8, 9, and 10 were respectively filled with 1 mL of lime peel MeOH:DMSO (1:1, v/v) extract in concentrations of 6.25, 12.5, 25, 50, 100, 200, 400, and 800 ppm. Furthermore, the ten tubes were added with 200 µl of the tested bacterial suspension. All tubes were vortexed to make them homogeneous, then 2 mL were taken to measure the Optical Density (OD) value of the bacteria using a wavelength 600 nm gamma spectrophotometer. The ten tubes were incubated for 18-24 hours at 37 ° C. The post-incubation OD value was measured again by taking 2 mL for the value's measurement using a wavelength 600 nm gamma spectrophotometer. If the difference between the OD value and the lowest concentration was negative, the value was assigned as Minimum Inhibitory Concentration (MIC). A positive ΔOD value indicated no decrease in ΔOD value, which means there was still an increase in the number of bacterial cells after incubation [17].

The MBC test was carried out using the pour plate method. Seven sterile empty Petri dishes and thawed MHA media were prepared. Lime peel MeOH:DMSO (1:1, v/v) extracted from the serial concentration and control of 1 mL each was inserted into the Petri dish, then the tested bacterial suspension was added. Each Petri dish was poured with 15 mL of MHA media, then homogenized and waited until the media solidified (± 15 minutes), then incubated at 37 degrees C for 18-24 hours. The presence or absence of bacterial colony growth was observed on MHA media. Total bacterial colonies were calculated using a colony counter. MBC was obtained if the MHA medium did not show the growth of bacterial colonies, or there was a 99.9% reduction from the original inoculum on the sub-culture. Data from the MBC test results were analyzed by a simple non-linear regression test to measure the effect of varying concentrations of lime peel MeOH:DMSO (1:1, v/v) extract on the number of MRSA bacterial colonies [17], [18], [32].

2.6. Statistical Analysis

Data were analyzed by simple linear regression and simple non-linear regression. Data Analysis performed using Statistic Package for Social Science Software, Version 17 (SPSS Inc. Chicago, IL, the USA). All data presented tables and graphics.

3. Results

Determination showed that the lime plant used in this study was the *Citrus aurantifolia* (Christm.) Swingle species from the Rutacea family. Results of the weight of each extract are shown in Table 1.

Table 1 Weight of each lime peel extract using graded extraction

Extract	Simplicia weight before extraction (g)	Extract weight (g)	Rendement value (%)
n-hexana	2500	12.86	0.51
Ethyl acetate	2500	25.53	1.02
Methanol	2500	98.38	3.94
MeOH:DMSO (1:1, v/v) before lyophilization	2500	1750.95	-
MeOH:DMSO (1:1, v/v) after lyophilization	2500	98.80	3.95

The results of the flavonoid screening test showed the presence of flavonoid compounds in MeOH:DMSO (1:1, v/v) extract of lime peel. Total flavonoid content in lime peel (*Citrus aurantifolia* (Christm.) Swingle) MeOH:DMSO (1:1, v/v) extract are presented in Table 2 and Fig. 1.

Table 2 The results of the absorbance measurement of the hesperidin standard solution at a wavelength of 415 nm

Concentration (ppm)	Absorbance (y)
10	0.025
20	0.048
30	0.074
40	0.099
50	0.138

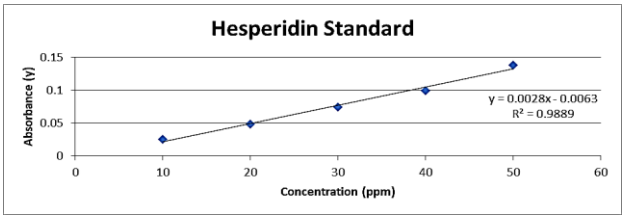

Fig. 1 Calibration curve of hesperidin at 415 nm wavelength

In the lime peel MeOH:DMSO (1:1, v/v) extract, the total flavonoid content was obtained at 6.7371 mgHSP/g extracts.

The results of measuring the Optical Density (OD) value on MIC using a spectrophotometer (wavelength 480 nm) are shown in Table 3.

Table 3 Measurement of OD value in MIC test of lime peel MeOH:DMSO extract (1:1) against MRSA bacteria

Treatments	OD values		ΔOD
	Pre-incubation	Post-incubation	
6.25 ppm	0.093	0.108	0.015
12.5 ppm	0.147	0.203	0.056
25 ppm	0.234	0.480	0.246
50 ppm	0.425	0.274	-0.151
100 ppm	0.606	0.387	-0.219
200 ppm	0.761	0.292	-0.469
400 ppm	0.788	0,205	-0.583
800 ppm	0.805	0.191	0.614
Positive control	0.180	0.055	-0.125
Negative control	0.048	0.327	0.279

The ΔOD value presented in Table 4 shows the negative value of the extract with a concentration of 50 ppm and 100 ppm, i.e., -0.151 and -0219, so that the MIC value lay in the extract concentration of 50 ppm.

Table 4 Minimum bactericidal concentration test for lime peel MeOH:DMSO (1:1, v/v) extract against MRSA bacteria

Treatment	Total colony (CFU/dish)
6.25 ppm	1261 ± 50
12.5 ppm	1059 ± 7
25 ppm	919 ± 9
50 ppm	354 ± 6
100 ppm	138 ± 8
200 ppm	128 ± 3
400 ppm	127 ± 1
800 ppm	126 ± 1
Positive control	48 ± 1
Negative control	1406 ± 32

None of the eight variations of lime peel MeOH:DMSO (1:1, v/v) extract concentrations was designated as MBC because, at a concentration of 6.25 to 100 ppm, the growth of bacterial colonies on MHA media was still found. The effect of the five concentrations of lime peel MeOH:DMSO (1:1, v/v) extracts on the number of MRSA bacterial colonies per Petri dish is shown in Fig. 2.

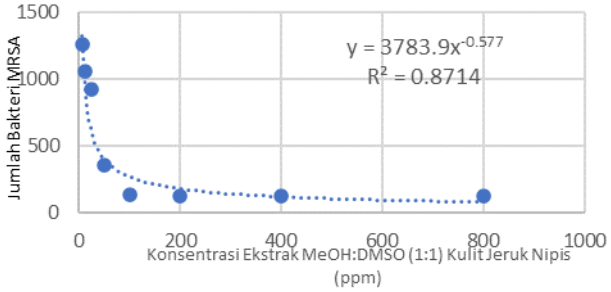

Fig. 2 Simple linear regression between eight variations of the concentration of lime peel MeOH:DMSO (1:1, v/v) extract against the number of MRSA bacterial colonies

We obtained the correlation values between the concentration of lime peel MeOH:DMSO (1:1, v/v) extract and the number of MRSA bacterial colonies per Petri dish, i.e. -6.377, with a significance of 0.001, which was less than alpha = 0.05. This means that increasing the concentration of the extract significantly reduced the number of MRSA bacterial colonies per Petri dish. The conclusion from the results of the simple non-linear regression test was that the variation in the concentration of lime peel MeOH:DMSO (1:1, v/v) extract (X) had an effect on the decrease in the number of MRSA bacteria (Y) with a total effect of 87.1%.

4. Discussion

This study used graded extraction using n-hexane as a solvent that could dissolve non-polar secondary metabolites. Ethyl acetate solvent can dissolve semi-polar secondary metabolites, while methanol (MeOH) and MeOH:DMSO (1:1, v/v) solvent can dissolve polar secondary metabolites.

The resulting extract can be affected by the polarity of the solvent used. The study showed that the yield of lime (*Citrus aurantifolia* (Christm.) Swingle) peel extract with polar solvents [methanol and MeOH:DMSO (1 : 1)] produced high yield values. The yield of lime (*Citrus aurantifolia* (Christm.) Swingle) peel extract with semi-polar solvent (ethyl acetate) was smaller than that of polar solvents (MeOH and MeOH:DMSO). The yield is the ratio of the extract obtained with the initial simplicia. The yield is given as a percentage. The higher the yield value produced, the higher the extract value produced [19]. In terms of time, to obtain more active substances, it takes a long time and a long process, because this method of extraction did not use heat as a support [20].

Previous studies have shown that, in the absence of other forces for immersion-only maceration methods, the osmosis of the solvent into the solid is static, even though the solvent has been replaced by a remaceration method. However, in terms of temperature, the maceration method is a cold extraction that is carried out at room temperature and is relatively safe to use for materials that are resistant or not resistant to heating. The results of other studies also suggest that most of the compounds can interact with cold extraction [21].

The flavonoid screening test is a preliminary stage in a phytochemical study with the aim of providing an overview of the class of compounds contained in the plant under study. Observation of the flavonoid screening test in lime peel MeOH:DMSO (1:1, v/v) extract showed a yellow-red color formation, which indicated that the lime peel contained flavonoids. Flavonoids are the main class of polyphenols with a C6-C3-C6 framework. To date, there are more than 8,000 known flavonoid molecules. Most of the flavonoid molecules have shown some biological activities, such as antioxidant, anti-inflammatory, anticancer, and cardiovascular protection [22]. Flavonoids can be classified into flavanones, flavones, and flavonols [23]. The composition of flavonoids in lime peel from the flavanone group includes eriocitrin, neoeriocitrin, narirutin, hesperidin, neohesperidin, neoponcirin, and poncirin. The composition of

flavonoids in lime peel from the flavone group include isorhoifolin, diosmin, neodiosmin, sinensetin, nobiletin, and tangeretin [24].

Flavanones such as nobiletin, tangeretin, and liquiritigenin have been reported as active agents responsible for the antibacterial activity of several medicinal plants. The substituent has a significant effect on the antibacterial activity of flavanones. Flavanones replaced by prenyl groups, at either the 6-C or 8-C chain positions, show antibacterial activity against *Staphylococcus aureus*. Prenylflavanones even show great activity against Methicillin-Resitant *Staphylococcus aureus* (MRSA) bacteria [25].

In this study, determination of total flavonoid levels in lime peel MeOH:DMSO (1:1, v/v) extract using UV-Vis spectrophotometry was carried out to determine total flavonoid levels in the extract. Analysis of total flavonoids was carried out using UV-Vis spectrophotometry because flavonoids contain conjugated aromatic systems so that they have strong absorption bands in the UV-Vis spectrum and visible light spectrum [15]. Quantitative analysis with UV-Vis spectrophotometry used a blank solution as a control which functions as a blank compound (multiplying with zero) that do not need to be analyzed [26].

In the measurement of total flavonoid compounds in this study, the sample extract solution was added with $AlCl_3$ which formed a complex, resulting in a shift in wavelength towards the visible area, which was marked by a solution that produces a yellow color and the addition of potassium acetate which aimed to maintain the wavelength in the visible area [26]. The incubation treatment performed 30–60 minutes before measurement is intended to maximize color intensity to ensure that the reaction runs smoothly [19].

One of the ingredients in lime peel that functions as an antimicrobial is hesperidin. Hesperidin is a bioflavonoid, flavanone glycoside. Hesperidin has multiple pharmacological actions, including antimicrobial, antifungal, anti-inflammatory, estrogenic, antiallergenic, and antioxidant properties. Hesperidin has specific antibacterial properties against the dominant bacteria found on the surface of wounds, *Staphylococcus aureus* [27].

The results of the lime peel MeOH:DMSO (1:1, v/v) extract bioactivity test against methicillin-resistant *Staphylococcus aureus* (MRSA) showed that the extract had bacteriostatic ability. The flavonoid content in the extract can inhibit the growth of MRSA by disrupting the permeability, stability, and formation of the membrane and cell walls [28].

The minimum bactericidal concentration (MBC) in this study could not be observed because MRSA bacteria were still able to survive at a concentration of 800 ppm, with an average number of colonies of 1.26 x 10^2 cfu/ml. Likewise, in penicillin, bacteria were still able to survive with an average colony of 4.8 x 10^1 cfu/ml. This was due to the breakdown of penetration of the target penicillin-binding protein and the presence of an outflow pump for beta-lactamase production. Therefore, a complex active ingredient mechanism was needed to inhibit the growth of MRSA strains [29].

The results of this study indicate the need for a combination of antibacterials. Where two antibacterials are used simultaneously, they can have synergistic effects. By combining herbal ingredients, this study aimed to find those formulas that exhibited the most antibacterial activity. When the extracts of several plants were combined together, they had a greater inhibitory effect against bacterial growth than a single plant extract [30].

Overall, this study has resulted in three discoveries of scientific novelty. To begin with, this research is the first to examine the antibacterial effect of MeOH:DMSO (1:1, v/v) extract of lime peel on inhibiting MRSA bacterial growth. Next, this study shows that MeOH:DMSO (1:1, v/v) extract of lime peel has potential as an antibacterial with bacteriostatic properties. Finally, this study builds on existing research relating to the determination of total flavonoid levels in MeOH:DMSO (1:1, v/v) extract of lime peel using hesperidin standard.

5. Conclusion

In conclusion, evaluation of the bioactivity of lime peel MeOH:DMSO (1:1, v/v) extract against MRSA bacteria showed that the extract contained total flavonoid levels of 6.7371 mgHSP/g. The MIC of MeOH:DMSO (1:1, v/v) extract of lime peel (*Citrus aurantifolia* (Christm.)) was 50 ppm, while none of the five variations in extract concentration was defined as the MBC.

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