

Combination Effect of Ethanol Extract of Pomegranate Peel (*Punica granatum* L.) and Lemongrass Stalk (*Cymbopogon citratus*) Against *Staphylococcus aureus* ATCC 6538

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ABSTRACT

Staphylococcus aureus is one of the bacteria that cause infectious diseases. Some cases were found to be antibiotic-resistant. That is why the development of natural antibiotics is necessary. The objectives of this study are to determine the effect of an antibacterial test from ethanol extract of pomegranate peel and lemongrass stalk, and its combination against the bacterium *Staphylococcus aureus* ATCC 6538. The Activities of antibacterial determined by using the method of microdilution to get value Concentration Inhibitory Minimum (MIC) and the Minimum Bactericidal Concentration (MBC), and diffusion methods to determine diameter of inhibition zone of each extract against *Staphylococcus aureus* ATCC 6538. Diffusion methods to test the extract combination using paper tape. The study results showed that the extract has antibacterial activity against *Staphylococcus aureus* ATCC 6538, the lowest value of the MIC found in ethanol extract of pomegranate peel. The combination of the extract against *Staphylococcus aureus* ATCC 6538 is additive. It can be concluded that each extract the effect of antibacterial against *Staphylococcus aureus* ATCC 6538, the use of extract combination were additive.

Keywords: MIC, MBC, pomegranate peel, lemongrass stalk

1. BACKGROUND

Infection is one of the major health problems in Indonesia and globally. According to the Ministry of Health of Indonesia (2011), the prevalence of infection in the hospital counted to be around 3-21% (9% on average) or over 1.4 million patients hospitalized inpatient due to infection all over the world. The prevalence of HAIs (Hospital Acquired Infection) in Indonesia reach 15,74% [1]. Addressing infectious disease need specific and proper antibiotics. Inappropriate use of antibiotics leads to antibiotics resistance.

According to the US Department of Health and Human Services and the Centers for Disease Control (CDC) in 2019, at least 35,900 people suffered death due to disease infections are accompanied by antibiotic resistance every year[2]. This number tends to be increased compared with the previous year 2013 where at least 23,000 people

suffered death due to disease infections accompanied by antibiotic resistance each year [3]. The raising of antibiotic resistance problems must be overcome by developing a new natural form of antibiotics.

One of the natural ingredients that have antibacterial activity is pomegranate peel and lemongrass stalk. Mostafa, et.al., has studied the antibacterial effect of pomegranate. Several studies in the literature already revealed that ethanol extract of pomegranate peel has the activity of antibacterial against *Staphylococcus aureus* [4]; While residual liquid ethanol extract of *Cymbopogon citratus* (lemongrass) has activity against *Staphylococcus aureus*[5]; methanol extract of *Cymbopogon citratus* have activity against *Staphylococcus aureus* [6]. However, the combination of extract pomegranate peel and extract lemongrass stalk is still insufficiently represented in the literature. The research aims are to determine the

effect of an antibacterial test from ethanol 90% extract of pomegranate peel and lemongrass stalk, and its combination against *Staphylococcus aureus* ATCC 6538.

2. METHODS

2.1 Materials

Lemongrass stalk, ethanol extract of pomegranate peel, distilled water, alcohol 70%, ethanol 90%, medium Mueller-Hinton order (MHA), medium Mueller-Hinton Broth (MHB), tetracycline, ethyl acetate, amyl alcohol, powdered magnesium, acid chloride, sodium hydroxide, sodium acetate, iron (III) chloride, gelatine, ether, chloroform, acid sulfate, ammonia, acid acetic glacial, potassium iodide.

2.2 Extract Processing

Fresh lemongrass stalk was obtained from Lembang, West Java and then cleaned, washed, drained, dried, and pulverized to be simplisia powder. The extract is made using the solvent ethanol 90% and extraction results are compressed with rotary evaporator. Ethanol extract of pomegranate peel was obtained from Azreen (a previous researcher).

Simplicia and Extract Characterization

Simplicia characterization was conducted in this research done by screening phytochemical (alkaloids, flavonoids, tannins, quinones, steroids/triterpenoids, saponins). While the extract characterization including shrinkage drying, extract ethanol-soluble levels, yield type weights, and phytochemical filtering (alkaloids, flavonoids, quinones, tannins, saponins, steroids/triterpenoids)[7].

2.3 Testing Bacteria

The testing bacteria used *Staphylococcus aureus* ATCC 6538 obtained from the Laboratory of Microbiology Analysis, School of Pharmacy, Bandung Institute of Technology (ITB). All the tools are sterilized using an autoclave at a temperature of 121°C with a pressure of 15 psi for 15 minutes. All bacteria-related work is done in an aseptic environment.

2.4 Media Creation, Inokulum and Extract Solution testing

A total of 21 grams of powder media Mueller-Hinton Broth (MHB) is suspended in 1 liter of distilled water, heat while stirring. The exact amount of 35 grams of powder media Muller-Hinton order (MHA) were suspended in 1 liter of distilled water, heat while stirring. All suspensions are placed on the erlenmeyer flask, closed with sterile gauze, and then

sterilized using autoclave at a temperature of 121°C for 15 minutes.

Inscribed the bacteria on an oblique using round ose needles, and then incubated in the incubator for 24 hours at 37°C. Bacteria that have been incredulity and grown on to tilt are taken with round ose needles and suspended at 5 mL of MHB media, then incrustrated for 24 hours at 37°C. The suspension of the bacteria is diluted with MHB until absorbance with a range of 0.08 - 0.13 ($1-2 \times 10^8$ CFU/mL) using a UV-Vis spectrophotometer at a wavelength of 625 nm (equivalent to 0.5 McFarland). Blanko used is MHB. After absorbance is produced in that range, the bacterial suspension is diluted again with MHB so that the final number of bacteria contained in each plate well is equivalent to approximately 5×10^5 CFU/mL (range $2-8 \times 10^5$ CFU/mL).

2.5 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Antibacterial activity of the test extract is determined by a microdilution method using a Microwell plate to determine the values of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). This microdilution method is a method recommended by the Clinical and Laboratory Standards Institute (CLSI). Microwell plate consists of 8 rows and 12 columns so that a total of 96 microplate wells. Column 1 is filled with 200 µL of sterile MHB media (negative control), column 2 is filled with 100 µL of sterile MHB media and 100 µL of bacterial suspension test, column 3 to 12 is filled with 100 µL of sterile MHB media. In the 12th column added 100 µL solution of test extract or antibiotic (tetracycline) with a certain concentration, then homogenized. Then, 100 µL is taken from the 12th column well, then transferred to the 11th column well and repeat until the results of dilution of the test extract/antibiotic have filled the 3rd column well. After that, the suspension of diluted test bacteria is inserted into all wells except in the negative control column located in the 1st column. Microwell plates are uncredited for 18-24 hours at $35 \pm 2^\circ\text{C}$. Minimum Inhibitory Concentration (MIC) is observed as the lowest concentration where there are no bacterial deposits at the bottom of the well (clear) which indicates inhibition of bacterial growth. This test is done on a triplo-basis.

The value of MBC is determined by being picked as much as 5 µL of microplate well solution from microdilution test results that show clear areas (no turbidity or bacterial deposits at the bottom of the well) and at concentrations above clear areas. Furthermore, this solution is scratched on sterile

MHA media that has been solid in a petri dish and incrustrated for 24 hours at a temperature of 37⁰C. MHA media for which there is no bacterial growth on its surface is designated as Minimum Bactericidal Concentration (MBC).

2.6 Determination of Diameter of Inhibition Zone

A total of 50 µL bacterial suspension (which has been prepared before) is inserted into a sterile 15 mL MHA that has not to solidify, vortexed until homogeneous, then poured into a sterile petri dish and allowed to solidify. Paper discs (disc diameter size of 5 mm) are dipped in test extracts/tetracyclines (which are suspended into DMSO10%) with concentrations of 2x MIC, 4x MIC, and 8x MIC, then dried and placed on top of MHA that already contain bacterial tests. In addition, paper discs were also dipped in a mixture of MHB and 10% DMSO as negative controls that proved to have no resistance. The petri dish was incantated at 37⁰C for 18-24 hours. Once inc incrustrated, the clear area around the paper disc is measured using the funnel term to

determine the diameter of inhibition zone against the test bacteria.

2.7 Determination of Combination Effect of Extract with Diffusion Methods to Use Paper Tape

Determination of the nature of the combination of test extracts is carried out by diffusion methods so that paper tape[8]. Pure culture of test bacteria is suspended in MHB and included for 18-24 hours at 37⁰C, then diluted using MHB. A total of 50 µL of bacterial suspension is added to the sterile 15 mL MHA that has not to solidify, then vortexed until homogeneous. This mixture is then poured into a sterile petri dish and allowed to solidify. One paper tape is dipped in a solution of ethanol extract of pomegranate peel and another paper tape is dipped in a solution of ethanol extract of lemongrass stalk. This dry paper tape is then placed on top of the MHA that has contained test bacteria with the meeting of this paper tape forming an angle of 90⁰. Petri dishes are incrustrated at 37⁰C for 18-24 hours. Obstacles at the meeting of the two paper tapes were observed.

3. RESULTS

Phytochemical filtering on ethanol extract of pomegranate peel and lemongrass stalk results are as follows:

Table 1. Phytochemical Screening Results

Compound Group	Ethanol extract	
	Lemongrass stalk	Pomegranate peel*
Alkaloid	+	+
Flavonoid	+	+
Tanin	+	+
Kuinon	+	-
Steroid/triterpenoid	+	-
Saponin	-	-

Note: *data obtained from Azreen Natasha Binti Azmi

Based on table 2, can be obtained the MIC value of pomegranate peel extract against *Staphylococcus aureus* ATCC 6538 is 256 µg/mL with MBC value >4096 µg/mL. The MIC value of lemongrass stalk extract against *Staphylococcus aureus* ATCC 6538 is 4096µg/mL with MBC value

>4096 µg/mL. Determination of MIC and MBC values is also done on tetracycline HCl as a comparison antibiotic. The MIC tetracycline value of HCl against *Staphylococcus aureus* ATCC 6538 is 0.5 µg/mL while the MBC value is 16 µg/mL.

Table 2. MIC and MBC Extracts Test against *Staphylococcus aureus* ATCC 6538

	MIC and MBC (µg/mL)	MIC and MBC (µg/mL)		
		Ethanol extract of pomegranate peel	Ethanol extract of lemongrass stalk	Tetracycline
<i>Staphylococcus aureus</i> ATCC 6538	MIC	256	4096	0,5
	MBC	>4096	>4096	16

Table 3. Diameter of Inhibition Zone of Test Extract against *Staphylococcus aureus* ATCC 6538

Concentration	Diameter of inhibition zone (mm)		
	Ethanol extract of pomegranate peel (MIC=256 µg/mL)	Ethanol extract of lemongrass stalk (MIC=4096 µg/mL)	Tetracycline (MIC = 0,5 µg/mL)
2xMIC	0,0±0,00	0,0±0,00	10,3±0,58
4xMIC	7,0±0,00	6,0±0,00	13,0±1,73
8xMIC	8,7±0,58	7,3±0,58	15,3±1,54

The diameter of inhibition zone of ethanol extract of pomegranate peel and ethanol extract of lemongrass stalk against *Staphylococcus aureus* ATCC 6538 is carried out using concentrations of 2xMIC, 4xMIC, and 8xMIC (Table 3). The result of diameter of inhibition zone of ethanol extract of pomegranate peel against *Staphylococcus aureus* ATCC 6538 at concentrations 2xMIC, 4xMIC, and 8xMIC is 0.0±0.00, 7.0±0.00, and 8.7±0.58 mm respectively. While the diameter of inhibition zone of ethanol extract of lemongrass stalk against *Staphylococcus aureus* ATCC 6538 at concentrations 2xMIC, 4xMIC, and 8xMIC is 0.0±0.00, 6.0±0.00, and 7.3±0.58 mm. The diameter of inhibition zone of tetracycline resistance to *Staphylococcus aureus* ATCC 6538 at concentrations 2 MIC, 4 MIC, and 8 MIC is 10.3±0.58, 13.0±1.73, and 15.3±1.54 mm.

4. DISCUSSION

Phytochemical filtering results of ethanol extract of lemongrass stalk show the presence of a group of alkaloid compounds, flavonoids, tannins, calcons, steroids/triterpenoids. In a study conducted by Umar, et al., (2016) ethanol extract of lemongrass contains a group of tannin compounds, flavonoids, alkaloids[9]. While Soraya, et.al., (2016) found ethanol extract of lemongrass contains a group of alkaloid compounds, terpenoids, saponins, flavonoids, tannins[10]. The results of the filtering of phytochemicals pomegranate peel extract showed the presence of a group of alkaloid compounds, flavonoids, and tannins. Another study conducted by Bhandary, et al., (2012) stated that pomegranate peel extract contains flavonoid compounds and tannins[11]. The difference in phytochemical screening results is caused by the source of the plant used comes from a different growing place so that the number of chemical components in it is also likely to be different.

Antibacterial activity Testing of pomegranate peel extract and lemongrass stalk extract against *Staphylococcus aureus* ATCC 6538 was conducted by determining Minimum Inhibitory Concentration (MIC) by microdilution method, determination of Minimum Bactericidal

Concentration (MBC), and measurement of diameter of inhibition zone through jelly diffusion methods. Determination of MIC with microdilution method has the advantages of small sample use, low cost, Reproducibility[12], quantitative [13], and easy to use[14]. The determination procedure of MBC value is done by scratching the test extract solution on the concentration of a clear solution from MIC test results to sterile Muller Hilton Agar (MHA) media that has solidified in petri dish. The agar concentration that indicates the absence of bacterial growth is expressed as MBC.

After the MIC value of each extract is obtained using the microdilution method, determination of the diameter of inhibition zone of the ethanol extract of pomegranate peel and the ethanol extract of lemongrass stalk against *Staphylococcus aureus* ATCC 6538 is also carried out. The negative controls used are a mixture of MHB and DMSO media 10% which is proven not to have a diameter of inhibition zone. The determination of this diameter of inhibition zone aims to see the sensitivity of antimicrobial activity of ethanol extract of pomegranate peel and ethanol extract of lemongrass stalk to bacteria. Determination of the hardness diameter is done by diffusion method to use paper discs that are 5 mm in diameter. Advantages of diffusion methods are that the method is simple, reproducible, easy to modify, low cost. This method also needs to pay attention to the diffusion of the compounds used[12]. In this study, it is suspected that most of the compounds contained in ethanol extracts of lemongrass stalk are nonpolar compounds (such as essential oils)[15]. While the medium used by these compounds to diffuse is polar so that nonpolar compounds will be difficult to diffuse [16]. Therefore, obtained the results of research on the diameter of inhibition zone of the ethanol extract of pomegranate peel and lemongrass stalk against *Staphylococcus aureus* ATCC 6538 can be seen in Table 3.

Based on the results of the study it can be seen that pomegranate peel extract and lemongrass stalk extract have antibacterial activity against *Staphylococcus aureus* ATCC 6538. Antibacterial activity of pomegranate peel extract is better than

lemongrass stalk extract. This is seen with a lower MIC value of ethanol extract of pomegranate peel compared to ethanol extract of lemongrass stalk.

Based on research conducted by Benguiar et al., (2020), ethanol extract of pomegranate peel has the main content of phenolic compounds and flavonoids [17]. Therefore, the effect of antibacterial activity on pomegranate peel extract is suspected due to the presence of the main phenolic compound [18]. While the content of compounds that are suspected to have antibacterial activity in lemongrass stalk is tannins by inhibiting the activity of protease enzymes from bacteria [6].

After getting MIC results of each extract of the samples against bacteria, then tested the combination effect of pomegranate peel extract and lemongrass stalk conducted on *Staphylococcus aureus* ATCC 6538 using the concentration of each extract test 8xMIC with the method of paper tape diffusion. The testing method used the diffusion method using paper tape. Testing methods used diffusion using a paper tape. The results of this test aim to find out the nature of the interaction between pomegranate peel extract and lemongrass stalk extract against *Staphylococcus aureus* ATCC 6538. The results of the combination of both extracts against *Staphylococcus aureus* ATCC 6538 showed that the diameter of inhibition zone of pomegranate peel extract and lemongrass stalk extract at the meeting angle of the paper tape showed no change in the diameter of inhibition zone compared to the diameter of inhibition zone on the side of the paper tape that did not meet. Therefore, the interaction between pomegranate peel extract and lemongrass stalk extract against *Staphylococcus aureus* ATCC 6538 can be concluded to have an additive effect where the use of pomegranate peel extract does not affect the activity of lemongrass stalk extract.

5. CONCLUSION

Ethanol extract of pomegranate and lemongrass stalk have antibacterial activity against *Staphylococcus aureus* ATCC 6538. A combination of test extracts against *Staphylococcus aureus* ATCC 6538 is an additive.

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