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Antibacterial Activity of Extract and Ethanol-Water Fraction of White Pomegranate Peel (*Punica granatum* L.) Against *Methicillin-resistant Staphylococcus aureus* (MRSA) and TLC-Densitometry Test

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Abstract

Methicillin-resistant Staphylococcus aureus (MRSA) is a bacterial infection that is resistant to the antibiotic methicillin and the *β*-lactam class of antibiotics. This study aims to determine the antibacterial activity of the extract and ethanol-water fraction of white pomegranate peel (*Punica granatum* L.) against MRSA bacteria and the TLC-Densitometry test. The results of this study are the antibacterial activity of the extract and the ethanol-water fraction at concentrations of 10%, 20%, and 30% were obtained: extract of 0,670 cm ± 0,010; 1,076 cm ± 0,010; 1,242 cm ± 0,010 and the ethanol-water fraction of 0,830 cm ± 0,010; 1,232cm ± 0,007; 1,419 cm ± 0,010. The gallic acid content in the extract was 3,13908 mg/ g sample and the ethanol-water fraction was 3,82457 mg/ g sample. The contents of phenolic compounds, tannins, and flavonoids can have the ability to inhibit MRSA bacteria, according to the results of a TLC bioautography test.

Keywords: white pomegranate, Staphylococcus aureus, TLC bioautography, TLC-densitometry, fraction

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1 Introduction

Infectious diseases are diseases caused by microorganisms such as bacteria and are currently still a health problem in many countries. Treatment of infectious diseases usually uses antibiotics. Currently, people often use antibiotics freely without a doctor's prescription and are used inappropriately. Therefore there is the growth of bacteria that are resistant to antibiotics [1]. One of the resistant bacteria is *Methicillin-resistant Staphylococcus aureus* (MRSA) which originates from a strain of *Staphylococcus aureus* that is resistant to the antibiotic methicillin and other *β*-lactam groups such as penicillin derivatives, cephalosporins, carbapenems, and monobactams [2].

One alternative that can be used to reduce the use of chemical drugs is by utilizing medicinal plants as antibacterial. Medicinal plants that can be used are white pomegranate peel (*Punica granatum* L.). Pomegranate peel contains gallic acid tannins which belong to the class of phenolic compounds but also contain other phenolic compounds such as flavonoids and anthocyanins which can be used to treat bacterial infections and antibiotic resistance [3], [4]. Pomegranate peel extract contains phenolic compounds that can inhibit the growth of *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus* at concentrations of 20%, 12,5%, and 6,25% [5]. Pomegranate peel extract with a concentration of 5%, 10%, 15%, and 20% is also able to inhibit the growth of *Methicillin-resistant Staphylococcus aureus* (MRSA) bacteria [6]. This study was conducted to determine the antibacterial activity of white pomegranate peel (*Punica granatum* L.) on the growth of *Methicillin-resistant Staphylococcus aureus* (MRSA) bacteria in fractions using the separating funnel method with *n*-hexane solvent to separate nonpolar-compounds-and-ethanol-water solvent (8:2) to separate polar compounds such as phenolic compounds so that they are expected to be extracted maximally.

2 Methods

2.1 Materials

The white pomegranate peel (*Punica granatum* L.) was obtained from Kendal, Central Java in March 2022. White Pomegranate was determined at the Pharmaceutical Biology Laboratory, Stifar Yayasan Pharmasi Semarang. The bacteria used were *Methicilin-Resistant Staphylococcus aureus* (MRSA) obtained from the Diponegoro National Hospital (RSND), Semarang, Central Java. Another material is gallic acid (Merck) as a standard substance.

2.2 Preparation of Extract and Fraction

White pomegranate peel (*Punica granatum* L.) was washed with water and cut into pieces. Dry it in the sun by covering it with a black cloth. After drying, blend until powder is obtained and sieved to 30/40 mesh.

White pomegranate peel powder (*Punica granatum* L.) weighed as much as 500 grams and was macerated for 4 days using 70% technical ethanol solvent. The maceration process is carried out by replacing a new solvent every 24 hours and stirring occasionally [7].

The viscous extract was fractionated using the liquid-liquid partition method by weighing 10 grams of it dissolved in ethanol p.a-water (8:2), adding 100 mL of *n*-hexane p.a, and then shaking it. Wait until the layers separate between the *n*-hexane fraction and the ethanol-water fraction. The fractionation process was carried out until the filtrate was clear [8].

2.3 Antibiotic Resistance Test

The resistance test was carried out using the well-diffusion method, in which 10 mL of Mannitol Salt Agar (MSA) medium was poured into a petri dish, allowed to solidify (first layer), and 4 cylinder-cups placed on top of the

solidified MSA media layer. A mixture of 20 mL of Mannitol Salt Agar (MSA) media containing 5 µL of *Methicillin-resistant Staphylococcus aureus* (MRSA) was added to a petri dish (second layer) and allowed to solidify. The cylinder cup was taken, and the wells formed were given 5 µL of amoxicillin, ampicillin, cefadroxil, and ciprofloxacin solution, then incubated at 37°C for 24 hours. The results show resistance if there is no zone of inhibition of bacterial growth around the wells.

2.4 Antibacterial Activity Test

Antibacterial activity testing was carried out using the well-diffusion method. 10 mL of Mannitol Salt Agar (MSA) media was poured into a petri dish and allowed to solidify (first layer) and 5 cylinder cups were placed on top of the solidified MSA media layer. 20 mL of Mannitol Salt Agar (MSA) media containing 5 µL of *Methicillin-resistant Staphylococcus aureus* (MRSA) suspension was added to a petri dish (second layer) and allowed to solidify. The cylinder cup was taken, and the wells formed were given 5 µL of the extracted sample and the ethanol-water fraction of white pomegranate peel (*Punica granatum* L.) with a concentration of 10%, 20%, and 30%, respectively. Ciprofloxacin solution as positive control and DMSO as negative control. Petri dishes were incubated at 37°C for 24 hours, and then the inhibition zone formed was measured using a vernier caliper [9].

2.5 TLC Bioautography Test

TLC bioautography test to determine compounds that have the potential as antibacterial seen from the inhibition zone formed on the media. Preparation of plates to be used in TLC bioautography tests for the TLC system for phenolic compounds, tannins, flavonoids, saponins, and triterpenoids using a mobile phase for each compound. The mobile phase for phenolic compounds is chloroform: ethyl acetate: n-butanol: formic acid (5: 2: 2: 1) [10], tannins are methanol: water (6: 4) [11], flavonoids are toluene: ethyl acetate: formic acid (4: 3: 0,4) [12], saponins and triterpenoids use the same eluent, namely toluene: ethyl acetate: formic acid (5: 4: 0,2) [13]. The bioautography test was carried out using 30 mL of Mannitol Salt Agar (MSA) media containing 5 µL of Methicillin-resistant Staphylococcus aureus (MRSA) bacterial suspension and poured into a petri dish, waiting for it to solidify. The plate that had been eluted using the mobile phase for each compound was attached to the surface of the media which had solidified and waited for 30 minutes. The plates were taken and incubated at 37°C for 24 hours. Observe the inhibition zone that forms on the media [14].

2.6 Qualitative and Quantitative Analysis by TLC-Densitometry

Determination of gallic acid levels by TLC-Densitometry [10]:

A standard gallic acid solution was prepared by weighing 100 mg of gallic acid dissolved in 100 mL of ethanol p.a then a series was made with a concentration of 25 ppm, 50 ppm, 75 ppm, 100 ppm, 200 ppm, 300 ppm, and 400 ppm from the standard gallic acid mother liquor and added up to 10 mL of ethanol p.a.

The sample solution was prepared by weighing 200 mg of extract and the ethanol-water fraction of white pomegranate peel (*Punica granatum* L.) each with ethanol p.a. The solution was sonicated at 50°C and added to 10 mL ethanol p.a.

Gallic acid content was determined by bottling 5 µL of the standard series and sample solution of the extract and the ethanol-water fraction of white pomegranate peel (*Punica granatum* L.) on GF 254 silica plates. The plates were eluted with chloroform: ethyl acetate: *n*-butanol: formic acid (5: 2: 2: 1) in a saturated chamber. Stains were detected in a 254 nm UV lamp. The plate is inserted into the *Densitometer Camag Linomat* 5 and a search for the maximum wavelength is carried out. Measure the Rf, AUC values, and calculate the levels.

2.7 Data Analysis

The results of measuring the diameter of the inhibition zone and the results of assays on the extract and ethanol-water fraction of white pomegranate peel (*Punica granatum* L.) were carried out statistical tests using SPSS (Statistical Product and Service Solution) version 23. Statistical tests for antibacterial testing were carried out using the two-way ANOVA test. The statistical test for the determination of levels was carried out using the Independent Samples T-Test.

3 Results and Discussions

Samples of white pomegranate peel powder were extracted by maceration method using 70% ethanol for 4 days with a new solvent every 1×24 hours to produce a yield of 56,89%. The results of the ethanol-free test showed that the extract and the ethanol-water fraction contained no ethanol as indicated by the formation of a raspberry red solution and no banana odor. The viscous extract was fractionated using the liquid-liquid partition method. The solvents used were *n*-hexane and ethanol-water (8:2). The *n*-hexane solvent is used to separate nonpolar compounds and the ethanol-water solvent is used to separate polar compounds. The results of the phytochemical screening and TLC tests showed that the extract and the ethanol-water fraction contained phenolic compounds, tannins, flavonoids, saponins, and triterpenoids, while the *n*-hexane fraction contained saponins and triterpenoids.

Antibacterial activity testing was carried out using the well-diffusion method on extract samples and the ethanol-water fraction, while the *n*-hexane fraction was not used because the yield produced was small, making it impossible for further testing. The medium used is Mannitol Salt Agar (MSA) because it is a selective medium for the growth of *Staphylococcus aureus* and contains mannitol and the indicator phenol red. *Staphylococcus aureus* will ferment mannitol to produce acid which can change the color of the phenol red indicator medium from red to yellow [15]. *Methicillin-resistant Staphylococcus aureus* (MRSA) bacteria were tested for resistance using *β*-lactam class antibiotics such as ampicillin, amoxicillin, and cefadroxil. The test results showed that the bacteria were resistant to these antibiotics.

Ciprofloxacin was used as a positive control. Ciprofloxacin is a fluoroquinolone class of antibiotics with a broad spectrum of activity and a mechanism of action that inhibits bacterial topoisomerase II and IV enzymes thereby inhibiting bacterial cell growth [16]. The concentrations used in the antibacterial test were 10%, 20%, and 30%. The results of the antibacterial activity test showed that the extract and the ethanol-water fraction had antibacterial activity against *Methicillin-resistant Staphylococcus aureus* (MRSA) forming an inhibition zone around the wells. The following results of the antibacterial activity test can be seen in Figure 1.

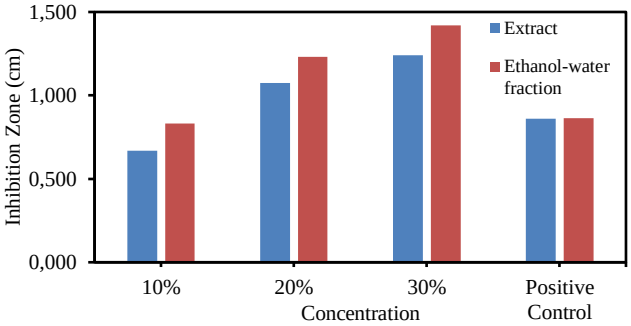

Figure 1 The Antibacterial Activity Test Results of Extracts and Ethanol-water Fractions of White Pomegranate Peel (*Punica granatum* L.)

The yield of the ethanol-water fraction was greater because it contained more specific compounds. After all, they had been separated based on their polarity, while the extract still contained many complex compound components. The results of the TLC bioautography test can be seen in Table 1 showing that phenolic compounds, tannins, and flavonoids have antibacterial potential while saponins and triterpenoid compounds do not have antibacterial activity.

Phenolic compounds have an antibacterial mechanism of action by breaking down cell walls and destroying bacterial enzymes. High concentrations of phenolic compounds can penetrate and destroy bacterial cell walls and precipitate bacterial cell proteins, while low concentrations of phenolic compounds can inactivate bacterial cell enzyme systems [17].

Table 1 TLC Bioautography Test Results of Extract and Ethanol-water Fraction of White Pomegranate Peel (*Punica granatum* L.)

Compound	Extract	Ethanol-water fraction
Phenolic	+	+
Tannin	+	+
Flavonoid	+	+
Saponin	-	-
Triterpenoid	-	-

(+) An inhibition zone is formed in the media.
(-) No inhibition zone is formed in the media.

Tannin compounds have an antibacterial mechanism of action by interfering with the transport of bacterial cell proteins which can inactivate microbial cell adhesion and enzymes and interfere with the formation of polypeptides and cell walls. Bacteria are then lysed due to physical and osmotic pressure which causes bacterial cell death [18].

Flavonoid compounds have an antibacterial mechanism of action by denaturing bacterial cell membrane proteins, damaging bacterial cell membranes, causing leakage of important metabolites, and deactivating bacterial enzymatic systems. This damage causes nucleotides and amino acids to leak out, preventing the drug from entering the cell. This condition can cause bacterial death [19].

Data on the diameter of the inhibition zone for testing antibacterial activity on extracts and the ethanol-water fraction were tested using SPSS (Statistical Product and Service Solution) version 23. The results of the normality test and homogeneity test showed that the data were normally distributed and homogeneous, followed by a two-way ANOVA test to see the difference between groups. The results of the two-way ANOVA test were seen from the significance value obtained by ≤0,05, so there were differences between groups, then continued with the Post Hoc test to find out the differences between groups. The results showed that between the extract groups, the ethanol-water fraction, and the positive control, there were significant differences marked by a sig value of ≤0,05. The 30% extract sample with the 20% ethanol-water fraction has a significance value of ≥0,05, which means that it is not significantly different or that it contains almost the same compounds that have the potential as antibacterial so that the resulting inhibition zones are almost the same.

Qualitative and quantitative analysis of extracts and ethanol-water fractions were performed using TLC-Densitometry. The eluent used was chloroform: ethyl acetate: *n*-butanol: formic acid (5:2:2:1) and the standard reference used was gallic acid. The maximum wavelength measurement in this study is 290 nm. The results of the qualitative analysis of the extract and the ethanol-water fraction in the form of a spectrum pattern were indicated by the presence of three peaks in the extract (A) with Rf values of 0,490; 0,700; 0,973 and three peaks in the ethanol-water fraction (B) with Rf values of 0,492; 0,705; 0,978. The peak shown in the spectrum pattern of the extract and the ethanol-water fraction with an Rf value of 0,458–0,522 is a gallic acid compound, which can be seen in Figure 2.

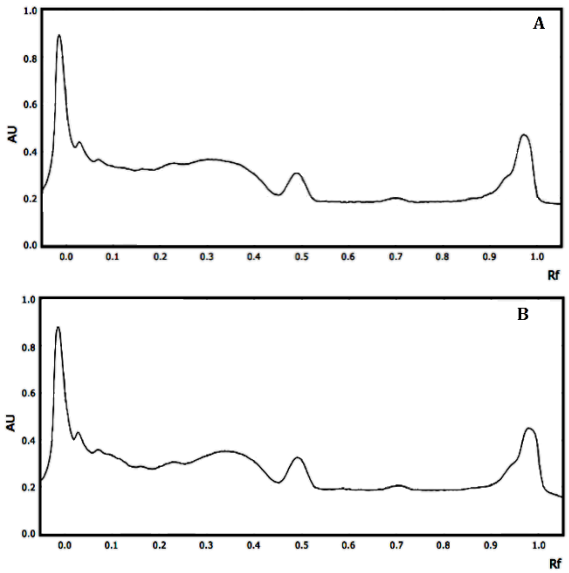

Figure 2 TLC-Densitometry Spectrum Results of Extract (A) and Ethanol-water Fraction (B) of White Pomegranate Peel (*Punica granatum* L.)

The results of quantitative analysis in the form of gallic acid levels obtained an average extract of 3,139082 mg/ g sample and an ethanol-water fraction of 3,824574 mg/ g sample. The results of the quantitative analysis of extract and ethanol-water fraction can be seen in Table 2.

Table 2 The Quantitative Analysis of Extract and Ethanol-water Fraction of White Pomegranate Peel (*Punica granatum* L.)

Replication	The gallic acid content in the sample (mg/ g sample)	
	Extract	Ethanol-water fraction
1	3,35042	3,60558
2	2,94261	3,59970
3	2,81876	3,52345
4	3,15367	3,97106
5	3,42995	4,42308
Mean ± SD	3,139082 ± 0,260139	3,824574 ± 0,377117

The results of the assay are related to testing the antibacterial activity. Phenolic compounds have the potential as antibacterial as evidenced by the results of the TLC bioautography test. The gallic acid contained in the ethanol-water fraction sample has a higher level than the extract, this is because the extract still contains many compounds. The ethanol-water fraction has a higher concentration because it has gone through a separation process so some other compounds that do not have antibacterial potential are attracted to the *n*-hexane fraction and produce a larger inhibition zone. The content of phenolic compounds affects the amount of antibacterial activity, namely the greater the phenolic compounds, the greater the antibacterial activity produced [20]. Data for the determination of gallic acid levels were carried out statistical tests using the Independent Samples T-Test to find out the difference in the mean of the two different groups. The results of the Independent Samples T-Test show the value of Sig. (2-tailed) ≤0,05, there is a difference in the data.

4 Conclusions

There was a significant difference in antibacterial activity between the extract and the ethanol-water fraction of white pomegranate peel (*Punica granatum* L.) against *Methicillin-resistant Staphylococcus aureus* (MRSA) and there was a non-significant difference between the 30% extract and the 20% ethanol-water fraction. Compounds that have antibacterial activity against *Methicillin-resistant Staphylococcus aureus* (MRSA) by TLC bioautography are phenolic compounds, tannins, and flavonoids. The results of the qualitative analysis using TLC-Densitometry obtained extract spectrum patterns with three peaks (Rf values 0,490; 0,700; 0,973) and ethanol-water fraction spectrum patterns with three peaks (Rf values 0,492; 0,705; 0,978). The results of quantitative analysis using TLC-Densitometry showed that the average gallic acid content in the extract was 3,139082 mg/ g sample and the ethanol-water fraction was 3,824574 mg/ g sample.

5 Declarations

5.1 Funding

This research was not supported by any funding sources.

5.2 Authors Contributions

- Venita Lutfia Ningrum: All research processes started from sampling, extraction, fractionation, phytochemical screening and TLC, antibacterial activity test, and TLC-Densitometry test.
- Endang Dwi Wulansari: TLC bioautography, TLC-Densitometry test.
- Yuvianti Dwi Franyoto: Antibacterial activity test.

5.3 Ethic

- EC Number: 370/YP-NA/KEPK/STIFAR/EC/V/2022.
- Institution : Stifar Yayasan Pharmasi Semarang.

5.4 Conflict of Interest

No conflict of interest.

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