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ANTIBACTERIAL ACTIVITY AND TLC-BIOAUTOGRAPHY ANALYSIS OF THE ACTIVE FRACTIONS OF *Muntingia calabura* L. LEAVES AGAINST *Staphylococcus aureus*

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ABSTRACT

This study aims to determine the antibacterial activity and TLC-bioautography profile of the active fractions of *Muntingia calabura* L. leaves against *Staphylococcus aureus*. *Muntingia calabura* L. leaves were macerated with ethanol 96% then fractionated with n-hexane, ethyl acetate, and methanol solvent, respectively. The antibacterial activity was tested by the Kirby-Bauer method to determine the most active fraction and the lowest concentration that inhibited the growth of *Staphylococcus aureus*. TLC-bioautography was tested using chloroform: ethyl acetate (2:8) as the mobile phase and silica gel F₂₅₄ as the stationary phase. Antibacterial activity test of n-hexane and ethyl acetate fractions at a concentration of 10% w/v showed activities with inhibition zone diameter of 0.33±0.288 and 9.66±5.77 mm, respectively. At the same time, the methanol fraction showed no activity. The lowest concentration of ethyl acetate fraction which still showed the inhibition zone was 0.312% w/v. The TLC-bioautography profile showed active spots with an R_f value of 0.82 and had an inhibitory zone diameter of 4.013±0.864 mm. It can be concluded that the ethyl acetate fraction was the most active fraction that inhibited the growth of *Staphylococcus aureus* and had one active spot on the bioautography test.

Keywords: antibacterial; *Muntingia calabura* L. leaf; *Staphylococcus aureus*; TLC-bioautography.

INTRODUCTION

Staphylococcus aureus is a commensal bacteria in human skin and mucosae but this bacteria often causes serious infections with high morbidity and mortality (Safitri *et al.*, 2018). Besides being frequently associated with skin and soft tissue infections, *Staphylococcus aureus* can also cause a variety of serious invasive infections such as osteomyelitis, necrotizing pneumonia, and bacteremia (Williamson *et al.*, 2013). *Staphylococcus aureus* is a serious problem because of the increased resistance of these bacteria to various types of antibiotics (Multi-Drug Resistance). *Staphylococcus aureus* has unique adaptability so that it can be resistant to many antibiotics (Afifurrahman *et al.*, 2014).

Therefore, it is necessary to explore new antibacterial compounds from various sources such as medicinal plants.

Muntingia calabura L. leaves have been shown to have antibacterial activity against both Gram-positive and Gram-negative bacteria (Arum *et al.*, 2012; Handoko *et al.*, 2019). Arum *et al.* (2012) reported that ethanol and methanol extracts of *Muntingia calabura* L. leaves have antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus*. Research done by Nanik *et al.* (2014) showed that ethanol extract of *Muntingia calabura* L. leaves has antibacterial activity against *Staphylococcus*

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aureus where the Minimum Bactericidal Concentration (MBC) value of ethanol extract is 1.25 mg/mL. The antibacterial activity of *Muntingia calabura* L. leaves is presumed to exist because it contains bioactive compositions such as flavonoids, tannins, and saponins (Zebua *et al.*, 2019).

The extract still contains a mixture of various compounds so that fractionation is needed to attract the active compound based on their polarity level. One way to detect the active compounds is by using the TLC-bioautography method. TLC-bioautography can show spots from Thin Layer Chromatography (TLC) that can inhibit bacterial growth (Muthadi *et al.*, 2012). This study aims to determine the antibacterial activity and TLC-Bioautographic profile of the active fractions of *Muntingia calabura* L. leaves against *Staphylococcus aureus*.

METHODS

Materials and Chemicals

Muntingia calabura L. leaves were collected from Sleman, Yogyakarta, Indonesia. *Staphylococcus aureus* isolates were collected from Center for Health Laboratory Yogyakarta. The other materials and chemicals used were Brain Heart Infusion (Oxoid), Mueller Hinton Agar (Oxoid) NaCl 0.9% (sterile), Mc Farland 0.5 (concentration 1.5×10^8 CFU/mL), ethanol 96%, n-hexane, ethyl acetate, methanol, paper disk (Oxoid), and TLC silica gel F₂₅₄ (Merck).

Extraction and Fractionation

Muntingia calabura L. leaves were dried using an oven at 45°C for four days then mashed by blending. The dry powder obtained was extracted by employing the maceration method. The dry powder of *Muntingia calabura* L. leaves (500 g) was extracted with 4000 mL ethanol 96% (1:8), stirred using a stirrer for 3 hours and then stored in 24 hours. Next, the macerated substance was filtered and concentrated using a rotary evaporator (Sulaiman *et al.*, 2017). The extract obtained was then suspended in warm water and fractionated with n-hexane, ethyl acetate, and methanol solvent, respectively using the

liquid-liquid partitioning method (Mulyani and Sukandar, 2011).

Media Preparation

Brain Heart Infusion (BHI) media was prepared by dissolving 3.7 g of BHI powder in 100 mL of distilled water while being heated and stirred until homogeneous. BHI media was sterilized using an autoclave at 121°C for 15 minutes (Lugan *et al.*, 2016).

Mueller Hinton Agar (MHA) media was prepared by dissolving 19 g of MHA powder in 500 mL distilled water while being heated and stirred until homogeneous. The media was sterilized using an autoclave at 121°C for 15 minutes and then put in a 20 mL petri dish and allowed to harden (Mahmudah *et al.*, 2017).

Bacterial Preparation

A total of 1 mL of *Staphylococcus aureus* stock was put into 1 mL of BHI media and incubated at 37°C for 24 hours. The bacteria culture was then taken for as much as 100 µL, put into 1 mL of BHI media, and incubated at 37°C for 3-5 hours. The bacterial culture was then taken for as much as 100 µL and was diluted using 0.9% NaCl sterile until the turbidity was the same as the Mc Farland 0.5 standard (1.5×10^8 CFU/mL).

Antibacterial Activity Test

The determination of the active fraction was carried out using the Kirby-Bauer method. A total of 20 µL of extract and fractions with a concentration of 10% w/v each were dripped on a 6 mm disc paper. The disc paper was then transferred aseptically to the MHA media that had been planted with test bacteria and then incubated using an incubator at 37°C for 24 hours. The most active fraction is the one that has the largest inhibitory zone (Sylvester *et al.*, 2015). The lowest concentration of the most active fraction that inhibited *Staphylococcus aureus* was determined using the same method. The most active fractions with concentrations of 10%, 5%, 2.5%, 1.25%, 0.625%, 0.312 and 0.156% were tested using the Kirby-Bauer method.

TLC-Bioautography

The test to find out the most active fraction was carried out by TLC method using chloroform:ethyl acetate (2:8) as the mobile phase and silica gel F₂₅₄ as the stationary phase. After elution, spots on the TLC plate were then observed under visible light and UV light with wavelengths of 254 and 366 nm. Furthermore, the chromatogram plate was contacted with the surface of MHA media which had been inoculated by *Staphylococcus aureus* for 30 minutes. The plate was removed and the culture was incubated at 37°C for 24 hours (Muthadi *et al.*, 2012).

Statistical Analysis

Inhibition zone data in the minimum inhibitory test were tested for their normality and homogeneity. The normality test was done using the Kolmogorov-Smirnov and Lilliefors, while the homogeneity test was done using One Way ANOVA and LSD with a confidence level of 95%. If the distribution is normal and homogeneous, then the correlation test will be done using the Pearson test. If the data are not normally distributed and are not homogeneous, further tests need to be performed using the Kruskal-Wallis and Mann-Whitney while the correlation test is performed using the Spearman test.

RESULTS AND DISCUSSION

In this study, *Muntingia calabura* L. leaves were extracted using the maceration method which obtained 19.07% extract yield. A non-heating extraction method was chosen to avoid damaging heat-resistant compounds that might be contained in the sample. Immersion of *Muntingia calabura* L. leaves in the solvent can make the compounds contained in cells soluble, and because of the differences in concentration between the solution of compounds inside and outside the cell, the compounds from high concentrations in the cell will diffuse out. In a previous study conducted by Puspitasari *et al.*, (2016), *Muntingia calabura* L. leaves extraction using the maceration method obtained a yield of 26.58%. Yield extraction results can be influenced by several factors, including biological factors and chemical factors. The

biological factors include harvesting time, location of growth, plant species and plant parts used while the chemical factors include extraction methods, size, hardness, dryness of the material, type of solvent used, and types and levels of active compounds contained in plant material (Prastiwi *et al.*, 2017).

The extract was fractionated successively using n-hexane, ethyl acetate and methanol solvents which aimed to separate the compounds based on their degree of polarity. The results of fractionation in this study showed that the yield of n-hexane, ethyl acetate, and methanol was 38.36%, 13.00%, and 7.70%, respectively. Non-polar compounds such as oil, carotenoids, steroids, and triterpenoids will tend to dissolve in non-polar solvents such as n-hexane. Semi-polar compounds such as aglycone flavonoids will tend to dissolve in semi-polar solvents such as ethyl acetate. Polar compounds such as flavonoids glycosides will tend to dissolve in polar solvents, such as methanol, according to the principle of 'like dissolves like' (Suryanto, 2012).

The most active fraction that inhibited the growth of *Staphylococcus aureus* was determined through antibacterial activity testing of each fraction using the Kirby-Bauer method. As a comparison, ethanol extract, negative control (96% ethanol) and positive control (Vancomycin Antimicrobial Susceptibility Disks 30 µg) were also tested. The results showed that the ethyl acetate fraction was the most active fraction associated with the formation of the largest inhibition zone diameter compared to the inhibition zone of the related fraction presented in (Figure 1). The inhibition zone of ethanol extract, n-hexane and ethyl acetate fractions in 3 replications were 3.33 ± 0.577 , 0.33 ± 0.288 and 9.66 ± 0.577 mm while the methanol fraction did not show inhibitory activity, as shown in (Table 1). The category of antibacterial activity can be distinguished based on its strength according to Rahmawati *et al.* (2014): if the diameter of antibacterial inhibition zone is more than 20 mm, the activity is classified into very strong; if it is between 11-20 mm, it is classified into

moderate; and if it is less than 5 mm, it is classified into weak. Based on this classification, the ethyl acetate which produced a clear zone of 9.66 ± 0.577 mm was classified into medium category.

The lowest concentration of ethyl acetate fraction that still showed the zone of inhibition

against *Staphylococcus aureus* was determined using the Kirby-Bauer disk diffusion method. The results showed that the ethyl acetate fraction could still inhibit the growth of *Staphylococcus aureus* bacteria at the lowest concentration of 0.312% (Figure 2) with the inhibition zone of 0.33 ± 0.288 (Table 2).

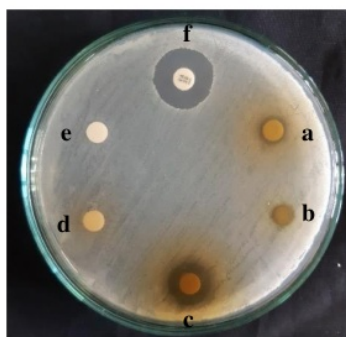


Figure 1. Screening of The Active Fractions; (a) 10% ethanol extract, (b) 10% n-hexane fraction, (c) 10% ethyl acetate fraction, (d) 10% methanol fraction, (e) negative control and (f) positive control.

Table 1. Inhibition zone diameters of the extract and fractions against *Staphylococcus aureus*.

Extract and Fractions	Inhibition Zone (mm)	Category
Ethanol Extract (10 % w/v)	3.33 ± 0.577	Weak
n-hexane Fraction (10 % w/v)	0.33 ± 0.288	Weak
Ethyl Acetate Fraction (10 % w/v)	9.66 ± 0.577	Medium
Methanol Fraction (10 % w/v)	0.00 ± 0.00	-
Negative Control (Ethanol 96%)	0.00 ± 0.00	-
Positive Control (Vancomycin 30 μ g)	12.33 ± 0.577	Strong

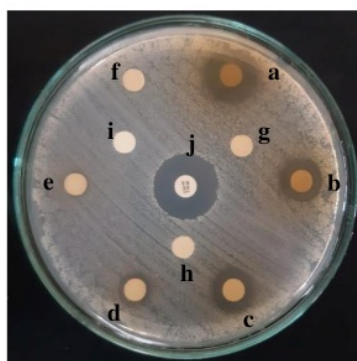


Figure 2. Testing the minimum inhibitory level of the ethyl acetate fraction with a concentration of: (a) 10%, (b) 5%, (c) 2.5%, (d) 1.25%, (e) 0.625%, (f) 0.312%, (g) 0.156%, (h) = 0.078%, (i) = negative control, (j) = positive control.

Table 2. Diameters of the inhibition zone of ethyl acetate fraction against *Staphylococcus aureus*.

Ethyl acetate fraction (% b/v)	Inhibition zone (mm)	Category
10	9.83±0.28	Medium
5	5.66±0.57	Medium
2.5	4.16±0.288	Weak
1.25	2.5±0.50	Weak
0.625	1.83±0.288	Weak
0.312	0.33±0.288	Weak
0.156	0.00±0.00	-
0.078	0.00±0.00	-
Negative Control (Ethanol 96%)	0.00±0.00	-
Positive Control (Vancomycin 30 µg)	12.33±0.577	Strong

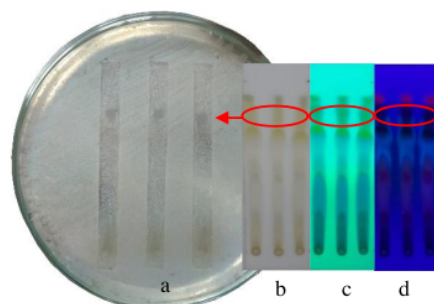
Statistical analysis showed that the inhibition zone data at the lowest level of ethyl acetate fraction testing was not normally distributed and not homogeneous. It was based on the significance value of <0.05 in Kolmogorov-Smirnov and Lilliefors test and the significance value of 0.019 <0.05 in One Way ANOVA and LSD test. Therefore, further testing was done using the Kruskal-Wallis test and the Mann-Whitney test. In the Kruskal-Wallis test, the result showed the significance of 0.002 <0.05. This shows that the treatment of the addition of the ethyl acetate fraction had a significant influence on the inhibition zones that emerged. Furthermore, the Mann-Whitney test showed that all concentration groups had significant differences. Therefore, it can be said that each concentration of ethyl acetate fraction- which consisted of 0.072%, 0.156%, 0.312%,

0.625%, 0.125%, 0.25%, 0.5% and 10% w / v- had different inhibitory zones. In addition, the Spearman correlation test showed the correlation coefficient value of 0.98. The value falls between range 0.81-1.00 and therefore it can be concluded that there was a very strong positive correlation where the higher the concentration of ethyl acetate fraction was, the greater the inhibitory zone that appeared.

1 Furthermore, to separate the content of compounds in the ethyl acetate fraction, Thin Layer Chromatography (TLC) technique was used. TLC was done by bottling 5 µL of ethyl acetate 10% onto the TLC plate. The results of the elution profile using mobile phase chloroform:ethyl acetate (2:8) and the stationary silica gel F₂₅₄ phase can be seen in figure 3. By viewing through visible light and UV light, there were eight spots detected during the observation (Table 3).

Table 3. The R_f value of the ethyl acetate fraction detected on the Thin Layer Chromatogram using chloroform:ethyl acetate (2:8) as the mobile phase.

No.	R _f	Visible Light	UV 254	UV 366
1	0.00	Brown	Brown	Black
2	0.21	Brown	Brown	Black
3	0.44	Green	Blue	Red Fluorescence
4	0.50	-	-	Blue Fluorescence
5	0.69	-	-	Blue Fluorescence
6	0.73	Yellow	Yellow	Black
7	0.82	Yellow	Yellow	Black
8	0.93	Green	Yellow	Red Fluorescence



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Figure 3. TLC-bioautography of ethyl acetate fraction, (a) TLC-bioautography of ethyl acetate fraction against *Staphylococcus aureus* by three replications; (b) TLC results viewed with visible light; (c) TLC results viewed with UV light 254 nm, (d) TLC results viewed with UV light 366 nm.

The bioautography test aims to determine which compound spots on TLC chromatograms have antibacterial activity. The active spots are marked by the formation of clear zones. The R_f value of the clear zone is calculated and matched to the R_f value of the chromatogram plate. TLC-Bioautographic test results of ethyl acetate fraction with three replications showed one active spot with an R_f value of 0.82 and had an inhibition zone diameter of 4.013 ± 0.864 mm as presented in figure 3. The inhibition zone formed was caused by the presence of active compounds from the chromatogram spot which diffused into the media and caused inhibition of bacterial growth in the diffusion site of the active compound (Muthadi *et al.*, 2012).

CONCLUSION

Ethyl acetate fraction is the most active fraction which inhibits the growth of *Staphylococcus aureus* bacteria and has the lowest levels of 0.312% w / v which still shows the inhibitory zone. Based on TLC-Bioautography testing using mobile phase of chloroform with ethyl acetate (2:8) and silica gel F₂₅₄ as a stationary phase, ethyl acetate fraction has one active spot with an R_f value of 0.82.

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