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Active antimicrobial substances of cherry leaf extracts (*Muntingia calabura* L.) against Methicillin-Resistant *Staphylococcus aureus* (MRSA) based on GC-MS analysis

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the bacteria that triggers nosocomial diseases. Bacterial resistance requires continuous exploration of active antimicrobial substances from various sources, including medicinal plants. Leaves of cherry (*Muntingia calabura* L.) reportedly contain three classes of compounds, namely, tannins, flavonoids, and saponins. This research was designed to identify active antimicrobial substances in cherry leaves that could inhibit the growth of MRSA. It employed the Kirby-Bauer test to examine the antimicrobial activities of the leaf extracts of *Muntingia calabura* L. (EMC) against MRSA. Through GC-MS, active substances were detected from the presence of active spots on TLC plates, as determined by direct-contact bioautography. The TLC used silica gel F254 as the stationary phase and chloroform:ethyl acetate (9:1) as the mobile phase. The antimicrobial activity test results showed that the zone of inhibition of 10% w/v EMC was 10.91±0.75 mm in diameter. At 5% w/v and 2.5% w/v, EMC created zones of inhibition with diameters of 8.5±0.25 mm and 7.25±0.25 mm, respectively. Meanwhile, at 1.25% w/v, it showed no inhibitory activities. Based on the TLC-Bioautography profile, the active spot that produced zones of inhibition was located at Rf 0.04 mm. The GC-MS analysis of this spot detected the presence of two compounds: the first compound had a similarity index of 35% with 3,11,13-triacetycynaratriol, and the second one had a similarity index of 80% with hexaborane-12. Cynaratriol is known to posses antimicrobial activity, whereas hexaborane is the opposite. In conclusion, the minimum inhibitory concentration of EMC for MRSA is 2.5% w/v. Also, the active compounds of EMC bear 35% similarities to 3,11,13-triacetycynaratriol.

Keywords: cherry leaf (*Muntingia calabura* L.), MRSA, TLC-Bioautography, GC-MS

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INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is the cause of various infectious diseases that are difficult to treat and have high levels of morbidity. The incidence of MRSA infections in Asian countries like Japan and Singapore is up to 50%, while, in the US, Australia, and some European countries, it ranges between 25% and 50 % (Green *et al*., 2012). In one of the big hospitals in Indonesia, MRSA exists with a high prevalence of approximately 45.3% (Erikawati *et al*., 2016). Due to its high incidence, researchers are encouraged to study resistant bacteria and discover potent antimicrobial agents from various sources, including medicinal plants that are suspected of having antibacterial ingredients, such as cherry (*Muntingia calabura* L.).

Cherry leaves are known to be active against, among others, *Streptococcus mutans*, viridans streptococci, *Streptococcus agalactiae*, *Staphylococcus aureus*, MRSA, *Escherichia coli, Pseudomonas aeruginosa, Candida albicans, Microsporum canis, Bacillus subtilis, Bacillus cereus, Klebsiella pneumoniae,* and *Proteus vulgaris* (Zakaria *et al*., 2010; Isnarianti *et al*., 2013; Sufian *et al*., 2013, Khasanah *et al*., 2014; Sulaiman *et al*., 2017). These activities are believed to be the results of three active compounds in the leaves, which are tannins, flavonoids, and saponins (Zakaria *et al*., 2006). Previous research has not been able to identify the active antimicrobial substances in cherry leaves, especially against MRSA. TLC-Bioautography is proposed to be a simple method of detecting active substances in extracts. Its output is TLC spots that mark the impeded growth of test bacteria, which are referred to as active spots (Muhtadi *et al*., 2012). Active spots can be further analyzed by, for example, Gas Chromatography-Mass Spectrometry (GC-MS).

GC-MS is the most widely used technique in recent metabolomics, chiefly to identify and even quantify metabolites. In this study, GC-MS was employed to discover compounds in the active spots produced by TLC-Bioautography analysis (Wang *et al*., 2015). GC-MS can be used to distinguish volatile compounds, including long-chain hydrocarbons, branched-chain hydrocarbons, alcohols, and esters. Samples analyzed by GC-MS must be able to evaporate at a temperature of below ~400 °C and stable when heated at >400 °C (Hermanto *et al*., 2008; Ruikar *et al*., 2009). Due to the paucity of information on the active antimicrobial agents of cherry leaves against MRSA, this study was conducted to discover such substances using a GC-MS analysis method.

MATERIALS AND METHODS

Materials

The cherry leaves (*Muntingia calabura* L.) were collected in July 2018, at Bantul, Yogyakarta, and identified by the Laboratory of Biology, Ahmad Dahlan University (No. 104/Lab.Bio/B/VIII/2018).

Methods

Preparation of *M. calabura* leaf extracts

Powdered *M. calabura* leaves were macerated with 96% ethanol. The extract was then evaporated using rotary evaporator to get concentrated extract.

Media preparation

The media used were Nutrient Agar (NA), Nutrient Broth (NB), and Mueller-Hinton Agar (MHA), all of which were prepared according to McKane (1996) and Komansilan *et al*. (2015).

Preparation of MRSA bacteria

One streak of pure MRSA culture (transferred with an inoculation loop) was dissolved in 1 mL of sterile NB media and incubated for 18-24 hours. Afterward, 100 µL of bacterial suspension was placed into a tube consisting of 1 mL of NB media and, then, incubated for 3-5 hours. The culture was added with 0.9% NaCl so as to create turbidity equivalent to the McFarland standards, i.e., 10^8

CFU/mL. Then, 100 µL of bacteria were diluted to a volume of 10 mL with the NB media to create a concentration of 10^6 CFU/mL (Abdulamir *et al.*, 2015).

Antibacterial activity testing

The antibacterial activities of cherry leaf extract (*Muntingia calabura* L.) against MRSA were tested by the Kirby-Bauer method. Twenty (20) µL of extracts with concentrations of 10, 5, 2.5, and 1.25% w/v were dropped into different 6-mm disk papers. Each disk paper was then transferred aseptically to a petri dish with MRSA-inoculated MHA media. Then, the petri dish was put into an incubator at 37°C for 24 hours. The zones of inhibition formed on the petri dish were observed and measured (Salni *et al.*, 2011).

TLC-bioautography

Active extraction was performed by TLC, with silica gel F254 as the stationary phase and chloroform:ethyl acetate (9:1) as the mobile phase. Spots on the TLC plate were observed under UV light at wavelengths of 254 and 366 nm. The chromatography plate was pressed against the surface of the MRSA-inoculated MHA media for 30 minutes. After the plate was removed, the culture was incubated at 37°C for 24 hours to observe any presence of TLC spots, which mark the inhibited growth of MRSA (Muhtadi *et al.*, 2012).

GC-MS analysis of active spots on TLC plates

The active spots created by the leaf extracts of cherrywere isolated by preparative TLC using silica gel F254 (stationary phase) and chloroform:ethyl acetate (9:1, mobile phase). They were removed from the TLC plate and dissolved in 96% ethanol and, then, filtered. The filtrate was analyzed in GC-MS QP2010 SE to find out the substances contained in the active spots. At the time of measurement, the condition of the GC-MS was set as follows: a column oven temperature of 40.0°C, injection temperature of 135.00°C, splitless injection mode, 1.00 min of sampling time, flow control and pressure mode, a pressure of 37.8 kPa, total flow of 173.9 mL/min, column flow of 0.85 mL/min, linear velocity of 33.3 cm/sec, purge flow at 3.0 mL/min, a split ratio of 200.0, equilibrium time of 3.0 min, ion source temperature of 220.00°C, interface temperature of 250.00°C, solvent cut time of 1.00 min, and MS scan speed at 625. Also, the GC-MS was started at m/z 75.00 and ended at m/z 250.00.

Data Analysis

The diameters of zone of inhibition were analyzed statistically by Kolmogorov-Smirnov and Lilliefors methods, One-Way ANOVA, and LDS and then followed by the Kruskal-Wallis and Mann-Whitney tests in SPSS 16.0 software for Windows.

RESULTS AND DISCUSSION

This study was aimed at detecting active antimicrobial substances in the ethanol extracts of *M. calabura* leaves using GC-MS. *M. calabura* leaf extracts were prepared by maceration, and the yield obtained was 24.7%. Maceration yields depend on the length of time of maceration, the presence or absence of stirring, and the number of re-macerations (Rahayu *et al.*, 2015). In addition to these physical factors, they are also affected by biological and chemical determinants. The former includes harvesting time, location of plants, plant species, and the plant parts used in the analysis, while the latter is comprised of extraction method, size, hardness, dryness of the material, the type of solvent used, and the type and content of active compounds in the ingredients (Yusuf and Candraningsih, 2017).

The Kirby-Bauer test determined the antimicrobial activities of *M. calabura* leaf against MRSA from its extracts at various concentrations, namely, 10, 5, 2.5, and 1.25% w/v. The test also examined a negative control (solvent extract) and a positive control that used 0.05% meropenem. As presented in Figure 1, the results showed the presence of the zone of inhibition in leaf extracts with concentrations of 10, 5, and 2.5% w/v. Three replications at these levels successively produced zones of inhibition

with the diameters of 4.92±0.75, 2.5±0.25, and 1.25±0.25 mm, respectively. On the contrary, at the concentration of 1.25% w/v, the leaf extract did not produce a zone of inhibition (Table I). According to Repi *et al.* (2016), microbial activities are categorized as weak if the diameter of the zone of inhibition is equal to or smaller than 5 mm, moderate with 6-10 mm diameter, strong with 11-20 mm diameter, and very strong with a diameter of larger than 20 mm. Therefore, the MRSA-inhibitory effects of *M. calabura* leaf extract at the concentrations of 10%, 5 %, and 2.5% w/v are categorically weak.

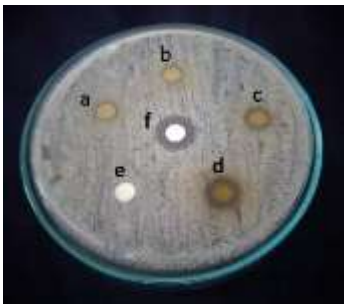


Figure 1. The antimicrobial activities of *M. calabura* leaf extracts against MRSA at concentrations of (a) 1.25% w/v, (b) 2.5% w/v, (c) 5% w/v, (d) 10% w/v, (e) negative (solvent) control, and (f) positive control (0.05% w/v meropenem)

Table I. The diameters of zones of inhibitions formed by *M. calabura* leaf extracts against MRSA

Extract Concentrations (% w/v)	Diameters of Zone of Inhibition (mm)	Categories
10	4.92±0.75[a]	Weak
5	2.5±0.25[b]	Weak
2.5	1.25±0.25[c]	Weak
1.25	0.00±0.00[d]	-
Positive Control (0.05 Meropenem)	5.34±1.45[a]	Moderate
Solvent Control (96% Ethanol)	0.00±0.00[d]	-

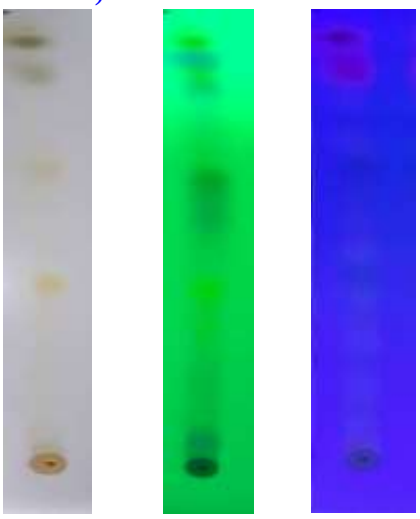
[a,b,c,d] shows significant differences in the diameter of zone of inhibition between extract concentrations; the comparison is made to concentrations marked with different letters. At 10% w/v, the extract has no significant difference to the positive control. All treatments show statistically substantial differences to the negative control and 1.25% w/v *M. calabura* leaf extract.

Several factors are known to influence the inhibitory capacity measured in the Kirby-Bauer diffusion method, namely, the length of time required to install the disk, the thickness of the inoculum, incubation time and temperature, disk plate size, agar thickness, distance between disks, and anti-microbes—believed to be a major determinant of the size of zone of inhibition in this method. Besides, active antibacterial substances and the composition of the media used also contributes to the resultant bacterial growth inhibition (Vandepitte *et al.*, 2003).

The statistical Kolmogorov-Smirnov and Lilliefors tests of the diameter of the zone of inhibition yielded a significance value of <0.05, meaning that the data are not normally distributed. Also, the One-way ANOVA and LDS with a 95% confidence level produced a significance value of 0.014<0.05, or, in other terms, the data are not homogeneous. For this reason, the Kruskal-Wallis test was used, and it produced a significance value of 0.014<0.05. In conclusion, the addition of the ethanol extract of *M. calabura* leaves affects the resultant zone of inhibition. Furthermore, the Mann-Whitney test results showed that all treatment groups or all concentrations examined in the study had significant differences. Therefore, at 1.25, 2.5, 5, and 10% w/v, the ethanol extracts of *M. calabura*

leaves produced zones of inhibition with varying sizes. Furthermore, the Spearman correlation test resulted in a coefficient of correlation (r) of 0.980, and because this value is in the range of 0.81-1.00, it represents a very strong positive correlation. In this context, the higher the concentration of the ethanol extract of *M. calabura* leaves, the larger the zone of inhibition will be.

TLC-Bioautography and GC-MS were employed to identify active substances in the extract. Through TLC, the study was able to separate the chemical contents in *M. calabura* leaf extract. Combined with TLC (a mixture of chloroform and ethyl acetate at 9:1 ratio as the mobile phase and silica gel F254 as the stationary phase), observations using visible light and UV rays detected as many as eleven (11) spots (Figure 2 and Table II).



a b c

Figure 2. The chromatogram (TLC) profiles of *M. calabura* leaf extracts, as observed under (a) visible light, (b) UV light at 254 nm, and (c) UV light at 366 nm

Table II. The Rf values of the spots detected by Thin-Layer Chromatography

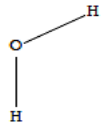
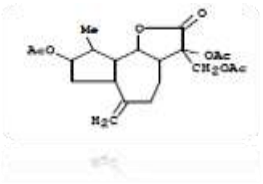
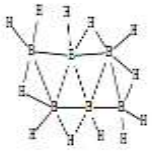
Rf	*M. calabura* leaf extracts		
	Visual	**UV 254**	**UV 366**
0.00	Brown	Quenching	Black
0.04	Green	Blue	Red Fluorescence
0.40	Yellow	Yellow	Black
0.52	-	Quenching	-
0.62	-	Quenching	-
0.66	Brown	-	Black
0.85	-	Blue	-
0.88	Gray	Yellow	Red Fluorescence
0.90	-	Blue	-
0.93	Green	Yellow	Black and Red Fluorescence
0.97	Yellow	-	-

After a direct-contact bioautography, the TLC plates produced a zone of inhibition against MRSA. This zone appeared in the spotting area with an Rf value of 0.04. Its appearance is attributable to the active compounds in the spots that are diffused into the media, leading to the death of bacteria in the diffusion site of this active substance (Muhtadi *et al.*, 2012).

Active substances were detected by removing the active spots from the TLC plates and analyzing them using GC-MS. The GC-MS analysis was intended to discover any compounds present in the active spots after the bioautographic examination, and the results are summarized in Table III. The active spots located at Rf 0.04 were dissolved in 96% ethanol and, then, filtered. The filtrate was

later analyzed using GC-MS, and the results showed five peaks with different retention times, namely, 1.902, 1.982, 2.010, 2.155, and 2.181 minutes. Three of them were the peaks of the solvents used, i.e., water (1.902 min) and ethanol (1.982 and 2.181 min). As seen in Figures 3 and 4, the third-highest peak (2.010 min) was identified as 3,11.13-triacetylcynaratriol with a similarity index of 35%, while the second-highest one (2.155 min) was defined as hexaborane-12 with a similarity index of 80%. Therefore, the source of the antimicrobial activities of *M. calabura* leaves is suspected to be one or both of these compounds, which bear similarities to 3,11,13-triacetylcynaratriol or hexaborane-12. *Cynara cardunculus* produces a sesquiterpene lactone, named cynaratriol, and this plant has been reported to possess antimicrobial properties (Kukić *et al*., 2008). As opposed to cynaratriol, hexaborane is a compound with no antibacterial effects (Gnanadeebam and Viswanathan, 2014). Cynaratriol-like compounds are thereby believed to be the reason for the antimicrobial activities of *M. calabura* leaves.

Table III. GC-MS data on *M. calabura* leaf extracts

Retention Time (minute)	Compounds	Abundance (%)	Similarity (%)	Compound Structures
1.902	Water (cas) ICE	2.44	98	
1.982	Ethanol (cas)	92.28	95	
2.010	3,11,13-triacetylcynaratriol	0.12	35	
2.155	Hexaborane-12	2.54	80	
2.181	Ethanol (cas) ethyl alcohol	2.62	94, 93, and 90	

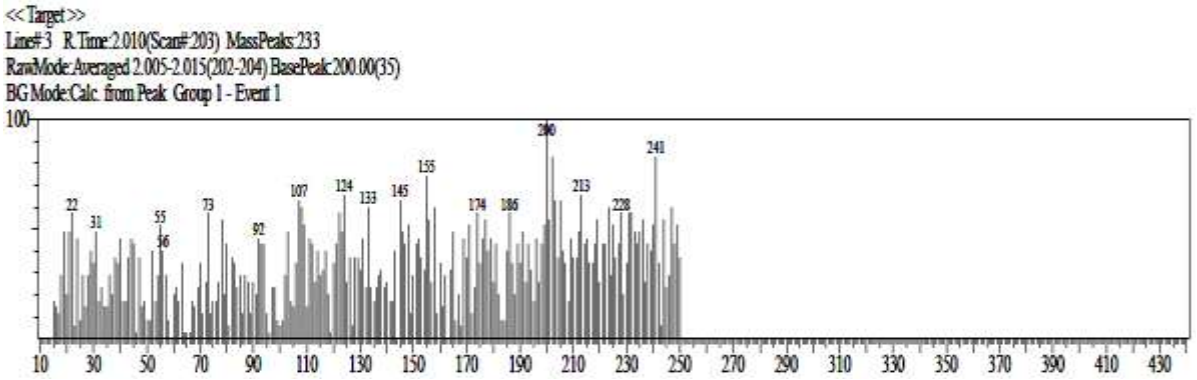


Figure 3. Mass spectra detected at the retention time of 2.010

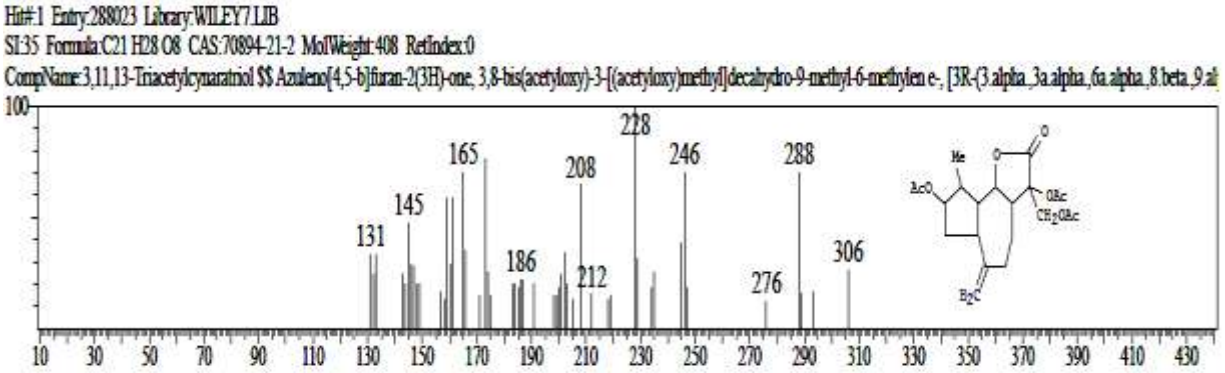


Figure 4. The spectra library of 3,11,13- triacetylcynaratriol (SHIMADZU)

CONCLUSION

M. calabura leaf extracts are proven to exhibit antimicrobial activities against MRSA. Based on the GC-MS analysis results, the antimicrobial compounds in the extracts have a similarity index of 35% with 3,11,13- triacetycynaratriol.

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