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Original Research Article

Cell leakage mechanism and time-kill studies on Staphylococcus aureus after exposure to ethanol leaf extract of *Muntingia calabura* L

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Abstract

Purpose: *To determine the effect of the ethanol leaf extract of M. calabura (EEMC) on cell leakage and time-kill against S. aureus.*

Methods: *The leaves were macerated with 96 % ethanol (1:8; w/v) for 27 h to produce EEMC. Chemical compounds of EEMC were analyzed using liquid chromatography with tandem mass spectrometry (LC-MS/MS). Various concentrations of EEMC (12.5; 25; 50; 100 mg/mL) were tested to determine the Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against S. aureus. Furthermore, EEMC was tested for its effect on cell leakage, changes in extracellular electrical conductivity, and time-kill against S. aureus. UV-spectrophotometer was used to test for leakages of nucleic acid, protein, DNA, and RNA, while atomic absorption spectrophotometer was used to evaluate leakage of potassium ion (K^+).*

Results: *The MIC and MBC of EEMC against S. aureus were 10 % w/v (100 mg/mL). The highest cell leakage occurred in S. aureus exposed to 2x MIC, with leakages of protein, DNA, RNA, and K^+ reaching 137.79 ± 58.99, 2298 ± 263.26, 1839 ± 210.61 and 770.86 ± 40.11 µg/mL respectively. The EEMC (1x MBC and 1.5x MBC) killed S. aureus in 24 h. Analysis of LC-MS/MS of EEMC showed that flavonoids (48.33 %) followed by anthraquinones (16.10 %) were the major classes of compounds present in the extract.*

Conclusion: *The ethanol leaf extract of M. calabura kills S. aureus by inducing cell leakage possibly due to flavonoids and anthraquinones contained in it. The extract should be further isolated and its active principles with potent antibacterial properties developed for therapeutic applications.*

Keywords: *Muntingia calabura, Cell leakage mechanism, Time-kill, Staphylococcus aureus*

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INTRODUCTION

Staphylococcus aureus is one of the Gram-positive bacteria that often cause infections in humans. Apart from skin and soft tissue infections, *S. aureus* is often associated with some severe invasive infections, such as osteomyelitis, necrotizing pneumonia, and bacteremia. These bacterial illnesses become increasingly serious because *S. aureus* has

extraordinary adaptability, so it is resistant to many antibiotics. The percentage resistance and multidrug resistant strains of *S. aureus* infection are on the increase, making the clinical treatment more difficult [1]. Therefore, it is essential to identify new antibacterial compounds from diverse sources such as medicinal plants.

Muntingia calabura L. is a plant that has been reported to have antibacterial potentials. Buhian *et al* [2] reported that *M. calabura* leave extracts exert antibacterial activity against *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Staphylococcus aureus,* and *Bacillus subtilis*. The leaves contain flavonoids, tannins, terpenoids, saponins, and polyphenols, which have been associated with antioxidant and antibacterial activities. Sufian *et al* [3] isolated three flavone compounds and one chalcone from *M. calabura* leaves. The three isolated flavone compounds were 5,7-dihydroxy-3,8-dimethoxy-flavone, 5-hydroxy-3,7-dimethoxyflavone, and 3,5,7 trihydroxy8-methoxyflavone, and the isolated chalcone compound was 2',4'-dihydroxychalcone. The 2', 4'-Dihydroxychalcone showed the strongest activity as an antibacterial agent against *methicillin-resistant S. aureus* (MRSA) and *methicillin-sensitive S. aureus* (MSSA) with minimum Inhibitory concentrations (MICs) of 100 and 50 µg/mL, respectively. Although the antibacterial property of *M. calabura* leaves has been extensively investigated, there is still limited information on the mechanism of its antibacterial action. Antibacterial compounds inhibit bacterial growth through several mechanisms, such as inhibition of the syntheses of bacterial cell walls, proteins, and nucleic acids and disrupting the function of bacterial cell membranes and bacterial cell metabolism. The level of damage to cell walls and cell membranes caused by antibacterial compounds can be determined by measuring the changes in extracellular electrical conductivity, and leakage of K^+ ions using an atomic absorption spectrophotometer and amount of nucleic acids and proteins using a UV spectrophotometer [4]. In addition, a time-kill kinetics assay is needed to provide information about the bacteria-killing effect [5]. Hence, this study aimed to determine the effect of ethanolic extract of *M. calabura* leaves (EEMC) on cell leakage, extracellular electrical conductivity, and time-kill of *S. aureus*.

EXPERIMENTAL

Materials and chemicals

M. calabura leaves were collected from Sleman, Yogyakarta, Indonesia on 3 November 2019. The plant was authenticated by Heri Setiyawan, a botanist from Biology Laboratory, Faculty of Applied Science and Technology, Universitas Ahmad Dahlan, Number: 107/Lab.Bio/B/XI/2019. *S. aureus* isolates were collected from the Center of Health Laboratory in Yogyakarta, Indonesia.

The growth media used were brain heart infusion and Mueller-Hinton agar (Oxoid, United Kingdom). The chemicals used were 0.9 % NaCl (Widatra, Indonesia), 96 % ethanol (Bratachem, Indonesia), 0.5 McFarland standard (concentration = 1.5 x 10^8 CFU/mL), phosphate buffer pH 7.4, 10 % DMSO, and 5 % glucose.

Extraction procedure

M. calabura leaves were dried in an oven at 45 °C for four days and then milled into powder. Approximately 500 g of powdered leaves was macerated in 4000 mL of 96 % ethanol (1:8; w/v) for 3 h with continuous stirring before being allowed to stand for 24 h. A Buchner funnel was used to filter the extract which was later concentrated using a rotary evaporator (Buchi®, Germany).

Liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis

Extract was analyzed using LC-MS/MS Acquity UPLC I-Class system with Xevo G2-XF QTof (Waters, United States). The sample (1 µL) was injected. The mobile phase consisted of 0.1 % FA/WA (solvent A) and acetonitrile + 0.1 FA (solvent B).

The flow rate was set at 0.3 mL/min and the gradient composition (solvent A: solvent B) was programmed as followed: 0 min (95:5); 1 min (95:5); 8 min (60:40); 11 min (0:100); 13 min (0:100) and 16 min (95:5). The column temperature was set at 40 ^oC.

Media preparation

The brain heart infusion (BHI) medium was prepared by dissolving 3.7 g of BHI powder in 100 mL of distilled water heated at a temperature of 50 °C and stirred until homogeneous for 5 min. The BHI broth was sterilized in an autoclave at a temperature of 121 °C for 15 min prior to use.

The Mueller-Hinton agar (MHA) was prepared by dissolving 19 g of MHA powder in 500 mL of distilled water at 50 °C and stirred until homogeneous for 5 min. The agar was sterilized in an autoclave at a temperature of 121 °C for 15 min, and then placed in a 10 mL petri dish and allowed to harden [6].

Preparation of bacterial stock

Pure culture of *S. aureus* was grown in 50 mL of BHI media and incubated at 37 °C for 24 h, then divided into Eppendorf tubes. Each tube containing 500 µL of bacterial suspension was then stored in the freezer as a bacterial culture stock.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays

A total of 100 µL of the bacterial stock was dispensed into a test tube containing 1 mL of the BHI broth and then incubated at 37 °C for 24 h. After incubation, 100 µL of the bacterial suspension was mixed with 1 mL of the BHI broth and incubated for 4 h at 37 °C. Later, it was diluted using 0.9 % of NaCl until the turbidity was the same as the 0.5 McFarland standard. Finally, the bacterial suspension was diluted 100 times with double-strength BHI broth.

Extract was prepared in the concentration of 200, 100, 50, and 25 mg/mL, through stratified dilutions. For each concentration of EEMC, 0.5 mL was transferred into a test tube, followed by 0.5 mL of the bacterial suspension, while the mixture was incubated for 24 h at 37 °C. Solvent control, bacterial control, drug control, and media control were also tested. The MIC value was determined by observing the turbidity of each tube and is considered when the tube appears clear at the lowest concentration of EEMC treatment. Content in each tube containing the treatment extract was then scratched onto the MHA and incubated for 24 h at 37 °C to determine the MBC value. The MBC was characterized by MHA that showed absence of colony growth at the lowest concentration of EEMC treatment [7].

Evaluation of cell leakage

The evaluation of cell leakage was performed using a Shimadzu UV-1800 UV-Vis Spectrophotometer (Shimadzu, Japan). Ten milliliters of bacterial suspension were centrifuged for 20 min at 3,500 rpm. The precipitate was collected and washed three times using a pH 7.4 phosphate buffer solution and then resuspended in the buffer followed by addition of EEMC at concentrations of MIC or 2 MIC. The mixture was incubated at 37 °C for 24 h, before being centrifuged for another 15 min at 3,500 rpm. Finally, the supernatant was collected to determine the cell leakage. The absorbance of the supernatant was measured at 260 and 280 nm to detect leakages of nucleic acid (DNA and RNA) and proteins. The absorbance ratio at 260 nm and 280 nm was calculated to estimate total nucleic acid and protein contents in the supernatant. The DNA and RNA concentrations were calculated with Eq 1 and Eq 2, respectively [8,9].

DNA concentration (µg/mL) = A260 × 50 …… (1)

RNA concentration (µg/mL) = A260 × 40 …… (2)

Determination of K^+ leakage

The sample was prepared with the same procedure as the one used in the cell leakage analysis. The leakage of K^+ was measured using Atomic Absorption Spectrophotometer- ContraAA 800 (Analytik Jena, Germany) at a wavelength of 766.5 nm [4].

Determination of the effect of EEMC on cell membrane permeability

The permeability of the cell membrane of *S. aureus* was measured using electric conductivity assay [4]. The test bacteria were centrifuged for 10 min at 5000 g, then washed with 5 % glucose until the conductivity approached that of 5 % glucose - a situation called isotonic bacteria. Furthermore, three samples of different concentrations (control, 1 MIC, and 2 MIC) were added to 5 % glucose, and their conductivities (L1) were measured. Subsequently, the three samples were added to the isotonic bacteria and then incubated at 37 °C for 24 h, and their conductivities (L2) were re-measured after 3, 5, and 24 h of incubation. The bacterial conductivity in 5 % glucose treated with boiling water for 5 min was used as control (L0). The permeability of the cell membrane was calculated using Eq 3.

Electrical conductivity (%) = {(L2-L1)/L0}100 .. (3)

Time-kill assay

The time-kill assay was conducted following the method described earlier [5]. The test bacteria in BHI broth (10^6 CFU/mL) was mixed with the extract at a concentration of 0.5 MBC, 1 MBC, or 1.5 MBC, and then incubated at 37 °C for 24 h. Approximately 0.1 mL of tested samples collected after 0, 2, 4, 6, 8, 10, 12, and 24 h of incubation were inoculated with a pour plate method using MHA and then incubated at 37 °C for 18 – 24 h. The growing bacterial colonies were counted and then time-kill curves were constructed by plotting $\log_{10}$ CFU/mL against time (h).

Statistical analysis

The experiments were conducted in triplicate, and the results are expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) and least significant differences (LSD) were performed using Statistical Package for the Social Sciences (SPSS) software in which *P*-value < 0.05 was considered statistically significant.

RESULTS

Antibacterial activity

Table 1 shows the MICs and MBCs of the EEMC prepared at different concentrations against *S. aureus*. Based on the turbidity and presence of bacterial colonies, the MIC and MBC of the EEMC were 10 % w/v (100 mg/mL). The result of the MBC experiment can be seen in Figure 1.

Table 1: Minimum Inhibitory Concentrations (MICs) and Minimum Bacterial Concentrations (MBCs) of the ethanolic extract of *Muntingia calabura* L. leaves (EEMC) against *S. aureus*

Concentrations of EEMC	Turbidity	Colony
10 % w/v (100 mg/mL)	Clear	-
5 % w/v (50 mg/mL)	Turbid	+
2.5% w/v (25 mg/mL)	Turbid	+
1.25% w/v (12.5 mg/mL)	Turbid	+
Solvent control	Clear	-
Media control	Clear	-
Drug control	Clear	-
Bacteria control	Turbid	+

***Note*:** Solvent control = 0.5 mL 10 % DMSO + 0.5 mL double-strength brain heart infusion (BHI-DS); Media control = 1 mL of BHI-DS; Drug control = 0.5 mL of 2 % vancomycin + 0.5 mL BHI-DS; Bacteria control = 0.5 mL of bacterial suspension in BHI-DS + 0.5 mL distilled water

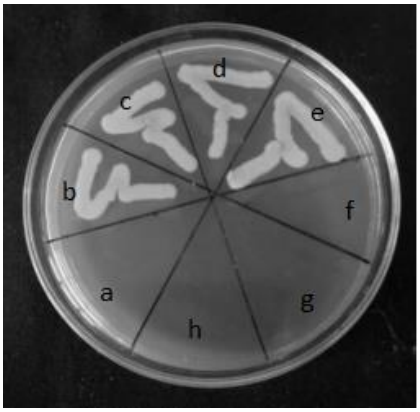

Figure 1: Minimum bactericidal concentration (MBC) of EEMC; (a) 10 %; (b) 5 %; (c) 2.5 %; (d) 1.25 %; (e) bacteria control; (f) solvent control; (g) media control and (h) drug control

Effect of EEMC on cell leakage

The leakages of cellular materials in *S. aureus* after exposure to EEMC were measured at 260 and 280 nm. The absorbance values for three different MICs and concentrations of EEMC are shown in Table 2.

Table 2: The MICs of EEMC that caused leakages of cellular constituents of *S. aureus*

Sample	Concentration of EEMC (mg/mL)	Wavelength (nm)	Absorbance
Control (0 MIC)		260	0.17±0.02
		280	0.13±0.02
1 MIC	100	260	0.23±0.08
		280	0.18±0.06
2 MIC	200	260	0.46±0.05*
		280	0.31±0.06*

Data are presented in mean ± standard deviation (n = 3). *Significantly different from controls ($p < 0.05$)

An increase in the absorbance value of the measured sample indicates an increase in the contents released from the cell. The amounts of nucleic acid and protein leaking from the bacterial cells after pretreatment with EEMC are shown in Table 3.

Effect of EEMC on the leakage of potassium ion (K^+)

Table 4 shows the effect of EEMC on the leakage of K^+ from *S. aureus*. The extract exerted a concentration-dependent effect wherein more K^+ ions ($p < 0.05$) leaked out of the cells as the concentration of EEMC added to the bacteria was increased.

Effect of EEMC on cell membrane permeability

Figure 2 shows the effect of EEMC on the relative electrical conductivity of *S. aureus*. The results indicate that the electrical conductivity increased with extract concentration and treatment time.

Effect of EEMC on the number of bacterial colonies

The effect of variations in extract concentration and incubation time on the number of *S. aureus* colonies was measured using the time-kill test. The EEMC at concentrations of 1 MBC and 1.5 MBC kills *S. aureus* at 24 h while EEMC with 0.5

Table 3: The amounts of nucleic acid and protein leaking from the *S. aureus* cell after exposure to EEMC

Parameter	Control (0 MIC)	1 MIC	2 MIC
Absorbance (280/260)	0.80±0.04	0.76±0.00	0.68±0.06
Absorbance (260/280)	1.25±0.07	1.31±0.01	1.49±0.15
Nucleic Acid (%)	6.60±1.13	7.83±0.29	12.68±5.06*
Protein (µg/mL)	82.07±18.57	99.59±34.06	137.78±58.99
DNA concentration (µg/mL)	830±86.60	1165±405.31	2298±263.26*
RNA concentration (µg/mL)	664±69.28	932±324.25	1839±210.61*

Data are presented as mean ± standard deviation (n = 3). *Significantly different from controls ($p < 0.05$)

Table 4: Effect of EEMC on the leakage of K^+ from *S. aureus*

Sample	Leakage of K^+(µg/mL)
Control (0 MIC)	298.60±8.89
1 MIC	708.60±35.95*
2 MIC	770.86±40.11*

Data are presented as mean ± standard deviation (n = 3). *Significantly different from controls ($p < 0.05$)

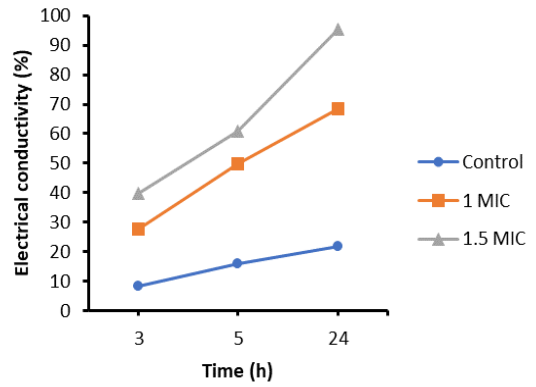

Figure 2: Effect of EEMC on the permeability of the *S. aureus* membrane, measured as relative electrical conductivity

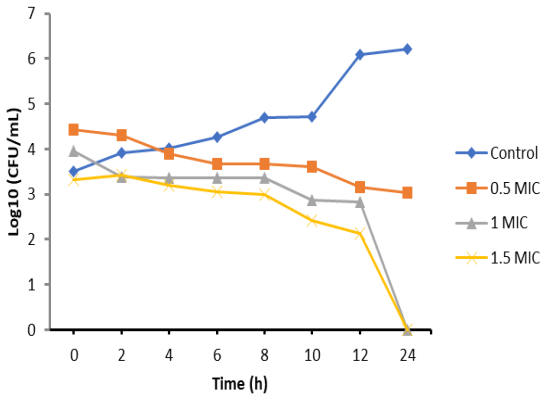

Figure 3: Effect of different concentrations of EEMC and incubation times on the time-kill of *S. aureus*

MBC only reduced the number of colonies. The results are shown in Figure 3.

Liquid chromatography - tandem mass spectrometry (LC-MS/MS) results

The LC-MS/MS analysis of EEMC showed the presence of several compounds, where the major classes of phytoconstituent detected were flavonoids (48.33 %) followed by anthraquinone (16.10 %). The flavonoid compounds detected were 3′,5-dihydroxy-7,4'-dimethoxy flavone; 3-hydroxy-7-methoxy baicalein; neobavachalcone; 3′,5-dihydroxy-7,4'-dimethoxy flavone; 3-Hydroxy-7-methoxy baicalein; genistein; liquiritigenin and quercetagetin-3,4'-dimethyl ether. The anthraquinone compounds detected were 1,4-Dihydroxy-2-methyl-anthraquinone and 1-Hydroxy-2-methyl-anthraquinone. The results of the LC-MS/MS analysis are presented in Table 5.

DISCUSSION

This study focused on the antibacterial effect of EEMC in inducing cell leakage against *S. aureus*. All the parameters measured indicate the occurrence of cell leakage. Prior to the cell leakage test, MIC and MBC tests were performed using the dilution method to provide a reference for concentration. This method ensures homogeneity between the growth media, test materials, and bacteria to allow for perfect interaction. The bacteriostatic and bactericidal activity were determined based on the MBC/MIC ratio. If the MBC/MIC ratio is ≤ 4, the effect of a tested agent is considered bactericidal, but if the MBC/MIC ratio is > 4, the effect is rather bacteriostatic [5]. The EEMC prepared in this research has an MBC/MIC ratio of < 4, signifying its bactericidal property.

The cell membrane can get damaged when the bacteria are exposed to antibacterial agents. Various necessary intracellular materials, including small ions such as K^+ and $(PO_4)^{3-}$, tend to escape from the cells, followed by leakage of large molecules, such as DNA and RNA [10]. DNA and RNA are nucleic acids that carry genetic information needed to make proteins.

Table 5: Chemical compounds in EEMC identified by LC-MS/MS analysis

Component	Observed m/z	Observed RT	Area (%)
1,4-Dihydroxy-2-methyl-anthraquinone	255.06	8.16	10.96
1-Hydroxy-2-methyl-anthraquinone	239.07	7.16	5.14
3′,5-Dihydroxy-7,4'-dimethoxy flavone	315.08	7.62	16.04
3-Hydroxy-7-methoxy baicalein	301.07	8.29	12.64
Neobavachalcone	299.09	9.25	3.69
3′,5-Dihydroxy-7,4'-dimethoxy flavone	315.08	8.67	3.15
3-Hydroxy-7-methoxy baicalein	301.07	8.41	5.51
Digiprolactone	197.11	5.52	1.48
Genistein	295.05	7.81	1.31
Liquiritigenin	257.08	6.16	1.66
Quercetagetin-3,4'-dimethyl ether	347.07	6.77	4.33
Sappanone B	303.08	7.02	4.20

Consequently, their release leads to bacterial cell death.

Proteins have an absorption spectrum at ultraviolet wavelengths. Their absorbance increases at a wavelength of 230 nm and is optimal at 190 nm. However, carboxylic acids, buffer ions, and alcohols also have an absorption spectrum in these wavelengths which may interfere with the measurements. It is, therefore, less specific to protein. In addition, proteins have absorption spectrum in the range of 275 – 280 nm because of the presence of tyrosine and tryptophan. Cellular components leaking out of the cell can be measured at a certain wavelength, i.e., 260 nm for DNA purines, pyrimidines, and ribonucleotides and 280 nm for tyrosine and tryptophan [8]. The UV light absorption increases with extract concentration, indicating an increase in the number of cell contents released from the cell.

The level of DNA purity correlates with the quality of DNA. In this study, the DNA quality was determined by calculating the ratio of absorbance at 260 (A260) to absorbance at 280 (A280) in the sample. DNA is said to be pure if the A260/A280 ratio is in the range of 1.8 – 2.0 [9]. A260/A280 ratio of < 1.8 indicates the presence of phenol or protein contamination. DNA is contaminated with RNA if it has an A260/A280 ratio of > 2.0. The DNA purity (A260/A280 ratio) after exposure to 10 and 20 % EEMC was below 1.8, indicating the presence of phenol or protein contamination [8,9]. In this study, it is suspected that the material released from *S. aureus* cells in response to EEMC contained not only DNA but also protein.

Potassium ion (K^+) is very important for every living cell because it is needed to activate various enzymes and maintain intracellular pH by resisting negatively charged nucleic acids [8]. The K^+ levels affect numerous physiological processes, such as membrane potential, acid-base homeostasis, fluid-electrolyte balance, and glucose metabolism. The intracellular environment is generally rich in K^+, making its presence in the extracellular medium an indication of serious and irreversible damage to the cytoplasmic membrane. Analysis of K^+ levels using the atomic absorbance spectrophotometer (AAS) showed that EEMC caused leakage of K^+ from *S. aureus*, as indicated by the increasing extracellular K^+ levels when the EEMC was introduced at the concentrations of 1 MIC and 2 MIC [4]. Leakage of intracellular components such as proteins, nucleic acids, and ions (K^+, Ca^{2+}, Na^+, and other intracellular materials) is also an indication of damage to the permeability of bacterial cell membranes [7]. Some extracellular fluid with elevated levels of ions most likely has increased electrical conductivity, causing the damage to the permeability. Moreover, the electrolytes in bacterial cells are needed to facilitate cell membrane function and maintain enzyme activity and normal metabolism; thus, leakage of electrolytes can lead to cell death. In the present study, EEMC disturbed the permeability, as indicated by the extracellular electrical conductivity that became higher when the extract was added at the concentrations of 1 MIC and 1.5 MIC. Such response may be linked to the increasing number of electrolytes resulting from damage to permeability [11].

Time-kill assay is the most appropriate method for ascertaining the bactericidal effect of an antibacterial agent. It is excellent for obtaining information about dynamic interactions between antimicrobial agents and microbial strains. Time testing reveals whether the antibacterial effect is time-dependent or concentration-dependent [7]. The result showed that EEMC kills *S. aureus* in 24 h when introduced at the concentrations of 1 MBC and 1.5 MBC but not 0.5 MBC. This suggests that the concentration affects the bacteriostatic and bactericidal activity of the EEMC. High enough concentrations induce a bactericidal effect and, consequently, cell leakage at a high rate. Apart from the

concentration, the contact time between the extract and the bacteria also determines the antibacterial activity. This was evident from the power of EEMC at 1 x MBC and 1.5 MBC to kill *S. aureus* in 24 h instead of 2 - 12 h. Antimicrobial agents become more effective in their action against bacterial cells over time, until the end of the incubation period [12].

The LC-MS/MS analysis of EEMC showed the presence of several compounds, such as anthraquinone (1,4-dihydroxy-2-methyl-anthraquinone; 1-hydroxy-2-methyl-anthraquinone). Among the two anthraquinones, 1-hydroxy-2-hydroxymethyl-anthraquinone has been reported to inhibit bacterial growth [13]. Anthraquinones have multiple antibacterial mechanisms, including simple cell wall destabilization. Wei *et al* [14] reported that 1,8-dihydroxy-anthraquinone has strong antibacterial activity against *S. aureus* with the mechanism of disrupting cell membrane permeability and leads to the leakage of cytoplasm and the deconstruction of cells. In addition to anthraquinones, the LC-MS/MS results also showed the presence of flavones (3′,5-dihydroxy-7,4'-dimethoxyflavone and 3′,5-Dihydroxy-7,4'-dimethoxyflavone) and a chalcone (Neobavachalcone). This is similar to the research conducted by Sufian *et al* [3] who succeeded in isolating three flavone compounds (5,7-dihydroxy-3,8-dimethoxyflavone, 5-hydroxy-3,7-dimethoxyflavone and 3,5,7-trihydroxy-8-methoxyflavone) and one chalcone (2′,4′-dihydroxychalcone) from *M. calabura* leaves, and showed that these compounds had antibacterial activity against *MRSA.* Some other flavonoids in EEMC detected by LC-MS/MS were sappanone B, liquiritigenin, and genistein. Sappanone B and liquiritigenin were reported to have antibacterial activity [15,16]. Genistein shows antibacterial activity through changes in cell morphology and inhibition of DNA, RNA and protein synthesis [17]. Flavonoids triggered cell perforation, inhibit nucleic acid synthesis, interfere with energy and coenzyme metabolism, and disrupt cell membrane integrity [18]. It can be suspected that EEMC kills *S. aureus* by inducing cell leakage possibly caused by the flavonoids and anthraquinones contained in it.

CONCLUSION

The ethanol leaf extract of *Muntingia calabura* L. (EEMC) induce leakages of cellular materials in *Staphylococcus aureus*, as indicated by increases in the levels of nucleic acid, protein, RNA, DNA, K^+ ions, and electrical conductivity in the extracellular medium. Anthraquinone, flavone, and chalcone compounds detected in EEMC are thought to be responsible for bacterial cell leakage and death. This study may serve as a reference for future studies aiming to investigate such topics as isolation of antibacterial active compounds and development of antibacterial drug products from *Muntingia calabura* leaves.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. The conceptual framework and methodologies were designed by Nanik Sulistyani. The experiment, data collection process and analyses as well as the writing up of the original manuscript were carried out by Rizki H Mawardi and Nanik Sulistyani. The manuscript draft was reviewed and edited by Nurkhasanah and Zainul Amiruddin Zakaria. All authors have read and agreed to the published version of the manuscript.

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