

## ANTIBACTERIAL ACTIVITY SCREENING OF *MUNTINGIA CALABURA L* LEAVES METHANOL EXTRACT ON THREE BACTERIAL PATHOGENS

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### Abstract

Screening of natural products for drug development, especially for antibacterial, is very important to find alternatives solution to overcome infectious diseases as well as antibiotic-resistance. *Muntingia calabura L* also known as Talok in Indonesia has been known to have a lot of health benefits. This study was conducted to screen the antibacterial activity of methanol extract of *Muntingia calabura L* leaves against *Staphylococcus aureus* FNCC 0047, *Staphylococcus epidermidis* FNCC 0048 and *Escherichia coli* FNCC 0091. Powdered air-dried plant material was extracted by maceration in methanol solvent. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined for the extracts that showed total growth inhibition using broth microdilution method. Phytochemical tests were carried out through Thin Layer Chromatography (TLC) method. The results showed that the MIC values of the methanol extract of *Muntingia calabura L* leaves on *Staphylococcus aureus* FNCC 0047, *Staphylococcus epidermidis* FNCC 0048, and *Escherichia coli* FNCC 0091 are 0.5 mg/mL, 1 mg/mL, and 2 mg/mL, respectively. While the MBC values are 1 mg/mL, 2 mg/mL, and 4 mg/mL, respectively. The phytochemical test of extract suggested the presence of flavonoid, tannin, as well as saponin. The overall results of this study indicate that the methanol extract of *Muntingia calabura L* leaves has potential as an antibacterial.

**Keywords:** Antibacterial, talok, methanol extract of *Muntingia calabura L* leaves

## Introduction

Infectious disease is one of the biggest problems in the health sector and is one of the causes of high morbidity and mortality rates in developing countries (1). According to WHO in 2011 one third of 25 million deaths worldwide are caused by infection and around 1.4 million patients worldwide experience nosocomial infections. While in Indonesia, the data of nosocomial infection prevalence is very limit. According to IMRAN study which is conducted in two Indonesian university hospitals in Java Island, Indonesia provided that the Surgical Site Infections (SSI) which is one kind of nosocomial infections is quite high than others (2).

Most infections are caused by pathogens such as bacteria, viruses, and fungi. Bacteria are one of the most common causes of infection, some of which are *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Escherichia coli*. *Staphylococcus epidermidis* is a normal flora of the skin. These bacteria can form biofilms on the surface of medical devices such as catheters and can cause urinary tract infections. The epidemiological percentage of some types of nosocomial infections are as follow: 34.5% urinary infection, infection at the insertion site for surgery 17.4%, infections in blood flow 14.2%, 13.2% pneumonia and other causes as much as 20% (3,4). These bacteria also often cause endocarditis in patients undergoing heart surgery for the installation of artificial intravascular devices such as artificial heart valves. Also, if these bacteria contaminate contact lenses, it can cause keratitis and endophthalmitis (5).

*Staphylococcus aureus* is a gram-positive bacterium found 20% -75% in the upper respiratory tract, hands, face, hair, and vagina. When someone is exposed to this bacterium, unique clinical manifestations will arise in the form of inflammation, necrosis, acne, folliculitis, and the formation of abscesses. The organ most commonly affected by *Staphylococcus aureus* bacteria is the skin because *Staphylococcus aureus* is a normal flora of the skin (6). These bacterial infections can enter the human body through open lesions as well as from infected tools. In addition to that, a hospital is also a place with the high risk of spreading these bacteria, especially places like the intensive care units, neonatal care, and operating room (7).

*Escherichia coli* is a rod-shaped gram-negative bacterium first discovered in 1885 (8). This bacterium normally resides in the digestive tract of humans and animals. But it can also be present in water and soil from contamination (9). The most common disease caused by this bacterium is diarrhea. In developing countries, especially tropical countries, diarrhea is one of the most common causes of mortality in children under five years old (10,11).

One of the pharmacological options to treat bacterial infections is antibiotics. But unfortunately, nowadays the development of antibiotic-resistance has become a major threat. *Staphylococcus aureus* and *Enterococcus* species are pathogens which are become resistance to multiple antibiotic classes (11). Most methicillin-resistant *Staphylococcus epidermidis* has multidrug-resistant to common antibiotics such as penicillin, amoxicillin, gentamycin, erythromycin, and doxycycline. Therefore, prior sensitivity testing is required when providing systemic treatment for these bacteria, because of their high resistance rates (8,12). These reasons lead to the need for newer antibiotics as an alternative to overcome the issues.

Indonesia has various types of plants that easily grows and is full of health benefits. *Muntingia calabura L.*, also known as cherry or talok have long been used as a traditional medicinal plant by the people of Indonesia. Kersen (*Muntingia calabura L*) leaves are widely used by Indonesian people as a traditional medicine such as to treat gout, diabetes, flu symptoms, cancer, and stomach cramp (13). Kersen leaf, which is green and hairy, is also effective as cough medicine, mucolytic, and antitumor. Peruvians have long used cherry leaves for drinking tea to relieve headaches and inflammation (14).

## Methods

### Materials

The tools used in this research, among others, were analytical balance (Mettler Toledo) glass beaker, measuring glass, Erlenmeyer, test tube, Petri dish disposable, 96-well microplate (Iwaki), micropipette (Transferpette), Laminar Air Flow (LAF) (ESCO), incubators (Memert), and other tools used for the process of extraction, a bacterium testing and others.

The material used in this research was the leaves of *Muntingia calabura L* obtained from Sleman, Yogyakarta. The chemicals used include methanol solvent, Aquades, Dimethylsulfoxide (DMSO) 10%, Alcohol 70%, Spiritus, NaCl 0.9%, Mc. Farland Standard 10<sup>8</sup> CFU/ml.

Bacteria used were *Staphylococcus aureus* FNCC 0047, *Staphylococcus epidermidis* FNCC 0048, and *Escherichia coli* FNCC 0091. All bacteria were obtained from Laboratorium Mikrobiologi Pascasarjana Universitas Gadjah Mada (UGM).

### Tool Sterilization

In this research, it is necessary to sterilize the tool to avoid any contamination that may affect the research result. The tools are sterilized using an autoclave for about two hours, while the Ose needle/round/straight is sterilized by means of being burned over bunsen to incandescent.

### Bacteria Breeding

Each bacterium was first cultured on Mueller Hinton Agar (MHA) media to multiple bacterial stocks. The stock

of each bacterium will be made up of three tubes with each tube containing 5 ml of MHA media. The formula to find the required amount of powder is:

$$\frac{38}{1000} \times 15 \text{ ml (aquadest)} = 0,57 \text{ gram}$$

Preparation of MHA media was done by dissolving 0,57 gram MHA powder with aquadest of 15 ml, then the solution was heated using microwave until clear but not to boil, then the media was put into the reaction tube, after which the tube was closed using cotton and aluminum foil and sterilized using autoclave for 2 hours.

The sterilized medium was placed in LAF with a 30° incline and then waited for the media to harden. Once the bacterial had hardened, media from the host stock was taken using ose and then scratched into the MHA medium in zigzag, the same was done on the other tubes. All the test tubes were incubated for 24 hours at 37° C in the incubator.

#### **Bacterial Suspension**

The bacteria from pre-made stocks were suspended into Mueller Hinton Broth (MHB) media. The formula to calculate the media that need was:

$$\frac{21}{1000} \times 3 \text{ ml (aquadest)} = 0,063 \text{ g}$$

MHB preparation was done by dissolving media as much as 0.063 gram with 3 ml of water and then heated using the microwave until clear but not to boil, then the media was put into the reaction tube, after which the tube was closed using cotton and aluminum foil and sterilized using the autoclave for 2 hours.

Sterilized bacteria were diluted inside LAF and wait until it was not hot, then take the bacteria from the stock of one or two colonies using sterilized ose then suspended into the MHB medium. The bacterial suspension was incubated using an incubator for 24 hours at 37°C.

#### **Extract dilution**

The largest concentration of extract used was 32 mg/mL. The stock prepared in this study was 64 mg / ml. This was done by weighing the extract using a watch glass as much as 64 mg, then extract was dissolved with 1 ml DMSO 10%. The diluted extract was put into a sterile vial.

In this research, the 10% DMSO solution was made as much as 4 ml, in which 1 ml was used to dissolve the methanol extract, 1 ml to dissolve the chloroform extract, and 2 ml for positive control on antibacterial testing. This 10% DMSO solution was obtained from mixing 400 µl of 100% DMSO with sterile distilled as much as 3600 µl.

#### **Microdilution test**

In the LAF the bacterial suspension was added 0.9% NaCl using micropipes to its turbidity equal to Mc Farland's standard for the bacterial concentration to be 10<sup>8</sup> CFU / ml. This bacterium was then taken 100 µl and

homogenized with MHB 9900 µl MHB so that can be obtained bacterial concentration of 10<sup>6</sup> CFU/ml.

For the test, 100 µl of bacterium 10<sup>6</sup> CFU/ml was taken using micropipette then put into 96-well microplate. Replication was made 3 times. Using a micropipette of 100 µl, the sample was taken and inserted into the well and then homogenized then 100 µl extract from the first well was taken and inserted in the second well so that the second pit concentration was halved. After the 2<sup>nd</sup> well has homogenized, pipette 100 µl of the mixture into the 3<sup>rd</sup> well and so on until the 8<sup>th</sup> well. After the 8<sup>th</sup> well, 100 µl was taken away. The concentrations of Kersen leaf extract were of 32 mg/ml, 16 mg/ml, 8 mg/ml, 4 mg/ml, 2 mg/ml, 1 mg/ml, 0,5 mg/ml, and 0,25 mg/ml respectively from the 1<sup>st</sup> to 8<sup>th</sup> wells.

In this test, there were three kinds of controls, in which the 10<sup>th</sup> well was a bacterial control containing 100 µl of bacteria 10<sup>6</sup>, the 11<sup>th</sup> well as a solvent control containing 90 µl of bacteria homogenized with 10 µl DMSO 10%, 12<sup>th</sup> well as control media containing 100 µl sterile MHB media.

#### **Subculture test in Petri dish**

96-well microplate which had been incubated for 24 hours was subcultured in the petri dish containing MHA media. In this study, one petri dish was divided into 6 zones. In one petri dish filled with MHA media as much as 20 ml. The formula for calculating media requirements was:

$$\frac{38}{1000} \times 360 \text{ ml (aquadest)} = 13,68 \text{ g}$$

Making MHA media was done by dissolving MHA powder as much as 13.68 gram with aquadest 60 ml, then the solution was heated using the microwave until clear but not to boil, then media put into Erlenmeyer, then Erlenmeyer closed using cotton and aluminium foil and sterilized using the autoclave for 2 hours.

Sterilized media was placed in LAF until warm then the media poured into 18 Petri dishes each approximately 20 ml and wait until the media hardens. Petri dishes were drawn into six zones and coded. After that, dip the sterile ose into well 1A then scratch it into the petri dish zigzag in accordance with the code that has been made and done on all the wells. The scraped petri dish wrapped in plastic wrap was then incubated at 37°C for 24 hours.

#### **Assessment of Test Result**

Conducted by observing bacterial growth in petri dishes that had been incubated for 24 hours. It was assessed that the determination of MIC and MBC, observation of bacterial growth on media control, solvent control and bacterial control. The determination of MIC and MBC was done by evaluation the bacterial growth on the media. If there was no bacterial growth in a particular zone in the petri dish, then this was called MBC.

Meanwhile, MIC was assessed by looking at zones where there was least bacterial growth.

The results of this study were valid and can be assessed if the incubation results in MHA agar in the bacterial control zone and the solvent controller had bacterial growth. While in the clear media control zone there was no bacterial growth.

## Results

The determination of Kersen plant consisting of stems, leaves, fruits, and flowers with plant morphology literature in biology laboratory of FMIPA UGM revealed the plant is *Muntingia calabura* L.

The extraction of *Muntingia calabura* L was done by using Soxhlet extractor. From this process, as much as 40 grams of kersen leaf simplisia yields a thickened methanol extract of 6.8 grams with the results of the calculation of the yield as follows:

$$\frac{\text{extract (6,8 gr)}}{\text{simplisia(40 gr)}} \times 100\% = 17 \%$$

### Phytochemical Screening

To find out whether the methanol extract of *Muntingia calabura* L leaves contains secondary metabolite compounds such as flavonoids, alkaloids, and tannins, then qualitative testing using Thin Layer Chromatography (TLC) method was done. To determine the secondary metabolite of Saponin quantitative method was done using spectrophotometry. The test results can be seen in table 1.

After the plate was sprayed using aluminum chloride reagent, on direct observation, there was a yellow stain with Rf 0.82. In addition, at observations with 356 nm UV light and 254 nm UV ray stains were also found. So, it can be concluded that the caustic methanolic extract contains flavonoid compounds (Figure 1A).

Reagents Dragendorff were used to know the content of alkaloid compounds. In this test after the reagent was spread, an orange coloured streak was expected. However, in this test, there was no spotting either on direct observation or using UV light. So, it can be concluded that the methanol extract of *Muntingia calabura* L leaves does not contain alkaloids (Figure 2).

### Antibacterial Activity

#### Results of Antibacterial Activity Test of Methanol Extract of *Muntingia calabura* L against *Staphylococcus epidermidis* FNCC 0048

The methanol concentration used for the tests was 32 mg/ml, 16mg/ml, 8mg/ml, 4mg/ml, 2mg/ml, 1mg/ml, 0.5 mg/ml, and 0.25 mg/ml, respectively. Accompanied by three kinds of controls, which were bacteria control, solvent control, and media control. The result of microplate incubation for 24 hours with temperature 37°C can be seen in figure 3.

Observations on changes in turbidity of the wells in 96-well microplate were done to determine the MIC. In figure 3 it can be seen at the wells number 6,7, and 8 had visible turbidity. Meanwhile, wells number 1,2,3,4, and 5 looked clear. So, it can be concluded that the MIC of methanol leaf extract of Kersen was found at the 6th well with concentration of 1 mg/ml. However, to reduce the subjectivity, the subculture of MHA media was performed. The result of the subculture can be seen in figure 4. The determination of MBC is done by observing the lowest concentration of bacterial growth on MHA media after incubation for 24 hours with temperature 38 C. Results of the MBC of kersen leaves methanol extract (*Muntingia calabura* L) against bacteria *Staphylococcus epidermidis* FNCC 0048 can also be seen in figure 4.

#### Results of Antibacterial Activity of *Muntingia calabura* L methanol extract against *Staphylococcus aureus* FNCC 0047

After incubation for 24 hours at 37 °C turbidity was visible at concentrations of 0.25 mg / ml. While at concentrations of 32 mg / ml, 16 mg / ml, 8 mg / ml, 4 mg / ml, 2 mg / ml, 1 mg / ml 0.5 mg / ml no turbidity was found. This suggests that the Kersen leaf methanol extract may inhibit the growth of *Staphylococcus aureus* at a concentration of 0.5 mg / ml to 32 mg / ml. Then the concentration of 0.5 mg / ml can be defined as the value of Minimum Inhibitory Concentration (MIC). Bacterial control can be seen with turbidity at the well 10A-C. Turbidity was also found at the control of solvents DMSO 10% at well 11A-C looks. This means the solvent has no activity in inhibiting the growth of *Staphylococcus aureus*. And media control can be seen cloudy in well 12A-C, indicating that the medium does not affect bacterial growth (figure 5). Results of sub culture on Mueller-Hinton media can be seen in figure 6.

#### Results of Antibacterial Activity of *Muntingia calabura* L methanol extract against *Escherichia coli* FNCC 0091

Figure 7 shows the turbidity at the 4th wells rows A through C that had the extract in the concentration of 2 mg / ml. Based on the subculture results in the petri dish, it was observed that in the first, second and third replications there was no bacterial growth in zone number 4 and bacterial growth was at least 5 (figure 8). Thus, obtained MBC value of 4 mg / ml and MIC of 2 mg / ml. Based on the three replications, it can be concluded that MBC of *Muntingia calabura* L methanolic extract against *Escherichia coli* bacteria is mostly 4 mg / ml. As for MIC, it is 2 mg / ml.

### Discussion

In this research, the Soxhlet extraction method was used to extract the simplisia. This method gave advantages in which it can extract a little amount of simplisia and solvents, cheap, and simple (15). This

research used polar methanol as solvent, so if yield of extract was calculated, we can measure how much polar substance is contained in the leaves of Kersen, because according to Azwanida (2015), if extraction is done then the substance having the same polarity with solvent will be soluble, while the different polarity substances will become residues (16). Based on the measurement of yields, the polar substance contained in the extract was approximately 17 %.

The choice of microdilution method in this research was because the bacteria can be homogenized with test substance thus there is more even direct contact between bacteria with the test substance, causing the results to be more sensitive than the diffusion method, the time required for the is short test, the cost is less expensive, and does not require a large number of samples (17,18). Microdilution method used in this study is identical to the guideline made by Stephen (2005) which is in accordance with National Committee for Clinical Laboratory Standards (NCCLS) (19).

This research revealed that the MIC and MBC of *Muntingia calabura* L methanol extract on *Staphylococcus epidermidis* FNCC 0048 is 1 mg/ml and 2 mg/ml; on *Staphylococcus aureus* FNCC 0047 is 1 mg/ml and 1250 µg/ml, and on *Escherichia coli* FNCC 0091 is 2 mg/ml and 4 mg/ml. The largest antibacterial activity is on *Staphylococcus aureus* FNCC 0047.

The MIC and MBC value found in this research are smaller than the ones found in a previous research by Zakaria, 2010 in which the MIC and MBC of Kersen leaf methanol extract against *Escherichia coli* FTCC 35218 were found >5000 µg/ml.

The ability of methanol extract of *Muntingia calabura* leaf to inhibit and eradicate the bacteria is supported by the presence of several active compounds that have antibacterial effects such as flavonoids, tannins, and saponins. Although this extract does not contain alkaloids, these three secondary metabolites are strong enough to provide antibacterial effects. As is known, that flavonoids have antibacterial activity in various ways, some of which is through the formation of protein complexes from hydrogen bonds and covalent bonds so as to disrupt the ability of bacterial attachment to the substrate, disrupt the permeability of bacterial cell walls so that transport of nutrients and organic components required by bacteria is disturbed. In addition, flavonoids also have a subdivision of flavones that are able to inhibit the formation of bacterial colonies and flavonoids have a ring B that can affect nucleic acid so as to inhibit DNA synthesis and bacterial RNA (20–22).

Tannins are also known to have toxic properties against bacterial cell walls. However, tannin also has several other mechanisms in inhibiting the growth of bacteria, one of which is by inhibiting oxidative

phosphorylation, reverse transcriptase enzymes, and DNA topoisomerase so that bacteria can not replicate. In addition, tannin can cause bacteria to lack the nutrients it needs because tannin would be hydrolyzed into gallotannin and inhibit the formation of glucose in bacteria. Tannin acid compounds can also inhibit bacteria from binding irons, whereas in aerobic conditions bacteria require iron to reduce RNA (23–25).

This finding certainly adds to the rows of traditional Indonesian medicinal plant findings that can be developed into drugs, especially as antibacterial. Thus, Indonesia is expected to develop its natural wealth so as to improve the welfare and health of people in Indonesia as well as the world.

### Conclusion

The results of screening of the three pathogens, *Staphylococcus epidermidis* FNCC 0048, *Staphylococcus aureus* FNCC 0047, and *Escherichia coli* FNCC 0091 has proven the antibacterial potential of Kersen leaves methanol extract.

### Acknowledgments

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Table 1. Results of Phytochemical Screening

Compounds	Results
Flavonoid	+
Tanin	+
Alkaloid	-
Saponin	8,82%b/b

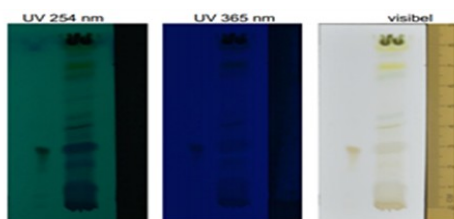


Figure 1A. Results of flavonoid TLC

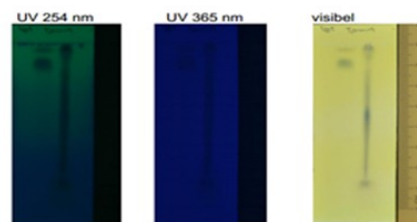


Figure 1B. Results of tannin TLC

For testing of tannin compound, after the plate was sprayed using reagent with the direct observation grayish blue spots with Rf 0.84 was found. In addition, in observations using 356 nm and 254 nm UV rays the same results of this spotting was also found (Figure 1B).

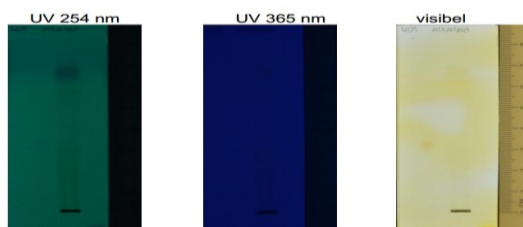
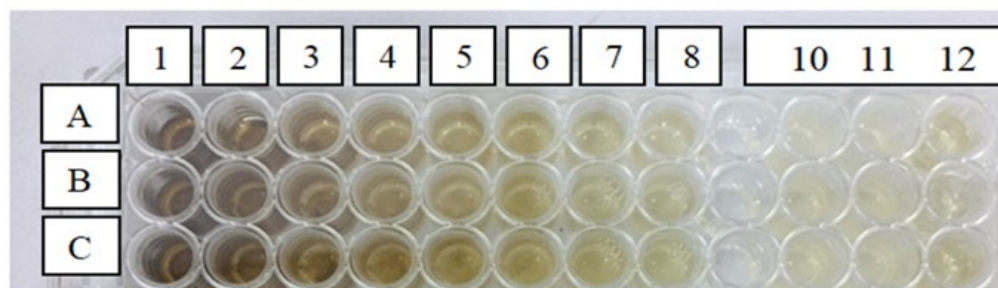


Figure 2. The results of Alkaloid TLC

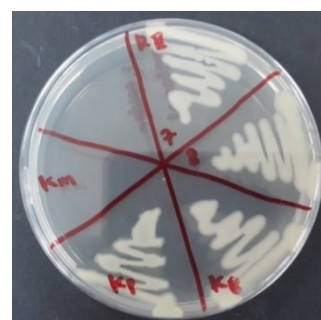
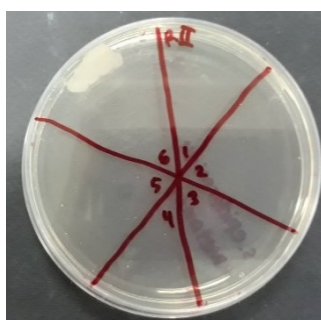
Figure 3. Results of microplate incubation on the methanol extract of *Muntingia calabura* L

Note: Extract concentrations were 32mg/ml (1A-C), 16mg/ml (2A-C), 8mg/ml (3A-C), 4mg/ml (4A-C), 2mg/ml (5A-C), 1mg/ml (6A-C), 0,5 mg/ml (7A-C), and 0,25 mg/ml (8A-C) accompanied by three control, bacteria control (10A-C), solvent control (11A-C), and media control (12A-C)

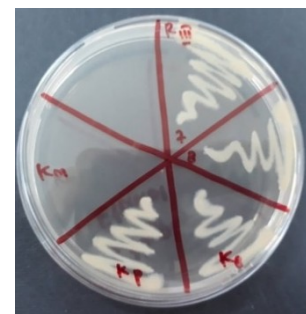
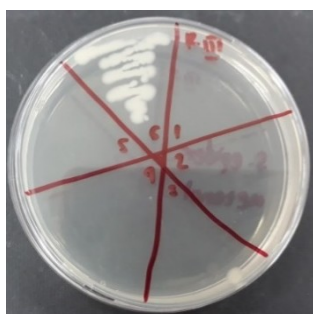
Replication I



Replication II



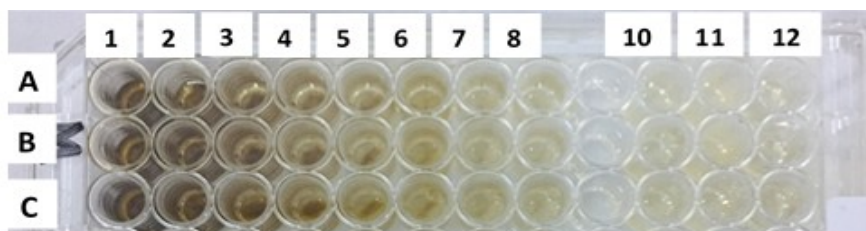
Replication III



**Figure 4.** Subculture results of Kersen leaves methanol extract (*Muntingia calabura* L) against *Staphylococcus epidermidis* FNCC 0048

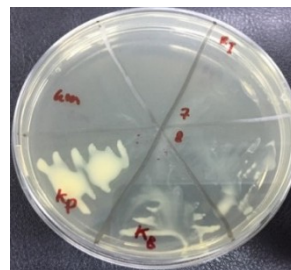
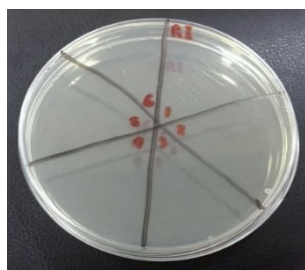
Figure 4 shows consistent results on the first, second, and third replication of bacteria not growing at concentrations of 32 mg / ml, 16 mg / ml, 8 mg / ml, 4 mg / ml, and 2 mg / ml. Meanwhile, at concentrations of 1 mg / ml there is bacterial growth. So it can be concluded that MIC and MBC of the kersen leaf methanol extract (*Muntingia calabura* L) against *Staphylococcus epidermidis* FNCC 0048 bacteria are 1 mg / ml and 2 mg / ml, respectively.



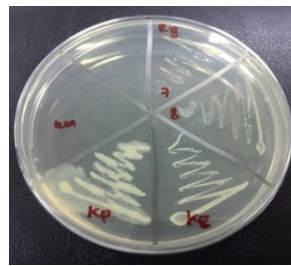
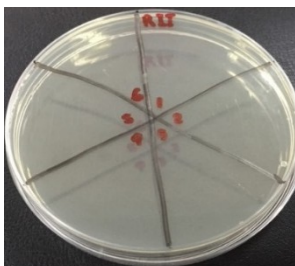


**Figure 5.** microdilution test of *Muntingia calabura L* methanol extract against *Staphylococcus aureus*  
 Note: Kersen leaves methanol extract in the concentration of 32 mg/ml (1A-C), 16 mg/ml (2A-C), 8 mg/ml (3A-C), 4 mg/ml (4A-C), 2 mg/ml (5A-C), 1 mg/ml (6A-C), 0,5 mg/ml (7A-C), 0,25 mg/ml (8A-C), bacteria control (10A-C), solvent control (11A-C) and media control (12A-C)

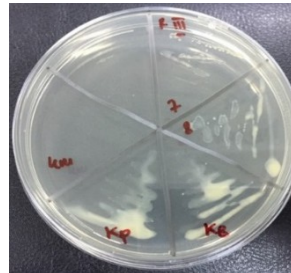
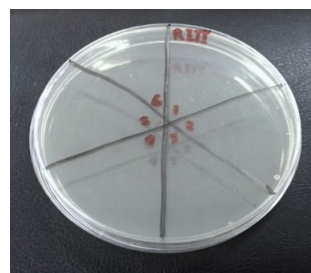
Replication I



Replication II



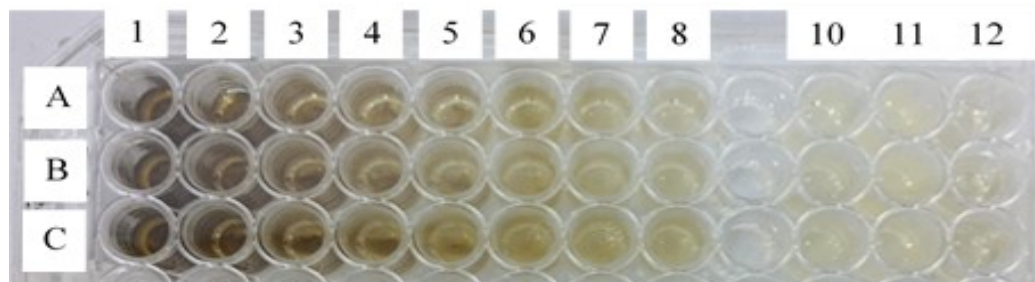
Replication III



**Figure 6.** Results of subculture of *Muntingia calabura L* methanol extract against *Staphylococcus aureus* FNCC 0047

Note: R1 (Replication 1), R2 (Replication 2), R3 (Replication 3), KB (Bacteria control), KM (Media control)

Figure 6 shows subculture results in MHA media that had no colony grown on methanol extract of *Muntingia calabura* leaf L was in the concentrations of 32 mg/ml, 16 mg/ml, 8 mg/ml, 4 mg/ml, 2 mg/ml, 1 mg/ml. The smallest concentration that did not show the growth of colonies of *Staphylococcus aureus* bacteria was 1 mg / ml which is the value of Minimum Bactericidal Concentration (MBC) Methanol Extract Leaf *Muntingia calabura L*. Meanwhile, in bacterial control and solvent control there is bacterial growth which means the media does not inhibit the growth of bacteria *Staphylococcus aureus*.



**Figure 7.** Results of Antibacterial activity of *Muntingia calabura* L methanol extract  
 Note: Kersen leaf methanol extract in concentration of 32mg/ml (1 A-C), 16mg/ml (2 A-C), 8mg/ml (3 A-C), 4mg/ml (4 A-C), 2mg/ml (5 A-C), 1mg/ml (6 A-C), 0,5 mg/ml (7 A-C), 0,25 mg/ml (8 A-C), bacteria control (10 A-C), solvent control (1 A-C) and media control (12 A-C)

