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Analysis of The Secondary Metabolite of Kersen Leaf Extracts (*Muntingia calabura* L.) and Its Potential as Anti-Bacteria to Inhibit *Aeromonas hydrophila*

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KEYWORDS

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Secondary metabolite

Abstract *Aeromonas hydrophila* is a gram-negative bacterium that massively attack aquatic organisms in fresh water, briny water and sea waters. Infection due to this bacterium caused septicemia in the host's body to the point of damaging the body's organs. Hence, alternative material is required in order to cope with this bacterium, which was by using natural material such as kersen leaf (*Muntingia calabura* L). The purpose of the research is to determine the effect of secondary metabolite contained in kersen leaf as anti-bacterial against *Aeromonas hydrophila*. This research used a method of MIC (*Minimum Inhibitory Concentration*) as an anti-bacterial test. In order to examine the secondary metabolite content, phytochemical screening and FTIR (*Fourier Transform Infrared Spectroscopy*) methods were used. The results obtained through the MIC test of 125 ppm is the minimum concentration capable of inhibiting the growth of *Aeromonas hydrophila*. Kersen leaf extracts is bacteriostatic anti-bacteria which only inhibit bacterial growth up to 24 hours. Phytochemical screening reported that kersen leaf extracts were positive of flavonoids, tannins, saponins, alkaloids and triterpenoids. Through FTIR test, it is known that phenol compounds were contained in kersen leaf extracts. This phenol is one of the metabolites with the function of anti-bacterial on this study.

Introduction

Aeromonas hydrophila is a massive gram-negative pathogenic bacterium that attacks aquatic organisms in fresh water, brackish water and marine water (Afrianto *et al.,* 2015). This bacterial attack is generally called *Motile Aeromonas Septicemia* (MAS), *Motile Aeromonas Infection* (MAI) and *Septicemia Hemorrhage*, they have a characteristic infection red spots on the fish's body (Camus *et al.,* 1998). The fish that is infected with this bacterium will show haemorrhage and septicemia on its skin and muscles with the signs of; wound on the skin, demaged fins, wound on the operculum up to the acute stage of severe ulcers and death (Maftuch and Dalimunthe, 2013). Therefore anti-bacteria is needed as inhibitor of the growth of *Aeromonas hydrophila*.

One natural anti-bacteria which can be used against *Aeromonas hydrophila* is from the kersen leaf (*Muntingia calabura* L.). These plants live in warm and tropical climates (Morton, 1987). Kersen was known to be originated from Latin America and was naturalized in Southeast Asia in the 19th century (Birasal, 2013). Kersen is a shrub plant that generally has a short size (Kosasih *et al.,* 2013). Kersen has long time ago been good for health, especially on its leaves (Andareto, 2015). Kersen leaf has secondary metabolite which has certain activities that are able to fight pathogenic bacteria as the anti-bacteria (Sibi *et al.,* 2012). Anti-bacterial is a certain substance that has the function of killing or inhibiting bacterial reproduction so that it is unable to grow and maturate (Sartika *et al.,* 2013).

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It is necessary to study the secondary metabolite produced by kersen leaf as a natural anti-bacterial to cope with diseases caused by *Aeromonas hydrophila* infection. The method used is dilution testing of *Minimum Inhibitory Concentration* (MIC). Phytochemical screening and FTIR (*Fourier Transform Infrared Spectroscopy*) tests are used to determine the content of secondary metabolite.

Materials and methods

Extraction

Kersen leaf powder macerated with a ratio of 1:7 (w/v) for 2 days using 96% ethanol, which as many as 100 g of kersen leaves are soaked with 700 ml of solvent on a closed erlenmeyer flask. After that, leave it for 48 hours and whisk it occasionally to dissolve the solvent with the samples. After 48 hours, the solution is filtered using a Buchner vacuum filter. Filtering results are collected in glass bottles, then evaporated using a rotary evaporator with a temperature of 45°C with a rotation speed of 80 rpm to obtain a thick crude extract.

Minimum Inhibitory Concentration (MIC) Test

- A test tube containing 5 ml sterile TSB (*Tryptic Soy Broth*) media was prepared.
- The 2 test tubes are used as positive and negative control. Positive control is given 5 ppm synthetic antibacterial (*Chloramphenicol*) and negative control is only given by *Aeromonas hydrophila* bacteria.
- Crude leaf extract is included in 10 test tubes with concentrations of 1000 ppm, 500 ppm, 250 ppm, 125 ppm, 62.5 ppm, 31.25 ppm, 15.6 ppm, 7.8 ppm, 3.9 ppm and 1.9 ppm.
- 1 ose loop isolate is added in each test tube.
- Then all tubes are incubated in an incubator at 37°C for 24 hours.
- The media is examined for its turbidity and the absorbance value is measured using a spectrophotometer.

Phytochemical Screening

- Flavonoid: 2 ml of crude leaf extract is dissolved on 10 ml of hot methanol. Then 0.1 grams of Magnesium and 5 drops of concentrated HCl are added. Brick red color reaction indicates positive flavonoids.
- Tannin: 2 ml of crude leaf extract is dissolved on 10 ml destilled water. It is then filtered and added with 3 drops of $FeCl_3$ 1%. Green-blackish color reaction indicates positive tannin.
- Saponin: 2 ml of crude leaf extract is dissolved on 10 ml boiling destilled water. Then it is shaken vigorously for 10 seconds. Stable foam reaction indicates positive saponins.
- Alkaloid (Alkaloid testing is carried out Dragendrof and Mayer reagents): 2 test tubes are prepared. 2 ml of crude leaf extract is dissolved and added 10 ml of hot destilled water. 6 drops of Dragendrof reagent is added on the first tube. The second tube was prepared with crude extract which is mixed with 10 ml of hot destilled water and given 6 drops of Mayer. The presence of white/yellow sediment (Mayer) and the orange color reaction (Dragendrof) indicates positive alkaloids.
- Triterpenoid: 2 ml of crude leaf extract is dissolved on 0.5 ml of chloroform and 0.5 ml of anhydrous acetic acid is the added. Finally, 2 ml H_2SO_4 concentrated is added on the tube wall. Orange/brownish color reaction indicates positive triterpenoids.

FTIR (Fourier Transform Infrared Spectroscopy) Test

As many as 1 mg of crude leaf extract is crushed with 100 mg KBr (*Potassium Bromide*) homogeneously. Then its infrared ray absorption is measured at a wavelength of 4000-400 cm^{-1} to determine the functional group of kersen leaf extracts.

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Results and discussion

Minimum Inhibitory Concentration (MIC) Analysis

The research result *Minimum Inhibitory Concentration* (MIC) using various concentrations test and measured using a spectrophotometer with a wavelength of 600 nm have obtained the data presented in Table 1.

Table 1. *Minimum Inhibitory Concentration* Test Results on TSB (*Tryptic Soy Broth*) Media for 24 hours Observation. (Positive Control (K+) Using 5 ppm *Chloramphenicol* and Negative Control (K-) Without Treatment (Only Bacteria)).

Concentration (ppm)	Absorbance	Media Condition
1000	0,125	Clear
500	0,121	Clear
250	0,114	Clear
125	0,110	Clear
62,5	0,289	Turbid
31,25	0,516	Turbid
15,6	1,102	Turbid
7,8	1,233	Turbid
3,9	1,241	Turbid
1,9	1,344	Turbid
Positive Control (K+)	0,025	Clear
Negative Control (K-)	2,286	Turbid

As test by growing a bacteria in the petri dish with the media TSA (*Tryptic Soy Agar*) as well as an time observation for 24, 48 and 72 hours respectively are carried out. To determine kersen leaf extracts (*Muntingia calabura* L.) as the bacteria growth inhibitor both bacteriostatic or bactericidal. The result of observation can be seen in Table 2.

Table 2. Bacterial Culture Test Results of *Minimum Inhibitory Concentration* on TSA (*Tryptic Soy Agar*) Media Tested for 24, 48 and 72 hours. (Positive (+) Grow Bacteria and Negative (-) doesn't Grow Bacteria).

Concentration (ppm)	Time (Hour)		
	24	48	72
1000	-	+	+
500	-	+	+
250	-	+	+
125	-	+	+
62,5	+	+	+
31,25	+	+	+
15,6	+	+	+
7,8	+	+	+
3,9	+	+	+
1,9	+	+	+

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Concentration (ppm)	Time (Hour)		
	24	48	72
Positive Control (K+)	-	-	-
Negative Control (K-)	+	+	+

Minimum Inhibitory Concentration test results have obtained 125 ppm concentration which can be said to inhibit bacterial growth. The reason is that, the condition of the media when viewed from the visual results are clear and the value of the arsobance is close to positive control (*Chloramphenicol* 5 ppm) (Table 1). After being tested into TSA (*Tryptic Soy Agar*), kersen leaf extracts with a minimum concentration of 125 ppm can inhibit bacterial growth for 24 hours and it is bacteriostatic anti-bacterial. The reason that it is only able to inhibit *Aeromonas hydrophila* for 24 hours. Bacteria begin to grow at 48 hours of observation (Table 2). Anti-bacteria are said to be bacteriostatic if they only inhibit bacterial growth without killing bacteria (Amin, 2014), Although it is different than bactericidal which is an anti-bacterial substance that has a direct mechanism to kill bacteria causing the bacteria never grow or to do proliferation (Kee and Hayes, 1996).

Phytochemical Screening

Results of screening of kersen leaf extracts (*Muntingia calabura* L.) using ethanol 96% on maceration show, positive secondary metabolites of flavonoids, saponins, tannins, alkaloids and triterpenoids. The result of observations can be seen in Table 3.

Table 3. Screening Phytocemical Kersen Leaf Extracts (*Muntingia calabura* L.)

Secondary Metabolite	Result	(+/-)
Flavonoid	Brick Red Color Reaction	+
Tannin	Green-Blackish Color Reaction	+
Saponin	Stable Foam	+
Alkaloid	Orange Color Reaction (Dragendrof)	+
	Yellow-Sediment Reaction (Mayer)	+
Triterpenoid	Brownish Color Reaction	+

The results of phytochemical screening have found secondary metabolite in kersen leaf extracts (*Muntingia calabura* L.) namely flavonoids, tannins, saponins, alkaloids and triterpenoids (Table 3). This is consistent with the statement made by Ragasa *et al.,* (2015) and Buhian *et al.,* (2016), that kersen leaf contain secondary metabolites of triterpenoids, phenols, alkaloids, flavonoids, saponins and tannins.

FTIR (Fourier Transform Infrared Spectroscopy) Analysis

The results of the analysis of the kersen leaf extracts with FTIR (Fig. 1) show wave number value data of 3412 cm^{-1} which is -OH group, 2927 cm^{-1} of a C-H function group. The absorption value is 1704 cm^{-1} which is the C=O function group, the absorption value of 1620 cm^{-1} is a functional group of C=C, the absorption value of 1451 cm^{-1} is a stretched C-C function group and an absorption number of 1042 cm^{-1} is a functional group of C-OH cyclic (Silverstein *et al.,* 2005; Konoz *et al.,* 2012; Maharani *et al.,* 2016 and Senthilkumar *et al.,* 2017).

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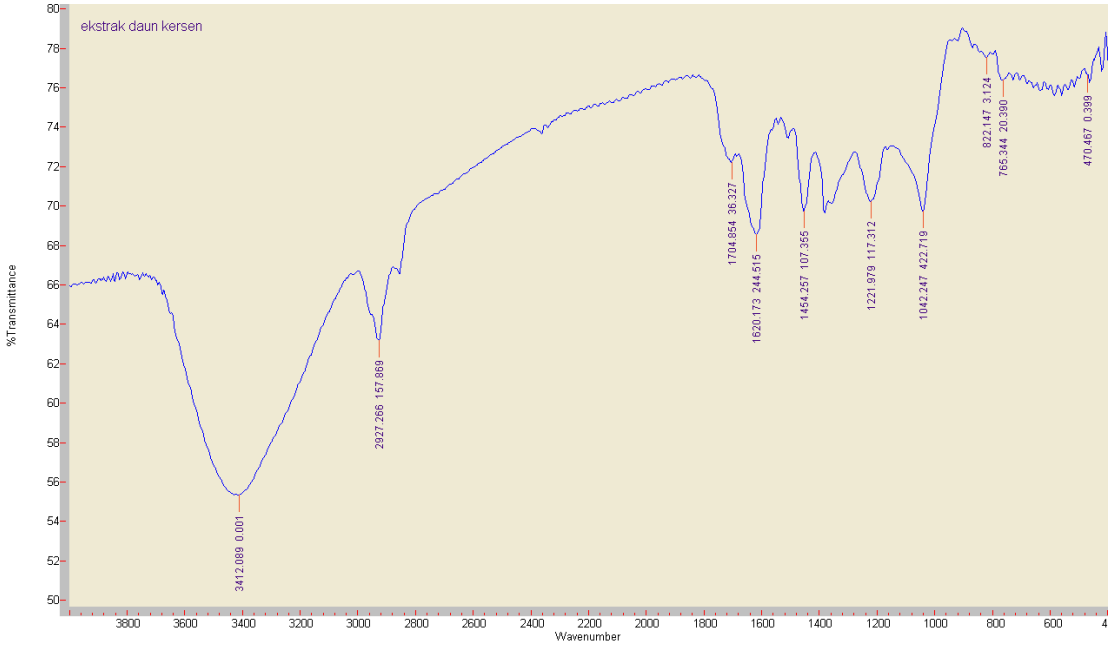

Figure 1. FTIR Test Results of Kersen (*Muntingia calabura* L.) Leaf Extract.

FTIR analysis of kersen leaf extracts (*Muntingia calabura* L.) has obtained data of wave value of 3412 cm^{-1} (Fig.1) with high and widened absorption which is part of -OH group. This group is derived from the phenol compound group (Harborne, 2006). Maharani *et al.* (2016), describe that the compounds with functional groups of -OH, C-H, C=C and C-OH cyclic are phenolic compounds. Hence, it can be suspected that phenol compounds are contained more in kersen leaf extracts.

The relation phytochemical screening and FTIR analysis have found that flavonoid and tannin are positively contained in the kersen leaf extracts (*Muntingia calabura* L.) which is a derivative of phenol. Phenol derivatives contained in plants are flavonoids and tannins which have anti-microbial content (Silalahi, 2006 and Mulyani, 2006). Phenol as anti-microbial is able to inhibit gram-positive and gram-negative bacteria up to mold and fungi through a series of anti-microbial tests (Merkl *et al.,* 2010). Phenol as anti-bacterial metabolites have a mechanism in inhibiting the growth of bacteria the occurrence of demaging process cytoplasmic membrane and the denaturation of protein membranes in bacterial cells. This can cause the metabolic system to be disrupted because the metabolic activities of bacterial cells are affected by enzymes which are proteins (Zare *et al.,* 2014).

Several other studies testing phenols and their derivatives as anti-bacteria from kersen leaf are; Buhian *et al.* (2016), who are explained the results of the content of phenol and flavonoid compounds extracts of kersen leaf capable of inhibiting the growth of *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Bacillus subtilis* through disc dilution test and *Minimum Inhibitory Concentration* test. Sibi *et al.* (2012), tannin and flavonoids compounds in leaf have been found to be capable to inhibit the growth of *Micrococcus luteus, Pseudomonas aeruginosa* and *Bacillus cereus* through inhibitory test (disc dilution). The research by Sulaiman *et al.* (2017), which is reported through tests of flavonoid and tannin with discs dilution test show that it can inhibit the growth of *Streptococcus viridians* colony. Hence, based on some of the studies described above and the results of the research, that has been carried out, it is known that phenolic

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compounds of kersen leaf extracts (*Muntingia calabura* L.) have characteristic as anti-bacterial.

Conclusion and suggestion

Based on the results of the research, it can be concluded that the kersen leaf extracts contains phenol compounds which have anti-bacterial function. *Minimum Inhibitory Concentration* test results obtained 125 ppm which is a minimum concentration that can inhibit the growth of *Aeromonas hydrophila*. Kersen leaf extracts are bacteriostatic anti-bacteria which can only inhibit bacterial growth for up to 24 hours.

Based on the results of the study, the researcher suggest to analyze phenol compounds specifically using LCMS (*Liquid Chromatography Mass Spectrometry*) instruments, which has an antibacterial function.

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