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Antibacterial Activity of Dayak Onion Bulbs (*Eleutherine palmifolia* (L) Merr) Ethanol Fraction against *Pseudomonas fluorescens* and Its Secondary Metabolite Analysis

Immaria Fransira, Annisa Fitri Anggreini, Uun Yanuhar, Maftuch

Fisheries and Marine Science Faculty, University of Brawijaya, Indonesia

Email address: immariafransira@gmail.com

KEYWORDS

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Abstract *Pseudomonas fluorescens* is a gram-negative pathogenic bacteria which is one of the problems in fish farming that can causes death in fish. The alternative to overcome this bacterial attack is by using of natural material such as Dayak onion bulbs (*Eleutherine palmifolia* (L) Merr), which are known to have secondary metabolites. The purpose of this research was to determine the antibacterial activity against *P. flourescens* that contained in the Dayak onion bulbs (*E. palmifolia* (L) Merr) ethanol fraction and the content of antibacterial compounds from the best Dayak onion bulbs (*E. palmifolia* (L) Merr) ethanol fraction. The method used in this research was antibacterial test with MIC (Minimum Inhibitory Concentration) followed by disc test and FTIR (Fourier Transform Infrared Spectroscopy) to analysis secondary metabolite. Fractionation was conducted using Chloroform: Methanol (9:1 v/v). MIC test result showed that 100 ppm was the minimum concentration that can inhibit the growth of *P. fluorescens*. Disc test reported that fraction 4 was the best fraction, with the dominant compounds that contained in the bulbs was phenolic compound based on FTIR test. So, the fraction of Dayak onion bulbs (*E. palmifolia* (L) Merr) can be used as potential leads to discover new drugs.

Introduction

Pseudomonas fluorescens is a gram-negative pathogenic bacteria which became one of the problems in fish farming, where these bacteria often attack fish farming. The infection of *P. fluorescens* will cause the surface of the fish body and fins turning red, thus triggering the appearance of red skin on fish (Younes *et al*., 2015). *P. fluorescens* also attacks the internal organs such as liver and kidney of fish, which effect the disruption of the work function of the organ until caused the death on fish (Kader and Balabel, 2017). The death of fish occurred as much as 65-75% for 7 days due to infection of *P. fluorescens* on fish. It caused severe economic losses for fish farmers and reduced the efficiency on cultivation condition (El-barbary and Hal, 2017).

Several ways can be done to overcome the attack of the *Pseudomonas fluorescens*, one of them is by using natural material such as herbal plants that contain antibacterial compounds (Hardi *et al*., 2017). One of the herbs that can be used is Dayak onion (*Eleutherine palmifolia* (L) Merr). Dayak onion (*E. palmifolia* (L) Merr) has light green leaf with 35.9 cm long of the leaves. The bulbs is oval with red color like red onion, but it doesn't smell (Galingging, 2006). Dayak onion (*E. palmifolia* (L) Merr) found a lot in Borneo and its application are already widely used by locals as a medicine (Arnida dan Sutomo, 2008; Febrinda *et al*., 2014). Dayak onion bulbs contained compounds such as flavonoid, saponin, tannin and other phenolic compounds that act as an antibacterial agent (Maftuch *et al.,* 2018; Harlita *et al*., 2018).

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The secondary metabolite compounds in onion Dayak bulbs (*E. palmifolia* (L) Merr) such as phenols and flavonoids, causes these onions can act as antibacterial agent. Secondary metabolites as antibacterial can affect microbial cells in several different ways (Nalini *et al*., 2018; Perez *et al*., 2016; Wu *et al*., 2016). However, no studies have reported the antibacterial activity of Dayak onion bulbs (*E. palmifolia* (L) Merr) ethanol fraction which can inhibit *P. flourescens*. Based on this, further analysis is needed regarding the antibacterial activity of Dayak onion bulbs (*E. palmifolia* (L) Merr) ethanol fraction and the presence of its secondary metabolites. Therefore, the purpose of this research was to determine the activity of antibacterial compounds against *P. flourescens* that contained in the Dayak onion bulbs (*E. palmifolia* (L) Merr) ethanol fraction, as well as the content of antibacterial compounds from the best Dayak onion bulbs (*E. palmifolia* (L) Merr) ethanol fraction.

Materials and methods

Preparation and Extraction

Dayak onion bulbs (*E. palmifolia* (L) Merr) was obtained from UPT Materia Medica Batu. Bulbs were washed and be dried, then blended and sifted. This examination was conducted in December 2018 at the Laboratory of Fish Health and Diseases (Brawijaya University, Malang). The extraction process with maceration was using the method according to Maftuch *et al*. (2018) with some modification, 100 gram of bulbs powder soaked with 600 ml ethanol (PA) solvent (1 : 6) for 3 x 24. Extracts are filtered using Whatman filter paper no. 42 and evaporated with a vacuum rotary evaporator (IKA RV10 Digital V) with 80 rpm at 50°C.

Phytochemical Screening of Extract

Phytochemical screening of onion Dayak bulbs (*E. palmifolia* (L) Merr) extract was carried out by qualitative test according to the standard method (Sembiring *et al*., 2013). This examination was conducted in January 2019 at the Laboratory of Phytochemistry (UPT Materia Medica Batu). Screening was done by testing the presence of flavonoid, alkaloid, tannin, terpenoid, phenol and saponin.

Fractionation

Fractionation of the Dayak onion bulbs (*E. palmifolia* (L) Merr) ethanol extract was analyzed in the stage of Thin Layer Chromatography (TLC) and Column Chromatography (CC) and carried out by method according to Putri *et al*. (2018). These examinations were conducted in January 2019 at the Laboratory of Pharmaceutical (Brawijaya University, Malang). The TLC analysis was used silica gel $60F_{254}$ paper and the comparison eluent was chloroform:methanol (7:3, 8:2, 9:1 v/v) and n-hexane:ethyl acetate (6:4, 7:3 v/v). The silica gel $60F_{254}$ paper which contained the stain then was observed under UV light (CAMAG UV Cabinet 4) at 254 nm and 366 nm. The appearance of the stain was marked with a pencil. The value of the Retardation factor (Rf) was calculated by comparing between distance of dissolved compounds and distance of eluent. The fraction compounds next was obtained based on color gradients.

Bacterial Preparation

Isolates of *Pseudomonas fluorescens* bacteria was obtained from the Brackishwater Aquaculture Center (BBPBAP) Jepara. This examination was conducted in January 2019 at the Laboratory of Fish Health and Diseases (University of Brawijaya, Malang). Bacteria which has been rejuvenated were re-cultured on TSB (*Trypticase Soy Broth*), then incubated (BINDER RI 115 Incubator) at 37°C for 24 hours. The bacterial density was 10^6 CFU.ml^{-1}.

Antibacterial Test of Fraction.

Antibacterial test in Dayak onion bulbs (*E. palmifolia* (L) Merr) fraction was analysed in the stages of MIC (Minimum Inhibitory

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Concentration) and disc test. This examination was conducted in February 2019 at the Laboratory of Fish Health and Diseases (Brawijaya University, Malang). On the MIC tes was done with method according to Fariestha *et al*. (2018), with the concentration of fraction that used was 1000 ppm, 500 ppm, 100 ppm, 10 ppm, 1 ppm, 0 ppm (negative control) and the concentration of chloramphenicol 30 ppm (positive control). The absorbance of each concentration was measured by a spectrophotometer (Thermo Scientific™ GENESYS™ 20).

The next stage was the disc test, to determine the fraction that has the best bacterial inhibition. This disc test was carried out using method according to Smahane *et al*. (2016). The paper discs were immersed in fraction with predetermined concentration based on MIC results and placed in the middle of the petri dish containing PSA (Pseudomonas Selective Agar) media. Furthermore, the clear zone that formed is measured by digital callipers (KRISBOW KW06-351).

FTIR (Fourier Transform Infrared Spectroscopy) Test of Fraction

The FTIR test of fraction was conducted in March 2019 at the Laboratory of Organic Chemistry (State Islamic University of Malang). Dry powder of onion Dayak bulbs (*E. palmifolia* (L) Merr) 2 mg was encapsulated in 100 mg KBr pellet. The sample was put into an FTIR spectrophotometer (Varian 1000 FT-IR Scimitar Series), with a scan range from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1} (Ashokkumar and Ramaswamy, 2014). The wavelength that appeared can be seen in the annotated spectrum. Infrared absorption spectrum then interpreted to determining the chemical bonds of molecule.

Results and discussions

Phytochemical Screening of Extracts

The results of phytochemical screening from Dayak onion bulbs (*E. palmifolia* (L) Merr) extract with ethanol (PA) solvents show positive results for flavonoid, tannin, triterpenoid, phenol along saponin and show negative results for alkaloid and steroid (Table 1). These results are in accordance with several previous studies that state the onion extract of Dayak onion bulbs contains flavonoid, tannin, triterpenoid, phenol and saponin (Maftuch, 2017; Harlita *et al*., 2018).

Table 1. Phytochemical Screening of Dayak Onion Bulbs (*E. palmifolia* (L) Merr) Extract

Identification of Compound	Parameter	Results
Flavonoid	Red Brick, Pink, Dark Red	+
Alkaloid (Meyer)	White Deposition	-
Alkaloid (Dragendrof)	Orange Deposition	-
Alkaloid (Bouchardat)	Brown Deposition	-
Tannin	Blackish Green, Blackish Blue, Blackish Brown	+
Steroid	Bluish Green	-
Triterpenoid	Orange	+
Phenol	Blackish Green, Blackish Blue	+
Saponin	Permanent Foam	+

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Fractionation

The TLC analysis is done to get the best eluent which will they be used in the CC analysis. The best eluent obtained from TLC can be seen by looking at the highest number of fluorescent stains point and also the highest total Rf value (Padhi and Panda, 2015). The results show the best eluent of Dayak onion bulbs (*E. palmifolia* (L) Merr) extract was a mixture of chloroform solvent:methanol solvent (9:1 v/v). This can be seen from the point of the stain which is 9 points that indicated by arrows (Figure 1). Besides that, it also shows the highest total value of Rf with value 4.78 in this eluent (Table 2). The comparison of chloroform eluent:methanol solvent (9:1 v/v) is then determined as the best eluent for CC because it produces more spots which are expected to break down the compounds of Dayak onion bulbs extract into fractions to be taken through CC analysis.

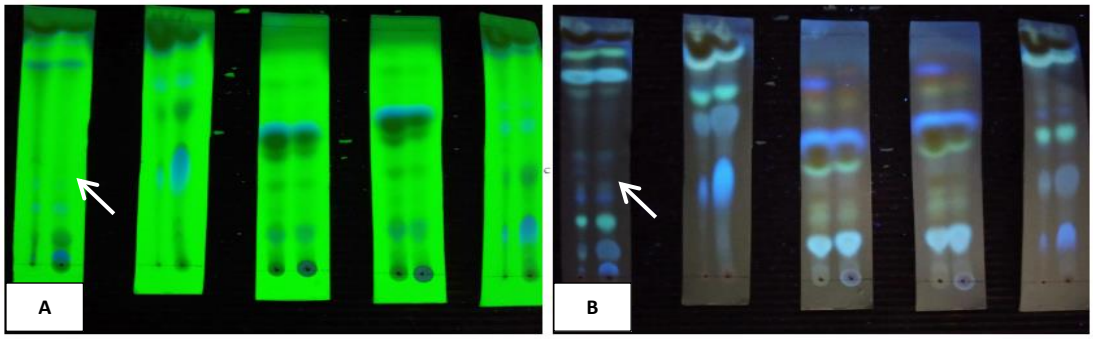

Figure 1. Thin Layer Chromatography (TLC) Analysis for Eluent Determination of Dayak Onion Bulbs (*E. palmifolia* (L) Merr) Ethanol Extract Irradiated by UV Rays (A) 254 nm, (B) 366 nm.

Table 2. Rf Value of Different Components on Dayak Onion Bulbs (*E. palmifolia* (L) Merr) Ethanol Extract

Mobile Phase	Number of Spots (by UV)	Rf Values	Total Nilai Rf
Chloroform: Methanol (7:3)	7	0.98; 0.92; 0.89; 0.75; 0.63; 0.4; 0.14	4.71
Chloroform: Methanol (8:2)	6	0.92; 0.76; 0.68; 0.6; 0.4; 0.2	3.56
Chloroform: Methanol (9:1)	9	0.98; 0.92; 0.82; 0.75; 0.49; 0.36; 0.32; 0.12; 0.02	4.78
N-Hexane: Ethyl Acetate (6:4)	8	0.85; 0.78; 0.65; 0.6; 0.55; 0.42; 0.3; 0.17	4.32
N-Hexane: Ethyl Acetate (7:3)	8	0.85; 0.766; 0.72; 0.55; 0.49; 0.34; 0.27; 0.15	4.13

Fractionation with CC stage using eluent with comparison of Chloroform:Methanol (9:1) and the stationary phase using silica gel powder G_{60} Merck. Analysis through column chromatography results in the fractions that released through the filtration column tubes. In this way, the separation of compounds from the mixture is reached and the compound is collected as a fraction (Febriyani *et al*., 2018). Through the results of fractionation of Dayak onion bulbs (*E. palmifolia* (L) Merr), obtained 6 fractions with varied colors that can be seen visually (Figure 2). Fraction 1 produces golden yellow, fraction 2 produces light golden brown, fraction 3 produces concentrated brown, fraction 4 produces brown brick, fraction 5 produces light brown and fraction 6 produces clear yellow.

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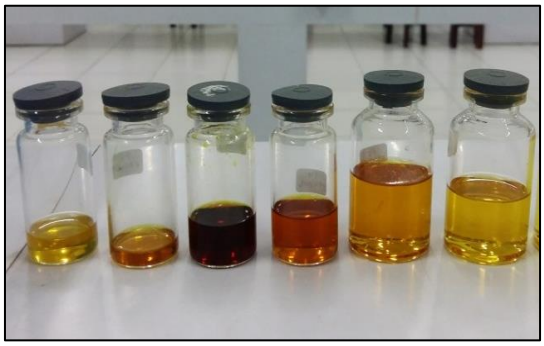

Figure 2. Results of Chromatographic Column of Dayak Onion Bulbs (*E. palmifolia* (L) Merr) Fraction

Antibacterial Test of Fraction

The results of MIC from each Dayak onion bulbs (*E. palmifolia* (L) Merr) fraction show different absorbance results on observations of spectrophotometers with wavelength of 600 nm (Table 3). Based on the results of the MIC, it can be seen that the concentration of 100 ppm in all fractions produces a quite clear solution, where the absorbance value is lower than 500 ppm and 1000 ppm. In addition, the absorbance value of 100 ppm was closest to the positive control absorbance value and much lower than the negative control. This results show that the concentration of 100 ppm fraction can inhibit *Pseudomonas fluorescens*. The contents of Dayak onion bulbs (*E. palmifolia* (L) Merr) extract such as phenolic compounds have antibacterial activity. So through result of MIC it can be seen that the lowest concentration can inhibit bacterial growth (Wicaksono *et al*., 2018).

Table 3. Results MIC Test of Dayak Onion Bulbs (*E. palmifolia* (L) Merr) Fraction

Concentration (ppm)	Absorbance of Fraction						Color
	F1	F2	F3	F4	F5	F6	
1000	0.451	0.454	0.402	0.703	0.485	0.471	Quite Clear
500	0.459	0.458	0.511	0.755	0.688	0.482	Quite Clear
100	0.443	0.420	0.401	0.695	0.476	0.447	Quite Clear
10	0.819	0.851	0.729	0.937	0.707	0.686	Turbid
1	1.094	0.974	1.042	1.105	1.006	0.974	Turbid
Negative Control (K-)	0.989	1.048	1.023	1.208	1.040	1.023	Turbid
Positive Control (K+)	0.052	0.073	0.052	0.059	0.045	0.043	Clear

Description: Positive Control (K+) using chloramphenicol 30 ppm and Negative Control (K-) Without treatment (0 ppm, only *Pseudomonas fluorescens*).

Antibacterial activity was then analyzed by disc test to determine the inhibitory of these fractions and determine the best fraction. Discs test are seen based on the diameter of the inhibitory zone (clear zone) formed. The categorial of bacterial growth was conducted diameter by inhibitory zone formed. The disc test results from each Dayak onion bulbs (*E. palmifolia* (L) Merr) show the presence of inhibitory zones formed around disc paper (Figure 3). The inhibitory zone formed from fractions 1 to 6, respectively 9.13 mm, 9.62 mm, 9.52 mm, 9.94 mm, 9.55 mm and 8.84 mm (Table 4). Based on the zone, fraction 4 was obtained as the best fraction to inhibit the growth of *P. fluorescens* bacteria. Secondary metabolites of Dayak onion bulbs are known to have antibacterial properties. The phenolic compounds and its derivatives that contained in onion Dayak bulbs were able to act as antibacterial, it can be seen from the disc test results that showed

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inhibition zone. The presence of phenol compounds can increase antibacterial activity, where these compounds will associate with other compounds to form conditions of complex compounds that will give maximum effect (Puspadewi *et al*., 2013; Suhartini, 2017).

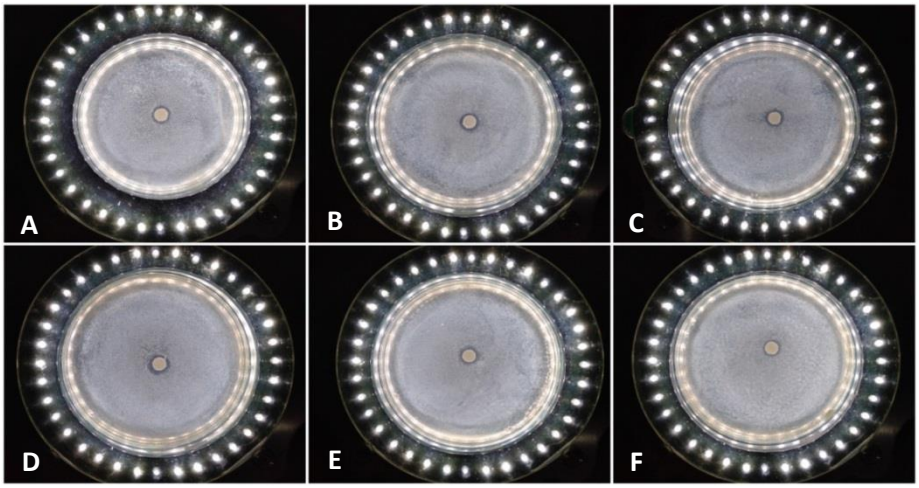

Figure 3. Disc Test Results of Dayak Onion Bulbs (*E. palmifolia* (L) Merr) Fraction, where (A) Fraction 1, (B) Fraction 2, (C) Fraction 3, (D) Fraction 4, (E) Fraction 5 dan (F) Fraction 6.

Table 4. Inhibitory Zone Diameter of Dayak Onion Bulbs (*E. palmifolia* (L) Merr) Fractions

Dayak Onion Bulbs (*E. palmifolia* (L) Merr) Fraction	Clear Zone Diameter (mm)	Categorial of Bacterial Inhibitory
Fraction 1	9.13	Medium
Fraction 2	9.62	Medium
Fraction 3	9.52	Medium
Fraction 4	9.94	Medium
Fraction 5	9.55	Medium
Fraction 6	8.84	Medium

FTIR (Fourier Transform Infrared Spectroscopy) (FTIR) Test of Fraction 4

FTIR test was carried out to determine the content of the compounds found in the best fraction of the Dayak onion bulbs (*E. palmifolia* (L) Merr) ethanol fraction. In the previous disc test, fraction 4 was found to be the best fraction, so this fraction was examined in the FTIR test. The results of FTIR analysis of Dayak onion bulbs (*E. palmifolia* (L) Merr) fraction 4 shows the presence of functional groups like phenol, aromatic, alkane, alkyne, alcohol, acid and stretch esters (Figure 4).

The peak at 3437 cm^{-1} indicates -OH group, the peak at 3430 cm^{-1} indicates -OH group, the peak at 2924 cm^{-1} indicates C-H aromatic group, the peak at 2852 cm^{-1} indicates C-H group, the peak at 1728 cm^{-1} indicates C=O group, the peak at 1656 cm^{-1} indicates C=C aromatic group and the peak at 1458 cm^{-1} indicates C=C aromatic group (Trifunschi *et al*., 2015; Divya *et al*., 2017; Senthilkumar *et al*., 2017). Based on these results it can be concluded that the fraction 4 Dayak of Dayak onion bulbs contains phenolic compound and its derivatives. This is indicated by the absorption that height and width is 3437 cm^{-1} which part of –OH group that belongs to the phenol group. In addition,

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the presence of C-H aromatic, C=O and C=C, indicates the presence of phenol compounds in Dayak onion bulbs (Otunola *et al*., 2017).

Figure 4. FTIR Results of Dayak Onion Bulbs (*E. palmifolia* (L) Merr) Fraction 4.

Phenolic compounds that present in fraction 4 from FTIR results coupled with the results of phytochemical screening Dayak onion bulbs (*E. palmifolia* (L) Merr) extract which showed positive results on phenol and its derivatives (such as flavonoid and tannin) before, proved that Dayak onion contained phenolic compounds. Phenolic compound which can be found on Dayak onion bulbs can inhibit bacterial growth by breaking down cell membranes and the content of peptidoglycan in bacteria (Suhartini, 2017). The antibacterial activity of phenolic compounds causes cytoplasmic leakage. The hydroxyl groups in phenol compounds interfere with the active site of bacterial enzymes (Janakat *et al*., 2015). Antibacterial compounds can interfere with lipid-protein interactions and increase membrane permeability, which causes changes in the membrane structure and accelerates intracellular leakage. This lead the inhibitory bacterial growth (Wu *et al*., 2016).

Conclusions and suggestions

Dayak Onion Bulbs (*Eleutherine palmifolia* (L) Merr) ethanol fraction showed the antibacterial activity, where 100 ppm was the minimum concentration to inhibit bacterial growth. The disc test in each fraction showed that fraction 4 was the best fraction with an inhibitory zone that formed at 9.94 mm. Based on FTIR results, fraction 4 showed the presence of phenolic compounds that are antibacterial. The fraction of the studied plants can be used as potential leads to discover new drugs to control another bacterial infection.

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