Antimicrobial Activity of Endophytic Fungi from Eleocharis Dulcis (Burm. f.) Henschell

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Antimicrobial Activity of Endophytic Fungi from Eleocharis Dulcis (Burm. f.) Henschell

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Keywords: *Eleocharis Dulcis,* Antibacterial Activity, Penicillium Citrinum, Alkaloid, Terpenoid, Phenol.

Abstract: Extracts of endophytic fungi isolated from *Eleocharis dulcis* were tested for antibacterial ability. Two isolates showed the strongest activity toward *Staphylococcus aureus, Escherichia coli* and *Salmonella typhi* than another isolates. The results of diffussion methods for antibacterial test of extract of DP1J1 isolate against *Staphylococcus aureus, Escherichia coli* and *Salmonella typhi* were 86.31%, 77.4%, and 83.15% respectively. MIC extract of DP1J1 isolate against *Staphylococcus aureus, Escherichia coli* and *Salmonella typhi* were 0,03%, 0,02% and 005%, respectively. Based on bioautography test, extract of DP1J1 isolate was contained alkaloid, phenol and terpenoid. DP1J1 identified as *Penicillium citrinum*.

1 INTRODUCTION

The search for antibacterial secondary metabolites from natural materials has been a lot of done. Relevant to the higher level of resistance of pathogenic bacteria to commercial antibacterial compounds. Bioactive plant compounds are potential source of medicinal ingredients. The use of plant as raw material for the production of antibacterial compounds generated new problems. Utilization of large amounts of plant biomass caused reduced plant biological resource (Alluruppa, et al, 2018).

Water chesnut (*Eleocharis dulcis* (Burm.f.) Henschell) are often found in South Sumatra swamps, especially in Ogan Ilir district. This plant adapted to acid swamp land conditions. The content of secondary metabolites is very interesting to know. The juice of the water chesnut tuber contains antibiotic compounds which are effective in inhibiting *Staphylococcus aureus*, *Escherichia coli*, and *Aerobacter aerogenes* (Asikin dan Thamrin, 2012). Ethanol, ethyl acetate and n-hexane extracts of water chestnut leaves have been known to contain triterpenoid compounds, tannins and flavonoids. These compounds are antibacterial activity (Baehaki *et al.,* 2018).

Endophytic fungi can be found in plants. Endophytic fungi was penetrate and live in the plant tissues which innoxious to the host. The fungi are able to produce the same bioactive compounds as plants due to genetic recombination between plants and fungi followed by coevolution. The ability of microbes to produce the same bioactive compounds as plants can be used as a substitute for taking up very large plant biomass. Endophytic fungi can produce functional compounds in the form of anticancer, antiviral, antibacterial, antifungal and growth hormone for plants (Tan and Zou, 2001; Noverita et al., 2009)

Plants which adapted to live in unique environments such as in swampy areas potentially produce bioactive compounds that are used to survive. Based on this, endophytic fungi from water chestnut have not been studied yet, so it is necessary to explore endophytic fungi from water chestnut that potentially produce antibacterial compounds against *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhi*.

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2 MATERIAL AND METHODS

2.1 Sampling

Water chestnut was obtained from the swamps along the road between Palembang and Indralaya by random sampling method. Sampling was carried out in two locations and the coordinate were S 03 ° 08'04.1 " E 104 ° 41 '57.3 "and S 03 ° 08 '39.2" E 104 ° 41 '36.5 ". The samples used in this study were old roots, stems and leaves with good quality as a source of fungi isolates

2.2 Isolation of Endophytic Fungi

The roots, stems and leaves are washed in running water to remove dirt. The part of plants were surface sterilized gradually by means of samples soaked in 70% alcohol and 0.1% NaOCl alternately then rinsed with sterile distilled water and dried on sterile tissue. The part of plants were split and placed in a PDA medium with the position of the surface of the sample attached to the agar and then incubated at room temperature (27°C). Pure fungal isolates were made into work culture and stock culture on slant tube medium.

2.3 Cultivation of Endophytic Fungi

Suspended endophytic fungal propagules of ± 10^6 propagules / mL were inoculated in PDB medium placed in a 1 liter cultivation bottle with two repetitions. Culture was incubated at room temperature until the medium undergoes a color change which indicates that secondary metabolite compounds have formed. The medium without the addition of endophytic fungi is used as a control. After the secondary metabolites are formed, the fungi biomass is separated from the medium. The liquid-liquid fractionation medium (partition) with ethyl acetate solvent and concentrated with a rotary evaporator.

2.4 Antibacterial Assay (Kirby Bauer Methods)

The test bacteria that have been equivalent to Mc Farland 0.5 were spread into the MHA medium. Disc paper that containing 2% antibacterial extract and 50 µl of them is placed on the surface of the MHA media. Tetracyclines with a 0.1% concentration of 50 µl were used as positive controls. The culture was incubated at 37 ° C for 1x24 hours (Rosyidah et al., 2010). Determination of the antibacterial activity of secondary metabolite extracts was carried out by measuring the diameter of inhibitory zones formed in secondary metabolite extracts of endophytic fungi compared diameter of inhibitory zones on standard antibiotics, tetracycline (Noverita et al., 2009; Balouiri et al, 2016). The activity of secondary metabolite extracts tested against standard antibiotics is determined by the following equation.

Percentage of ntibacterial activity = $\frac{A}{B}$ x 100%

A= inhibition zone (mm) of extract

B= inhibition zone (mm) tetracycline (Chan, 2007).

2.5 Minimum Inhibition Concentration (MIC)

Extracts of secondary metabolites of endophytic fungi were made in different concentrations of 2%, 1%, 0.5%, 0.25%, 0.125%, 0.06% and control. The extract was dripped on disc paper and placed on a bacterial inoculated MHA medium and then incubated at 37 ° C for 24 hours. The diameter of inhibition zone formed is observed and measured. If the lowest concentration was still capable to inhibit bacterial growth so the extract concentration is reduced and tested again to the lowest concentration that was unable to inhibit bacterial growth (Ruangpan, 2004; Fatisa, 2013).

2.6 Bioautografi Assay

The secondary metabolite extract of endophytic fungi which showed the highest antibacterial ability was dissolved with ethyl acetate solvent. Extracts of secondary metabolites were dripped on TLC plates and developed with n-hexane: ethyl acetate (8: 2). TLC plates were sprayed with 10% H2SO4. The color formed indicates the group of secondary metabolites.

TLC plates that have been developed are placed on top of a dense bacterial culture. The spots/stains on the chromatogram are attached to the medium so that the compound can diffuse into the agar medium. The culture was incubated for 24 hours and then a clear zone was observed which was due to inhibition of the active compound (Salni *et al*., 2011; Balouiri et al, 2016).

2.7 Characterization and Identification of Endophytic Fungi

Endophytic fungi of water chestnut with highest antibacterial ability are characterized based on

macroscopic and microscopic morphology. Macroscopic morphological characters observed included colony growth, colony diameter, color of colony and media and reverse colony. The fungi is grown in different media (PDA, MEA and Czapex Dox Agar). Preparation of fungi was observed under a microscope. Morphological characters of microscopic fungi were hyphae, hyphae colors, conidial shapes, conidia colors, and conidial sizes. Both macroscopic and microscopic characters were compared with the books (Barnett & Hunter, 2006; Houbraken et al., 2010).

3 RESULTS AND DISCUSSION

3.1 Isolation of Endophytic Fungi

A total of 9 endophytic fungal isolates were obtained from the parts of water chestnut (*Eleocharis dulcis* (Burm.f) Trinius ex. Henschell). The isolates had 2 isolates from the root, 3 isolates from the stem, and 4 isolates from the leaf. The highest number of endophytic fungi isolates was found in the leaf tissue of plants. Endophytic fungi that were isolated from each of the rat purun tissue have different numbers and types according to the adaptation of endophytic fungi to their host plants. According to Noverita et al., 2009) more than one type of endophytic fungi can grow from one plant tissue due to the physiological factors of the host plant, so that various endophytic fungi are produced.

Table 1: Isolation and Purification of Endophytic Fungi from Water Chestnut (*Eleocharis dulcis* (Burm.f) Trinius ex. Henschell).

Sample	Code	Total
Root	AP_1J_1, AP_1J_2	2
Stem	BP_1J_2, BP_1J_3, BP_1J_5	3
Leaf	DP_1J_1, DP_1J_2, DP_1J_3, DP_1J_4	4
Total		9

3.2 Antibacterial Activity

Secondary metabolites extract from endophytic fungi were tested for their antibacterial activity towards *S. aureus, E. coli* dan *S. typhi*. Extracts of secondary metabolites of endophytic fungi were tested at 2%. The result of this study was inhibitory zones formed around the paper disk. The diameter of inhibition zone formed in testing antibacterial activity can be seen in Figure 1.

Based on table 2, it is known that all extracts of endophytic fungal isolates have different antibacterial activity. This is evidenced by the formation of inhibition zones around the paper disk. Based on the criteria for antibacterial activity (Chan et al., 2007) showed that the result of the percentage of antibacterial activity of the secondary metabolite extract of DP1J1 fungi isolates towards three test bacteria was strongest than the other fungal isolates. Based on Table 2, the result showed that the antibacterial activity of DP1J1 isolates against *Escherichia coli, Staphylococcus aureus,* and *Salmonella typhi* were 77.4%, 86.31%, and 83.15% , respectively.

A large percentage of antibacterial activity of the secondary metabolite extract was obtained from the area of inhibition zone formed by each isolate. The diameter of the inhibited zone measured was the amount of antibacterial compounds that inhibit bacteria (Balouiri et al., 2016). The higher antibacterial activity of secondary metabolite extract of endophytic fungi, the greater of inhibition zone.

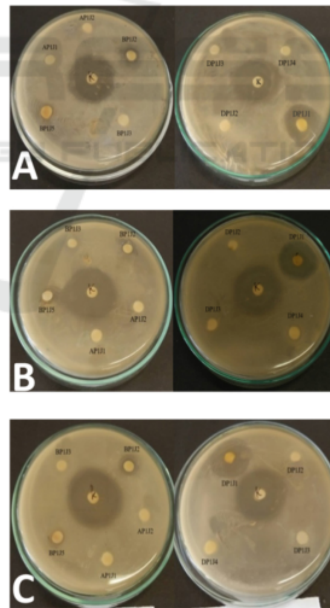

Figure 1: Antibacterial Activity Test for Secondary Metabolite Extracts (a) *Escherichia coli* (b) *Staphylococcus aureus* and (c) *Salmonella typhi*.

Table 2: Antibacterial Activity of Metabolite Secondary Extract of Endophytic Fungi.

Isolate	Origin of sample	Concentration (%)	Antibacterial activity (%)			Criteria
			E. coli	*S. aureus*	*S. typhi*	
AP_1J_1	Root	2	6,63	4,06	6,31	weak
AP_1J_2		2	8,84	5,07	12,63	weak
BP_1J_2	Stem	2	17,69	22,33	23,15	weak
BP_1J_3		2	11,06	13,20	13,68	weak
BP_1J_5		2	6,63	19,29	8,42	weak
DP_1J_1	**Leaf**	**2**	**77,40**	**86,31**	**83,15**	**strong**
DP_1J_2		2	13,27	10,15	18,94	weak
DP_1J_3		2	15,48	11,16	10,52	weak
DP_1J_4		2	11,06	18,27	13,68	weak
Tetracycline		**0,1**				

Criteria of anticaterial activity : <50% (weak), 50-74% (medium), ≥75% (strong) (Chan *et al*., 2007).

3.3 Minimum Inhibitory Concentration (MIC)

The aimed of determination of Minimum Inhibitory Concentration (MIC) was to know the lowest secondary metabolite extract concentration value capable to inhibit the growth of test bacteria (Andrews, 2001). The extract concentration were 2%, 1%, 0.5%, 0.25%, 0.125%, and 0.06%. The MIC value of secondary metabolite extract of DP1J1 isolates was determined because of strongest antibacterial activity against the three test bacteria.

Table 3: Minimum Inhibitory Concentration (MIC) of secondary metabolite extract towards *E. coli, S aureus* and *S. typhi*.

Isolate	(%)	Diameter of inhibition zone (mm)		
		E. coli	*S. aureus*	*S. typhi*
DP_1J_1	2	10	15	18
	1	8,5	10	15
	0,5	6,5	10	10
	0,25	5	9	7
	0,125	5	7	6
	0,06	4	5	4
	Control	0	0	0
	0,06	3,5	2	3
	0,05	3	2	**2**
	0,04	2,5	1,5	0
	0,03	**2**	1	0
	0,02	0	**1**	0
	0,01	0	0	0
	Control	0	0	0

Based on Table 3, the results showed that inhibition of *E. coli* growth to ensue at 0.06% - 2% of secondary metabolite extract DP1J1 isolates. In 0.06% of secondary metabolite extracts were still capable to inhibit test bacteria, so the concentration was reduced from 0.06% to 0.01%. Andrews (2001) certify that antibacterial compounds at the smallest levels with clear inhibition zone without the growth of test bacteria are determined as MIC. The MIC value obtained from the secondary metabolite extract of DP1J1 isolates against *E. coli* was 0.03%. The secondary metabolite extract of DP1J1 isolates was still able to inhibit the *S. aureus* at a concentration of 0.06%, so the concentration was reduced from 0.06% to 0.01%. The MIC value of DP1J1 secondary metabolite extract after concentration reduction was obtained by 0.02%. The secondary metabolite extract of DP1J1 isolates at a concentration decrease of 0.06% was still able to inhibit the *S. typhi*. Therefore the concentration of DP1J1 isolates was reduced in concentration of DP1J1 isolates from 0.06% to 0.01%. The results of the MIC value of the secondary metabolite extract of DP1J1 isolates were 0.05%.

The secondary metabolite extract of DP1J1 isolates had different MIC values for different types of bacteria. The MIC value of metabolite extracts secondary to the *Staphylococcus aureus* was lowest than other bacteria because of differences in sensitivity of gram-positive bacteria to gram-negative bacteria against antibacterial. According to Ullah & Ali (2017) differences in sensitivity of gram-positive and gram-negative bacteria due to differences in the structure of the cell wall where gram-negative bacteria have a relatively more complex cell wall structure, while gram-positive bacteria that have a simpler cell wall structure so that the test compounds which is active as an antibacterial more easily enter the cell..

S. aureus is a Gram-positive bacterium with thick peptidoglycan on cell wall. Based on the results of the study, the MIC value of *S. aureus* was lowest, so it can be assumed that the secondary

metabolite content of DP1J1 fungi isolates was easier to inhibit Gram-positive bacteria. The greatest MIC value of secondary metabolite extract was *S. typhi* compared to the other bacteria. It has been known that *S. typhi* was pathogenic, so on the cells contain a certain protein that interacts with antibiotics (Lee et al., 2019)

3.4 Bioautography Test of Secondary Metabolite Extracts

The result of bioautographic test of secondary metabolite extracts using thin layer chromatography (TLC) method showed that the secondary metabolite extracts of endophytic fungi isolate DP1J1 contained antibacterial compounds. Wardhani and Sulistyani (2012) have determined the class of active compounds which are potentially antibacterial using thin layer chromatography (TLC). Based on the research results obtained by the price of Rf (retondation factor) antibacterial active compounds in extracts of secondary metabolites of endophytic fungi are presented in Table 4.

Determination of the class of active compound of secondary metabolite extract was carried out on a chromatogram sprayed with 10% H_2SO_4. If the cromatogram appeared purple stains which were thought to be terpenoid group antibacterial compounds. The active compound on the chromatogram is attached to the agar medium, after incubating it shows the results of the formation of a clear zone around the chromatogram stain as in Figure 2.

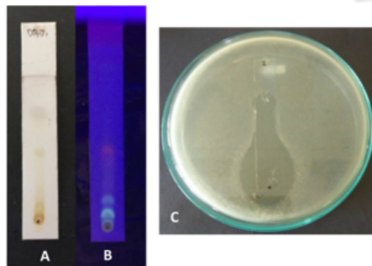

Figure 2: Bioautography test of secondary metabolite extract. (a) Under visible light, (b) Under UV light, (c) Bioautography test on agar medium

Table 4: Bioautographic Test Results and Rf Values of Endophytic Fungal Secondary Metabolite Extracts.

Isolate	Total compounds	*Rf*	color	class of compounds
DP_1J_1	3	0,14	brown	Alkaloid
		0,52	yellow	Fenol
		0,62	violet	Terpenoid

Based on the results of the study, it was found that DP1J1 isolate had 3 active compounds according to the number of stains formed on the chromatogram. The clear zone formed in the bioautographic test was found in the second stain which was suspected to be phenol group with an Rf value of 0.52.

3.5 Characterization and Identification of Endophytic Fungi

DP1J1 isolate was identified based on macroscopic and microscopic morphological characters. DP1J1 isolate was grown on three fungi growth media, namely Czapek Agar, MEA, and PDA. The colony color on the PDA was different from the other media. Colony color on PDA showed white colony and slow growth with a colony diameter of ± 1 cm. On the other hand, colony color on MEA showed deep green color with a white edge, the opposite color of the brownish yellow colony and a colony diameter of ± 1.8 cm. On CDA medium, the color of the colony formed was bluish green with a white edge and a diameter of ± 1.5 cm (Figure 3).

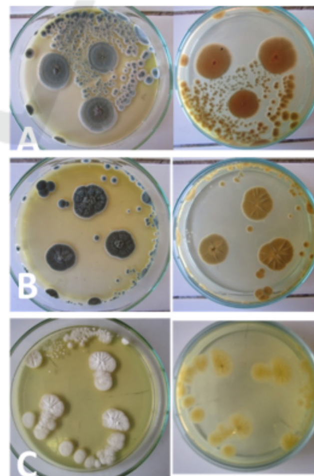

Figure 3: Macroscopic morphology of DP1J1 isolate. (a) PDA (top and reverse), (b) MEA (top and reverse), (c) Czapek (top and reverse).

Microscopic morphology characters were branched conidiophore, biverticillate, round conidia, light brown conidia, and phialid flask-shaped (Figure 4). Based on the microscopic character, suspected DP1J1 isolate as *Penicillium citrinum* (Barnett & Hunter, 2006; Houbraken et al., 2010).

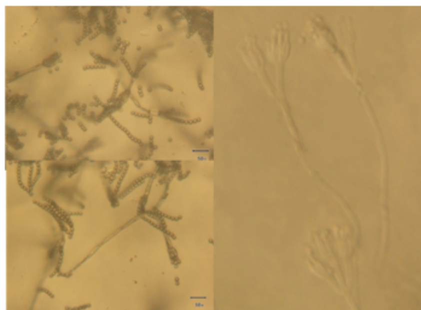

Figure 4: Microscopic morphology of DP1J1 isolate.

The content of compound in the secondary metabolite *Penicilium citrinum* were alkaloid, phenol, and terpenoid compounds. *Penicilium citrinum* have the ability to produce antibacterial compounds which consist of three active compounds. This study was compatible with the research of Zhan et al (2014) which declare that the result of chromatography test the *Eleocharis dulcis* ethyl acetate fraction has been obtained bioactive compounds in the form of flavonoids. Thus, endophytic fungi isolates DP1J1, identified as *Penicilium citrinum,* which were isolated from water chestnut (*Eleocharis dulcis*) had the potential to produce antibacterial compounds in all three test bacteria. That is important to know is the group of compounds that are active as antibacterial. Therefore further examination of each class of these compounds should be done.

4 CONCLUSION

Based on the results of the research, from 9 endophytic fungal isolates of water chestnut (*Eleocharis dulcis* (Burm.f) Trinius ex. Henschell), it was found that DP1J1 isolates showed strong antibacterial activity against *E. coli* ATCC8739, *S. aureus* ATCC6538, and *S. typhi* IPBCC B.11.669. MIC value of DP_1J_1 isolate were 30 µg/ml, 20 µg/ml and 50 µg/ml towards *E. coli* ATCC8739, *S. aureus* ATCC6538 and *S. typhi* IPBCC B.11.669, respectively. DP_1J_1 isolate has been identified as *Penicillium citrinum*.

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