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Antioxidant activity of the secondary metabolites produced by endophytic fungi isolated from Jeruju (*Acanthus ilicifolius* L.) plant

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Abstract *:*

Endophtic fungi can produce secondary metabolites that are same as the host. Twelve endophytic fungi were isolated from Jeruju plant. Three of them have the strongest antioxidant activity, namely DJS1, DJS2, and BJS4. The fungi can produce secondary metabolites that have the potential as antioxidant compounds. This study aims to isolate the antioxidant compound using a chromatography column and test the antioxidant activity using DPPH method. Isolation of secondary metabolites produced 40 eluates. The IC50 value of DJS111 and BJS42 eluates were 10.20 µg/ml and 20.89 µg/ml, which is an antioxidant compound with very strong activity. The IC50 value of DJS24 eluate was 54.45 µg/ml, which is strong antioxidant activity. DJS111 eluate has antioxidant activity that approaches to ascorbic acid as a standart antioxidant compound (7.76µg/ml). From TLC, DJS111 contains flavonoid compound and DJS24 and BJS42 contain terpenoid compound.

Keywords: Endophytic fungi, Jeruju, antioxidant, DPPH, flavonoid, terpenoid

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1. Introduction

Antioxidants are compounds that can reduce free radicals. Free radicals are produced naturally in the body as a residual metabolic process. Antioxidants will donate electrons so that free radicals become stable and prevent chain reactions in the body that can cause various diseases [1]. Efforts to find alternative sources of antioxidants continue to be done. One source of antioxidants can be produced in endophytic fungi [2].

Biosynthesis of secondary metabolites from endophytic fungi that have the potential as an in vitro antioxidant compound (DPPH method) has been extensively studied before, endophytic fungi from Jamblang leaves can produce very strong antioxidant compounds, with IC50 values of 20 µg/ml [3]. *Smilax leucocarpa* also has a very strong antioxidant activity, namely with an IC50 value of 45.22 µg/ml [4]. Besides, the leaves of *Excoecaria agallocha, Rhizophora mucronata* leaves, and *Kandelia candel* root, which have strong antioxidant activity, with IC50 values of 67.2 µg/ml, 80.1 µg/ml, and 45.4 µg/ml, respectively [2]. This proves that endophytic fungi can produce alternative antioxidant compounds that have strong activity.

The endophytic fungi were isolated from the stem, leaves, and roots of the Jeruju plant. This study aims to reveal the antioxidant activity of the endophytic fungi using the DPPH method. Besides, this study also aims to find secondary metabolite compounds that act as antioxidant compounds by using antioxidant bioautography and thin layer chromatography. The results are expected to provide information on alternative antioxidant compounds produced from endophytic fungi.

2. Materials and Methods

2.1 Isolation and culturing of the endophytic fungi

Jeruju plant collected from Sungsang, Kabupaten

Banyuasin, Sumatera Selatan. The fresh and mature leaves, stem, and root were used for the isolation of endophytic fungi. The samples were stored in the sealed plastic bags at 4°C until processed. The samples were sterilized by dipping them in 75% ethanol, followed by immersing in 3% sodium hypochlorite, then rinsed in sterile distilled water, and finally dried on sterile filter paper on a petri dish. A piece of each sample was removed with a sterile scalpel then cut into small pieces about 0.5x2 cm, each piece was put on a petri dish containing Potato Dextrose Agar (PDA) medium and incubated at room temperature (28°C) to promote fungal growth and sporulation [5], [6].

After 7 days, individual hyphal tips of actively growing fungi were picked up for subculturing by inoculating it onto new PDA individually and incubated at room temperature for one week. The purified fungal isolates were labeled for further use.

2.2 Cultivation and extraction of the secondary metabolites of the endophytic fungi

Agar contained mycelium was cut using cork borer and inoculated to 500ml Potato Dextrose Broth (PDB), the condition must be sterile. The cultures were incubated at room temperature for 30 days. Sterile PDB was used as a control. The medium (PDB) is separated from the fungal using filter paper. The fungal biomass and dried biomass were weighed [7].

The medium was mixed with ethyl acetate to extract the secondary metabolites, ethyl acetate use aims to attract polar and non-polar compounds. The mixture was shaken and, the medium and ethyl acetate formed two layers. The ethyl acetate layer that contains the secondary metabolites was extracted using a rotary evaporator at 80°C that we get the viscous extract of the endophytic fungi [8].

2.3 Detection of antioxidant potential by TLC bioautography assay

The antioxidant potential of the extracts was tested by thin-layer chromatography (TLC) bioautography method. Each extract was diluted by methanol and pasted on the plate (TLC silica gel 60 $_{F254}$), and let the methanol evaporated. The plate was sprayed by 0.2% 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (diluted by methanol) in dark room. The plate was observed, the yellow/orange color show the isolate has antioxidant compound [4], [9]. Besides, a quantity test that is to measure the absorbance and DPPH scavenging effect (%) of the extract was also carried out [10]. After that, the three most active extracts as antioxidants were obtained.

2.4 Isolation of antioxidant compounds

The secondary metabolites in extracts were isolated by column chromatography. Silica gel powder poured into the column and stirred to avoid the air cavity in the middle of the column. The silica gel powder in the column reached 14 cm and added the extract. The n-hexane: ethyl acetate and ethyl acetate: methanol solvent with the ratio of 9:1; 8:2; 7:3; 6:4; 5:5; 4:6; 7:3; 8:2; 9:1 as much as 50mL were poured into the column gradually, with the column tap opened. The solvent from the column tap was stored in a vial per 20 mL and evaporated using a hair dryer until eluate was obtained [11].

Each eluate will be tested by TLC bioautography to find eluate that has antioxidant compounds [4], [9]. The three most active eluates were pasted on the plate and eluted by eluent (a mixture of organic solvent), and let it evaporated. The plate was observed on UV light (254nm), if only one spot appeared, it shows that the eluate contains the pure compound. The plate (had eluted) also was sprayed by 0.2% DPPH, this is needed to be sure that antioxidant activity caused by the compound [12].

2.5 Evaluation of antioxidant activity by DPPH method

The antioxidant activity of the three most active eluates was determined by the DPPH method. In brief, 0.1 mM solution of DPPH in methanol was prepared. 1 ml DPPH was added to 3 ml of different eluates in DMSO at different concentration (15.63; 31.25; 62.5; 125; 250; 500; 1000 µg/ml). The mixture was shaken vigorously and placed at dark room for 30 minutes, and absorbance was measured at 517 nm by using a spectrophotometer (UV-VIS Shimadzu). Reference standard antioxidant compound that is often used is ascorbic acid [10].

The IC_{50} value of the eluates, which is the concentration of the sample required to inhibit 50% of the DPPH free radical, was calculated using the Log dose inhibition curve. Lower absorbance of the reaction mixture indicated higher free radical activity. The percent DPPH scavenging effect (% inhibition) was calculated by using the following equation:

$$\% \text{ inhibition} = \frac{A_0 - A_1}{A_0} \; x \; 100$$

Where A_0 was the absorbance of control reaction and A_1 was the absorbance in the presence of test or standard sample [13].

2.6 Thin Layer Chromatography

The three most active eluats were diluted using methanol and pasted on the plate, and let the methanol evaporated. The plate was eluted by eluent (mixture of organic solvent). The plate was observed on UV light (254nm). Then, the plate was sprayed with 10% H_2SO_4 and heated on hot plate. The color spot appeared was observed and the Rf value was determined using this formula [14]:

$$Rf = \frac{\text{Distance travelled by component}}{\text{Distance travelled by solvent}}$$

2.7 Identification of the endophytic fungi

The endophytic fungi were identified at Genetika Lab Division, PT. Genetika Science Indonesia, Jakarta Barat. Identification using molecular technique involving Polymerase Chain Reaction (PCR) and DNA sequencing was performed. The identification used ±700bp DNA fragments and ITS primer.

3. Results and Discussion

A total of twelve endophytes were found in Jeruju plant. One from the roots (AJS1), six from the leaves (DJS1-6), and five from the stem (BJS1-5). This shows that each part of vascular plants must have endophytic fungi. This was consistent with previous reports that all the plant samples were found to harbor various endophytic fungi with different colonization rates [15], [16]. All endophytic fungi from Jeruju can be seen in Figure 1.

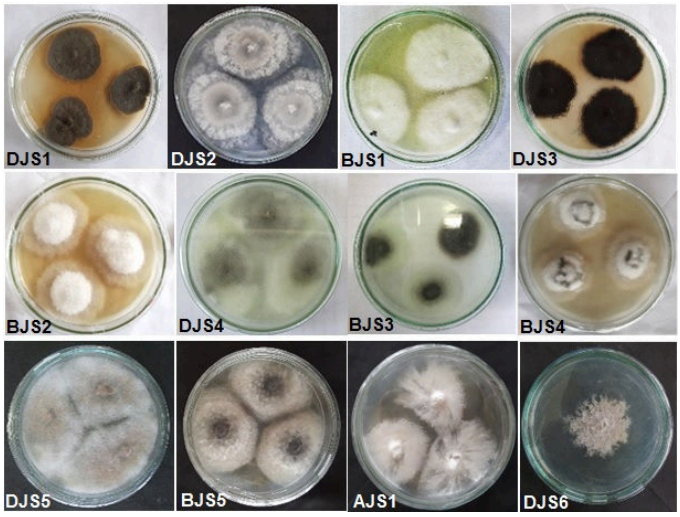

Figure 1. The endophytic fungi isolated from Jeruju

The cultivation aims for the endophytic fungi can produce the secondary metabolites. The secondary metabolites contain many various compund that has antioxidant activity [1]. After 30 days, fungal mycelium in the medium was increased, it means the fungal grew. The fungal grew because succesfully adapted on the medium. The adaptation made the fungal able to absorb the nutrient namely carbon. The adaptation can harmonize the fungal physiology on the environmental conditions of the medium [17]. The PDB color also was changed. The changes in the PDB are caused the fungi cultivated produce secondary metabolites as a result of their metabolic processes, the secondary metabolites are produced by fungi at the end of the stationary phase. The secondary metabolite synthesis will begin when there is limited nutrition in the growth medium [18]. The changes of PDB can be seen in Figure 2.

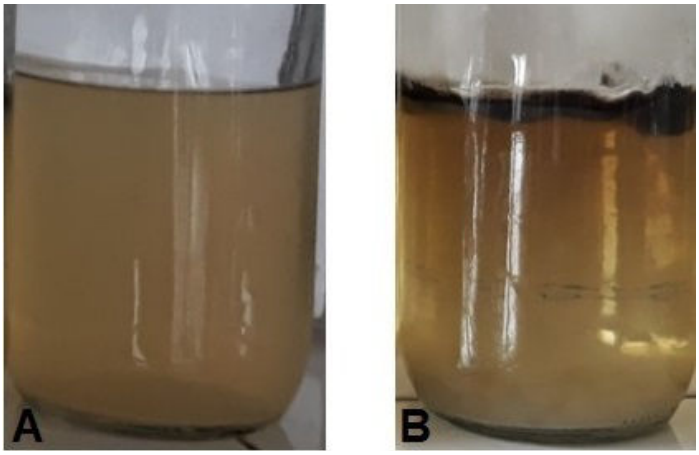

Figure 2. Control PDB (A); PDB after 30 days (B)

Detection of antioxidant potential by TLC bioautography assay (on extracts)

The twelve of the fungi extracts have yellow or orange color after sprayed with 0.2% DPPH (Figure 3). The yellow or orange color shows that the extracts have antioxidant potential. The intensity of yellow or orange color that appears is different. These differences indicate different levels of antioxidants. This was consistent with previous reports that the more yellow the extract color, the stronger the antioxidant compound in the extract [4], [9].

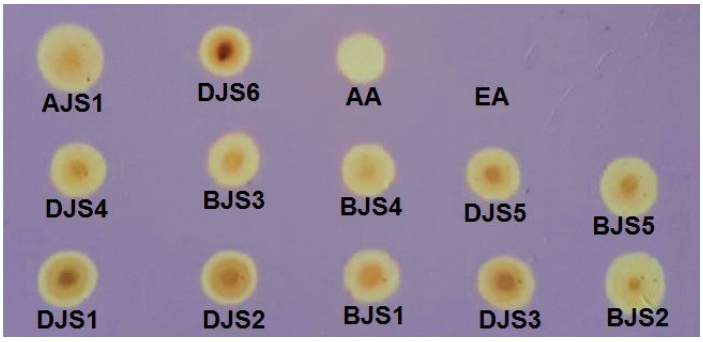

Figure 3. The result of TLC Bioautography on extracts; AA is ascorbic acid; EA is ethyl acetate

Isolation of antioxidant compounds

The result of theantioxidant test shows that DJS1, BJS4, and DJS2 have the highest absorbance values of 78.25%, 75.48%, and 74.24%. DJS1 has the closest absorbance value to ascorbic acid (84.76%).

Table 1. The result of antioxidant quantity test

SAMPEL	ABS	%
BLANKO	0.722	-
DJS1	**0.157**	**78.25%**
DJS2	**0.186**	**74.24%**
BJS1	0.322	40%
DJS3	0.212	51%
BJS2	0.293	42.90%
DJS4	0.190	73.68%
BJS3	0.493	31.72%
BJS4	**0.177**	**75.48%**
DJS5	0.195	72.94%
BJS5	0.222	41.45%
AJS1	0.210	43.11%
DJS6	0.191	73.55%
AA	0.110	84.76%

% is percentage of inhibition

There are 40 eluates isolated from the fungi. DJS1 have 14 eluates ($DJS1_{1-14}$), BJS4 have 13 eluates ($BJS4_{1-13}$), and DJS2 have 13 eluates ($DJS2_{1-13}$). Each eluate is thought to contain a compound which has antioxidant potential. The compound can separate caused the chromatography column separating compound based on polarity [19].

Detection of antioxidant activity by TLC bioautography assay (on eluates)

The 40 eluats were sprayed by 0.2% DPPH, the yellow color showed the eluat contains compound that had antioxidant activity. In Figure 4, $DJS1_{11}$, $DJS2_4$, and $BJS4_2$ had yellow color on purple plate, this is shows the eluats had antioxidant activity. The pasted eluat will be detained and attached to the plate, because the plate is stationary phase that can hold substances at a predetermined point [12]. The sprayed DPPH will be detained and attached to the plate too, and the previously eluat reacted with the DPPH. The presence of antioxidant compounds causes the dark purple color of DPPH to decrease and changes to yellow [20].

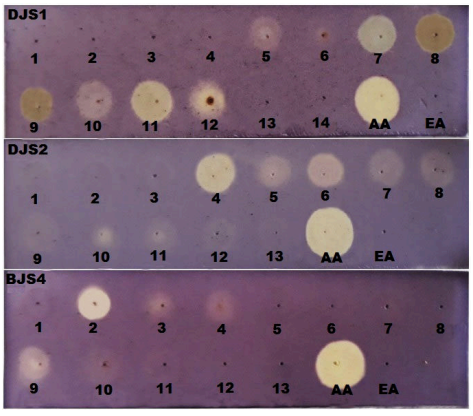

Figure 4. The result of TLC Bioautography on eluates; AA is Ascorbic acid; EA is Ethyl acetate

Evaluation of antioxidant activity by DPPH method

In Table 2, the IC_{50} value of $DJS1_{11}$, $DJS2_4$, and $BJS4_2$ are 10.70 µg/ml, 54.45 µg/ml, 20.89 µg/ml. The $DJS1_{11}$ has the lowest IC_{50} and the $DJS2_4$ has the highest IC_{50}. The lower the IC_{50} value, the greater the antioxidant activity [21].

Table 2. IC_{50} value, Rf, and compound class of $DJS1_{11}$, $DJS2_4$, and $BJS4_2$ eluats and ascobic acid

Eluat	Endophytic Fungi	IC_{50} (µg/ml)	Rf	Color	Compound
$DJS1_{11}$	DJS1	10.70	0.22	Dark yellow	Flavonoid
$DJS2_4$	DJS2	54.45	0.62	Purple	Terpenoid
$BJS4_2$	BJS4	20.89	0.35	Purple	Terpenoid
Ascorbic acid	-	7.76	-	-	-

The IC_{50} value was measured based on the DPPH absorbance. DPPH in methanol has dark purple color. When DPPH acts with antioxidant compound (H-A), the DPPH structure will change from DPPH to DPPH-H. The DPPH color decreases and becomes yellow. The antioxidant compounds can donate the electron (-H) to DPPH, and this can reduce (stabilize) the DPPH [21]. This method is simple and easy to use in the process of finding sources of antioxidants.

The $DJS1_{11}$ and $BJS4_2$ have very strong antioxidant activity, and $DJS2_4$ has strong antioxidant activity. This was consistent with previous reports that the IC_{50} values below 50, have very strong antioxidant activity, and between 50 to 100, have strong antioxidant activity [21]. The $DJS1_{11}$ has the IC_{50} value which the closest to the AA (7,76 µg/ml). This proves that endophytic fungi from Jeruju plant can produce the alternative antioxidant compounds that have very strong activity. This was consistent with previous study that endophytic fungi from *Syzygium cumini* and *Smilax leucocarpa* can produce very strong antioxidant compounds, with IC_{50} values of 20 µg/ml [3] and 45.22 µg/ml [4].

Thin Layer Chromatography

The $DJS1_{11}$, $DJS2_4$, and $BJS4_2$ eluates have various Rf value. The Rf value of $DJS1_{11}$ was 0.22, using mobile phase n-hexane : ethyl acetate (7:3). The $DJS1_{11}$ eluate showed dark yellow color after sprayed with 10% H_2SO_4, it identified that the $DJS1_{11}$ eluate contains flavonoid compound [22]. The Rf value of $DJS2_4$ was 0.62, using mobile phase etlyl acetate (100%) and the Rf value of $BJS4_2$ was 0.35, using mobile phase n-hexane : ethyl acetate (5:5), the $DJS2_4$ and $BJS4_2$ eluates showed purple color after sprayed with 10% H_2SO_4, it identified that $DJS2_4$ and $BJS4_2$ contain terpenoid compounds [23], [24]. Endophytic fungi can produce secondary metabolites which contain antioxidant compound [1]. Many studies stated flavonoid dan terpenoid as antioxidant compounds, cause they can donate the

electron and stabilize the free radical [25], [26]. The color of the compound can be seen in Figure5.

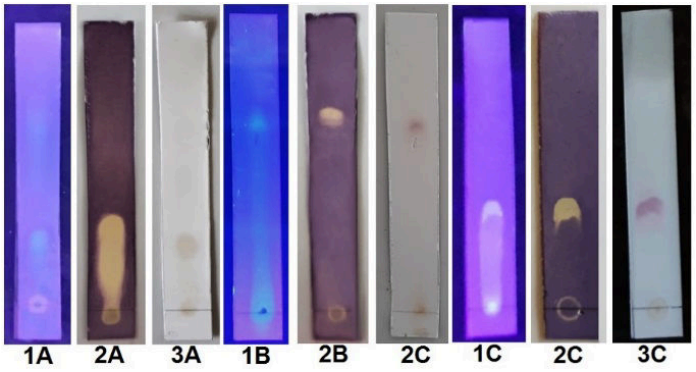

Figure 5. TLC-antioxidant bioautography; $DJS1_{11}$ (A), $DJS2_4$ (B), and $BJS4_2$ (C); in UV light 256 nm (1); sprayed 0.2% DPPH (2); sprayed 10% H_2SO_4 (3)

In Figure 5, each plate showed one spot, and it means $DJS1_{11}$, $DJS2_4$, and $BJS4_2$ contain the pure compound. As the previous study, TLC-antioxidant bioautography can detect the antioxidant compounds [4], [9], [12], and chromatography column method can isolate the compound [11].

Identification of the endophytic fungi

The DJS1 is identified as *Culvularia lunata*, the DJS2 is identified as *Diaporthe phaseolorum*, and the BJS4 is identified as *Colletotrichum siamense*.

4. Conclusion

This study showed antioxidant activities of secondary metabolites from Jeruju's endophytic fungi. *Culvularia lunata*, *Diaporthe phaseolorum*, and *Colletotrichum siamense* were the endophytic fungi which have antioxidant activities. The fungi can produce the antioxidant compounds, namely flavonoid and terpenoid compounds. The flavonoid and terpenoid have strong antioxidant activity.

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