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Study of Phytochemistry and Potential of Endophyte Fungi Extract in *Avicennia marina* Roots as Antioxidants Inhibiting Early Aging

Muhammad Andhika Pratama[1], Mochammad Agung Seno Pambudi[1], Eri Bachtiar[2], Mochamad Rudyansyah Ismail[2], Yeni Mulyani[2], Sulastri Arsad[3] and Fiddy Semba Prasetiya[2,4*]

[1]Study Programme of Marine Science, Faculty of Fisheries and Marine Sciences, Universitas Padjadjaran, Jl. Raya Bandung Sumedang, Jatinangor, Sumedang 45363, Indonesia
[2]Marine Science Department, Faculty of Fisheries and Marine Sciences, Universitas Padjadjaran, Jl. Raya Bandung Sumedang, Jatinangor, Sumedang 45363, Indonesia
[3]Aquatic Resources Management Study Program, Faculty of Fisheries and Marine Sciences, Universitas Brawijaya, Jl. Veteran, Ketawanggede, Lowokwaru, Malang, East Java 60235, Indonesia
[4]Research Center of Biotechnology and Bioinformatics, Jl. Singaperbangsa 2, Bandung, West Java 40132, Indonesia

*Correspondence :
fiddy.semba.prasetiya@unpad.ac.id

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Abstract

Mangrove plants have long been utilized as traditional medicinal ingredients for treatments of various diseases in favor of bioactive components from its secondary metabolites. Mangroves may contain endophyte fungi in their tissues that are capable of producing secondary metabolites. In theory, endophyte fungi isolated from a plant can produce secondary metabolites similar to those of the original plants or even in relatively high numbers. In this research, mangrove species *Avicennia marina* was sampled from Blanakan, Subang District, West Java. Endophyte fungi were isolated from the mangrove roots that had been obtained. The experimental method was used to test antioxidant activity using DPPH (1,1-diphenyl-2-picrylhidrazyl) method and phytochemical content, with three replicates for each test. Fungal isolates were coded according to the distance of the roots from the main mangrove tree, namely STAD and STAJ that represents "close" and "far" roots. Our results showed that the environmental parameters, namely salinity, dissolved oxygen, and temperature meet the quality standards and mangrove life in the tropics. The *A. marina* type of substrate muddy soil which did not meet the quality standards for mangrove waters. As for antioxidant activity, both extracts could reduce DPPH free radicals. Further determination of the IC_{50} value of the two extracts showed that the endophytic fungi STAD and STAJ had IC_{50} values of 97.8 ppm (classified as strong) and 822.56 ppm (classified as very weak) in the DPPH radical immersion method. The phytochemical test revealed that the two extracts of fungal isolates contain bioactive compounds, where flavonoids and alkaloids were identified in isolate STAD, whereas STAJ was positive for tannins and alkaloids content.

INTRODUCTION

Subang Regency is one of the areas where mangrove vegetation is spread in the northern part of West Java (BIG, 2005). Mangrove roots of *Avicennia marina* contain secondary metabolite compounds such as alkaloids, saponins, tannins, flavonoids, triterpenoids, and glycosides (Wibowo *et al*., 2009). These bioactive compounds can be used as an inhibitor of premature aging (Kadir *et al*., 2019). Antioxidants are substances that can provide endogenous protection and exogenous oxidative stress by capturing free radicals that can cause premature aging (Lai-Cheong and McGrath, 2017).

The utilization of endophyte fungi in mangrove tissue is effective for obtaining secondary metabolite content in mangroves (Thatoi *et al*., 2013). Endophyte fungi from mangrove stems and roots of *A. marina* have high antioxidant activity with an IC_{50} value of (±1.1 ppm) containing phenolic compounds (Rahmawati *et al*., 2019).

Research on endophytes is generally shown to isolate and identify endophyte fungi (Strobel and Daisy, 2003). Furthermore, this study was carried out to screen potential isolates that could produce antibiotic, antiviral, anticancer, antibacterial, and antioxidant compounds (Tan and Zou, 2001). Isolation and purification of endophyte fungi are carried out as a first step to select endophytic fungi isolates to be used to produce metabolites and bioactive materials.

In this research, an initial screening of bioactive compounds from endophyte fungi isolated from mangrove *A. marina* in Blanakan Village was carried out as well as their antioxidant activity. Information about antioxidant activity and content of secondary metabolites from the endophytes fungi from *A. marina* is essential to provide initial information for the development of natural pharmacological drugs.

METHODOLOGY

Place and Time

This research was conducted from August 2019 - December 2020. Samples of *A. marina* roots were taken from Blanakan Village, Blanakan District, Subang Regency. The isolation of endophyte fungi, phytochemical screening, and antioxidant activity analysis was carried out at the Laboratory of Marine Biotechnology, Faculty of Fisheries and Marine Sciences, Universitas Padjadjaran. Liquid fermentation and extraction of endophytic fungi at the Laboratory of Marine Biotechnology, Building 4, Faculty of Fisheries and Marine Sciences, Universitas Padjadjaran.

Research Materials

The materials used in this study were *A. marina* mangrove roots collected from Blanakan Village in August 2019, sterile distilled water, 70% ethanol, 5.25% sodium hypochlorite, Potato Dextrose Agar (PDA), Potato Dextrose Broth (PDB), chloramphenicol, methanol, ascorbic acid extract, DPPH solution, Mayer and Wagner's reagent, Mg powder reagent, concentrated HCl solution, 1% $FeCl_3$ reagent, acetic anhydride reagent, and H_2SO_4.

The tools used in this study were plastic bag, vacuum machine, coolbox, refractometer, DO meter, pH meter, thermometer, ruler, cutter, rotary evaporator, and UV-Vis spectro-photometer.

Research Design

This research was conducted using laboratory exploration methods for in vitro testing. The number of endophyte fungi used in this study was two, and each sample was tested for antioxidant activity and phytochemical tests.

In the antioxidant activity test, the concentrations used in the two samples of endophyte fungi, at concentration 100, 200, 300, 400, 600 ppm and the positive control (ascorbic acid), at concentrations

2, 4, 6, 8, and 10 ppm. The phytochemical tests were carried out to identify the presence of bioactive compounds such as alkaloids, flavonoids, tannins, triterpenoids, and steroids.

Work Procedures

Sampling was done by purposive random sampling where the samples were collected from the desired location. Sample on. The roots of *A. marina* were collected as many as 10 mangrove roots with a length of 10-15 cm (50 gr wet mass).

Root sampling was carried out at roots near and far from the stem of the host. Then the sample was washed with clean water and put in a plastic bag, then vacuum packed. Then put it in the coolbox to be taken to the laboratory. Measurement of water parameters including salinity, temperature, Dissolve Oxygen (DO) and pH were carried out as well as checking the substrate in the sampling area (Radji, 2011).

Isolation of endophyte fungi was carried out based on the method described by Radji (2011) and Posangi and Bara (2014). Root samples of *A. marina* were rinsed with sterile distilled water and then were cut into 2 cm lengths of 3 pieces. After that, the surface was sterilized by consecutively immersing in 70% ethanol for 1 minute, 5.25% sodium hypochlorite solution for 5 minutes, 70% ethanol for 40 seconds, and sterile distilled water for 1 minute. In Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB) powders used, to be a solution to be used is dissolved using sterile distilled water. The sterile sample was then stored onto PDA medium which had been added with 500 ppm of chloramphenicol. The sample was then incubated at room temperature for two weeks. The growing endophyte fungi colonies were then subcultured several times to PDA to obtain a single colony.

Single colonies of endophyte fungi were cultivated in a 300 mL PDB medium at room temperature. After two weeks, the culture including the biomass was extracted three times by maceration for 24 hours with the same volume of ethyl acetate. The extracts were placed together and the solvents were removed using a rotary evaporator to obtain a concentrated extract (Nursid *et al*., 2010).

The antioxidant activity of the endophyte fungi extract was expressed as the immersion activity of the DPPH radical by the DPPH method. The extract solution in methanol as much as 2 mL (100, 200, 300, 400, and 600 ppm) was added to the test tube. The concentration of the extract solution was applied, which is intended to see on a large scale the difference in activity resulting from high concentrations of endophyte fungi extracts compared to using an ascorbic acid extract which has been tested as an effective antioxidant activity (Mantle *et al*., 2000).

Added 0.5 mL (500 ppm) of DPPH solution. Using positive control in the form of ascorbic acid (2, 4, 6, 8, and 10 ppm). Extracts with DPPH radical immersion activity will change the purple color from DPPH to yellow. To determine the secondary metabolites in the extract that are responsible for antioxidant activity, phytochemical tests were carried out on the extract of endophyte fungi.

The absorbance measurements were carried out with a UV-Vis spectrophotometer with a wavelength of 517 nm on the DPPH blank and the sample after incubation for 30 minutes. Calculation of the percentage of antioxidant activity is calculated using the formula according to Habib *et al*. (2016), as follows:

$$\%\text{Antioxidant} = \left(\frac{\{\text{Abs. Blank} - \text{Abs. Sample}\}}{\text{Abs. Blank}}\right) \times 100\%$$

The 50% inhibition concentration (IC_{50}) was calculated from the graph of the relationship between percentage inhibition (antioxidant activity) and extract concentration. After finding the percentage value of inhibition of each concentration, the IC_{50} value was found using a linear regression equation. After obtaining the IC_{50} value, determine the group based on the strength category of the compound (Rahman *et al*., 2017).

With the equation according to Habib *et al*. (2016), as follows:

$Y = a + bX$

Where:

Y = percentage of inhibition (i.e., 50)

X = value IC_{50}

Each sample methanol extract (1000 ppm) was tested for alkaloids, flavonoids, tannins, terpenoids, and steroids. The alkaloid test was performed by using 3-5 drops of Mayer and Wagner's reagent, the positive results of the change were white precipitate in Mayer's reagent and brown precipitate in Wagner's reagent. Moreover, the flavonoid test was conducted using 0.025 g of Mg powder reagent and 5 drops of concentrated HCl solution, the positive results change to red, orange, or yellow. Tannin test was carried out using 0.5 mL of 1% $FeCl_3$ reagent, the positive results change to blue or green. Terpenoid and steroid tests were conducted using 2 drops of acetic anhydride reagent and 1 drop of H_2SO_4, with positive results changing to purple or orange on terpenoids and turning green on steroids (Harborne, 2006).

Data Analysis

The environmental parameters from the study sites were compared with the quality standard of mangrove waters. Then the observation results of two pure isolates of endophyte fungi are then displayed in the form of images, which show the characteristics of PDA media. A qualitative antioxidant activity test was performed by observing color changes before and after partitioning, which then were documented and described using comparative analysis. Quantitative antioxidant activity test was analyzed using comparative descriptive analysis comparing with the standard of antioxidant strength of a compound. Results from phytochemical analysis of the obtained endophyte fungi were and analyzed descriptively using comparative analysis.

RESULTS AND DISCUSSION

The water quality and the type of substrate from the study site are presented in Table 1. In the mangrove waters in Blanakan Village, Blanakan District, Subang District, direct measurements (*in situ*) were carried out with the characteristic of closed waters (after being used as a silvofishery-based pond). The salinity of mangrove waters is 5 ppt. This value is still in the range of seawater quality standards for mangrove habitat (0-34 ppt) (MENLH, 2004). The temperature of the mangrove waters at the research location was 28.1 °C. Mangroves can grow well in tropical areas with temperatures above 20 °C (Schaduw, 2018).

Table 1. Water quality of study site in Mangrove Blanakan Village.

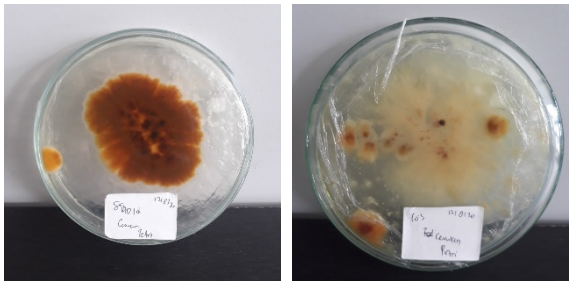
Parameter	Measurement Result	Quality Standards (MENLH, 2004)
Salinity	5 ppt	0-34 ppt
Temperature	28.1°C	>20°C
DO	8.1 mg/L	>5 mg/L
pH	6.8	7.5-8
Type of Substrate	Muddy Land	Mud

The type of substrate in the study site was muddy soil. In turbid water, light penetration into the water column can be difficult, thus resulting in phytoplankton being unable to photosynthesize optimally, as a result, the amount of dissolved oxygen produced is lower (Poedjirahajoe *et al*., 2017). The measured DO was 8.1 mg/L, in other words, the DO value in mangrove waters meets the quality standards, which is >5 mg/L (MENLH, 2004).

The pH condition at the research location was 6.8, this condition doesn't meet the quality standard in the range (7.5-8) (MENLH, 2004). This value is

strongly influenced by the oceanographic and geomorphological characteristics of the area. Open water tends to have a higher pH than closed water. Closed waters tend to accommodate a lot of water intake from river flow because the storage area is relatively small, so it tends to reduce the pH to become acidic (Poedjirahajoe *et al*., 2017).

The isolates of endophyte fungi from the roots of *A. marina* in Blanakan Village were two isolates. Two isolates were used for this research because they represent one branch root, namely the near (STAD) and distant roots of the same stem (STAJ). The endophyte fungi isolate stored at the Central Laboratory, UNPAD, Jatinangor, Sumedang. The macroscopic appearance of both isolates (Figure 1).

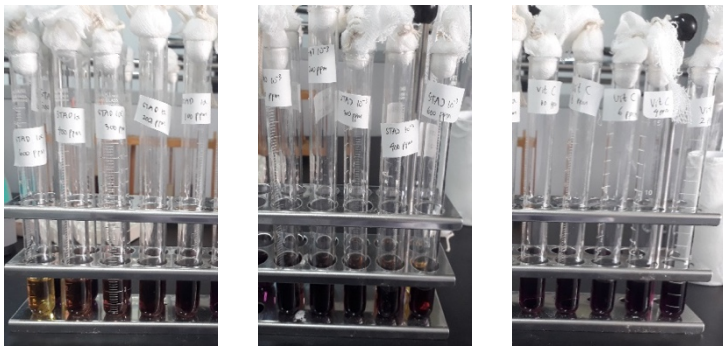


A B

Figure 1. Endophyte fungi STAD (A) and STAJ (B) isolates.

The isolation of endophyte fungi associated with *A. Marina* roots collected from Blanakan Village showed growth in the PDA medium. These endophyte fungi are microscopic organisms and are present in plant tissue systems such as leaves, flowers, fruits, tubers, twigs, and roots (Murdiyah, 2017).

Antioxidant activity test to extracts of endophyte fungi showed that the extracts STAD and STAJ could reduce DPPH radicals in tests with the DPPH method. The results of antioxidant activity showed differences in activity to DPPH radicals (is presented in Figures 2 and 3). The two extracts of endophyte fungi showed activity as antioxidants on screening with the DPPH method.



A B C

Figure 2. Color changes in antioxidant activity STAD (A), STAJ (B), and Ascorbic Acid (C).

The extract STAD showed prominent activity compared to extract STAJ against DPPH radicals. The immersion reaction is characterized by a dark purple color change in the DPPH free radical to form a clear yellow non-radical stable compound. The unpaired electrons in the presence of hydrogen atoms from antioxidants form reduced DPPH-H (2,2-

Diphenyl-1-picrylhydrazyl (Bougatef *et al.*, 2009).

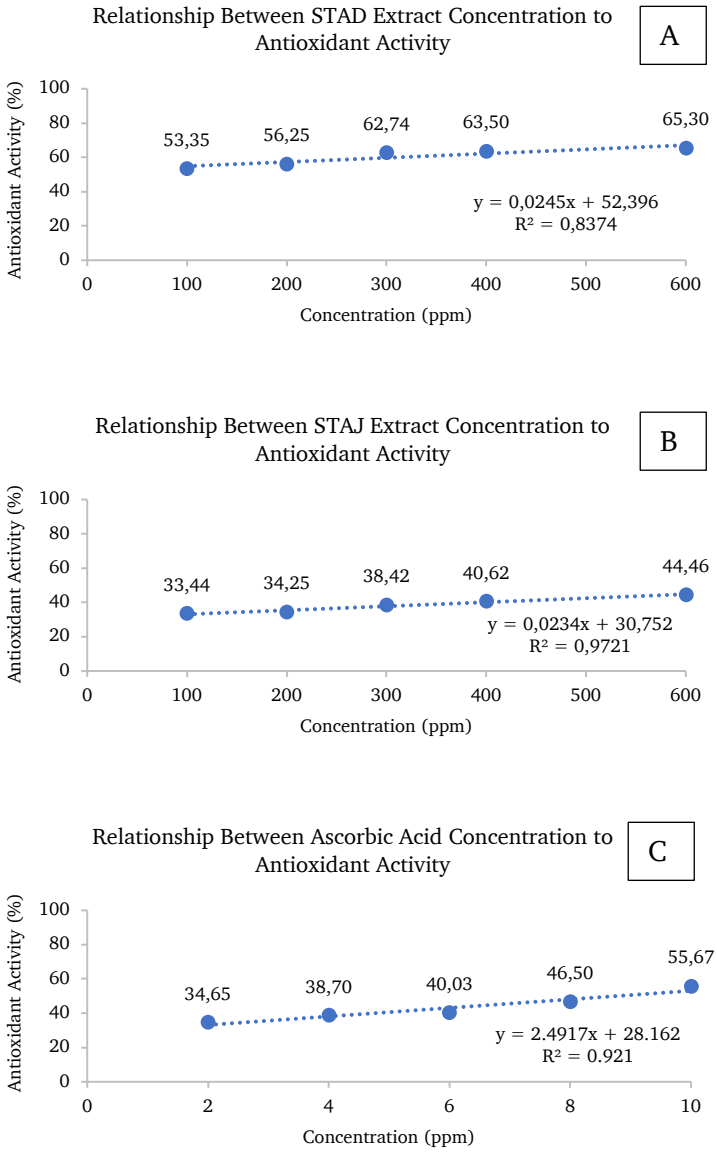


Figure 3. Relationship between concentration and antioxidant activity of STAD (A), STAJ (B), and Ascorbic Acid (C).

A decrease in absorbance indicates an increase in the ability to reduce free radicals from DPPH (Amrun *et al.*, 2007). High concentrations indicate high antioxidant activity. The antioxidant activity of each sample is namely a percentage of antioxidant activity.

In both extracts calculated the IC_{50} value, obtained for STAD was 97.8 ppm and for STAJ was 822.56 ppm. Through category determination, it was determined that STAD was classified as a strong antioxidant and STAJ was classified as a very weak antioxidant (Table 3).

Table 2. Category of compound strength as an antioxidant (Rahman *et al*., 2017).

Category	IC_{50} Value (ppm)
Very Strong	< 50
Strong	51-100
Medium	101-150
Week	151-200
Very Week	> 200

Table 3. Regression and sample IC_{50} value.

Sample	Value R^2	IC_{50} (ppm)	Antioxidant Activity
STAD	0.84	97.8	Strong
STAJ	0.97	822.56	Very Weak
Ascorbic Acid	0.92	8.76	Very Strong

In both extracts, the IC_{50} value was calculated results obtained for STAD was 97.8 ppm and for STAJ was 822.56 ppm. Meanwhile, a study from Rahmawati *et al*. (2019) demonstrated that samples of endophyte fungi from mangrove roots of *A. marina* from mangrove forests in Serang, Banten, show similar bioactivity. Two types of isolates were obtained with the IC_{50} value obtained for the Sr-A-Ak K01 and Sr-A-Ak K02 isolate was 18 and 1 ppm, respectively.

Compared with the study from Rahmawati *et al*. (2019), the IC_{50} value of the samples in Blanakan Village (this study) had a higher value, which resulted in less effective free radical binding activity. Indicating that the activity against free radical binding is less effective. Substances with high antioxidant activity have a low IC_{50} or EC_{50} value and vice versa. The smaller the IC_{50} value, the tested compound has better effectiveness as a free radical scavenger (Cholisoh and Utami, 2008).

The phytochemical test showed that the secondary metabolites were contained in the two extracts of endophyte fungi. The result is that STAD and STAJ extract has active compounds groups (Table 4, Figure 4).

Table 4. Phytochemical test of endophyte fungi samples.

Sample	Compound	Result	Form of reaction
STAD	Alkaloid: Mayer Wagner	+	There are white deposits (Mayer) and brown deposits (Wagner)
	Flavonoid	+	Change color to pink
	Tannin	-	
	Terpenoid and Steroid	-	
STAJ	Alkaloid: Mayer Wagner	+	There are white deposits (Mayer) and brown deposits (Wagner)
	Flavonoid	-	
	Tannin	+	Changes color to greenish-yellow
	Terpenoid and Steroid	-	

Extract STAD has active compounds from the alkaloid and flavonoid groups, whereas extract STAJ has active compounds from the alkaloid and tannin groups. Compounds that have an important effect on antioxidant activity are flavonoid and tannin (Ali, 2008; Mustafa *et al*., 2010).

Figure 4. Change in color of samples in the phytochemical test.

The antioxidant activity of flavonoid and tannin is because these compounds are phenolic compounds. Phenolic compounds are compounds with -OH groups attached to the aromatic ring carbon. This phenolic compound can donate hydrogen atoms so that DPPH radicals can be reduced to a more stable form (Sudirman, 2011). The hydroxyl group of phenolics can capture free radicals, able to reduce the radical properties of reactive oxygen compounds such as superoxides, peroxide radicals, hydroxyl radicals, and peroxynitrite (Sirait, 2007).

CONCLUSION

The two extracts of endophyte fungi associated with *A. marina* mangrove roots from Blanakan Village had different levels of antioxidant activity. The endophyte fungi extract of STAD showed strong DPPH antiradical activity with an IC50 was 97.8 ppm, while in STAJ it showed very weak activity with an IC50 was 822.56 ppm. Each endophyte fungi extract has active compounds from different groups. The STAD extract has active compounds from the alkaloid and flavonoid groups, the STAJ extract has active compounds from the alkaloid and tannin groups. From the results obtained, the STAD extract is potential for further research regarding the content and structure of secondary metabolites with the flavonoid compounds. Such Thin Layer Chromatography (TLC) method to strengthen the arguments of the phytochemical test results can be applied, as well as *in vivo* test using animals.

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