Antioxidant Activities of Mangrove Fruits Endophytic Fungus from Segara Anakan Lagoon, Indonesia

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Abstract. Mangrove derived endophytic fungi are particular interest because they constitute the second-largest environmental group of marine fungi and are adapted to extreme conditions, which make them a rich source for novel metabolite and enzyme discovery. Especially the bioactivities of endophytic fungi from mangrove’s fruits have not been explored yet. We took our samples from The Segara Anakan lagoon which is situated at the southern coast of Central Java. The mangrove forest is semi-enclosed because there were Nusa Kambangan Island and its connection to the Indian Ocean. The endophytic fungus from 4 species mangrove’s fruits which are Acacia mangium Wild, Avicennia alba Blume, Pongamia pinnata (L.) Pierre, Bruguiera gymnorrhiza (L.) Lam have been isolated, and antioxidant analysis have been done. Its resulted that the extract from endophytic fungi isolated from Bruguiera gymnorrhiza showed the highest antioxidant activities among others even though the extract yield showed the opposite. From profiling of secondary metabolite from endophytic fungi extract, we obtained data that the extract contained Cyclohexane, 1-hexadecanol, 1H-Indene as and farnesene that might be contributed to antioxidant activities of ethyl acetate extract from isolated fungi from Bruguiera gymnorrhiza fruits

Keyword: Antioxidant, Endophytic fungus, Mangrove fruits, Segara Anakan Lagoon

1. Introduction

Segara Anakan Lagoon is located in Central Java, especially on the southern coast. The mangrove forest is semi-enclosed because there were Nusa Kambangan Island and its connection to the Indian Ocean. The lagoon's area mainly is mangrove forest, three-quarters approximately, and the remaining is water [1]. Mangrove forest in Segara Anakan Lagoon is the most extensive mangroves forest in Java Island. There numerous species of mangroves tree can be found, such as Avicennia marina, A. alba, Aegiceras corniculatum, Bruguiera gymnorrhiza, B. cylindrica, Rhizopora mucronata, and so on [2]. Therefore, research about mangrove in Segara Anakan is interesting to be done.

Mangrove is the halophytic salt-tolerant plant forest which locating in intertidal regions between sea and land. These forests are ecologically stressful environment (abiotic stresses) because of high humidity, high salinity, low oxygen, and tend to be unstable because of high wind and temperature [3]. Consequently, a limited of plant communities such as trees, herb, and shrubs can tolerate those conditions. Various abiotic stresses were challenging mangrove plants to survive and trigger changes in physiological, biochemical, morphological, and molecular [4]. These abiotic stresses enhanced the production of ROS (Reactive Oxygen Species), which make mangrove should produce antioxidant
agents to survive [5]. Many researches have been done for bioactive compounds that work as antioxidant in mangrove trees such as Janmanchi et al. (2017)[6] reported gallic acid in *Aegiceras corniculatum*, Rahim et al. (2008)[7] reported catechin in *Rhizophora apiculata*, Assaw et al. (2019)[8] reported naphthofuranquinones in *Avicennia officinalis*, and many more. The antioxidant compounds is very interesting because its system has many therapeutic values for several diseases such as inflammation, viral infections, neurodegenerative disorders, digestive system disorders and autoimmune pathologies that require additional sources of antioxidants to fight an excess production of free radical, especially in the human body [9]. Unfortunately, deforestation of mangrove because of human activities become worst each year. The mangrove plants are currently being protected, so the use of bioactive components from mangrove plants directly is very difficult. Therefore, one alternative to produce secondary metabolite from mangrove is the use of endophytic fungus from mangrove plants.

Mangrove endophytic fungus grows within mangrove as their plant host without causing any disease symptoms, furthermore metabolic interaction between fungus and host continue as they grow together in this habitat [10]. It makes endophytic fungus, especially from mangroves rich and reliable sources of bioactive and chemically compounds for medicinal purposes [11]. Tan and Zou (2001) [10] recently reviewed the diversity of metabolites that have been isolated from an endophytic fungus, and it is known that secondary metabolites from endophytes are synthesized via various metabolic pathways such as phenols, xanthones, terpenoid alkaloid, flavonoid, amino acid derivation, and so on. Mangrove plants inhibiting a stressful environment condition that could enhance ROS production. Consequently, mangrove and its endophytic fungus should produce an active and high concentration of antioxidative compounds to neutralize the ROS. Thereafter, the research of antioxidant compounds from endophytic mangroves is exciting to be explored.

In addition, until now there is no report about endophytic fungus from fruits of mangroves, moreover, report about secondary metabolite from mangrove fruits itself also very limited. Therefore, we would like to explore endophytic fungus that isolated from mangrove fruits, especially mangroves from Segara Anakan Lagoon, Indonesia, and its bioactivity as an antioxidant.

2. Method

2.1. Sampling

Sampling was located in Segara Anakan Lagoon, Cilacap City, East Java, Indonesia. The samples taken were fruits of several mangrove trees, that located in LS 7° 42′ 55″ BT 108° 52′ 44″. The fruit samples were sprayed by 70% ethanol, and cut areas were wrapped by parafilm to prevent contact with air. All samples were kept in a ziploc bag and kept in the cool box with ice to the laboratory for isolation preparation. For identification of mangrove trees, we kept the leaves, flower, and fruits of mangrove trees. The mangrove trees were dried and identified by Herbarium Bogoriense, Research Center for Biology, Indonesian Institute of Sciences.

2.2. Isolation of Mangroves Derived Endophytic Fungus

Preparation of media using PDA (Potato Dextrose Agar) for isolation of fungus. Suspend 39 g of PDA in 1000 mL distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving in 121˚C for 15 minutes. Plated the media on a sterilized plate in laminar airflow, after that kept until firm and ready to use for isolation.

The isolation was beginning by rinsed all fruit samples used running water then dried by a paper towel. Then in the laminar airflow, the samples were cleaned using three steps by soaked in 70% ethanol for 1 minute, NaOCl for 5 minutes and another 30 seconds in 70% ethanol. After that, the samples were cut using sterilized forceps and placed in PDA. The incubation was done until 14 days. Meanwhile, the observation was done every two days. Following, the fungi that exposed from the samples were subculture and plated onto fresh PDA plate then incubated for another maximum 14 days. Once it was an axenic fungus and had grown sufficiently for inoculation of liquid media (fermentation), they were wrapped with parafilm and placed in a refrigerated incubator at 4˚C until needed.
2.3. Fermentation and Extraction of Mangroves derived Endophytic Fungus Secondary Metabolites
Isolated endophytic fungus were grown on PDA medium for 14 days in petri dishes. Five (5) pieces of inoculums were selected using sterile cork punch and placed into liquid fermentation Potato Dextrose Broth media (100 ml media in Erlenmeyer flask 250 ml). Then further fermentation was done on a shaker for the next 12 days at room temperature with a rotating velocity of 130 rpm. The supernatant separated from biomass via centrifugation at -4°C rotating at 2000 rpm for 20 min and extracted with EtOAc (1:1) for 3 times. The extract was then evaporated until lyophilized extract powder was obtained. The extracts were used for antioxidant assay and profiling secondary metabolites of the extract using GCMS.

2.4. Antioxidant of Mangroves derived Endophytic Fungus Secondary Metabolites Extracts
Antioxidant activities were measured using the ABTS method. Radical ABTS solution was prepared the day before, by mixed 5 ml of 19.2 mg ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) with 88 µL 140 mM potassium persulfate in distilled water and kept for 12-16 h. The mixed solvents were diluted using 99% of EtOH until 200 mL. Control solvent was prepared using 99% EtOH. Determination of antioxidants was started by incubated 1 mL of ABTS' that reacted with 1 mL extracts or standard for 6 minutes at 37˚C in a water bath. The antioxidant activity was measured using spectrophotometer using absorbance 680 nm. Inhibition of ABTS' calculated based on IC_50 value using the equation:

Percentage of ABTS radical scavenger activities = [(A_con - A_test)/ A_con]*100%
A_con is control absorbance
A_test is extract absorbance

2.5. Profiling of Secondary Metabolites from Mangroves derived Endophytic Fungus
Ethyl acetate extracts of mangrove fruits endophytic fungus (1 ppm) were diluted in an analytical grade of ethyl acetate solvent, then injected into GC-MS Shimadzu QP2010 with column RTX-5MS (30 m x 0,15 mm ID x 0,25 µm) using Electron Impact (EI) and Negative Chemical Ionization (NCI) for ionization method under conditions:

| Condition                        | Value         |
|----------------------------------|---------------|
| Column Oven Temperature          | 60˚C          |
| Injection Temperature            | 200˚C         |
| Pressure                         | 9.2 psi       |
| Total Flow                       | 54 mL/min     |
| Column Flow                      | 1.08 mL/min   |
| Linear Velocity                  | 37.8 cm/sec   |
| Purge Flow                       | 3 mL/min      |

Ion Temp. Program:
- Temp 60˚C for 3 min
- 10 min increased to 235˚C
- Temp 235˚C for 20 min

Interface Temperature: 250˚C
Gas sources: Helium

3. Result and Discussion

3.1. Species of mangrove trees

Table 1. Species of mangrove trees as the sample for isolation endophytic fungus

| No. | Code | Species                  | Genus              |
|-----|------|--------------------------|--------------------|
| 1.  | C    | Acacia mangium Willd     | Leguminosae/ Fabaceae |
| 2.  | F    | Avicennia alba Blume     | Acanthaceae        |
| 3.  | G    | Pongamia pinnata (L.) Pierre | Leguminosae/ Fabaceae |
4. **H** *Bruguiera gymnorrhiza* (L.) Lam. Rhizophoraceae

FAO reported that Indonesia has 67% area of *A. mangium* plantations in the world [12]. *A. mangium* is a tree species that has the ability to fast-growing and usually uses as pulp, particleboard, paper, crates, and woodchips. In addition, *A. mangium* also can grow in a wide range of area, including in marginal sites such as mangroves [13]. *A. mangium Wild* known has a high level of flavonoid and tannin, which are compounds that have strong antioxidants. Pereira et al. in 2017 [14] showed that ethanolic extract of *A. mangium* has a strong antioxidant activity in ABTS and β-carotene assays. Different from *A. mangium*, the wood of *Avicennia* commonly used as construction and fuel. Meanwhile, the high tannin content in the bark of *Avicennia* is used for leather production and dyeing. Leaves of *Avicennia* also use as food for cattle, also used for the preparation of mats and doors. Last, the fruits are used for insect repellent [3]. Recent pharmacological investigations on *Avicennia* reported that extract of *Avicennia* could be used for against cancer, HIV, diabetes, inflammation, diarrhea, oxidative stress-related diseases, and so on. Furthermore, several metabolites, such as alkaloids, flavonoids, phenols, tannins, terpenoids, have been identified from *Avicennia*, which could be contributed as medicinal properties of this plant [15]. Patra et al. (2014) [16] reported that water extract of *Avicennia alba* obtained higher antioxidant activities on NO scavenging, DPPH scavenging, and metal chelating than other extracts.

*Pongamia pinnata* belong to Leguminaceae family is a tree that underutilized. The tree can be used to produce biodiesel, poultry feed, and soap because containing oil. It’s commonly used traditionally as lubrication, illuminating oil, and medicine [17]. The Fruits of *Pongamia pinnata* were used for abdominal tumors, useful in ailments of the female genital tract, ulcer, and so on [18].

*B. gymnorrhiza* is well known as mangroves tree with so many bioactivities, especially for traditional medicine. The fruit of *B. gymnorrhiza* also has been applied for traditional medicine diarrhea treatment in China. Roy et al. (2018) [19] reported the n-hexane extract of the fruit of *B. gymnorrhiza* showed the presence of many bioactive compounds such as like flavonoid, saponins, phenolic compounds, glycosides, tannins, terpenoids, as the major secondary metabolites [19].

3.2. **Isolation of Mangroves Derived Endophytic Fungus**

![Figure 1. Isolated of endophytic fungus from several species of mangrove fruits. CBU: Isolated fungi from the fruit of *Acacia mangium* Wild; F2BU: Isolated fungi from the fruit of *Avicennia alba* Blume; GBU: Isolated fungi from the fruit of *Pongamia pinnata* (L.) Pierre; HBU: Isolated fungi from the fruit of *Bruguiera gymnorrhiza* (L.) Lam.](image)

We have 50 isolated endophytic fungi from four species of mangroves in Segara Anakan Lagoon. The fungi were isolated from flower, fruit, stem, leaf and root of mangrove trees. Based on antioxidant activity screening qualitatively using a TLC plate known that these four isolates from fruit of mangrove trees have antioxidant activities. Therefore, isolated fungi from the fruit of *Acacia mangium Wild*, isolated fungi from the fruit of *Avicennia alba Blume*, isolated fungi from the fruit of *Pongamia pinnata* (L.) Pierre and isolated fungi from the fruit of *Bruguiera gymnorrhiza* (L.) Lam was then through further analysis for extract yield and antioxidant activities. From morphology of isolated fungus, it showed that one fungus to another has different morphology. However, it still needs further analysis for the identification of the isolated fungus.

3.3. **Extract Yield of Mangroves Fruits Endophytic Fungus Secondary Metabolites**
Figure 2. Extract yield of isolates of endophytic fungus from several species of mangrove fruits extraction using ethyl acetate as solvent. CBU: Isolated fungi from the fruit of *Acacia mangium* Willd; F2BU: Isolated fungi from the fruit of *Avicennia alba* Blume; GBU: Isolated fungi from the fruit of *Pongamia pinnata* (L.) Pierre; HBU: Isolated fungi from the fruit of *Bruguiera gymnorrhiza* (l.) Lam.

Figure 2. showed that the highest extract yield was obtained by isolated fungi from the fruit of *Pongamia pinnata* (L.) Pierre, and the lowest extract yield was obtained by isolated fungi from the fruit of *Bruguiera gymnorrhiza* (l.) Lam. We suspected that the highest extract yield obtained by fungi from *Pongamia pinnata* (L.) Pierre because the mangrove plant contains mainly oil bases that can easily be extracted by ethyl acetate. Therefore, besides secondary metabolites such as phenolic compounds, terpenoid, alkaloid, so on, oil also extracted inside the ethyl acetate extract from endophytic fungi derived mangrove fruits. However, it still needs further analysis to know the compounds inside the extracts.

### 3.4. Antioxidant of Mangroves Fruits Endophytic Fungus Secondary Metabolites Extracts

#### Table 2. Table of antioxidant activities of Mangroves fruits Endophytic Fungus Ethyl Acetate Extracts

| Code | Type of fungus | Equation | IC<sub>50</sub> |
|------|----------------|----------|----------------|
| CBU  | Isolated fungi from the fruit of *Acacia mangium* Willd. | y = 0.0012x + 0.1598 | 136.62 |
| F2BU | Isolated fungi from the fruit of *Avicennia alba* Blume. | y = 0.0011x + 0.0992 | 200.05 |
| GBU2 | Isolated fungi from the fruit of *Pongamia pinnata* (L.) Pierre. | y = 0.0017x - 0.0207 | 206.44 |
| HBU  | Isolated fungi from the fruit of *Bruguiera gymnorrhiza* (l.) Lam. | y = 0.0015x + 0.168 | 92.67 |

*Note: CBU: Isolated fungi from the fruit of *Acacia mangium* Willd; F2BU: Isolated fungi from the fruit of *Avicennia alba* Blume; GBU: Isolated fungi from the fruit of *Pongamia pinnata* (L.) Pierre; HBU: Isolated fungi from the fruit of *Bruguiera gymnorrhiza* (l.) Lam.*

The highest antioxidant activities were obtained by the extract from isolated fungi from the fruit of *Bruguiera gymnorrhiza* (l.) Lam. Information about mangroves plants phytochemical compounds such as Anthocyanins, coumarins, flavonoid, isoﬂavone, flavones, catechin and so on, which those compounds known as sources of antioxidant, were well established. Mangrove plants inhibiting a stressful environment condition that could enhance ROS production. Consequently, mangrove and its endophytic fungus should produce an active and high concentration of antioxidative compounds to neutralize the ROS [5]. However, the three other extracts (CBU, F2BU, and GBU2) from isolated fungi from fruits of different mangrove species were categorized as an antioxidant with medium activities. All data of antioxidant activities can be seen in Table 2.
3.5. Profiling of Secondary Metabolites from Mangroves fruits Endophytic Fungus

Table 3 showed main of phytoconstituent of ethyl acetate extracts of Isolated fungi from the fruit of *Bruguiera gymnorrhiza* (l.) Lam is Cyclohexane that known as antimicrobial [20], 1-hexadecanol known as antimicrobial [21], 1H-Indene as anti-Alzheimer agent [22], and farnesene known as a common volatile component of many higher plants also as a pheromone in several insect species. Even though the report about those compounds as antioxidant very rare but there is a possibility that it gives activities on reactive oxygen species.

**Table 3.** Phytoconstituents in ethyl acetate extract of Isolated fungi from the fruit of *Bruguiera gymnorrhiza* (l.) Lam

| No. | R. Time | Area (%) | Compounds | Similarity Index |
|-----|---------|----------|-----------|-----------------|
| 1.  | 3.047   | 1.02     | Heptane, 3-methyl       | 81              |
| 2.  | 3.171   | 9.73     | Cyclohexane, 1,3-dimethyl- | 95              |
| 3.  | 3.299   | 3.36     | Cyclopentane, 1-ethyl-3-methyl, | 93              |
| 4.  | 3.355   | 4.66     | Cyclopentane, 1-ethyl-2-methyl, cis- | 89              |
| 5.  | 3.461   | 2.44     | Cyclohexane, 1,2-dimethyl-, trans- | 91              |
| 6.  | 10.650  | 9.29     | 1H-Indene, 1-methylene    | 95              |
| 7.  | 13.129  | 5.08     | Trans-3-methylpent-3-ene-5-ol | 80              |
| 8.  | 16.289  | 1.41     | Cyclopentane, 1,1,3-trimethyl- | 87              |
| 9.  | 16.372  | 2.65     | Decane, 3,7-dimethyl-    | 89              |
| 10. | 17.443  | 2.92     | 1,3,6,10-Dodecatetraene,3,7,11-trimethyl-,(Z | 75              |
| 11. | 17.959  | 1.64     | Cycloheptane              | 75              |
| 12. | 18.411  | 1.31     | Oxalic acid, allyl nonyl ester | 89              |
| 13. | 18.568  | 5.05     | Salicyl hydrazide         | 73              |
| 14. | 18.731  | 5.43     | Octane,2,3,3-trymethyl-   | 89              |
| 15. | 20.125  | 1.71     | Butanoic acid, 2-methyl-  | 82              |
| 16. | 20.360  | 3.90     | Dodecane, 1-chloro-       | 70              |
| 17. | 20.409  | 3.57     | Pyrrolol[1,2-a]pyrazine-1,4-dione,Hexahydro-3 | 80              |
| 18. | 20.654  | 2.70     | Benzoic acid,2-(1-oxopropyl)- | 85              |
| 19. | 20.813  | 4.97     | 1-Hexadecanol            | 88              |
| 20. | 20.873  | 3.78     | Hexane, 3,3-dimethyl-     | 87              |
| 21. | 20.984  | 8.99     | Farnesene epoxide, E-     | 75              |
| 22. | 21.426  | 2.36     | Benzoic acid, 3-(2-methylpropyl)oxy-, methyl | 66              |
| 23. | 23.316  | 3.52     | Oxirane, tetradecyl-      | 79              |
4. Conclusion

The endophytic fungus from 4 species mangrove's fruit has been isolated, and antioxidant analysis has been done. Its resulted that the extract from endophytic fungi isolated from Bruguiera gymnorrhiza showed the highest antioxidant activities, among others, even though the extract yield showed the opposite. From profiling of secondary metabolite from endophytic fungi extract, we obtained data that the extract contained Cyclohexane, 1-hexadecanol, 1H-Indene as and farnesene that might be contributed to antioxidant activities of ethyl acetate extract from isolated fungi from Bruguiera gymnorrhiza fruits. Furthermore, the identification of mangrove derived endophytic fungi is needed to give more information for the mass production of antioxidant agents in the near future.

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6. References

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