Antioxidant and cytotoxic activity of endophytic bacteria isolated from mangrove species

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Abstract. Mangroves have proven to possess strong bioactivities to survive their complex environmental condition. However, apart from the primary function of mangroves as an ecosystem keeper, mangroves are often overexploited for construction materials. Hence, their medicinal purposes are often overlooked, and the direct exploration of pharmacologically active compounds derived from mangrove species seems illogical. Endophytic bacteria are found capable of producing secondary metabolites similar or even the same as their host plant. Therefore, exploration of endophytic bacteria from mangroves becomes an excellent alternative for bioproduction of certain plant-specific bioactive compounds. Moreover, mangroves live in an extreme living environment, and therefore, may contain interesting endophytic bacteria that possess remarkable bioactivities, particularly antioxidant and cytotoxic activity. Endophytic bacteria were isolated from mangrove species collected from the Segara Anakan Lagoon, on the south coast of Java, Indonesia. Isolated bacteria were cultivated to produce secondary metabolites. Fermentation liquid was then extracted and investigated for its antioxidant activity and cytotoxic activity. Secondary metabolites from isolated bacteria showed to have potentials to be used as an antioxidant as well as a cytotoxic agent.

Keywords: endophytic bacteria, mangrove, antioxidant, cytotoxicity

1. Introduction
Mangroves are salt resistant marine tidal forests ecosystems in tropical and subtropical climates [1, 2]. Mangroves have proven to possess strong antioxidant activity to survive their complex environmental condition [3]. A large number of mangroves have been used traditionally as medicinal plants, including three mangrove species studied in this research; Aegiceras corniculatum, Sonneratia alba, and Avicennia alba. A. corniculatum, known as the black mangrove, belongs to the Myrsinaceae family and are traditionally used as a cure for asthma, rheumatism, and diabetes [4]. The leaves are also used to treat a boil, earache, and smallpox as well as to tackle fish poison [5]. S. alba is a species from the family Sonneratiaceae and has been traditionally used to treat hemorrhage, swelling, intestinal parasites, and coughs [6]. A. alba, a member of Avicenniaceae family, is used traditionally for skin diseases, ulcers, and antifertility treatment [3].

Despite the high potential for their bioactivity and medicinal purposes, mangroves primary purpose is to protect shorelines from erosion through remediation of terrestrial [7]. Located in the intertidal zone, mangroves known as the bridge between the marine and the terrestrial and therefore, serve the purpose as a protection system in the coastal area [1, 2]. Hence, its medicinal purposes are often overlooked, and the scientific reports about the bioactivity activity of mangroves are considered scarce [1]. Apart from the primary function of mangroves as an ecosystem keeper, the increase of mangroves

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deforestation for construction materials [8] is adding more reasons why the direct exploration of pharmacologically active compounds derived from mangrove species seems illogical [9]. In recent years, research regarding endophytes has been slowly gaining interest worldwide. Endophytes are a term for microbes, whether fungi or bacteria, that live within plant tissues without causing disturbances or harmful effects to the host plant [10, 11]. The presence of these microbes is found to promote the growth of plants and help their adaptation to the environment as well as contribute to plant defense mechanism against environmental stress [10, 12-15]. Exploration of endophytes has attracted many attentions of researchers. Especially after the discovery of endophytic fungus, *Taxomyces andreanae*, which produces taxol, a secondary metabolite known as anti-cancer agent due to its potent cytotoxic and antioxidant activity [16]. This compound is previously known exclusive to its host plant, *Taxus brevifolia* [17]. Both endophytes and host plant formed a mutual symbiosis with complex interaction, enabling them to share genetic information which may allow endophytes to produce secondary metabolites that are exclusive to host plant [18, 19].

The research of endophytes, especially endophytic bacteria, is highly regarded as an excellent alternative for bioproduction of certain plant-specific bioactive compounds [10, 13, 20], due to the relatively small sample size needed when compared to direct extraction from the host plant [6, 7]. This alternative becomes highly crucial for extinct or rare plants, as well as plants with the primary purpose for conservation and alike, such as mangroves [21, 22]. In addition to that, Mangroves live in such an extreme environment with physical stress including, sharp variation in changing tides and salt-moisture concentration as well as biological stress, such as abundant living microorganisms or insects [2]. Endophytic bacteria reside in mangroves may be different from those in marine or land. Due to this fact, we hypothesized that mangroves might contain interesting endophytic bacteria that possess remarkable bioactivities, particularly antioxidant and cytotoxic activity, as their defense mechanism against their extreme living environment. Therefore, the study of endophytic bacteria from mangroves became interesting and essential to study. Despite its high potential, research regarding endophytic bacteria from mangroves is still limited. To date, there are not many reports on the biological activity of endophytic bacteria isolated from *A. corniculatum*, *S. alba*, and *A. alba*. The current study performed as preliminary research on the exploration of bioactivity of secondary metabolites derived from mangroves endophytic bacteria.

2. Materials and Methods

2.1. Plants Materials

Various parts, such as leaves, stems, fruits, and roots, of three mangrove species (*A. corniculatum*, *S. alba*, and *A. alba*) were obtained from the Segara Anakan Lagoon, located on the south coast of Java, Indonesia. The materials collected were immediately sealed with parafilm to avoid contamination. The sample was transported in zipper bags and kept in an icebox. Sample identification was carried out in Herbarium Bogoriense, Pusat Penelitian Biologi, Lembaga Ilmu Pengetahuan Indonesia (LIPI), Cibinong, Bogor, West Java, Indonesia.

2.2. Surface sterilization of the samples and Isolation of endophytic mangroves

Dust and debris removal from the sample was carried out with running tap water and followed by distilled water for [23]. Surface sterilization was performed using ethanol 70% for 1 min and sodium hypochlorite for 5 min before finally rinsed by ethanol 70% for 30 sec [24]. Afterward, the plant tissues were rinsed thrice in sterilized distilled water. Washed materials were dried on sterilized filter paper. Following the surface sterilization, each sample was crushed and cut into smaller fragments to obtain the inner tissue of each sample [25]. It was then laid and slightly pressed upon the surface of the culture medium, Nutrient Agar (NA), and incubate at 30°C for 48 h. The cultures were monitored daily to check the growth of bacteria. The cultivated colonies were streaked on a new sterile plate for re-culturing by incubating for another 48 h at 30°C [26, 27]. Preculture was carried out by transferring the selected colony into 100 mL liquid medium, Nutrient Broth (NB). After 24 h incubation at room
temperature on a rotary shaker, the culture was then transferred into 900 mL NB for secondary metabolite production. The culture was further incubated for five days using the same condition [27].

2.3. Extraction of bioactive compounds
Vacuum filtration using Buchner funnel was carried out before the extraction of secondary metabolites from endophytic bacteria. The filtrate was extracted three times with ethyl acetate (1:1) by liquid-liquid extraction (LLE) method using separating funnel. The organic layer from each extraction was collected and evaporated at 40°C using rotary vacuum evaporation at 180 rpm. The dry extract was weighted to determine the extraction yield [28].

2.4. Biological Activity Assay

2.4.1. Antioxidant activity. The antioxidant activity assay was carried out using ABTS⁺ (2,2′azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)) radical scavenging activity. ABTS⁺ was prepared using the method reported by Miller et al. with slight modifications [29]. ABTS⁺ produced by reacting 5 ml of 7.46 mM ABTS stock solution with 88 μL of 140 mM potassium persulfate in distilled water and left in the dark at room temperature for 16 h before use. ABTS⁺ solution was diluted in ethanol absolute until 200 mL to achieve absorbance of around 0.6–0.7 ± 0.02 at 680 nm. Determinations were carried out by reacting ABTS working solution with extracts or standard (1:1 v/v). The positive control used in this study was Ascorbic acid (Vit C) in concentrations of 1, 2, 3, 4, and 5 mg/L. Each extract was made in concentrations of 50, 100, 150, 200, and 250 mg/L. Incubation of all samples, control, and standard were carried out at 37 °C for 6 minutes. Measurement was carried out using spectrophotometer (Hitachi U-3900H) at 680 nm. The equation of the standard curve obtained from the OD values of those five concentrations. The results expressed as IC₅₀ values (mg extract per mL) for comparison.

2.4.2. Cytotoxic activity. Cytotoxic activity was measured using the Brine Shrimp Lethality Test (BSLT). BSLT was performed following the method described by Meyer et al. with slight modification [30]. The hatching eggs of Artemia salina Leach were prepared in seawater for 48 h under the light at room temperature and aerator to obtain mature shrimp called nauplii. Preparation of samples carried out by dissolving them in seawater. The nauplii of A. salina were added to experimental and control vials. After 24 h of exposure, percent of mortality calculated from the dead nauplii. The results expressed as LC₅₀ (lethal concentration 50%) values, obtained by the probit analysis using SPSS from the mortality of nauplii in three leveled concentrations (10, 100, and 1000 mg/L).

3. Results and Discussion

3.1. Isolation of Endophytic Bacteria
Three mangrove species collected were identified as follow Aegiceras corniculatum (L.) Blanco, Sonneratia alba Sm. and Avicennia alba Blume. Various parts of each species were planted in NA culture medium. Even though the growth of endophytic bacteria was observed in several parts from each plant; however, only endophytic bacteria from the fruit of A. corniculatum (D₃₀₃₀), the stem of S. alba (E₉₉) and the leaf of A. alba (F₁₃) was successfully cultivated (Fig. 1). Hence, only these isolates were further cultured for their secondary metabolites and investigated for their bioactivities.
3.2. Extraction yield
The extraction yield for each extract was calculated as a percentage of dry extract (mg) per 1000 mL cultivation medium and plotted in Fig. 2 below. Highest extraction yield obtained by isolate ER and followed by isolate FD, whereas isolate DBH showed the lowest extraction yield.

3.3. Antioxidant activity
Comparison of IC50 value from each extract can be seen in Table 1 below. Isolate FD showed much lower IC50 value than isolate DBH and ER. Both isolate DBH and ER yielded the relatively same IC50 value; however, the later showed slightly lower IC50 value than the former. However, neither of the extracts showed IC50 value similar to that of positive control. The antioxidant properties inversely correlated with IC50 values.
Table 1. Antioxidant activity expressed in IC\(_{50}\) value.

| Sample                | Linear regression equation | R\(^2\) | IC\(_{50}\) (mg/mL) |
|-----------------------|----------------------------|---------|---------------------|
| Positive control (Vit C) | y = 0.1175x - 0.0008       | 0.9639  | 2.85                |
| Isolate DBH           | y = 0.0009x + 0.1267       | 0.9696  | 207.00              |
| Isolate ER            | y = 0.0014x + 0.0149       | 0.9985  | 212.04              |
| Isolate FD            | y = 0.001x + 0.2532        | 0.9619  | 69.05               |

3.4. Cytotoxic activity

As displayed in Table 2, isolate ER yielded a very high LC\(_{50}\) value, most top out of three isolates. Isolate DBH showed much lower LC\(_{50}\) value. Similar to IC\(_{50}\) values in antioxidant activity, LC\(_{50}\) values inversely correlated with cytotoxicity.

Table 2. Cytotoxic activity expressed in LC\(_{50}\) value.

| Sample | LC\(_{50}\) (µg/mL) |
|--------|---------------------|
| Isolate DBH | 69.198               |
| Isolate ER   | 7,707.932            |
| Isolate FD   | 358.037              |

In the present study, various parts of Aegiceras corniculatum, Sonneratia alba and Avicennia alba, including leaves, stems, roots, as well as fruits and flower (if available) were planted on culture medium. The growth of endophytic bacteria was observed in many parts. However, only some were successfully cultivated. Endophytic bacteria are known to be not as easily cultivable as rhizospheric bacteria, for example, due to the need for specific conditions [31]. Moreover, according to the study of Torsvik and Øvreås [32], only up to 1% of the endophytes present in plant tissues are cultivable, despite their viability. In addition to that, the high salinity and complex living environment of mangroves [2] are increasing the difficulty in cultivating endophytic bacteria from mangroves. Hence, only three isolates were able to be cultivated in the current study.

Endophytic bacteria observed from the fruit of A. corniculatum, from now on will be called as isolate DBH, whereas endophytic bacteria isolated from the stem of S. alba and the leaf of A. alba will be called isolate ER and isolate FD, respectively. Extracted secondary metabolites were assessed for their antioxidant and cytotoxic activity.

Endophytic bacteria are said to be beneficial under more extreme conditions [33]. Mangroves are inhabiting challenging environmental condition with physical stress such as drought, salinity, and other extreme condition that might culminate into the increase of reactive oxygen species (ROS) formation [2]. The high level of ROS levels may result in significant damage to cell structures, known as oxidative stress. In order to survive such a living condition, mangrove species and their endophytes should possess a strong antioxidant activity.

According to Minami et al., compounds can be categorized into three, based on their antioxidant activity; very active (IC\(_{50}\) < 10 mg/L), active (IC\(_{50}\) < 100 mg/L), and inactive (IC\(_{50}\) > 100 mg/L) [34]. Isolate DBH and ER are categorized as inactive; isolate FD is categorized as active; whereas none of the isolates is categorized as very active compounds. Despite none of the isolates possesses similar antioxidant activity to Vit C as the positive control, isolate FD from the leaf of A. alba showed the best antioxidant activity compared to two other isolates by having an IC\(_{50}\) value of 69.05 mg/L. A study
from Banerjee et al. [3] reported the methanol extract of *A. alba* showed moderate antioxidant activity. The same study also reported a strong anti-radical activity possessed by *A. corniculatum*. Unfortunately, the same cannot be said about the bacteria isolated from the fruit of *A. corniculatum* in this study which possess a low antioxidant activity.

Slightly different from antioxidant activity assay, two isolates possess cytotoxic activity. It is considered toxic to brine shrimps if the sample had LC$_{50}$ less than 1000 μg/mL [30]. In the current research, only Isolate E$_R$ is excluded in that category. Both isolate D$_{BH}$ and F$_D$ are considered toxic by having an LC$_{50}$ value of 69.198 and 358.037 μg/mL, respectively. Previous studies also corroborated this result; endophytes isolated from *Avicennia* sp. and *A. corniculatum* were found to show cytotoxic activity due to their polyketides [35, 36].

Isolate E$_R$ from the stem of *S. alba* failed to show notable bioactivities, neither as an antioxidant nor cytotoxic agent, performed in this study. However, it does not mean that endophytic bacteria from the stem of *S. alba* possess no bioactivity at all. According to Hardoim et al., not every endophyte is found to have beneficial effects on the host plants. Some endophytes that have no apparent effects on plant performance are termed commensal endophytes [33].

Study of Kjer et al. reported that endophytes from *S. alba* showed positive antibiotic activity against multidrug-resistant *S. aureus* [37]. In line with this finding, members of the Sonneratiaceae family, including *S. alba*, are known as a rich source of tannins, a group of compounds with antimicrobial activity [38]. Hence, there is indeed a linear correlation in the bioactivity of endophytes and their host plant.

The differences in their bioactivity potential may occur due to their metabolite profiles. Bioactive compounds isolated from endophytic bacteria which have been reported over the years are including flavonoids, phenols, alkaloids, terpenoids, as well as aliphatic compounds [12]. Extract with higher antioxidant activity may contain more flavonoids and phenolic compounds [39]. These compounds act as reducing agents and single electron donors to stabilize ROS [40]. Meanwhile, polyketides, alkaloids phenylpropanoids and terpenoids possess the best activity for cytotoxic activity [41, 42].

Antioxidant and cytotoxic activity exhibited by endophytic bacteria isolated from the leaf of *A. alba* and the fruit of *A. corniculatum* could be very helpful as preliminary data in the search for potential bioactive compounds from mangroves endophytic bacteria. Further work needs to be carried out to establish their metabolic profiles and the correlation with their bioactivity.

4. Conclusion
This study evaluates the antioxidant and cytotoxic activity of secondary metabolites extracted from endophytic bacteria derived from various parts of mangrove species, namely *Aegiceras corniculatum* (L.) Blanco, *Sonneratia alba* Sm. and *Avicennia alba* Blume. Endophytic bacteria isolated from the stem of *S. alba* showed the highest yield of extraction; however, its bioactivities were proven to be the lowest among other isolates. Isolate from the leaf of *A. alba* showed the best antioxidant activity, whereas isolate from the fruit of *A. corniculatum* yielded the best cytotoxic activity. Future studies are needed to establish the correlation of their metabolic profiles and their bioactivity as well as further pharmacological activity possessed by each endophyte.

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