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# Endophytic microbes and antioxidant activities of secondary metabolites from mangroves *Avicennia marina* and *Xylocarpus granatum*

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**Abstract.** The utilization of mangrove plants as traditional medicinal ingredients has long been utilized by the community for treatment of various diseases in favour of bioactive components from its secondary metabolites. Secondary metabolites from mangroves could act as an antioxidant to prevent oxidative stress. Mangrove may contain endophytic microbes in its tissues that are capable of producing secondary metabolites. In theory, endophytic microbes isolated from a plant can produce secondary metabolites similar to those of the original plants or even in relatively high numbers. In this research, two mangrove species *Avicennia marina* and *Xylocarpus granatum* from Serang, Banten province, samples have been taken to investigate the antioxidant activities of its extracts. Furthermore, isolation of endophytic microbes from both mangroves had been done. This research succeeded to isolate six fungi and three bacteria as endophytic microbes. Meanwhile, the highest extraction yield was obtained by the leaves of *A. marina* that is approximately 3% (w/w), whereas mangroves' endophytic microbe highest extract yield was obtained by bacteria from fruits of *A. marina* 18 mg/100 mL media. For antioxidant activities, the highest activities were obtained by fungi within the stems and the roots of *A. marina*, also additionally fungi in the leaves of *X. granatum*.

**Keywords:** antioxidant, mangrove, microbe

## 1. Introduction

Mangroves are one of the tropical forests that quickly grow and that have not been utilized much. It is supported by the fact that globally there are only 0.4% of mangroves which have been investigated for their chemical contents [1]. Mangroves in Indonesia are the most extensive mangrove forests in the world, both regarding area (± 42,550 km$^2$) and the number of mangroves species (± 45 species) [2].

Recently, Indonesia has lost mangrove forests for about 40% [3]. It means that Indonesia has the biggest mangrove deforestation among all country in the world. The main reason for mangrove deforestation in Indonesia was the conversion of mangrove forests to shrimp ponds (in Sumatra, Sulawesi, and East Java), illegal logging and land conversion to agriculture purposes (in Java and Sulawesi) as well as degradation because of pollution (in East Borneo) [3].

Mangroves are one kind of forest that has salt-tolerant ecosystems, which situated between terrestrial also marine environments of tropical and subtropical regions [4]. The unique and dynamic environment in mangroves ecosystems are because of its geochemical characteristics for example humidity, salinity,

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and also nutrient concentration that modified by tidal flooding periodically [5]. Characterization of mangrove forests is depending on their high biological productivity. Mangrove also the second most productive marine ecosystems after coral reefs in marine [4]. Mangrove ecosystems have a close relationship in the cycle and conservation of nutrients in microorganisms also plants [6],

Mangroves are one example of traditional medicinal materials, which contain bioactive compounds such as steroids, alkaloids, terpenoids, saponins and tannins with various bioactivities such as insecticides, antitumor, antiviral, antifungal and antimicrobial [7]. The utilization of mangrove plants as traditional medicinal ingredients has long been done by the community in the treatment of various diseases, especially gastroenteritis and so on [8].

Bioactive compounds resulted mainly from secondary metabolites of mangrove. Secondary metabolite means compounds that were not produced for metabolism (primary metabolite), its common work as a defense mechanism from other plants, animals, parasites and also extreme environmental condition. Mangroves can survive in environments with high salinity levels, in addition to extreme humidity and pH levels make secondary metabolites from mangrove are varied and have good bioactivity especially as medicine that is used as an antibacterial, anti-inflammatory, antioxidants, antiviral and anti-cancer [9].

High-level plants, for example, mangroves, may contain some endophytic microbes in their tissues that are capable of producing biological or secondary metabolites. In theory, endophytic microbes isolated from a plant can produce secondary metabolites similar to those of the original plants or even in relatively high numbers [10]. Mangrove-derived endophytic microbes are particular interest because they are the second largest ecological group of marine microbes. They also could adapt to extreme conditions, which make them a good resource for novel metabolite and also for the discovery of a new enzyme. Microbes, especially fungi, are abundance in the microbial community of mangrove forests, and also playing essential roles to support mangrove ecosystem especially in the nutritive cycle [11]. Mangrove ecosystem has a special condition such as an abundance of fungal biodiversity, low pH, aeration, rich organic matter, and moisture condition [12-16].

Endophytic microbes are the prolific source for the discovery of active metabolites that have structurally-interesting and biologically active [17-19]. Recently, endophytic microbes especially growing in the mangroves area received much attention from medicinal researcher because the unique ecosystem that makes them rich of bioactive metabolite which very useful for human health [20].

In addition, the production of secondary metabolite using endophytic microbes have several advantages such as short life cycles while mangroves plants need years to grow. Also, for mass production of a secondary metabolite by mangrove endophytic microbe may be easier because it would only need a bioreactor that does not require a much place, while mangroves would require a large place for plantation. Therefore, endophytic microbes have a good prospect in the production of bioactive compounds as raw materials for medicines [21].

The mangrove plants are currently being protected due to illegal logging for making charcoal, so the use of bioactive components from mangrove plants directly is challenging. Therefore, one of the paths that can be taken is the utilization of endophytic microbes as a source of natural medicine to reduce the environmental damages caused by the harvesting of medicinal plants in large quantities. Moreover, endophytic microbes' ability to produce many bioactive components is an opportunity in the provision of medicinal raw materials. Breeding of endophytic microbial cultures can be done in a considerable number and a short harvest time. Thus, the use of endophytic microbes as a source of medicinal raw materials is more economical [22].

This research is preliminary research on the exploration of mangrove endophytic microbes as a source of raw material for medicines. As for mangroves themselves, they are known to have secondary metabolites that have high bioactivity as medicines. So, mangrove endophytic microbes are expected

to produce secondary metabolites that have bioactivities similar to or even in relatively higher numbers than mangroves themselves.

Mangrove endophytic microbes are attracting significant attention due to their potential of producing novel metabolites as a source of raw material for medicines. Mangrove endophytic microbes are very attractive because they are the most extensive ecological group of marine fungi, even though living in the mangrove area. They also live in special conditions, such as high pH, humidity and high salinity, which make them a good source for research area especially on novel metabolite and enzyme discovery. Endophytic fungi is a prolific source for the discovery of active metabolites, because of structurally interesting and biologically active as medicine for human health [17-23]. Among plant-derived fungi, those associated with trees growing up in mangrove areas have received much attention from medicinal chemists owing to the unique ecosystem [20]. An example for bioactive metabolites from the mangrove endophytic fungi, four eremophilane sesquiterpenes from *Xylaria* sp. BL321 that have activity on alpha-glucosidase as antidiabetic assay [24].

Mangrove endophytic microbes are attracting significant attention due to their potential of producing novel metabolites. Several studies have shown the mechanism of endophytes activate host stress responses to various abiotic stresses such as temperature, salt, drought [25-27], and some biotic stresses that can be caused by several fungal pathogens [28]. Those active responses were shown by secondary metabolites, especially those produced by themselves or associated with endophytic microbes. For example, secondary metabolite namely Paeciloxin A *was* isolated from the bark of mangrove that not been identified in Taiwan have cytotoxicity on cancer.

Unfortunately, in Indonesia, the studies about mangrove endophytic microbes were very rare, even though Indonesia's mangrove forests are the largest in the world. Few studies have been reported in the Indonesian language about secondary metabolites from mangrove endophytic microbes as antibacterial [29], while the reports about secondary metabolites from mangroves themselves as a plant have been much more explored [30-32]. Therefore, research about the antioxidant of mangrove endophytic microbes is fascinating to be done.

## 2. Material and Methods

### *2.1. Collection of samples*

Sample collection was done in a mangrove forest in Serang, Banten Province. We took samples from leaves, branches, fruits and roots of two different mangrove plant species, *Avicennia marina* (*A. marina*) and *Xylocarpus granatum* (*X. granatum*). The collected plant parts were sealed with a sterile plastic cover and kept in an ice box. Washing procedure of the samples was carried out with tap water and follow by distilled water for removal of the free-coating organisms. To remove the epiphytes using 70% ethanol. Isolation and antioxidant analysis were done in the Research Center for Biotechnology, Cibinong, Bogor. Dried plant samples were prepared for identification in Herbarium Bogoriense, Research Center for Biology, Indonesia Institute of Science, Cibinong, Bogor.

### *2.2. Isolation of endophytic mangroves*

The leaves, branches, fruits, and roots of the sampled mangrove plants were removed from the outer tissues by crushing them with the application of minimal pressure using a sterile mortar and pestle. After the inner tissues were excised, about 2–3 segments of crushed leaves placed onto petri plates containing potato dextrose agar (PDA) media for fungi and potato dextrose broth (PDB) for bacteria, then they were incubated at 30°C. Observations were carried out daily until the growth of endophytic microbes were observed. Next step was a purification, selection, and preservation of endophytic microbes. After incubation, fungal colonies were selected and streaked on PDA (fungi) and PDB (bacteria) plates for incubation at 30°C for about 72 hours, approximately. Then the isolated endophytic microbes were characterized morphologically by shape, colony color, texture, and topography.

*2.3. Extraction of bioactive compounds*

We have done two extraction steps which were the extraction of mangrove samples and endophytic microbe mangroves. For collected samples from leaves, branches, fruits and roots of mangrove plants were sundried for 12 hours. After that, the samples were pulverized to make powder samples. The extraction was done by sonication for 10 minutes then continued by maceration for 3x24 hours and ended by sonication for 10 minutes. Filtration and evaporation were done for all samples to get a dried extract and yield of extraction. Next step was the extraction of secondary metabolites from the mangrove endophytic microbes. The antioxidant potential of active strain was extracted using ethyl acetate solvent by liquid-liquid extraction method. The active strain was inoculated in Malt Extract Broth (MEB) and then proceeded with the addition of peptone, then incubated at 30°C for seven days fermentation. The broth of fermentation was centrifuged at 3,000 rpm for 30 minutes. Then centrifugation, the supernatant was then recovered by using a filtrate with Whatman No.1 filter paper. After filtering, 1 L of ethyl acetate was mixed with 1 L of supernatant for antibacterial potential. The ratio was maintained at 1:1(w/v) and shaken vigorously for completed the extraction. Using separating funnel, the organic layer of the extract was separated from the aqueous layer. The organic layer was then evaporated at 40°C using vacuum evaporation. After evaporation, dry crude compounds were collected and determined for antioxidant assay.

*2.4. The antioxidant assay using DPPH assay*

DPPH radical scavenging activities were determinate by using a 1 mM DPPH solution in ethanol and preparation of extract for concentration 1 mg/1 ml in ethanol was prepared. The extract solution for 1.5 mL was added to the DPPH solution for 1.5 mL also. Then the absorbance was measured at wavelength 517 m against the corresponding blank solution using ethanol as blank. The analysis was measured in triplicates. The antioxidant activity was calculated based on $IC_{50}$ of free radical inhibition using the equation (1) below:

$$\text{Percentage of DPPH scavenger} = [(A_{con} - A_{test})/ A_{con}]*100\% \quad (1)$$

$A_{con}$ = control absorbance

$A_{test}$ = extract absorbance

*2.5. Statistical analysis*

Results were described the mean ± standard deviation (SD) based on triplicates determination and all analysis under the same experimental conditions. The $IC_{50}$ values for antioxidant activities were calculated using the linear regression equation in Microsoft Excel. For significant ($p \leq 0.05$) coefficients of determination ($r^2$) were determined by the relationships between pairs of variables using MS Excel. Furthermore, statistical differences were calculated using SPSS, between groups of treatments were analyzed by ANOVA and followed by Tukey's multiple comparisons. The differences were decided as significantly different with condition $p < 0.05$ ($\alpha = 0.05$).

## 3. Results and Discussion

*3.1. Isolation of endophytic microbes from A. marina and X. granatum*

*A. marina* is known as a grey mangrove and belongs to the family of Avicenniaceae. *A. marina* grow about 10 m in height in tropical areas. It several branches and also thick leaves. Furthermore, A. marina has aerial roots that could grow until 20 cm with a diameter of 1 cm. The function of roots is to fulfill oxygen absorption since in mangrove the oxygen is deficient. Another function of roots is to provide firm support in regular tidal waves, so the plant still survives. *A. marina* has white to golden yellow flower color for the amount of three to five in a branch. Its fruits surround the new seedling stem. If the saline condition is extreme, the growth of *A. marina* was prone to stunt. Nevertheless, in extreme salinity also, A. marina via leaves can excrete salts [33].

*A. marina* contains benefit as ecologically and economically [34]. Several parts of the mangrove have been proven to have ethnomedicine uses in treating various diseases like diabetes. Notably, the pharmacological investigations on the *A. marina* reveal antioxidant, anti-inflammatory, antiviral, anti-

microbial and antidiabetic activities [35-36]. It has unique bioactive compounds, such as terpenoids, glycosides, tannins, saponins, phenols, flavonoids, and alkaloids [37]. This has ensured traditional medicine practitioners to utilize its products to develop drugs from the biologically active phytochemical.

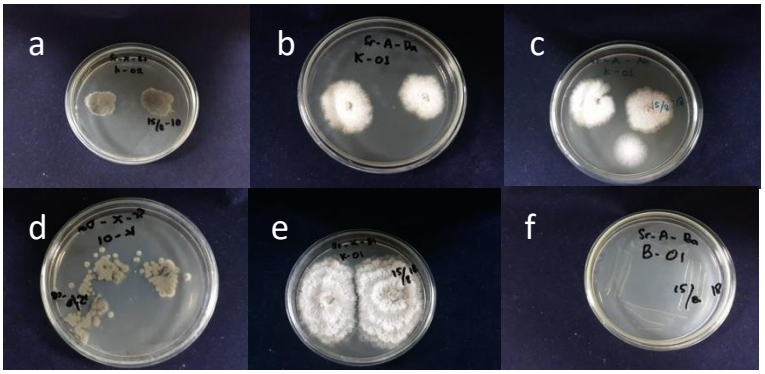



**Figure 1**. Isolated Mangrove Endophytic Microbes from stems, leaves, fruits, and roots *A. marina* and *X. granatum*. Whereas [a] Sr-X-Bt K02 Fungi from steams of *X. granatum*; [b] Sr-X-Bt K01 Fungi from steams of *X. granatum*; [c] Sr-A-Ak K01 is fungi from roots of *A. marina*; [d] Sr-X-Da K01 Fungi from leaves of *X. granatum*; [e] Sr-A-Da K01 is fungi from leaves of *A.marina*; [f] Sr-A-Ba B01 is bacteria from fruits of *A. marina.*

*X. granatum*, is one of the mangroves species that can grow as tall as 10-20 m. This species has a root board, and stems are often hollow, especially in older trees. The bark is light yellow-brown, thin and peeling, while in young branches the barks are wrinkled. The fruits dangle with the arrangement of seeds randomly in them.

Mangrove *X. granatum* has seeds, fruits, and barks which are useful as a medicine for various types of diseases because they contain secondary metabolites. Based on this statement, seeds, fruit, and barks of *X. granatum* contain secondary metabolites which have been used as raw materials for medicines, but the content of the secondary metabolites is not yet known.

We succeeded in isolating a few endophyte microbes, mostly fungi, as much as six isolates and three bacteria from roots, stems, leaves, and fruits. As seen in figure 1, the morphology of fungi and bacteria have different characteristics. From figure 1 can indicate that the species of those fungi and bacteria were different from one another. Further identification is needed to decide the species of those microbes, such as using DNA identification. From the isolation, we have got many fungi because mangroves are the right place for fungi to live due to their moist conditions, plenty of organic matter, low of aeration, and the low pH of mangrove ecosystems [13, 14].

*3.2. Yield of A. marina and X. granatum and its endophytic microbes*

The extraction of mangroves was done by using ethanol with sonication and maceration method. This kind of extraction was expected to yield more extract. As seen in figure 2, the highest extraction yield was obtained by a sample of leaves *A. marina* that is 3% (w/w dry base), approximately. Leaves are a weaker tissue than stems and roots that have higher lignin on their cell wall, so it is easier to extract bioactive compounds from leaves inside the powdered sample. The reduction in the size of the particle can increase the superficial area for mass transfer that in turn increases the extraction yield.

In figure 3 we can see the highest ethyl acetate extract yield was obtained from Sr-A-Ba B01 which are bacteria from the fruits of *A. marina*. The advantage of using bacteria compared to fungi is that bacteria only need less than 12 hours to harvest, while fungi need more than seven days to harvest depending on the media used. Sr-A-Ak K02 are fungi from the roots of *A. marina* which yielded the least ethyl acetate extract. Even though the value of the yield does not always relate to the bioactivity, as it requires further research to know the bioactive compounds contained in the extracts.

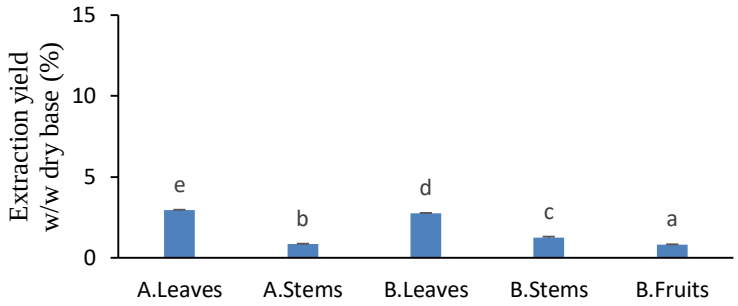



**Figure 2.** Extraction yield from stems, leaves, and fruits *A. marina* [A] and *X. granatum* [B]. Data showed in the mean ± SD (n = 3). Different letters above the bars showed significantly difference value ($P < 0.05$).

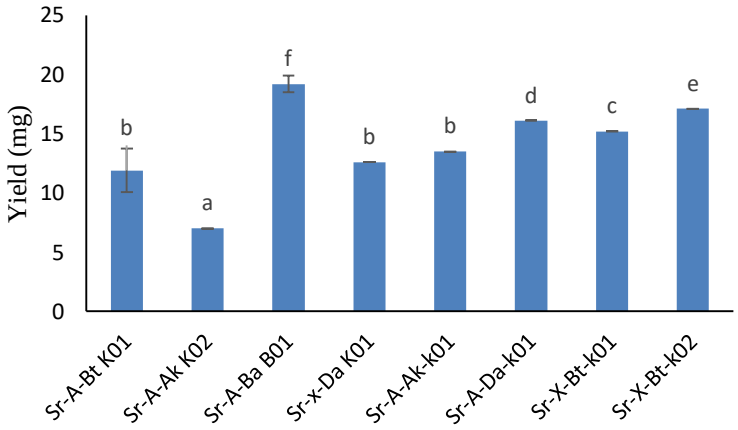



**Figure 3.** Extract yield of secondary metabolites mangroves endophytic microbes by ethyl acetate liquid extraction, whereas Sr-A-Bt K01 is fungi from stems of *A. marina*; Sr-A-Ak K02 is fungi from roots of *A. marina*; Sr-A-Ba B01 is bacteria from fruits of *A.marina*; Sr-X-Da K01 Fungi from leaves of *X. granatum*; Sr-A-Ak K01 is fungi from roots of *A. marina*; Sr-A-Da K01 is fungi from leaves of *A. marina*; Sr-X-Bt K01 Fungi from stems of *X. granatum*; Sr-X-Bt K02 Fungi from stems of *X. granatum*. Data showed in the mean ± SD (n = 3). Different letters above the bars showed significantly difference value ($P < 0.05$).

*3.3. Antioxidant activities of mangroves and its endophytic microbe extracts*

Antioxidants are commonly available in plants, vegetables, and fruits, and often use as traditional medicine. An antioxidant is known as promising agents used as a treatment for aging, cardiovascular, neurodegenerative diseases and cancer [38]. The biggest antioxidant constituents from endophytes microbes came from phenolic compounds [39]. On research in India, *Phomopsis amygdale*, an endophytic fungus that isolated from mangrove, showed potent antioxidant activity against DPPH radicals [40]. Endophytic colonization of *Trichoderma* was found to be higher in mangrove leaves of *Aegiceras corniculatum* than the other mangroves and was demonstrated to have a potential for antioxidant activity [41]. Two new resveratrol derivatives, namely, resveratrol dehydes A and C, isolated from the endophytic fungus *Alternaria sp*. R6, obtained from *Myoporum bontioides* A. Gray roots, also showed moderate antioxidant activity by DPPH antioxidant assay [42].

DPPH is commonly used as a substrate to determine antioxidant activity, because of their radical activities. In the present study, DPPH assays of mangrove plant extract showed maximum antioxidant activity in fruits of *X. granatum*. Also, its endophytic microbes which occurrence of $IC_{50}$ of antioxidant activities was the lowest that means the highest activity of antioxidant. The increase in endophytic fungi observed in other studies, it related to the increase in host tolerance of stresses range [26-28]. If we compared the $IC_{50}$ of mangrove extracts and its endophytic microbes, the result that we would get is

that on average the antioxidant activities of endophytes were higher than in mangroves themselves. So, we can conclude that endophytic microbes are a potential source for an antioxidant agent in the future.

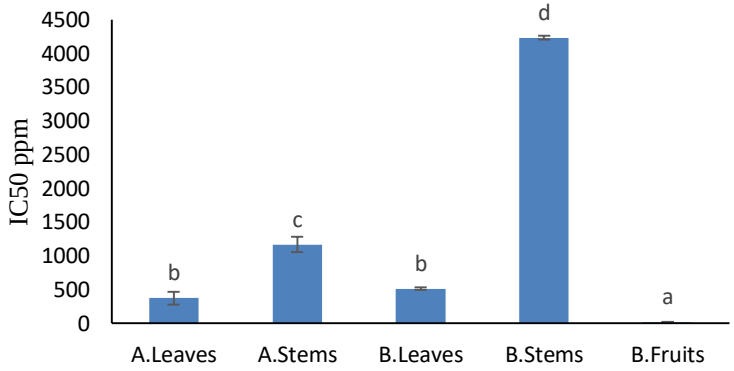



**Figure 4.** Antioxidant activities from stems, leaves, and fruits *A. marina* [A] and *X. granatum* [B] extracts. Data showed in the mean ± SD (n = 3). Different letters above the bars showed significantly difference value ($P < 0.05$).

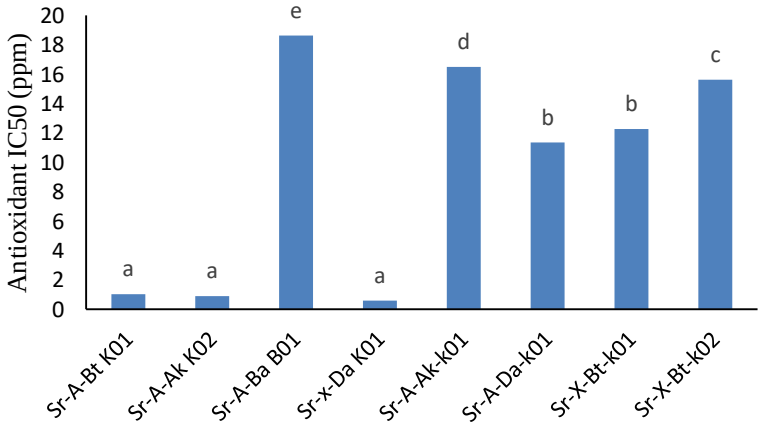



**Figure 5.** Antioxidant activities of secondary metabolites mangroves endophytic microbes by ethyl acetate liquid extraction, whereas Sr-A-Bt K01 is fungi from stems of *A. marina*; Sr-A-Ak K02 is fungi from roots of *A. marina*; Sr-A-Ba B01 is bacteria from fruits of *A. marina*; Sr-X-Da K01 Fungi from leaves of *X. granatum*; Sr-A-Ak K01 is fungi from roots of *A. marina*; Sr-A-Da K01 is fungi from leaves of *A. marina*; Sr-X-Bt K01 Fungi from steams of *X. granatum*; Sr-X-Bt K02 Fungi from steams of *X. granatum*. Data showed in the mean ± SD (n = 3). Different letters above the bars showed significantly difference value ($P < 0.05$).

Most abiotic and biotic stresses in mangrove ecosystem will increase highly reactive molecules, production of reactive oxygen species, also can give damage on cell structures and alter their functions, which can result in oxidative stress. As the rise of oxidative stress lead to many pathological conditions and diseases, including diabetes, neurological disorders, cancer, atherosclerosis, acute respiratory distress syndrome, and so on [43]. Control of oxidative stress is attaining by antioxidative systems. The defense mechanism is involving secondary metabolites, especially in plants [28]. Mutualistic relation between endophytes microbes and plants improve the host plant defense responses toward various biotic also abiotic stresses [25-28]. For this reason, the activities of antioxidant that resulted from mangrove endophytic microbes may come from their response to various abiotic stresses like temperature, salt, drought, and biotic stresses caused by pathogens [26-29].

## 4. Conclusion

In conclusion, this research succeeded in isolating six fungi and three bacteria as endophytic microbes from two mangrove species, namely *A. marina* and *X. granatum*. The yield of ethanol extracts from different parts of mangrove and shown by a sample of leaves of *A. marina*. On the other hand, the highest yield of a secondary metabolite by ethyl acetate extraction was obtained from bacteria from fruits of *A. marina*. For antioxidant activities, the highest activities were shown by fungi from the stems and roots of *A. marina*, also fungi from the leaves of *X. granatum*. We conclude that endophytic microbes, especially fungi, are a potential source for an antioxidant agent. For further research, the bioactivity of resulting extracts by profiling extracted secondary metabolites should be explored. Moreover, the identification of endophytic fungi is needed to give more information for the next steps of bioproduction in the near future.

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