Antibacterial activity of fungi endophytic isolated from leaves the mangrove *Acanthus ilicifolius* L

Nur Arfa Yanti1*, Jamili1, Ardiansyah1 and Syaiful Anwarrudin1

1 Department of Biology, Faculty of Mathematic and Natural Science, Halu Oleo University Indonesia. 93232

nur.yanti@uho.ac.id (corresponding author)

Abstract. Endophytic fungi are able to synthesize secondary metabolites that act as antimicrobial compounds to protect the host plant from pathogenic microbes. In the present study, endophytic fungi isolated from leaves of *Acanthus ilicifolius* was characterized and their antibacterial activity was assessed. Characterization of endophytic fungi isolates was carried out based on morphological characteristics of macroscopic (colony) and microscopic. Antibacterial activity test was conducted by well diffusion method using 2 bacterial indicators, namely *Escherichia coli* (Gram negative bacteria) and *Staphylococcus aureus* (Gram positive bacteria). Six endophytic fungi were isolated from *A. ilicifolius* leaves and 5 isolates among them had antibacterial activity, namely isolates DT1, DT3, DT4, DT6 and DST1 with broad-spectrum activity. Five endophytic fungi identified belonged to 3 genera; DT1 and DT4 are identical to the Genus *Geotricum*, DST1 and DT6 are identical to the Genus *Humicola* and DT3 is identical to the Genus *Aspergillus*. This study demonstrates an interesting bottom-up approach to the discovery of new antibacterial compounds.

1. Introduction

*Acanthus ilicifolius* L. is one of the mangroves that has been used by coastal communities as traditional medicine including the bark, flowers, fruit and leaves. The Leaves extract of *A. ilicifolius* is used as a medicine for diarrhea. The leaves of the *A. ilicifolius* contain bioactive compounds including saponins, flavonoids, alkaloids and phenols as antibacterial agents [1,2]. Singh & Aeri [1] reported that n-hexane and chloroform extract of the *A. ilicifolius* leaves has inhibitory activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Proteus vulgaris*.

Intake of bioactive compounds from leaf extracts requires a lot of biomass and it is destructive [3]. The solution of these problems is by taking bioactive compounds from symbiotic organisms such as endophytic fungi [3,4]. Endophytic fungi naturally live and form colonies without endangering the host [4].

Endophytic fungi is all fungus inhabiting plant organs that at some time in their life can colonize internal plant tissues without causing apparent harm to the host [4]. Hilarinoet al. [5] states that the distribution of endophytic fungi on leaves is different for each age of the leaf and plant species. Endophytic fungi will synthesize secondary metabolites that are the same as the host as an antimicrobial agent to protect plants from pathogenic microbes [3]. Several previous studies on endophytic fungi from mangrove plants that have potential as antibacterial activity, include *Avicennia marina* [6], *Rhizophoraracemosa* [7], *Avicennia* sp. [8], *Sesbaniagrandifolia* [9], *Acanthus ilicifolius* [10], and *Sonneratiagriffithii*[11]. *A. ilicifolius* leaves contain bioactive compound so that the opportunity of finding endophytic fungi with high bioactivities is possible. Therefore, the present study explored endophytic fungi in mangrove *A. ilicifolius* leaves to evaluate their antibacterial potential.
2. Methods

2.1. Leaves sample collection and Surface Sterilization
Leaves of mangrove Acanthus ilicifolius were collected from Kendari bay mangrove forest in Southeast Sulawesi, Indonesia. Mangrove leaves were used as samples were young, mature and old leaves that are intact and in good condition. Determination of young, mature and old leaves, based on the location of the leaves sitting on the stem. Young leaves are located in the second leaf of the shoot, mature leaves are located in the middle, namely in the sixth leaf of the shoot, while the old leaves are located at the base of the branches and the leaves have yellowed. Leaves were washed in running tap water and were surface sterilized by dipping in 70% ethanol for 1 min., NaOCl 5.3% for 5 min, followed by rinsing in sterile distilled water for 2 min[11]. The surface sterilization procedure was validated by imprinting the surface sterilized plant part onto nutrient media and was maintained as control.

2.2. Isolation of Endophytic Fungi
Each sterilized Leaf of A. ilicifolius, was cut into approximately 1 cm squares and placed on petri plates containing potato dextrose agar medium (PDA). Then it was monitored every day for growth of endophytic fungal colonies. Fungi growing out from the samples were subsequently transferred on to fresh PDA plates.

2.3. Preparation of the indicator bacteria
The indicator bacteria were used in this study, included the Gram-negative bacteria, Escherichia coli ATCC 35218 and Gram-positive bacteria, Staphylococcus aureus ATCC 25923. Indicator bacteria were made a suspension in 0.9% NaCl solution, by means of a loopfull bacterial colony was diluted in 0.9% NaCl solution with the help of inoculation loop and then homogenized. Turbidity of bacterial suspension was compared with McFarland 0.5 standard (density 1.5 x 10^8 cfu/mL) on a black and white background and bright light.

2.4. Preparation of Crude Extract of Endophytic Fungal
Fungal endophytes growing out from the cut ends were isolated and sub cultured. Pure cultures were prepared from isolates with different morphology. These isolates were mass cultured in 10 ml potato dextrose broth at 28°C for 7-10 days in a rotary shaker. Mycelia were separated by filtering using whatman No.1 filter paper. The supernatants of endophytic fungi were checked for antibacterial activity.

2.5. Antibacterial Assay
The agar well diffusion method was used to evaluate antimicrobial activity of the supernatans of the endophytic fungi. An initial volume of 50 ul crude extract was transferred to wells bored on Nutrient Agar plates inoculated with different bacterial pathogens. After an incubation period of 24 hrs the diameter of zone of inhibition was noted. Sterilized aquadest was used as negative control and Streptomycin was used as positive control.

2.6. Characterization of Endophytic Fungi
The characterization of fungi was done using cultural and microscopic characteristics such as shape, colour and pattern arrangement of mycelium. Microscopic characteristics include types of spores, types of hyphae and conidial arrangement. Microscopic characteristics of endophytic fungi were observed using slide culture technique. Obtained data were then compared with the descriptions of fungi species in the identification of tropical mold by Gandjar et al. (in Indonesian) [12], Samson et al. [13] and Pitt & Hocking [14] and matches were recorded [15, 16, 17].
3. Result and Discussion

3.1. Endophytic Fungi Isolate from Acanthus ilicifolius Leaves
A total 6 endophytic fungi were isolated from leaves of mangrove Acanthus ilicifolius and 5 isolates showed antibacterial activities. Within each leaves, 4 (66,67%) active isolates out of 5 isolates were cultured from old leaves and 1 (16,67%) active isolates from mature leaves, but no fungi isolate from young leaves (Fig. 1). Hilarino et al. [5] and Gonzales et al. [18] also reported that endophytic fungi were found more in the old leaves than younger leaves.

Figure 1 showed that endophytic fungi are predominantly obtained from old leaves. This is due to the fact that in old leaves many cells have died, so the fungi can get food by degrading organic compounds from the dead cells. Several studies have shown that old leaves support more endophytes than younger leaves [5,18]. Hilarino et al. [5] explained that dead cells on old leaves produce organic material that supports fungal growth.

3.2. Antibacterial Activity
Five Fungal isolates from mangrove A. ilicifolius were active against 2 indicator bacterial, namely Gram positive bacteria Staphylococcus aureus and Gram negative bacteria Escherichia coli. The ability of endophytic fungi isolates to inhibit both Gram positive and Gram negative bacteria indicates that fungal isolates have a broad-spectrum of antibiotics. Acar [19] states that antibiotics can inhibit the growth of Gram-positive and Gram-negative bacteria are broad-spectrum antibiotics.

Table 1. Antibacterial activities of endophytic fungi from A. ilicifolius leaves

| Endophytic fungi isolates | Staphylococcus aureus | Escherichia coli |
|--------------------------|-----------------------|-----------------|
| DT1                      | 11,72                 | 10,87           |
| DT3                      | 13,70                 | 12,70           |
| DT4                      | 17,07                 | 15,97           |
| DT6                      | 15,15                 | 15,67           |
| DST1                     | 12,65                 | 13,12           |
| Positive control          | 18,67                 | 18,47           |
The antibacterial activity is expressed by the diameter (d) of inhibition zone (mm).

Control positive is streptomycin, DT is fungal isolate from old leaves, DST fungal isolate from mature leaves.

Table 1 shows that DT4 isolate showed the highest antibacterial activity on *S. aureus* (17.07/18.67 mm for positive control) and *E. coli* (15.97/18.47 mm for positive control) whereas the lowest activity was recorded by DT1 isolate on *S. aureus* and *E. coli* (11.72/18.67 mm and 10.87/18.47 mm, respectively). The results also showed that the Gram-positive bacteria (*S. aureus*) were more sensitive than Gram-negative bacteria (*E. coli*) from endophytic fungi activities. Shebany [6] reported that Gram positive bacteria was more sensitive than Gram negative bacteria from endophytic fungi activities. The highest sensitivity of Gram positive bacteria may be due to its cell wall structure and outer membrane [20,21,22]. A possible explanation for this observation may be due to presence of an outer membrane and a unique periplasmic space in Gram negative and not found in Gram-positive bacteria [23].

3.3. Morphological characteristics of endophytic fungi

Morphological characteristics of endophytic fungi observed included macroscopic and microscopic characteristics. Observation of macroscopic characteristics endophytic fungi isolates by studying their colony performance on the media PDA and microscopic characteristics was done using the slide culture technique. The identification of fungal strains was done under the basis of macroscopic (colonies) and microscopic observations of cultural characters (Figure 2).

![Figure 2. Morphotypic characteristics of endophytic fungi isolates by colony performance on media PDA at 7 days incubation (a) and light microscopic photographs 400x (b).](image)

Macroscopic characterization of colony such as colour, diameter, colony growth, colony reverse. The observation was done for seven days during the fungal culturing. DT1 isolate has a colony of
obverse and reverse are white with orange in the center colony, colony diameter is 84 mm and fastest growing. DT3 isolate has a colony of obverse and reverse are white, colony diameter is 79 mm and fastest growing. DT4 isolate has a obverse colony is white but reverse colony is white and yellowish in the center colony, colony diameter is 73 mm and fastest growing. DT6 and DST1 isolates have a obverse colony are white and the reverse colony are white with black in the center colony, colony diameter are 54 mm and 31 mm, respectively and slow growth rate.

Microscopic characterization was done by observing shape of conidia, and hyphae. Observation of conidia including its arrangements (singular, chain or cluster). Observation of hyphae was also performed on the presence or absence of septa in hypha and hypha’s pigment (hyaline/non pigmented). DT1 and DT4 isolates have a cylindrical conidia and arrangement are cluster, their hyphae are septate and hyaline. DT3 isolate has a globular conidia and arrangement is cluster, its hyphae is septate and hyaline. DT6 and DST1 isolates have a oval conidia and arrangement are chain, their hyphae are septate and hyaline.

Identification of fungi can be done conventionally by observing the morphological characters and compare it to descriptions from the literature or monograph. Based on the descriptions of endophytic fungi isolates and refer to Gandjar et al. [12], Samson et al. [13] and Pitt & Hocking [14], it is known that the DT1 and DT4 isolates are a group of genus Geotrichum, DT3 isolate is a group of Aspergillus whereas DT6 and DST1 isolates are a group of Humicola.

Some species of endophytic fungi that have been identified are also found as endophytic fungi in the other plants. Jariwala & Desai [16] reported that several Geotrichum species found as endophytic fungi in the leaves of medicine plants. Ariole&Akinduyite [7] also isolated Geotrichum species from leaves of red mangrove (Rhizophora racemose). Several studies have reported that species belonging to the genus Aspergillus are endophytic fungi in the roots of the mangrove plant Avicennia marina [6], in mangrove Avicennia sp. [8], in the mangrove plant Sonneratiaagriffithii Kurz [11], in the leaves of medicinal plants [16] and in the leaves of Acanthus ilicifolius [24]. Endophytic fungi members of genus Humicola was found in the leaves of Calopogonium mucunoides [15] and in the stem of Elaeocarpusphaericus [25]. Several previous studies reported that strains belonging to the genera Geotrichum, Aspergillus and Humicola are endophytic fungi that have antibacterial activity [7,8,11,26].

4. Conclusion
Based on research conducted, it can be concluded that endophytic fungi derived from leaves of mangrove Acanthus ilicifolius are a source of antibiotic compound. The endophytic fungi from leaves A. ilicifolius are members of genera Geotrichum, Aspergillus and Humicola.

Acknowledgment
This work was financially supported by the Directorate General of Research and Development of the Ministry of Research, Technology, and Higher Education in project of “Hibah penelitian Dasar Unggulan Perguruan Tinggi” 2019.

References
[1] Singh, D. and Aeri, V. 2013. Phytochemical and pharmacological potential of Acanthus ilicifolius J Pharm Bioallied Sci. 5(1): 17–20.
[2] Saranya A, Ramanathan T, Kesavanarayanan KS. and Adam A. 2015. Traditional Medicinal Uses, Chemical Constituents and Biological Activities of a Mangrove Plant, Acanthus ilicifolius Linn. : A Brief Review, American-Eurasian J. Agric. & Environ. Sci., 15 (2): 243-250.
[3] Selim, KA, El-Beih, A.A, AbdEl-Rahman, TM, and El-Diwany, AI. 2012. Biology of endophytic fungi. Curr. Res. Environ. Appl. Mycol. 2(1):31–82.
[4] Padhy L, Mohanta YK and Panda SK. 2013. Endphytic Fungi with Promises : A Review. Journal of Advanced Pharmacy Education & Research3 (3) : 152-170.
[5] Hilarino, MPA, Silveira, FAO, Oki Y, Rodrigues L, Santos JC, Junior, AC, Fernandes GW and Rosa CA. 2011. Distribution of the endophytic fungi community in leaves of Bauhinia brevipes (Fabaceae). Acta Botanica Brasiliaca 25(4): 815-821.

[6] Shebany, YM. 2012. Antimicrobial Activity of Endophytic Fungi Isolated from Avicennia marina Plant, Red Sea, Egypt. Egypt. J. Microbiol. 47: 141-152.

[7] Ariole CN., Akinduyite AE. 2016. Antibacterial potential of indigenous red mangrove (Rhizophora racemosa) fungal endophytes and bioactive compounds identification, International Journal of Microbiology and Mycology 4 (4): 12-24.

[8] Ling OM, Teen LP, Mujahid A, Proksch P. & Muller M. 2016. Initial Screening of Mangrove Endophytic Fungi for Antimicrobial Compounds and Heavy Metal Biosorption Potential, Sains Malaysiana 45 (7): 1063–1071.

[9] Powthong, P., Jantrapanukorn B., Tongmee A., dan Suntornthiticharoen, 2013, Screening of Antimicrobial Activities of the Endophytic Fungi Isolated from Sesbania grandiflora (L.) Pers, Journal Agricultural Sciences Technology, 1(5): 1513-1522.

[10] Chi WC, Pang KL, Chen WL, Wang GJ. and Lee TH. 2019. Antimicrobial and iNOS inhibitory activities of the endophytic fungi isolated from the mangrove plant Acanthus ilicifolius var. xiamenensis, Botanical studies 60 (4) : 1-8.

[11] Handayani D, Rivai H, Hutabarat M, Rasyid R. 2017. Antibacterial activity of endophytic fungi isolated from mango tree Sonneratia griffithii Kurz. J Appl Pharm Sci 7 (4): 209–212.

[12] Gandjar I, Samson RA, Tweel-Vermeulen KV, Oetari, A dan Santoso, I. 1999. Pengenalan kapang tropik umum, Yayasan Obor Indonesia, Jakarta. (in Indonesian).

[13] Samson, R.A., Hoekstra, A.S., Van Oorschot, C.A. 1984. Introduction to Food-Borne Fungi, Second. ed. Institute of The Royal Netherlands, Netherlands.

[14] Pitt, J.I., Hocking, A.D., 1985. Fungi and Food Spoilage, Food Science and Technology. Academic Press, Australia.

[15] Fitriarni D. and Kasiamdari RS. 2018. Isolation and Identification of Endophytic Fungi from Leave and Stem of Calopogonium mucunoides, J. Trop. Biodiv. Biotech. 3 : 30—36.

[16] Jariwala B. and Desai B. 2018. Isolation and Identification of Endophytic Fungi from Various Medicinal Plants. Bmr Microbiology, 4(1). 1-7.

[17] Shebany YM, El-Magraby OMO, Abdel Wahab MA., 2014. Isolation and Identification of Endophytic Fungi from Leaves and Roots of Althea rosea. International Journal of Life Science Res. 2, 48–57.

[18] Gonzales RCL, Cornelio SG, Garcia SCD, Garrido E, Mariano OO, Heil M, Martinez LPP. 2017. The age of lime bean leaves influences the richness and diversity of the endophytic fungal community, but not the antagonistic effect of endophytes against Colletotrichum lindemuthianum, Fungal Ecology 26 : 1-10.

[19] Acar J. 1997. Broad- and narrow-spectrum antibiotics: an unhelpful categorization, Clinical Microbiology and Infection. 3 (4): 395-396.

[20] Ceylan E. and Fung DYC. 2004. Antimicrobial activity of spices. Journal of Rapid Methods and Automation in Microbiology, 12 : 1- 55.

[21] Lopez, P., Sanchez, C., Batlle, R. and Nerin, C. 2005. Solid and vapor-phase antimicrobial activities of six essential oils: susceptibility of selected foodborne bacterial and fungal strains. Journal of Agricultural and Food Chemistry, 53 : 6939-6946.

[22] Kohanski MA, Dwyer DJ & Collins JJ. 2010. How antibiotics kill bacteria: from targets to networks, Nature Reviews Microbiology 8 (6): 423–435.

[23] Duffy, C.F. and Power, R.F. 2001. Antioxidant and antimicrobial properties of some Chinese plant extracts, International Journal of Antimicrobial Agents, 17 : 527- 529.

[24] Priyadharsini R, Ambikapathi V. and Panneerselvam A. 2015. Isolation and Identification of Endophytic Fungi from Acanthus ilicifolius in Muthupet Mangroves, Botany 4 (9) : 169-171.

[25] Shukla AK, Yongam Y. and Tripathi P. 2012. Distribution of Endophytic Fungi in Different Parts of Rudraksh (Elaeocarpus sphaericus) Plants, Microbes: Diversity and Biotechnology
[26] Gong LJ. and Guo SX. 2009. Endophytic fungi from Dracaena cambodiana and Aquilaria sinensis and their antimicrobial activity. African Journal of Biotechnology 8 (5) : 731-736.