Isolation and Identification of *Acanthus ilicifolius*-Associated Fungi and Investigation of Their Antibacterial Activity

Farras Daffa Imtiyaz[1], Ferisa Lestari Nugrahayu[1], Kawidian Putri Bayu Alam[2], Insan Shiddiq[1] and Hermin Pancasakti Kusumaningrum[1]

[1]*Department of Biology, Faculty of Science and Mathematics, Diponegoro University, Semarang, Indonesia*
[2] *Faculty of Public Health, Diponegoro University, Semarang, Indonesia*

Keywords: *Acanthus ilicifolius*-Associated Fungi, Antibacterial, Bioactive compounds, Biopharmaceutical

Abstract: Antibacterial resistance is one of the biggest threats to the global health security today. Therefore, there is an urgency to find alternative treatment to combat the bacteria. This paper aimed to identify and assess the biological activity of *Acanthus ilicifolius* endophytic fungi, a medicinal plant originated from Asia used in ancient medicine. In this experiment, two strains of bacterial were used, namely *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The tree samples were obtained from Jepara, Indonesia. Surface sterilization and purification were applied to isolate the fungal. Moreover, their antibacterial ability was tested using agar plug diffusion method. The study found that three isolates were broadly active against two types of pathogenic bacteria; one of them was AB1-isolate. In the identification of the colony, AB1-fungal have both black stain in the basal part and the outside layer, with rough structure and planted strongly to the basal medium. The other fungal isolates, namely AD1 and AB2, showed a bacteriostatic effect against bacterial test as well. Our results suggested that AB1-isolate, obtained from the stems, shows a good prospect to be a pharmaceutical candidate to inhibit bacterial growth that normally present in the infection cases.

1 INTRODUCTION

Endophyte associates each other with the host plant to protect them from pathogens (Vasundhara *et al*., 2016); and the majorities are fungi (Strobel and Daisy, 2003). Endophytic fungi belong to mitosporic and meiosporic ascomycetes that reside beneath the epidermal cell layer without causing damages. These microorganisms are important sources of natural products with pharmaceutical potential (Porras-Alvaro and Bayman, 2011). They contain more than 40% of new chemical compounds from microorganisms (Sieber and Marahiel, 2005) and have wide-ranging applications (Zhao *et al*., 2011).

Acanthus ilicifolius is a type of mangrove plant in Central Java, Indonesia, used as a traditional medicine due to their abilities. Moreover, the plant has anti-inflammatory, antioxidant and antimicrobial activities. Those abilities are affected by microorganisms beneath the plant tissues (Faeth and Fagan, 2002). Since the plants are directly exploited, the wild *A. ilicifolius* will quickly decrease, therefore, the preferred technique to take the plant's pharmacological abilities is by exploring its fungal endophyte beneath the tissues. Fungal can be grown in the laboratory, enable us to utilize them without exploiting the plant directly. In this paper, we were exploring all the endophytic fungi ability to combat two bacteria.

2 METHODS

2.1 Sample Area and Process of Collecting

Acanthus ilicifolius samples were collected from the growing areas named Mecok. The area is exposed to ocean spray, mists and tides along Teluk Awur coast, Jepara, Indonesia (S 6°37′7.86″, W 110°38′41.16″). Plant species found in the ground were preferentially selected. Multiple numbers of samples (n = 20) from *Acanthus ilicifolius*, locally recognized as *jeruju*, were washed using tap running water to remove the dust and dirt. Samples were

Imtiyaz, F., Nugrahayu, F., Alam, K., Shiddiq, I. and Sari, H.
Isolation and Identification of Acanthus ilicifolius-Associated Fungi and Investigation of Their Antibacterial Activity.
DOI: 10.5220/0007545504840488
In *Proceedings of the 2nd International Conference Postgraduate School (ICPS 2018)*, pages 484-488
ISBN: 978-989-758-348-3

stored in the airless polythene bags within a cooler bag until processing.

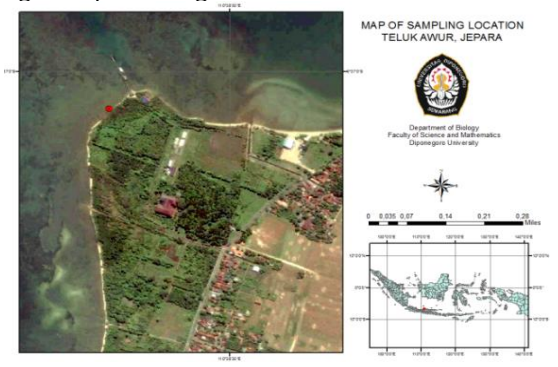

Figure 1: Map of the sampling area

2.2 Isolation and Identification

Endophytic fungi were isolated as described previously (Ezra *et al*., 2004) with minor modification. After sampling, samples were rinsed by tap water to remove soil or dust in a beaker glass containing distilled water. Stems, roots and leaves surface were sterilized and impregnated by 90% ethanol for 1 minute, 5% sodium hypochlorite for 1 minute, sterile double distilled water (DDW) for 1 minute, 70% ethanol for 1 minute, and a quick rinse in sterile DDW. The Potato Dextrose Agar (PDA) media (Becton, Dickinson & Co; Sparks, MD) was diluted by seawater in the Erlenmeyer glass. The media was autoclaved at 121^{O} C for 15 minutes, and then mixed with 50 mg of Chloramphenicol in order to suppress the epiphyte bacteria.

The outer tissues of the plant samples were removed after drying the tissues under sterile laminar airflow and passing through the flame. The internal tissues were cut into pieces of 0.5 inch and plated on petri dishes containing PDA media that had been made. Each petri dish was divided into four segments, with each segment containing plant samples from the same organ. The plates were sealed and incubated at room temperature and examined for emerging fungi every 2 - 3 days. As fungi emerged, they were transferred to the new PDA plates.

The fungi were identified based on characteristics according to the methods described by Kong and Qi (1985). Colony descriptions were based on observations on PDA under ambient daylight conditions after 4–7 days of incubation. Identification was performed according to the taxonomic key, including colony diameter, texture and colour.

2.3 Agar Plug Diffusion Method

Strains of bacteria species used to test antibacterial activity of isolated fungi were *Staphylococcus aureus* and *Pseudomonas aeruginosa*. These bacterial strains were taken from Kariyadi Hospital, Indonesia. The bacterial inoculum was prepared by mixing a few bacterial colonies (1 mL) with 9 mL of sterile 1.5 % NaCl and compared the turbidity with the standard 0.5 McFarland solution. The sterile swab was dipped properly into the mix solutions to adjust the inoculum. In order to decrease the liquid, the swab was pressed with gentle rotation to the inner surface of the test tube. To obtain a perfect growth, the entire Muller Hinton Agar (MHA) media (Merck) surface was swabbed uniformly. The inoculated plates were stored at room temperature for 3-5 minutes.

The antibacterial test used agar plug diffusion method proposed by Elleuch *et al.* (2010). In this method, the fungal inoculum was prepared as that of disc diffusion method. The fungal mycelia were cultured in PDA for 7 days at temperature of 30^{o}C. After the fungal growth, the wells were prepared on the plate by using sterile corks borer. The agar plugs were transferred carefully to MHA media which were already cultivated by bacterial test and allowed to diffuse for 2 hours. The cultures were incubated at 37^{o}C for 24-48 hours, and the plate was examined for the presence of inhibition zones next to the fungal plugs. After 24 hours of incubation, the inhibition zone around each well was recorded.

3 RESULTS

Twelve segments from three parts of the plant were used to obtain the endophyte. A total of eight fungi isolates were obtained in the first screening, but only four isolates were successfully re-cultivated. The sampling type and the total number of isolates are summarized in Table 1. Those fungi belong to four genera of filamentous fungi. They are *Fusarium* sp. (Putih), *Ulocladium* sp. (AB1), *Cylindrocarpon* sp. (AD1), and *Acremonium* sp. (AB2).

Table 1: Quantity of fungi obtained from different part.

Sampling Types	Segments	Isolates
Leaves	4	1
Roots	4	1
Stems	4	1

The isolated strains were cultivated in PDA for eight days in the ambient of light. We performed observation on aerial mycelium and substrate mycelium, as mycelium serves as the key of fungi identification, including the colours and the form of it. Morphological observation revealed that both aerial and vegetative hyphae were abundant, well developed, and had a varied characteristics of mycelium. The colour of aerial and substrate mycelium of most strains varied from white to black.

Several isolated fungi inhibited at least one bacterial test with agar plug diffusion method and created inhibition zones (Table 2). Among the fungal endophyte, "putih" strain did not exhibit any inhibited zone to the bacteria in the MHA medium. The greatest antibacterial activity was shown by AB1 isolate.

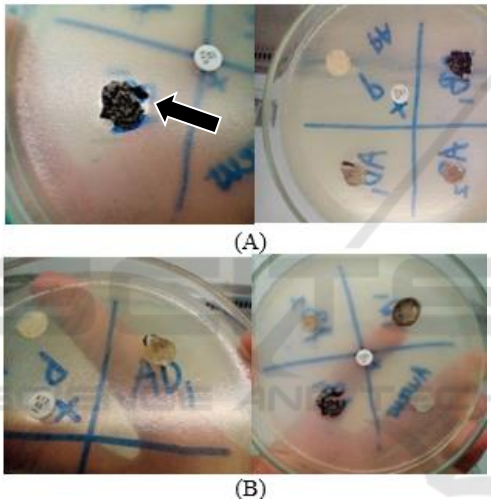

Figure 2: Antibacterial test to (a) *Staphylococcus aureus* and (b) *Pseudomonas aeruginosa*.

Table 2: Antibacterial activity of endophytic fungi isolates

Isolates	*P. aeruginosa*	*S. aureus*
AB2	+	+
AB1	+	+
AD1	+	-
Putih	-	-
Chloramphinacol	+	+

Notes: + = Positive and - = Negative

4 DISCUSSION

Mangrove is one of the vital ecosystems in the world. In addition to becoming a home to various organisms, it plays an important role as the source of medicinal plants. The bioactive compound of *Acanthus* species has been studied expansively. However, there is still limited information regarding endophytic fungi derived from the plant. Two previous studies limitedly explained about the ability of *Aspergillus flavipes* from *Acanthus* (Bai *et al.*, 2014) and the endophyte diversity (Ananda and Sridhar, 2002).

In this article, four endophytic fungi derived from *A. ilicifolius* plant were isolated and investigated for their antibacterial activity. However, only three fungi showed antibacterial activity against the bacterial test. AD1 did not show antibacterial activity against *Staphylococcus aureus*, whereas AB1 and AB2 show antibacterial activities. The inhibitory activities of those two fungi were higher than the chloramphenicol as the positive control. The fungal plugs "Putih" did not show any antibacterial activity. This observation was also reported in several studies, in which endophytic fungi showed activity against bacteria (Santos *et al.*, 2015). The potency of the fungi is attributed to possible contribution of the type of media and culture condition in the biosynthesis of active metabolites (De Siqueira *et al.*, 2011; Dos Santos *et al.*, 2015). The antibacterial activity exhibited by the A. *ilicifolius* endophyte can be said to be relevant with the leaf activity against *S. aureus* and *P. aeruginosa* (Govindasamy and Arulpriya, 2013).

Acremonium sp. in this study showed antibacterial activity against two bacterial test. It was relevant to the other *Acremonium* species obtained from marine sediment (Samuel *et al.*, 2011) and *Antiaris toxicaria* organism (Dai *et al.*, 2009). They have been reported to be a source of diverse antibacterial compounds. Efrapeptin G and RHM1, isolated from *Acremonium* sp. 021172C associating with sponge *Axinella* sp, was reported to have a potent antibacterial activity (Boot *et al.*, 2006). *Acremonium* sp. endophyte of *Taxus baccata* was the source of leuesnostatin A which exhibited effectiveness against *Pythium ultimum* (Yu *et al.*, 2010). Moreover, amides of the corresponding amino alcohols and amino aldehydes of D-allo- and L-isoleucine of an intertidal *Acremonium furcatum* can inhibit *B. subtilis*, *S. aureus* and *E. coli* (Gallardo *et al.*, 2006). Moderate inhibitory activity against *S. aureus* was exhibited by the 19-O-Methyl-pyrrocidine B, some pyridine alkaloids isolated from *Cylindrocarpon*, which is an endophytic fungus of *Sapium ellipticum*, with 25 μM MIC (Ramsay *et al.*, 2018).

Ulocladium sp. is the typical pathogenic fungi that may be responsible for Allergic fungal sinusitis and nasal polyposis (Kaur *et al.*, 2010). However,

the fungal compounds exhibit various abilities for medical benefits and form the inhibitory zone around the fungal plates. Ophiobolin T, isolated from *Ulocladium sp.* associating with *Everniastrum sp.*, showed moderate antibacterial activity against *B. subtilis* and MRSA (Wang *et al.*, 2013). *Ulocladium botrytis* from *Callyspongia vaginalis* sponge produces ulocladol, a new tyrosine kinase inhibitor and antimicrobial, to inhibit *Myxilla incrustans* (Holler *et al*., 1999). Surprisingly, 1-hydroxy-6-methyl-8-(hydroxyl methyl) from the fungi has antifungal activity (Konig *et al*., 2005).

5 CONCLUSIONS

Marine environment is proven to provide sources of antibacterial compounds, particularly produced by fungi. A total of 4 endophytic fungi isolated from *Acanthus ilicifolius* showed different activities against 2 bacteria. However, in order to find the most effective compounds, further studies are needed to obtain comprehensive data regarding those antibacterial activities.

REFERENCES

Ananda, K and Sridhar, KR. 2002. Diversity of endophytic fungi in the roots of mangrove species on the west coast of India. *Can J Microbiol*. 48(10): 871-78.

Bai, ZQ., Lin, X., Wang, Y., Wang, J., Zhou, X., Yang, B., Liu, J., Yang, X., Wang, Y., and Liu, Y. 2014. New phenyl derivatives from endophytic fungus *Aspergillus flavipes* AIL8 derived of mangrove plant *Acanthus ilicifolius*. *Fitoterapia*. 95:194-202.

Boot, CM., Tenney, K., Valeriote, FA., and Crews, P. 2006. Highly N-Methylated Linear Peptides Produced by an Atypical Sponge-Derived *Acremonium* sp. *J Nat Prod*. 69(1): 83-92.

Dai, H., Gan, Y., Que, D., Wu, J., Wen, Z., and Mei, W. 2009. A new cytotoxic 19-Nor-cardenolide from the latex of *Antiaris toxicaria*. *Molecules*. 14: 3694-3699.

De Siqueira, VM., Conti, R., De Araújo, JM., and Souza-Motta, CM. 2011. Endophytic fungi from the medicinal plant *Lippia sidoides* Cham. and their antimicrobial activity. *Symbiosis*. 53(2):89-95.

Dos Santos, IP., da Silva, LC., da Silva, MV., de Araújo, JM., Cavalcanti, MS., and Lima, VL. 2015. Antibacterial activity of endophytic fungi from leaves of *Indigofera suffruticosa* Miller (Fabaceae). *Front Microbiol*. 6:350.

Elleuch, L., Shaaban, M., Smaoui, S., Karray-Rebai I., Fourati-Ben, FL., Shaaban, KA., and Laatsch, H. 2010. Bioactive secondary metabolites from a new terrestrial *Streptomyces* sp. TN262. *Appl Biochem Biotechnol*. 162(2): 579–593.

Ezra, D., Hess, WH., and Strobel, GA. 2004. New endophytic isolates of *Muscodor albus*, a volatile-antibiotic-producing fungus. *Microbiol*. 150:4023–31.

Faeth, SH and Fagan, WF. 2002. Fungal endophytes: common host plant symbionts but uncommon mutualists. *Integr Comp Biol*. 42(2): 360–368.

Gallardo, GL., Butler, M., Gallo, ML., Rodriguez, MA., Eberlin, MN., and Cabrera, GM. 2006. Antimicrobial metabolites produced by an intertidal *Acremonium furcatum*. *Phytochemistry*. 67(21):2403–10.

Govindasamy, C and Arulpriya, M. 2013. Antimicrobial Activity of *Acanthus Ilicifolius*: Skin Infection Pathogens. *Asian Pac J Trop Dis*. 3(3): 180–183.

Holler, U., Konig, G., and Wright, AD. 1999. A new tyrosine kinase inhibitor from marine isolate of *Ulocladium botrytis* and new metabolites from the marine fungi *asteromyces cruciatus* and *varicosporina ramulosa*. *Eur j org chem*. 11: 2949-2955.

Kaur, R., Wadhwa, A., Gulati, A., Agrawal, A. 2010. An unusual phaeoid fungi: *Ulocladium*, as a cause of chronic allergic fungal sinusitis. *Iran J Microbiol*. 2(2):95-97.

Kong, H and Qi, Z. 1985. Some new records and rare taxa of *Aspergillus* of China. *Bulletin of Botanical Research*. 5(3):147–152.

Konig, GM., Kehraus, S., Seibert, SF., Abdel-lateff, A., and Muller, D. 2005. Natural Product from Marine Organisms and Their Associated Microbes. *Chembiochem*. 7(2):229-238.

Porras-Alfaro, A and Bayman, P. 2011. Hidden Fungi, Emergent Properties: Endophytes and Microbiomes. *Annu Rev Phytopathol*. 49:291–315.

Ramsay, STK., Wafo, P., Nidja, R., Lasse, VG., Simon-Patrick, H., Tim-Oliver, K., Christoph, J., Parichat, S., Matthias, UK., Wenhan, L., Rainer, K., Zhen, L., and Peter, P. 2018. Metabolites from the endophytic fungus *Cylindrocarpon* sp. isolated from tropical plant *Sapium ellipticum*. *Fitoterapia*. 128: 175–179.

Samuel, P., Prince, L., and Prabakaran, P. 2011. Antibacterial Activity of Marine derived Fungi Collected from South East Coast of Tamilnadu, India. *J Microbiol Biotech Res*. 1(4):86-94.

Santos, IP., Silva, LCN., Silva, MV., Araújo, JM., Cavalcanti, MS., and Lima, VLM. 2015. Antibacterial activity of endophytic fungi from leaves of *Indigofera suffruticosa* Miller (Fabaceae). *Front Microbiol*. 6:350.

Sieber, SA and Marahiel, MA. 2005. Molecular Mechanisms Underlying Nonribosomal Peptide Synthesis: Approaches to New Antibiotics. *Chem Rev*. 105(2):715–738.

Strobel, G and Daisy, B. 2003. Bioprospecting for microbial endophytes and their natural products. *Microbiol Mol Biol Rev*. 67(4):491–502.

Vasundhara, M., Kumar, A., and Reddy, MS. 2016. Molecular Approaches to Screen Bioactive Compounds from Endophytic Fungi. *Front Microbiol*. 7:1774.

Wang, QX., Bao, L., Yang, XL., Liu, DL., Guo, H., Dai, HQ., Song, FH., Zhang, LX., Guo, LD., Li, SJ., Liu, HW. 2013. Ophiobolins P-T, five new cytotoxic and antibacterial sesterterpenes from the endolichenic fungus *Ulocladium* sp. *Fitoterapia*. 90:220–7.

Yu H., Zhang L., Li L., Zheng C., Guo L., Li W., Sun P., Qin L. 2010. Recent developments and future prospects of antimicrobial metabolites produced endophytes. *Microbiol Res.* 165(6):437-449.

Zhao, J., Shan, T., Mou, M., and Zhou, L. 2011. Plant-derived bioactive compounds produced by endophytic fungi. *Mini Rev Med Chem.* 11(2):159–168.