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Endophytic Fungi from Mangrove Plant *Acanthus ilicifolius* L.: Antimicrobial, Anticancer, and Species Determination

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Abstract

The current study aimed to screen, identify and evaluate the biological activity of fungi strains from the mangrove plant *Acanthus ilicifolius* L, which has proven to be a rich source of fungal species. Thirteen fungi were screened from the leaves, stem bark, and roots. Each fungal strain was cultivated in rice as media, and then the secondary metabolites of the fungus were extracted with ethyl acetate. The extracts were tested for antimicrobial and cytotoxic activities. Next, a phylogenetic tree was reconstructed based on the sequence similarities of each strain. The fungal extract AIA1 displayed excellent antimicrobial activity in a concentration of 5 % against some pathogenic bacterial strains with an inhibition zone in the range of 11.87 ± 0.87 to 15.42 ± 0.51 mm. The brine shrimp lethality test (BSLT) showed that strains AIA1 and AIKB3 were toxic against *Artemisia salina* L with an LC_{50} value of less than 80 ppm. The MTT assay determined the cytotoxicity against the human ductal breast epithelial tumor (T47D) cell line. Based on molecular identification, AIA1, AIKB3, and AID1 showed maximum similarity (> 95 %) with *Pestalotiopsis* sp. AIA1, *Trichoderma yunnanense* AIKB3, and *Schizopyllum commune* AID1, respectively. The ethyl acetate extracts of fungi strains from the mangrove plant *A. ilicifolius* showed strong antibacterial and moderate cytotoxic activities, which make them good candidates for secondary metabolite isolation.

Keywords: MTT assay, Internal transcribed spacer (ITS), *Pestalotiopsis* sp, *Schizopyllum commune, Trichoderma yunnanense*

Introduction

The mangrove ecosystem is large in the tropical region, especially in the Indo-Pacific region. The chemical compounds from these mangrove plants represent a rich biological application in medicine [1]. *A. ilicifolius* is a mangrove species traditionally utilized for human remedies. This mangrove's leaf, stem bark, and roots have been used as herbal drugs for headaches, asthma, rheumatic, and skin infection [2].

Endophytic fungi are microorganisms that live in the host plant's internal tissues and produce abundant bioactive natural compounds [3]. Due to their unique symbiotic nature, mangrove-associated fungi have attracted special interest in the study of these bioactive compounds. [4]. Active constituents from this mangrove displayed various pharmacological activities such as antitumor, anticancer [5], anti-inflammation [6], and antimicrobial [7].

This research is part of our study, which focused on discovering bacteria and fungal strains from the marine sponges and mangrove plants from the West Sumatra area that may contain antibacterial and anticancer compounds [8-10]. In the present study, endophytic fungi associated with the mangrove plant *A. ilicifolius* L. were isolated, identified, and analyzed for their biological activities.

Materials and methods

Collection mangrove plant

A. ilicifolius L (**Figure 1**) was collected from Mangrove Forest, Pariaman, West Sumatera, Indonesia (0°36.0970'S, 100°6.5986'E) (**Figure 2**). The ANDA Herbarium, Department of Biology, Faculty of

Mathematics and Natural Science, Universitas Andalas, West Sumatera, Indonesia, identified the plant. The plant was identified as *Acanthus ilicifolius* L, which belongs to the Acanthaceae family.

Figure 1 Mangrove plant Jeruju (*Acanthus ilicifolius* L.).

Figure 2 Location of *A. ilicifolius* L in Mangrove Forest Tourism Park, Pariaman, West Sumatera (0°36.0970'S, 100°6.5986'E).

Isolation and identification of the fungi

The endophytic fungi were screened from the stem bark, roots, and leaf of the *A. ilicifolius* L. The fungal isolation process of the fungi was carried out following the previous research [9]. Each fungal strain was purified based on the morphology characteristic. The fungi were identified at the Indonesian Center of Biodiversity and Biotechnology (ICBB), Bogor, Indonesia, using the internal transcribed spacer (ITS) DNA fragment.

Culture and extraction of the fungal

The fungal strain culture was carried on a 100 g rice medium supplemented with 110 mL sterile seawater and then incubated at 25 °C for 30 days [11]. The extraction procedure was conducted after our recently published paper [9].

Assay of antimicrobial activity

The antimicrobial activity of fungi was tested against indicator pathogenic microbial strains *Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC25922, *Candida albicans*, clinical strain Methicillin-Resistant *Staphylococcus aureus* (MRSA), and Multi-Drug-Resistant *Pseudomonas aeruginosa* (MDRPA).

Each ethyl acetate extract of fungal strains was diluted in dimethylsulfoxide (DMSO) to a 5 mg/mL concentration. The antimicrobial activity assay followed the previously described method [9]. Onto the 6 mm sterile disk (Advantec®) was dripped with 10 µL of each extract and placed on Nutrient Agar (Merck®) containing 0.5 Mc Farland of bacterial suspension and Sabouraud Dextrose Agar (Merck®) containing 0.75 Mc Farland of fungal suspension. Chloramphenicol (Oxoid®) in a 30 µg/mL concentration was used as a positive control for antibacterial activity assay, and nystatin (Oxoid®) in 100 units/disk for antifungal activity assay. The plate was incubated in an incubator at 37 °C overnight. The clear zone was measured, and the data was displayed as inhibition activity (mm).

Cytotoxic and toxicity screening

Brine Shrimp Lethality Test (BSLT) method was used as a preliminary study to evaluate the cytotoxic activity of the fungal extracts and was conducted based on our previous way [12]. This assay was carried out in triplicate. The toxic extract (with LC50 < 100 ppm) was then tested against T47D (human ductal breast epithelial tumor) cell lines (MTT Assay). The fungal extracts were made in serial concentrations of 1,000, 500, 250, 125, 62.5, and 31.25 ppm. The IC50 value was obtained from regression equations.

Phytochemical assay

Phytochemical screening of ethyl acetate fungus extract was carried out to determine the content of alkaloids, flavonoids, steroids, triterpenoids, saponins, and phenolics using the method of Handayani *et al.* [8].

Microscopic examination

The bioactive fungi that have the potential to be developed as antimicrobials and anticancer were subjected to morphological examination under a microscope. One loop of fungi was placed on a glass object, dripped with lactophenol, covered with cover glass, and observed under a microscope with a magnification of 2,000× [13].

Molecular identification

The DNA extraction procedure was done by modifying Fatimawali *et al.* [14]. Three endophytic fungal strains with the highest antimicrobial and cytotoxic activity were identified using the internal transcribed spacer (ITS1 and ITS2) DNA barcode fragments. The amplification reaction was conducted according to the method described by Ferrer *et al.* [15]. The PCR products were sent to First Base Malaysia for sequencing. The phylogenetic tree was constructed on MEGA 7.0 software using the neighbor-joining method, and the p-distance model was set with a bootstrap value of 1,000 replications [16]. The nucleotide sequence data of potential endophytic fungal strains were deposited in GenBank to obtain an accession number.

Results and discussion

Antimicrobial activity of endophytic fungi

Thirteen fungal endophytes were straind from the leaves, stem bark, and roots of the mangrove plant *A. ilicifolius*, originating from West Sumatra, Indonesia. Five fungi were straind from the roots, 4 fungi from stem bark, and 4 fungi from the leaves of the mangrove plant and coded as AIA, AIKB, and AID, respectively. The ethyl acetate extracts' weight ranged from 1.20 - 8.20 g.

The antibacterial assay showed that fungal extracts had various zone inhibition on selected pathogenic bacterial and indicator fungal strains. The fungal AIA1 extract displayed an excellent antimicrobial activity in a 5 % concentration against *S. aureus, E. coli*, and *C. albicans* with 15.42 ± 0.51, 11.87 ± 0.87, and 12.50 ± 1.15 mm inhibition zones, respectively. The fungal extracts of AIKB3 and AID4 also showed excellent

inhibition zones against bacterial indicator strains, although they showed no inhibitory activity against *C. albicans* (**Table 1**). Based on David and Stout (1971), inhibition activity was categorized firmly if the inhibition zona diameter ranges from 10 to 20 mm. The fungal extract code AIA1, AIKB3, AIKB4, and AID3 were tested against resistant pathogenic bacterial strains MRSA and MDRPA. The limited number of extracts obtained from cultivation prevented further testing of all extracts against resistant bacteria.

Table 1 Antimicrobial activity of endophytic fungi from *A.ilicifolius* extracts against human pathogenic bacteria.

No	Code of extracts	Inhibition zone (mm) ± Deviation standard (SD)				
		S. aureus	***E. coli***	***C. albicans***	**MRSA**	**MDRPA**
1	AIA1	15.42 ± 0.51	11.87 ± 0.87	12.50 ± 1.15	13.79 ± 0.9	8.46 ± 0.14
2	AIA2	7.83 ± 0.26	9.69 ± 0.76	-	n	n
3	AIA3	-	-	-	n	n
4	AIA4	9.71 ± 0.40	9.62 ± 1.30	-	n	n
5	AIA5	-	-	-	n	n
6	AIKB1	8.45 ± 0.88	-	-	n	n
7	AIKB2	8.70 ± 0.83	9.33 ± 0.97	-	n	n
8	AIKB3	13.95 ± 0.85	8.96 ± 1.06	-	9.08 ± 0.69	8.29 ± 0.72
9	AIKB4	11.08 ± 0.62	9.5 ± 1.14	-	9.88 ± 2.17	8.13 ± 0.98
10	AID1	14.58 ± 0.68	7.04 ± 0.19	-	n	n
11	AID2	7.04 ± 0.64	7.25 ± 0.75	-	n	n
12	AID3	11.25 ± 0.51	7.46 ± 0.90	-	7.92 ± 0.47	7.88 ± 0.66
13	AID4	11.75 ± 1.29	8.21 ± 0.82	-	n	n
14	Positive control	30.62	25.75	30.75	24.0	8.75
15	Negative control	-	-	-	-	-
- = no inhibition						
n = no data						

Note: The value is expressed as the mean ± standard deviation; n = 3

Cytotoxic activity of endophytic fungi

The preliminary screening for cytotoxic activity was performed using the BSLT method. Meyer *et al*. categorized the toxicity of the extracts based on LC_{50} value. The LC_{50} value below 1,000 ppm is classified as toxic; if greater than 1,000 ppm, it is classified as non-toxic [18]. An average of 92.30 % of fungal extracts had cytotoxic activity (**Figure 3**).

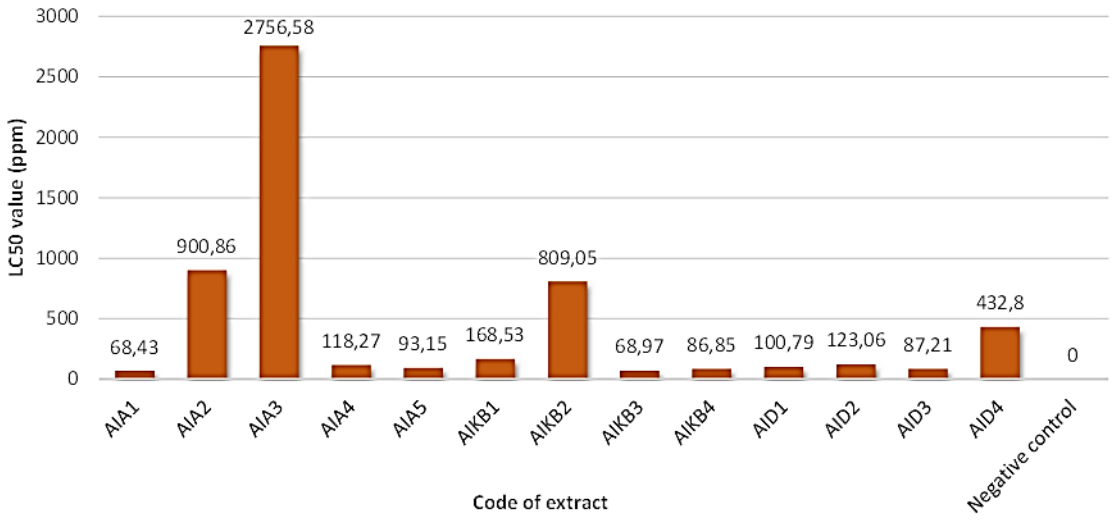

Figure 3 The cytotoxic activity of ethyl acetate extract of endophytic fungi straind from *A.ilicifolius*.

Further testing for toxicity activity was conducted by MTT assay against the T47D cell line. The assay was only carried out on the extracts with an LC_{50} value of less than 100 ppm. Five extracts meet these criteria, namely AIA1, AIA5, AID3, AIKB3, and AIKB4. Data showed no fungal extracts had an excellent IC_{50} value (**Table 2**).

Table 2 The cytotoxic activity of selected extract of endophytic fungi straind from *A.ilicifolius* on the T47D cell line.

No.	Code of extract	The IC_{50} value of the T47D cell line (ppm)
1	AIA1	485.84
2	AIA5	4,076.03
3	AID3	2,671.9
4	AIKB4	177.2
5	AIKB3	512.57
6	Doxorubicin	10.05

Phytochemical assay

The phytochemical examination was a preliminary assay to screen the chemical constituents obtained before the isolation process of the bioactive compound of the fungi. Five bioactive fungal extracts were examined for secondary metabolite compounds. The phytochemical analysis showed that extracts contained terpenoid, steroid, phenolic, and flavonoid (**Table 3**).

Table 3 Phytochemical screening result of selected extracts of endophytic fungi, straind from *A.ilicifolius*.

Code of extract	Alkaloid	Flavonoid	Triterpenoid/steroid	Saponin	Phenolic
AIA1	−	−	+	−	+
AIA5	−	+	+	−	−
AIKB3	−	−	−	−	+
AIKB4	−	−	+	−	−
AID3	−	−	+	−	−
+ = positive reaction					
− = negative reaction					

Identification of potential fungi

The macroscopic and microscopic observations of some fungi are shown in **Figure 4**. We only identified 3 fungal codes, namely AID1, AIA1, and AIKB3. Data on antibacterial activity led us to choose this particular fungus. This fungus, straind from several regions of the mangrove plant, was distinct from our previous strains. The macroscopic examination was carried out by observing the characteristics of the colony surface, surface, texture, color, and hyphae. The microscopic examination included the spores or conidia features and reproductive structure under a light microscope. The macroscopic characteristics of the fungal colonies that strain AIA1 were yellowish-white, rough surface, and wavy edges. This fungus had prolonged oval hyphae and conidia. Molecular identification based on ITS showed that this fungus had an identity of 100 % with *Pestalotiopsis* sp strain 1-1-3-1 on BLAST analysis (**Figure 5**).

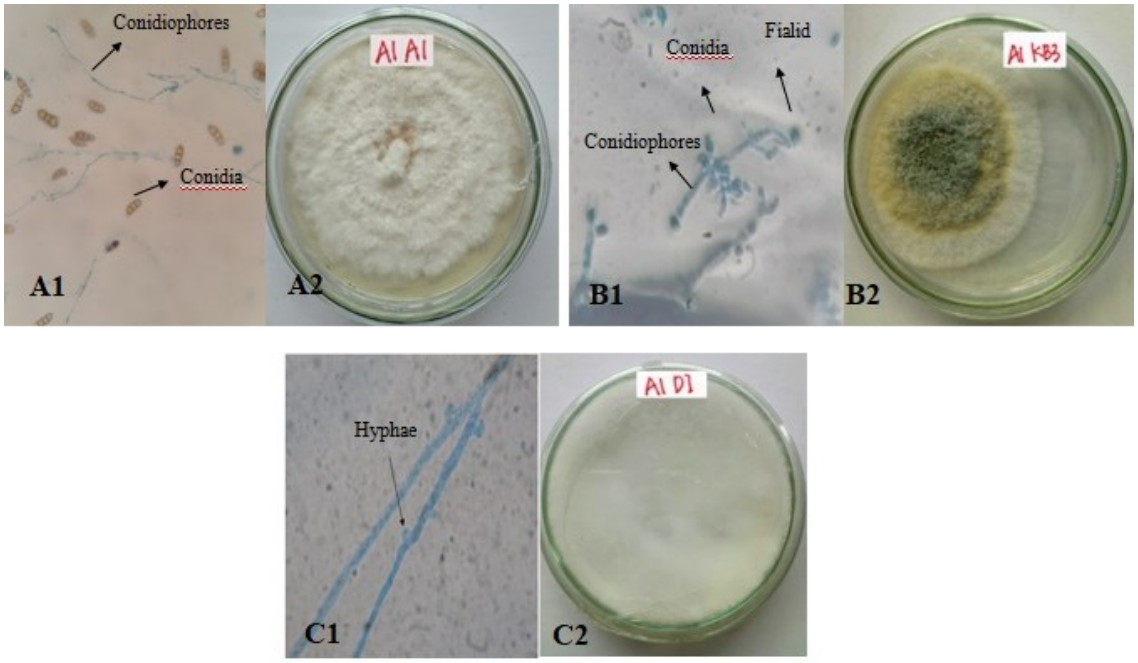

Figure 4 The macroscopic (right side) and microscopic (left side) fungi observations. A1 and A2 are fungal strain AIA1 (*Pestalotiopsis* sp. MN989978). B1 and B2 are fungal strain AIKB3 (*T. yunnanense* MN989979), and C1 and C3 are fungal strain AID1 (*S. commune* MN989980).

Figure 5 Neighbor-joining phylogenetic tree analysis of the strain fungal-derived mangrove plant *A. ilicifolius*.

The next fungus was strain AIKB3. Macroscopically, this strain had a yellowish-green and white wavy edge colony. It had a straight and branched conidiophore, short conidia, and thick phialides, which followed the research by Qiao *et al.* [19]. Molecular identification showed that AIKB3 had a 100 % identity with *T. yunnanense* (**Figure 5**).

The strain AID1 had a 100 % homology with *Schizophyllum commune* strain HLJ 20 (**Figure 5**). The fungus had a white colony, cotton-shaped, and wavy edges on macroscopic observation. Microscopically,

this fungus did not have conidia, but hyaline hyphae connected by a bulkhead (**Figure 4**) which was similar to the results of research by Castro *et al.* [20].

The GenBank accession number of endophytic fungus strains from mangrove *A. ilicifolius* is indicated in the brackets. Three potential endophytic fungal strains i.e., AIA1, AIKB3, and AID1 were identified as *Pestalotiopsis* sp (MN989978), *T. yunnanense* (MN989979), and *Schizopyllum commune* (MN989980), respectively.

The research on natural product discovery nowadays is focused on plant endophytic fungi. There was a balanced system between endophytic fungi and host plant defense [21]. This symbiotic relationship can be explored further by searching the secondary metabolites without disturbing the biodiversity of the plants. The fungi associated with the mangrove plant have become an interesting focus of interest in recent years. The mangrove *A. ilicifolius* is widely distributed in southeastern Asia and is traditionally used in Indian and Chinese medicine [2]. Chemical investigations revealed that this plant contained alkaloids [22], triterpenoids, saponin [23], coumaric acid derivatives [24], and many others.

The mangrove ecosystem is a rich repository of unusual fungal endophytes. The environment influences the endophyte population that a plant may survive. Chi *et al.* discovered a high diversity of fungi associated with leaves of *A. ilicifolius* var. xiamenensis, with 110 taxa recovered from the isolation and metabarcoding methods [25]. This diversity is affected by the environment where this mangrove habitat, such as a variety of ph, temperature, salinity, and other factors. Bai *et al.* reported new phenyl derivatives isolated from endophytic fungi, *Aspergillus flavipes* AIL8, from the mangrove plant *A. ilicifolius* [26].

Based on the antibacterial activity assay results, the fungal extracts were generally more sensitive to Gram-positive indicator bacterial strains [27,28]. It might be due to the differences in the cell wall composition of each group. Gram-negative bacteria possess an external impermeable membrane, causing antibiotics not readily cross the membrane [29]. These differences impacted the difficulty of finding specific antibiotics for negative gram bacteria. Much related research has been conducted on the antibacterial potential of fungi-associated mangrove plants. Endophytic fungi from *A. ilicifolius* showed a broad spectrum of antimicrobial activities [30,31].

Endophytic species of Pestalotiopsis are generally isolated from tropical plants. Many studies reported the potential pharmacological activities of the secondary metabolites of this fungus. Xu *et al.* successfully isolated 6 new chromones, a pestalotiopsones A-F, from the mycelia and culture filtrate *Rhizophora mucronata* endophytic fungus *Pestalotiopsis* sp [32]. This fungus is repeatedly reported to be cytotoxicity [33], antifungal [34,35], antibacterial [36-38], and anti-inflammation [39]. Phytochemical screening of crude extract of *Pestalotiopsis* sp. showed a different secondary metabolite group [40,41]. The secondary metabolites produced by plants depend on the plants' source, environment, and solvent used in the extraction procedure. Pestalotiopsis species have gained much attention as they have been found to possess ambuic acid, pestacin, and isopestacin as antifungal activity [42,43].

Little is known about a chemical constituent produced by *T. yunnanense*. However, Saravanakumar *et al*. reported the 16-metylheptadecanoic acid as an anticancer agent [44]. The fungus *S. commune* is more abundant in terrestrial plants and mangroves, and lesser was found in little sea gutters [45]. The various bioactive compounds have been reported successfully isolated from *S. commune*, such as polysaccharides, phenolic compounds, terpenoids, and ergosterol. Besides, *S. commune* polysaccharide (SPGs), also known as Schizophyllan, has received much attention for its biological properties, including immunomodulator, anticancer, antitumor, and antioxidant activities [46].

Conclusions

Based on the potential antimicrobial and cytotoxic activities, 3 fungi have been successfully isolated from the leaves, stem bark, and roots of mangrove *A. ilicifolius*. All fungi were identified as *Pestalotiopsis* sp. AIA1, *Trichoderma yunnanense* AIKB3, and *Schizopyllum commune* AID1, respectively. Further investigation on their bioactive compounds is needed to elaborate more on their potential as promising antibacterial and cytotoxic agents in pharmaceutical industries.

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