

# Cytotoxic and antimicrobial activities of ethyl acetate extract of mangrove plant *Scyphiphora hydrophyllacea* C. F. Gaertn—Associated fungi

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## ABSTRACT

Association between mangrove plants with fungi has been explored for many biological activities. The purpose of this study was to determine the antimicrobial and cytotoxic activities of endophytic fungi isolated from mangrove plants *Scyphiphora hydrophyllacea* C. F. Gaertn. The isolation of endophytic fungus from the mangrove has been carried out by the direct planting method on Sabouraud Dextrose Agar medium. Eleven fungal isolates were recovered and purified. The isolates were cultivated on the rice medium for 1 month and extracted using ethyl acetate. Eleven ethyl acetate extracts were screened for cytotoxic activity by *Brine Shrimp Lethality* Test method and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and for antimicrobial activity toward some human pathogenic microbes. The results showed that five (45.5%) isolates of endophytic fungi had antimicrobial activity against the growth of *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* with a diameter from 9.1 to 21 mm. Furthermore, four extracts (36.4%) were cytotoxic (cell viability < 50%) against T47D cells. Isolate ADSh2 which exhibited the highest cytotoxic activity was identified molecularly as *Penicillium chrysogenum*. The results of this study indicate that the ethyl acetate of endophytic fungi associated with *Scyphiphora hydrophyllacea* has the potential to be developed as antimicrobial and anticancer compounds.

## INTRODUCTION

Endophytic fungi from the mangrove plant are categorized as the second biggest ecology after marine endophyte fungi (Khrueayu and Apiradee, 2012). The host and endophytic plants have symbiotic mutualism in which the host plants provide nutrients for endophytes and function as a shelter, while endophytes produce bioactive substances to increase host growth and competitiveness in their natural habitat (Sun *et al.*, 2014). Endophytes can produce various bioactive compounds that are useful in the research and can be used in the development of anticancer and antimicrobial drugs (Li *et al.*, 2018).

The endophytic fungi from mangroves are reported to produce various types of secondary metabolites (Firáková *et al.*, 2007). The natural products compound produced by the endophytic fungi from mangroves has been screened for antimicrobial agents against many pathogenic bacteria and fungi and also for cytotoxic effect in many cell cancer lines. This research was focused on determining the potency of fungal extracts isolated from the mangrove plant *S. hydrophyllacea* C. F. Gaertn collected from West Sumatra, Indonesia in continuation with our research on bioactive natural compounds derived from marine and mangrove origins (Handayani and Artasasta 2017; Handayani and Aminah 2017; Handayani *et al.*, 2015a; 2015b; 2016; 2017; 2018; 2019).

## MATERIALS AND METHODS

### Sample preparation

Mangrove plant was collected from Teluk Pandan, Bungus (1°1'54.17" S; 100°25'42.3"E) West Sumatra, Indonesia.

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The leaves, bark, and roots of *S. hydrophyllacea* C. F. Gaertn were taken as much as ±100 g, put into the sterile plastic bag and then transferred to the laboratory of Sumatran Biota, University of Andalas, Padang, West Sumatera, Indonesia.

### Isolation, cultivation, and extraction of mangrove plant *S. hydrophyllacea* C. F. Gaertn-associated fungi

The leaves, bark, and roots were surface-sterilized by rinsing with running water for 10 minutes, and then rinsed with ethanol 70% for 1 minute. Thereafter, the samples were soaked in bleach solvent (NaOCl 5.3%) for 5 minutes and rinsed again with ethanol 70% for 30 seconds. The isolation process was conducted under Laminar Air Flow. The sterile samples were cut into small pieces. Each piece was inoculated on Sabouraud Dextrose Agar (SDA) medium and incubated at 25°C (3–5 days). The fungal colonies which grew on SDA medium then purified gradually. The colonies that have different shape and colors regarded as different isolates.

Pure isolates of endophytic fungi were cultivated in 100-g rice medium and then incubated at 25°C for 30 days. The cultivation process was conducted based on Kjer *et al.* (2010). The extraction method of the secondary metabolite has been carried out following the previous research (Handayani *et al.*, 2018).

### Testing for antimicrobial activity

*Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Candida Albicans*, which are used as test microbes, were obtained from the Microbiology Laboratory, M. Djamil General Hospital Padang, West Sumatra, Indonesia. Testing for this activity was carried out by the agar diffusion method in the concentration 5, 3, and 1 mg/ml in dimethyl sulfoxide (DMSO). Tetracyclin HCl (300 µg/ml) (PT Phapros, Indonesia), and ketoconazole (20 mg/ml) (PT Kimia Farma, Indonesia) in distilled water were used as a positive control. The antimicrobial screening was conducted based on the method described previously (Handayani *et al.*, 2015a; 2017; 2018).

### Screening for cytotoxic activity

#### BSLT method

The *nauplii* of brine shrimp (*Artemia salina* L.: Artemiidae) were used for cytotoxic activity assay. The eggs were hatched in 500 ml of filtered seawater under constant aeration for 48 hours at 27°C ± 2°C. After hatching, active *nauplii* free from eggshells were collected and used for the assay. The final concentrations of the extracts used in this assay were 1,000, 100, and 10 µg/ml in triplicate. As negative controls are used filtered seawater and DMSO. Cytotoxic activity was determined based on the LC<sub>50</sub> value determined using the curve method based on probit analysis (Meyer *et al.*, 1982).

### MTT assay

Two cell lines, T47D as human ductal breast epithelial tumor cell line, and Vero as normal cell line were prepared for cytotoxic assay. All the cell lines were obtained from the Laboratory of Parasitology at Gajah Mada University, Yogyakarta, Indonesia. The stages of the research method for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay have been

carried out as written in the research that we have done before (Handayani *et al.*, 2017; 2018; Permanasari *et al.*, 2013).

### Molecular identification of mangrove-associated fungi

The characterization of endophytic fungi was performed in macroscopic examination included surface, colors, and the edge of each colony. The molecular identification was performed out using internal transcribed spacer (ITS) DNA barcode with special primer pairs for fungi, by following the procedures carried out by Handayani *et al.* (2019). The ITS region covers internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and large subunit ribosomal RNA. PCR product was sequenced in first Base, Malaysia. The sequencing result was processed following the procedure performed by Tallei and Kolondam (2015) and subjected to BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) at NCBI (<https://www.ncbi.nlm.nih.gov/>) for species identification. The identification result was further verified using pairwise sequence identification at MycoBank (<http://mycobank.org>). Analysis of the phylogenetic tree is formed by arranging the neighbor-joining tree method with a value of 1,000 bootstrap replicate by using MEGA 7.0 developed by Kumar *et al.* (2016).

## RESULTS AND DISCUSSION

Endophytes are a group of microorganisms that live in healthy plant tissues without causing negative impacts on host plants. The types of endophyte mainly include fungal, bacterial, and actinomycetes endophytes. Based on previous research reports, endophytic an important source producer of natural compounds for coexistence between endophyte and host plants create complex relationships, so the endophyte is able to produce the same bioactive secondary metabolites by plants, such as antimicrobial and cytotoxic agents (Li *et al.*, 2018). Endophytes can also be found in mangrove plants tissue.

### Antimicrobial activity of fungal extracts

In this study, 11 pure endophytic fungi have been isolated from the mangrove *S. hydrophyllacea* C. F. Gaertn. The leaves contained most of the endophytic fungi than bark and root. The isolation of endophytic fungi from the different part of the host plant can obtain different fungal species. Endophyte which grows in the stressful condition area experiences many physical and chemical challenges, so they become the potential sources of bioactive substances (Raghukumar, 2008). All of the isolates were distinguished with each other based on the difference of color, surface, and the edge of the colony. Each fungal isolates was cultivated in 100 mg rice medium for 30 days. Some factors can give effects in the growth process of microorganisms, such as inoculum size, nutrition ingredients in the substrate, water content, pH, and temperature. After being cultivated, the fungal isolates were extracted using ethyl acetate solvent to obtain the secondary metabolite.

The screening of antimicrobial activity of all fungi extracts was conducted against some pathogenic microorganisms, such as *S. aureus*, *E. coli*, and *C. albicans*. Based on this screening result, four ethyl acetate extracts of endophytic fungi inhibited the growth of *S. aureus* with a diameter zone of >9 mm (at concentration 5%), four extracts inhibited *E. coli* and two extracts inhibited the growth of *C. albicans*. The fungal isolates with

code DNSH3 exhibited good activities against all the reference pathogenic microorganisms with a diameter of inhibitory zone 21, 21, and 10.2 mm, respectively (Fig. 1).

### Cytotoxic activity of fungal extracts

The Brine Shrimp Lethality Test (BSLT) method was used as a preliminary test for cytotoxic activity screening. The results showed that four isolates of the fungus (36.4% of total fungal isolates) have cytotoxic activity below 100  $\mu\text{g/ml}$ . The cytotoxic isolates were ADSH1, ADSH2, DNSH2, and DNSH3. The isolate which showed the highest cytotoxic activity was ADSH2 with  $\text{LC}_{50}$  of 45.6  $\mu\text{g/ml}$  (Fig. 2). According to Meyer *et al.* (1982), the extracts are considered very toxic when it has an  $\text{LC}_{50}$  value  $<30$   $\mu\text{g/ml}$ , and toxic if it has a value of  $\text{LC}_{50}$  value 30–1,000  $\mu\text{g/ml}$ .

Further testing on the cytotoxic test was conducted by MTT assay. This assay is a popular method to assess cytotoxicity and cell viability of the living cell. The test principle is an enzymatic reduction of the lightly colored tetrazolium salt to its formazan, whereas changing the color solution in microtiter plate from intense purple to blue color. Absorbance measurements are obtained at the end of the assay (Grela *et al.*, 2018; Stockert *et al.*, 2012). The determination of  $\text{IC}_{50}$  value begins by determining the percentage viability of cancer cells in the culture medium after adding endophytic fungi extract with a concentration of 100  $\mu\text{g/ml}$ .

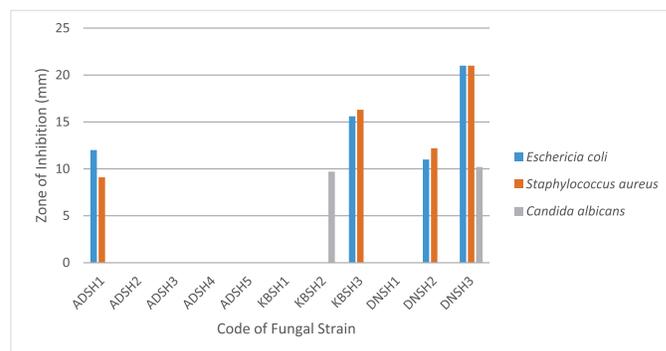
The fungal extract of ADSH3, KBSH1, KBSH2, KBSH3, and DNSH2 are the extracts with the lowest percentage of viability ( $<50\%$ ) against T47D cancer cells. The EtOAc extracts of DNSH2 was the best selectively cytotoxic on T47D ductal breast cancer cells (the percentage of viability of 11.75%), and has low toxicity on Vero normal cells (the percentage of viability of 80.61%) (Fig. 3).

### Molecular identification of selected fungal isolates

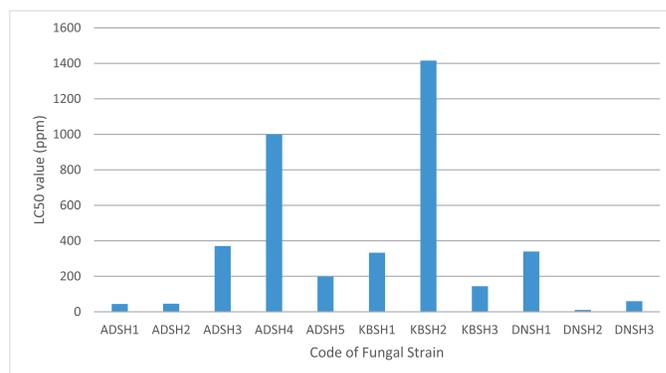
The isolate ADSH2, one of the potential fungi in producing cytotoxic agent, was then identified morphologically and molecularly. The ITS region was chosen for the molecular identification. The colony of isolate ADSH2 was brown-colored with wavy edges (Fig. 4). The BLAST identification showed that isolate ADSH2 has 100% identity with *Penicillium chrysogenum* isolate AYMA5 (Accession number: MK643348) with *E* value 0.0. The closer the *E*-value is to zero, the more significant the match is considered to be. Using pairwise sequence identification at MycoBank, isolate ADSH2 has 100% similarity with *Penicillium chrysogenum* isolate 2OI2F1T (Accession number: KY524459). The phylogenetic tree was constructed by MEGA (version 7.0) using a neighbor-joining *p*-distance algorithm method with a bootstrap value of 1,000 (Fig. 5). The tree shows the position of the sample together with the species it belongs to.

The fungus *P. chrysogenum* has been explored in other previous research. A novel chaetoglobosin, Penochalasin I, was isolated from the culture of endophytic fungus *P. chrysogenum* inhabiting the vein of mangrove *Myoporum bontioides* in Guangdong Province, China. This compound displayed cytotoxic activities toward MDA-MB-435 and SGC-7901 cell lines ( $\text{IC}_{50}$   $<$

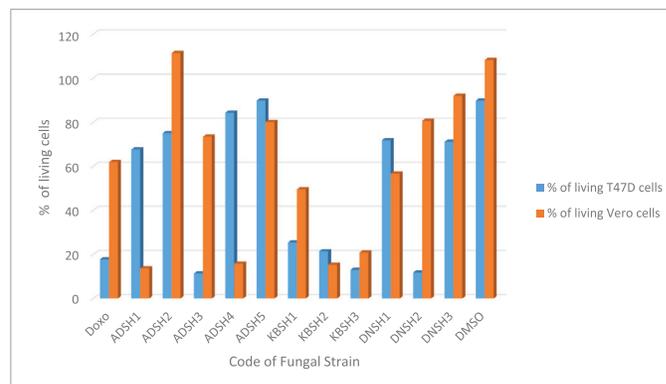
10  $\mu\text{M}$ ) (Huang *et al.*, 2018). New polyketides, penicichrosogenins A–L, were isolated from the solid substrate fermentation culture of *P. chrysogenum* MT-12, an endophytic fungus from a Chinese club moss (*Huperzia serrata*). All of the isolated compounds were able to inhibit the production of nitric oxide in lipopolysaccharide-stimulated RAW264.7 macrophage cells ( $\text{IC}_{50}$  = 17.5–98.4  $\mu\text{M}$ ) (Qi *et al.*, 2017).



**Figure 1.** The antimicrobial activity of ethyl acetate extract of endophytic fungi isolated from *S. hydrophyllacea*.



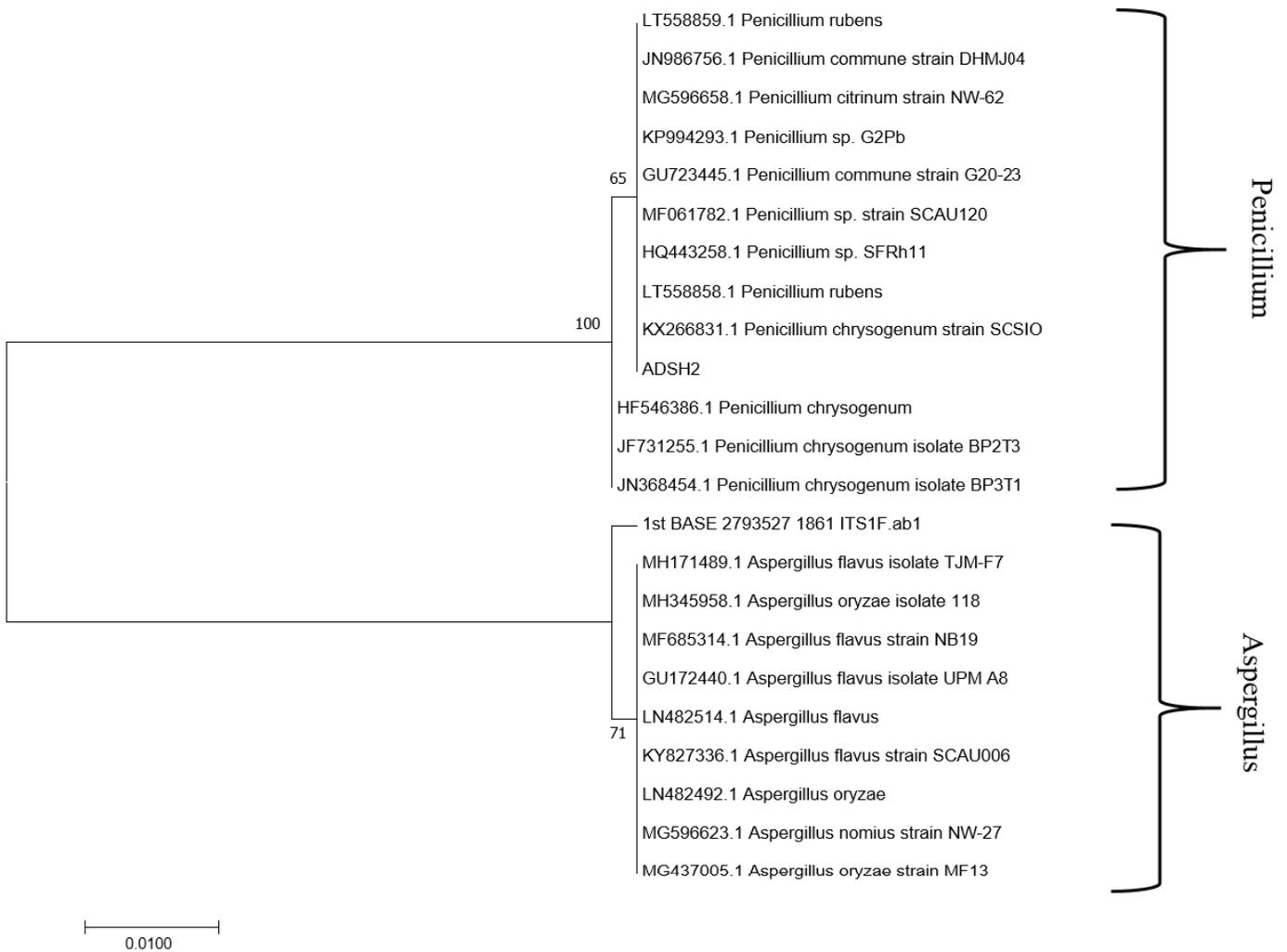
**Figure 2.** The cytotoxic activity of ethyl acetate extract of endophytic fungi isolated from *S. hydrophyllacea*.



**Figure 3.** The cytotoxic activity of ethyl acetate extract of endophytic fungi isolated from *S. hydrophyllacea*.



**Figure 4.** Macroscopic characteristics from endophytic fungus isolate ADSH2 of *Scyphiphora hydrophyllacea* C. F. Gaertn.



**Figure 5.** The phylogenetic tree inferred using the neighbor-joining method of ITS sequence of endophytic fungus isolate ADSH2 of *Scyphiphora hydrophyllacea* C. F. Gaertn and its allied taxa.

## CONCLUSION

Based on the results of this study, it can be concluded that the endophytic fungi from mangrove plants *S. hydrophyllacea* C. F. Gaertn are the potential source for antibiotics and anticancer agents. The result indicated that the isolate ADSH2 shows the potential to be used as a source of new anticancer compounds in the future. However, further research needs to be done, for the stages of purification and elucidation of the structure of antimicrobial and cytotoxic compounds produced by the fungi.

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## CONFLICT OF INTEREST

There are no conflicts of interest declared by the authors.

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