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Antimicrobial and Cytotoxic Activities of Endophytic Fungi Isolated from Mangrove Plant *Sonneratia alba* Sm

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ABSTRACT

Endophytic fungi are those that grow intra- or intercellular within the tissues of higher plants without causing a disease. This study aims to evaluated antimicrobial and cytotoxic activities of endophytic fungi from leaf, bark and root of mangrove *Sonneratia alba* Sm, collected from Bungus, West Sumatra, Indonesia. The isolation of endophytic fungi was done by using direct planting method with sabouraud dextrose agar (SDA) as growth medium. Thirteen isolates fungi strains were obtained from this mangrove. They were cultivated on unpolished rice as medium for ± 4 weeks, and extracted with ethyl acetate. The ethyl acetate extracts were analyzed for antimicrobial and cytotoxic activities by using agar diffusion method and MTT assay on T47D and Vero cells. The study revealed nine (69%) of the total extract had antimicrobial activity against pathogenic bacteria and fungal such as, *Staphylococcus aureus, Escherichia coli* and *Candida albicans*. While nine extracts (69%) were cytotoxic (Percentage of cell viability < 50) against T47D cells. Based on the results of antibacterial activity screening, three fungal isolates were selected as the most active against *S. aureus, E. coli* and *C. albicans*. This selected fungal isolates were first macroscopically and microscopically characterized and later molecularly identified as *Trichoderma koningiopsis, Aspergillus sydowii* and *Trichoderma lixii* respectively. This study concluded that the endophytic fungi of *Sonneratia alba* Sm can be developed as a new source of antibiotic and anticancer compounds.

INTRODUCTION

Terrestrial endophytic fungi found on leaves, stems, and roots of mangrove were more numerous than marine fungi (Latha and Mitra 1998). The endophytic fungi are known as a source of abundant secondary metabolites. Many endophytic fungi produce secondary metabolites which are very attractive in terms of their activity and chemical structure. The secondary metabolite such as alkaloids, phenolic derivatives, terpenoids and steroids plays an important role as a potential candidate of drug compounds, and useful in agro-chemical industries (Strobel and Daisy, 2003; Alfaro and Boyman, 2011).

Research on secondary metabolites produced by endophytic fungi from mangroves as a drug-producing compound has been widely performed. In continuation of our work on natural substances of terrestrial and marine origins (Handayani and Artasasta, 2017; Handayani and Aminah, 2017; Handayani *et al.*, 2016, Handayani *et al.*, 2015), we examined the endophytic fungi from the mangrove *S. griffithi* Kurz in producing antibacterial compounds (Handayani *et al.*, 2017). Based on these potentials, continuous research on screening for antimicrobial and cytotoxic activities of other *Sonneratia* species, such as *S. alba* Sm has been performed. Screenings of antimicrobial and cytotoxic activities were performed on fungal extract isolated from leaves, bark, and roots of *S. alba* Sm collected from West Sumatra, Indonesia.

MATERIAL AND METHODS

Material

To isolate the endophytic fungi, fresh leaves, barks, and roots of *S. alba* Sm were collected from Nirwana Beach, West

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Sumatra, Indonesia. Samples of leaves, bark, and roots were taken as much as ± 100 grams and then put in a clean and sterile plastic container and transported to the laboratory.

Isolation, cultivation, and extraction of secondary metabolites from of endophytic fungi

The research method for the isolation and cultivation of endophytic fungi have been carried out as written in the research that we have done before (Kjer *et al.*, 2010; Handayani *et al.*, 2017).

Screening for antimicrobial activity

Screening of antimicrobial activity of the EtOAc extracts of endophytic fungi was performed by the disk diffusion method (Bauer *et al.*, 1966). The testing was done against pathogenic bacterial and fungal such as *Staphylococcus aureus, Escherichia coli,* and *Candida albicans*. The EtOAc extract was prepared to the concentration (in DMSO) 5, 3 and 1 mg/ml. Tetracycline HCl and Ketoconazole as a reference compound were prepared at 300 µg/ml and 20 mg/ml in distilled water, respectively. Each 10 µl of above reference and extracts were dropped onto 6 mm sterile paper disk on the surface of the medium containing bacteria and fungi test strain. Each plate was incubated at 37ºC for 24 hours for bacteria and at a temperature of 25ºC-27ºC for 5-7 days for fungi. Inhibition zones were measured and recorded. Screening of antibacterial activity was experimented in triplicate, and mean value ± standard deviation was also determined.

Screening for cytotoxic activity

The cell line of T47D (human ductal breast epithelial tumor) and Vero (normal cell) have been prepared for cytotoxic assay using MTT. All cell lines were obtained from Laboratory of Parasitology at UGM. T47D was cultured in RPMI 1650 and Vero was cultured in M199 Medium. All cells were subcultured after mild trypsinization with trypsin-EDTA (Sigma-Aldrich, USA), and then determined the cell number and viability. The cells were seeds in 96-well plates at density 6x103 cells/well in 100 µl medium and incubated overnight. All media were supplemented with 10% with fetal bovine serum (Gibco) and streptomycin and penicillin (2%, Sigma-Aldrich, USA). The cell line was kept at 37ºC, 98% relative humidity with 5% CO_2 atmosphere.

A stock solution was prepared by dissolving the samples in DMSO and was given 100.000 ppm concentration. Cells that had been incubated 24 hours, then divided into several groups, namely treatment, positive control, cell control and media control (blank). Removed medium and washed using PBS sterile which each well was added 100 µl PBS. Then, 100 µl of each material (extract) added to each well with one concentration (100 ppm). As control positive was used with doxorubicin. Then it was incubated for 24 hours in an incubator at 37ºC, 5% CO_2.

Cells that had been treated and incubated 24 hours later dumped throughout the medium and washed using sterile PBS. Then in each well was added 100 mL of MTT (5mg/ml) followed by 4-hour incubation in an incubator at 37ºC, 5% CO_2. To each well was added 100µl of 10% SDS to dissolve the formazan crystals formed and incubated one night at room temperature. The plates were then read by ELISA reader at 540 nm (Permanasari *et al.*, 2016).

% Cell viability then was calculated by the equation

$$\frac{\text{OD of treatmnet} - \text{OD of blank}}{\text{OD of control} - \text{OD of blank}} \times 100\%$$

The aim of this screening was to identify which sample was given cytotoxic activity in the cell line.

Identification of fungal cultures

Three endophytic fungal strains (SaKB1, SaAK3, and SaKB4) which have the greatest antibacterial activity were identified according to the molecular biological protocol with DNA amplification and sequencing of the ITS region (White, Bruns, and Lee 1990). The sequence data were submitted to GenBank. By comparing molecular sequences in National Center for Biotechnology Information. The fungal strains were kept in one of the author's labs (D.H.).

RESULTS AND DISCUSSION

Antibacterial activity of fungal extracts

In this study, thirteen endophytic fungal from leaves, roots, and bark of *S. alba* have been isolated. Observation of the fungus colony was done macroscopically (visual) based on color, surface, and the edge of the colony. The same criteria are considered to be the same isolates, and the criteria showing differences are considered to be different isolates. Each fungus isolate was then cultivated in rice medium for 30 days and extraction using ethyl acetate, with the aim of obtaining more secondary metabolites. The screening of antibacterial activity of this extract against *S. aureus*, *E. coli*, and *C. albicans* has been performed. Based on the screening results, it is known that seven endophytic fungal extracts (at concentration 5%) can inhibit the growth of bacteria *E. coli* with a diameter of > 10 mm, nine extracts had inhibition against *S. aureus* and two extracts were active against *C. albicans*. The extract of SaKB1 had an average of the highest diameter of growth inhibitory against *S. aureus* (diameter inhibitory of 22.83 mm), while the extract of SaAK3 and SaKB4 had the highest inhibitory effects on the growth of *E. coli* and *C. albicans* with diameter inhibitory of 15.33 and 14.57 mm, respectively (Table 1).

Cytotoxic activity of fungal extracts

The cytotoxic activity screening of all extracts on T47D and Vero cell lines was evaluated as presented in Figure 1. The results revealed that 9 (69.2%) out of 13 extracts tested were cytotoxic and exhibited a percentage of viability cell value ≤ 50%. Of these, 6 extracts (46.1%) from all extracts were found to have a percentage of viability cell value ≤ 20%. Fungi extract with the lowest percentage of viability (≤ 50%) especially against the T47D cancer cells are extracted SaDa1, SaDa2, SaDa3, SaDa5, SaDa6 and SaKB3 (Figure 1). The percentage of viability extracts against T47D cancer cells is highly variable and some of which are not toxic to the normal cells (Vero).

Table 1: Antimicrobial activity of endophytic fungi extracts isolated from mangrove *S. alba* SM.

No	Sample Code	Concentrations	Zone of Inhibition (mm) ± Deviation Standard (SD)		
			SA	EC	CA
1	SaDa1	1%	6.10 ± 0.01	6.56 ± 0	7.58 ± 0.01
		3%	-	6.60 ± 0	11.80 ± 0.00
		5%	9.75 ± 0.07	8.53 ± 0.03	14.08 ± 0.00
2	SaDa2	1%	9.07 ± 0.20	9.90 ± 0.14	-
		3%	11.58 ± 0.11	11.38 ± 0.39	-
		5%	15.58 ± 0.00	10.82 ± 0.01	-
3	SaDa3	1%	-	-	-
		3%	-	-	-
		5%	8.35 ± 0.00	5.57 ± 0.01	6.62 ± 0.00
4	SaDa4	1%	7.88 ± 1.67	6.85 ± 0.01	7.55 ± 0.00
		3%	6.86 ± 0.04	6.06 ± 0.00	-
		5%	-	-	-
5	SaDa5	1%	7.58 ± 0.10	-	-
		3%	5.17 ± 0.00	-	-
		5%	14.15 ± 0.00	7.11 ± 0.00	7.10 ± 0.00
6	SaDa6	1%	12.06 ± 0.00	10.83 ± 0.04	6.10 ± 0.00
		3%	12.82 ± 0.01	12.65 ± 0.01	6.10 ± 0.00
		5%	11.96 ± 0.14	14.12 ± 0.00	8.05 ± 0.00
7	SaDa7	1%	13.915 ± 0.01	8.45 ± 0.00	-
		3%	15.60 ± 0.00	14.85 ± 0.00	8.86 ± 0.00
		5%	22.83 ± 0.00	13.82 ± 0.01	11.8 ± 0.00
8	SaKB2	1%	-	-	-
		3%	6.64 ± 0.01	7.50 ± 0.00	-
		5%	10.08 ± 0.00	9.95±0.01	-
9	SaKB3	1%	7.70 ± 0.00	-	-
		3%	8.67 ± 0.01	8.17 ± 0.00	7.81 ± 0.34
		5%	13.57 ± 0.00	13.15 ± 0.00	7.81 ± 0.35
10	SaKB4	1%	11.07 ± 0.00	11.00 ± 0.00	10.10 ± 0.00
		3%	11.68 ± 0.01	11.57 ± 0.02	14.15 ± 0.00
		5%	14.37 ± 0.01	13.65 ± 0.00	14.57 ± 0.00
11	SaAK1	1%	9.24 ± 0.01	9.13 ± 0.04	-
		3%	10.53 ± 0.01	10.20 ± 0.00	6.81 ± 0.01
		5%	14.37 ± 0.00	15.15 ± 0.00	6.83 ± 0.01
12	SaAK2	1%	-	-	-
		3%	-	8.05 ± 0.00	7.75 ± 0.00
		5%	9.86 ± 0.01	9.33 ± 0.04	-
13	SaAK3	1%	9.08 ± 0.00	9.15 ± 0.00	-
		3%	9.84 ± 0.01	11.10 ± 0.00	7.10 ± 0.00
		5%	11.80 ± 0.01	15.33 ± 0.00	9.60 ± 0.00

SA: *Staphylococcus aureus*, EC: *Escherichia coli,* CA: *Candida albicans.*

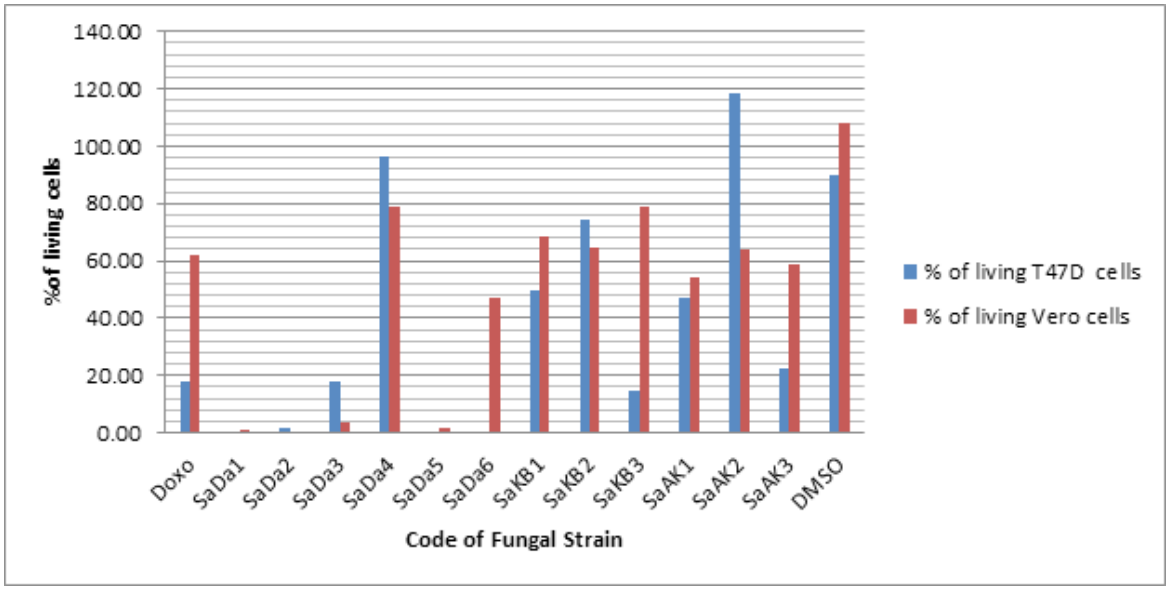

Fig. 1: Cytotoxic activity of endophytic fungi extracts of *S. alba* SM on T47D and Vero cells.

Molecular Identification of selected fungal extracts

The three active antibacterial and cytotoxic extracts above were selected for characterization and identification based on its molecular characterization. Based on BLAST results on the NCBI database on the 18S sequence ribosomal RNA gene sample of SaKB1, obtained a 100% homology with *Trichoderma koningiopsis* TVD fungal-culture 18418S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

Based on BLAST results on the NCBI database on the 18S sequence ribosomal RNA gene sample of SaAK3 obtained a 99% homology with RNA gene of *Aspergillus sydowii* strain FJAT-30991 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

Based on BLAST results on the NCBI database on the 18S sequence ribosomal RNA gene sample of SaKB4 obtained a 99% homology with *Trichoderma lixii* isolate F481RSF1518S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

Based on research reported that on each plant can be found one or more types of endophytic microbes (Strobel and Daisy, 2003). The endophytic microbe has grown to colonize the stem, root, petiole, the leaf segments, flowering weeds, fruit, buds and seeds (Stępniewska and Kuzniar, 2013). According to Dudeja and Giri, 2014, the population of endophyte on plant species varies widely and depends on the various components, such as the host species, stage of development of the host, inoculum density and environmental conditions.

The endophytic fungi have been known to produce some potential antibiotic and anticancer drugs. Fungal cytotoxic to numerous cell lines metabolite such as Penicillenols, was isolated from *Penicillium* sp. The most effective and successful anticancer drug extracted from endophytic fungi to date is Taxol, isolated from *Taxomyces andreanae*. Another compound such as Clavatol (*Torreya mairei*), sordaricin (*Fusarium* sp.), jesterone (*Pestalotiopsis jesteri*), and javanicin (*Chloridium* sp.) are all known to possess strong antibacterial and antifungal properties against numerous foodborne infectious agents (Jalgaonwala *et al.*, 2011).

Our research on endophytic fungi isolated from leaves, roots, and bark of *S. alba* was succesful to select 3 species of potential fungi producing antibacterial and cytotoxic compounds; *Trichoderma koningiopsis, Trichoderma lixii* and *Aspergillus sydowii.*

The fungal genus *Trichoderma* (Ascomycetes, Hyprocreales) was reported to have an ability in producing different metabolites depended on ecological factors, and that strains showed varying effects on pathogens. Marine fungus of *Trichoderma* species is reportedly significant in silico anti-cancer activity against human skin and breast cancer protein. Marine fungus of *Trichoderma harzianum* isolated from the sedimentary coast of Arab gulf in Fawregion in southern Iraq has the potential for antibacterial and antifungal activities (Jafar *et al.*, 2016). The koninginins N-Q, four new fungal polyketides, and four known analogs were isolated from the endophytic fungus *Trichoderma koningiopsis* YIM PH30002 from *Panax notoginseng* (Liu *et al.*, 2016).

The fungal genus of *Aspergillus* is well known as a potential source of bioactive compounds in the pharmaceutical field. Aspergifuranone and isocoumarin derivatives were produced by mangrove endophytic fungus *Aspergillus* sp. 16-5B. The fungus was isolated from the leaves of *Sonneratia apetala* from Dongzhaigang Mangrove National Nature Reserve in Hainan Island, China. All isolated compounds were evaluated for their α-glycosidase inhibitory activities, and Aspergifuranone

showed significant inhibitory activity with an IC_{50} value of 9.05 ± 0.60 μM (Liu *et al.*, 2015). *A. sydowii* has been reported mainly from marine sources and produce some biologically active compounds. One new alkaloid, named as acremolin C, was isolated from static culture of Antarctic fungus, *Aspergillus sydowii* SP-1, and displayed weak inhibition activities against MRSA and MRSE (Li *et al.*, 2017).

CONCLUSION

The results obtained indicate that endophytic fungi derived from mangrove *Sonneratia alba* Sm are an important source of antibiotic and anticancer compounds. Three selected endophytic fungi *Trichoderma koningiopsis*, *Aspergillus sydowii* and *Trichoderma lixii* have been isolated from the root and bark of mangrove. The results of this study indicate that endophytic fungi need to be explored further and continuous research should be done to purify and identify the bioactive compounds as well as optimization of the fermentation process.

CONFLICT OF INTERESTS

The authors declare that no conflict of interest is associated with this work.

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