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Antibacterial Activity of Ethyl Acetate Extracts from Mangrove Plants *Rhizophora apiculata* and *Sonneratia alba* — Associated Fungi

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ABSTRACT
This study aims to explore the source of antibacterial compounds from mangrove endophytic fungi. Nine fungi were isolated from the leaves, barks, and roots of mangrove *Rhizophora apiculata* and *Sonneratia alba* from West Sumatera, Indonesia. The fungal isolate was cultivated in rice for 4-6 weeks, and subsequently, its secondary metabolites were extracted using ethyl acetate. The ethyl acetate extracts were tested against pathogenic bacteria; *Bacillus cereus*, Multi-Drug Resistant *Pseudomonas aeuruginosa*, *Staphylococcus aureus*, and *Escherichia coli*, and *Mycobacterium tuberculosis*. Seven extracts showed antibacterial activity at 5% (b/v %) concentration in the diffusion method. The highest inhibition was exhibited by the extract of RAA1 with the range of inhibition zone of 21.87 ± 0.81 mm – 26.12 ± 0.21 mm. Six fungal extracts (RAA2, RAA3, RAA4, RAK2, SaDa1, SaDa2) showed sensitivity to *M. tuberculosis* at concentrations of 3% and 5%. The potential fungi were identified molecularly by DNA amplification and sequencing of the fungal ITS region. The results of identification showed RAA1 as *Aspergillus flavus*, RAA4 as *Aspergillus aculeatus*, and SaDa1 as *Penicillium rubens*.

Keywords: *Mycobacterium tuberculosis, Aspergillus flavus, Aspergillus aculeatus, Penicillium rubens.*

1. INTRODUCTION

Bacteria and pathogens constantly evolve and leading to reduced antibacterial sensitivity. Antibacterial resistance has become a global issue and caused 700.000 deaths each year [1], [2]. *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus pneumonia*, and Enterobacteriaceae are the common group of bacterial found to have been resistant towards one or more antibiotics [2]. Tuberculosis (TB) caused by *M. tuberculosis* is one of the top 10 causes of death worldwide. Current TB treatment needs multiple regiment dose and takes a long time duration of treatment that cause the increase in toxicity and adverse effects [3]. Therefore, the research on antibacterial drug discovery is a solution to solve the antibacterial resistance issue and obtain the more effective and less toxic anti-TB drug.

Mangrove endophytic fungi are a promising source of bioactive compounds. Mangrove endophytic fungi have unique structures, metabolic pathways, reproductive systems, and durability because they have adapted to extreme environments [4]. Secondary metabolites isolated from mangrove endophytic fungi have been reported to be rich in biological activity such as cytotoxic [5], antibacterial and antimycobacterial [6], antifungal [7], antiviral [8], and antioxidant activity [9]. This study was conducted to isolate endophytic fungi associate with mangrove from West Sumatera and examine their antibacterial activity against several pathogens including *M. tuberculosis.*

2. METHODS

2.1. Sample Collections

Mangrove *Rhizophora apiculata* was collected at Mandeh Beach, West Sumatra. Roots, barks, and leaves from the mangrove that have been surface sterilized by sterile seawater were used for endophytic fungi isolation. The culture stock of endophytic fungi from *Sonneratia alba* in the previous research was obtained from Sumatran Biota Laboratory [10].

2.2. Isolation, Cultivation, and Extraction of Endophytic Fungi

Method for fungi isolation and cultivation was carried out in previous research [11]. The sample was cut into small pieces. 10 gr of the sample was dispersed in 100 mL of sterile seawater. The filtrate was diluted using the multilevel dilution method and transferred 1 mL of each dilution into Sabouraud Dextrose Agar (SDA) medium

(Merck®) in a petri dish. The fungi were purified by isolating each fungal into the fresh medium. Endophytic fungi of *S. alba* from culture stock were inoculated using a sterile loop into an SDA medium. Furthermore, the fungal isolate was cultivated in rice medium for 4-6 weeks. Fungal culture medium that has been overgrown by fungi was macerated using ethyl acetate (EtOAc) at a 1:1 ratio of fungi and solvent with three repetitions. The extract was subsequently evaporated in vacuo using a rotary evaporator.

2.3. Evaluation of Antibacterial Activity

Antibacterial activity evaluation was carried out using the disc diffusion method [12]. EtOAc extracts from endophytic fungi were tested against clinical isolate of *Bacillus cereus* and Multi-Drug Resistant *Pseudomonas aeuruginosa*, *Staphylococcus aureus* ATCC25923, and *Escherichia coli* ATCC25922. The disc was prepared with 10 µl of 5% EtOAc extract in DMSO that was equivalent to 500 µg extract in each disc. Chloramphenicol 30 µg/disc was used as positive control and DMSO as a negative control. The discs were placed on the surface of a nutrient agar medium that has been inoculated with bacteria, then the medium was incubated for 18-24 hours at 37°C. The test was performed with three repetitions. The diameter of the inhibition zone and the standard deviation of the test were measured.

The method for testing antibacterial activity against *Mycobacterium tuberculosis* H37Rv was followed the previous study [14]. The fungal extract was dissolved in 5 ml of Lowenstein-Jensen (LJ) medium with a concentration of 1%, 3%, and 5% (% b/v). Bacterial was suspended in distilled water and adjusted to 0,5 McFarland standard. Inoculated 0.2 - 0.4 ml bacterial suspension on to LJ medium. Rifampin was used as a positive control. The cultured medium was incubated at 37°C. The growth of bacteria in the culture was observed within 8 weeks. The test was performed without repetitions Extracts are considered sensitive to bacteria if there is no bacterial growth or medium discoloration. The extract is assessed resistance if there is bacterial growth or medium discoloration occurs.

2.4. Phytochemical screening

Screening of chemical constituents to detect the presence of alkaloid, flavonoid, phenolic, tannin, and saponin in the selected fungal extracts was conducted using the standard method [15].

2.5. Identification of fungi

2.5.1. Macroscopic and microscopic evaluation of fungi isolate

Macroscopic characteristics of fungi observing by visualization of fungal colony. Lactophenol cotton blue was used as a fungal staining agent and microscopic features of fungi were observed under a light microscope [16].

2.5.2. Molecular identification

The DNA extraction method was conducted by the following method of Atashpaz et al. Cell biomass of the pure fungal isolate on SDA was transferred into 500µL lysis buffer in a microtube and the mixture was subsequently centrifuged at 18.000 rpm for 5 minutes at 4°C. The DNA pellet was dissolved in 50 µL TE buffer pH 8.0 [17].

Molecular identification was carried out using an internal transcribed spacer (ITS)1 (F 5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 primer (R 5'-TCC TCC GCT TAT TGA TAT GC-3'). The amplification ran in PCR was conducted by 34 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 1 minute, and step extension at 72°C for 1 minute.

The sequencing of the PCR product was done in first base Malaysia [18]. Furthermore, the sequenced were subjected to the BLAST program on NCBI for the identification of fungal species. The phylogenetic tree was constructing using the Neighbor-Joining method by MEGA 6.0 software with 1,000 bootstrap replications [19]

3. RESULTS AND DISCUSSION

Mangrove forests have extensive biodiversity and are rich in host diversity for endophytic fungi [20,21]. The aeration roots of mangroves are below sea level so that fungi and other marine organisms occupy the bottom of the mangrove while the stem is above sea level and occupied by fungi from the terrestrial [22]. Many endophytic fungi from mangroves are known to produce the antibacterial compound. Asperterpenoid from *Aspergillus* sp. was reported to inhibit protein tyrosine phosphatase B (mPTPB) of *M. tuberculosis* with an IC_{50} value of 2.2µM [23]. *Nigrospora* sp from mangrove *Pongamia pinnata* was produced 2,3-dihydro-19a-hydroxy-14-epicochlioquinone B that exhibit significant antibacterial activity against MRSA, *E. coli*, *P. aeruginosa*, *P. fluorescent*, and *S. epidermidis*, with MIC values of 16.5, 8.2, 8.2, 1.0, and 1.0 µM respectively [24]. In this study, here we report seven endophytic fungal isolates from *R. apiculata* and antibacterial activity of mangrove endophytic fungi from *R. apiculata*, and two fungal isolates from *S.alba*.

Antibacterial activity from seven *R. apiculata* was coded as RAA1, RAA2, RAA3, RAA4, RAK1, RAK2, RAD1 and two endophytic fungi of *S. alba* with the code SaDa1 and SaDa2 showed different results (Table 1 and Table 2). Seven isolates showed a zone of bacterial growth inhibition in the agar medium. The diameter of the inhibition zone showed in table 1. RAA1 at 5% concentration showed the strongest inhibition toward all bacteria testing with diameter inhibition zone above 21 mm and followed by SaDa1 and RAA4 at 5% concentration with diameter inhibition zone above 10 mm.

RAA2, SaDa2, RAK2, RAA3, RAA4, and SaDa1 in 3% and 5% concentrations are considered sensitive toward *M. tuberculosis* because of no bacterial growth or medium discoloration. LJ medium of some testing tubes was contaminated, resulting in the antituberculosis activity of those extracts cannot be determined. Figure 1 showed there is no bacterial growth or discoloration in the culture of *M. tuberculosis* in LJ medium containing extract of RAA4 in the observation at the eighth week. The complete antituberculosis activity of fungal isolate extracts is listed in table 2.

Based on antibacterial activity RAA1, RAA4, and SaDa1 are the most interesting fungi for further identification and phytochemical screening. Macroscopic and microscopic identification of the fungi was shown in figure 2. Phytochemical screening of potential fungi extract showed in table 3.

RAA1 was yellowish-green with cotton white edges and yellowish-white at the reverse colony. Molecular identification of fungi revealed that RAA1 was *Aspergillus flavus*. In comparison using BLAST, the sequence of RAA1 was 100% identical to *Aspergillus flavus* MT420620. The various biological active compound has been isolated from endophytic fungi *A. flavus* such as 5-hydroxy-pyrone and alkaloid that showed cytotoxic activity and kojic acid that exhibit antibacterial activity and widely used as UV B protection agent [25]. Endophytic fungus *A. flavus* has been isolated from mangrove *Sonneratia griffiti* and showed antibacterial activity against *Escherichia coli* [26]. Research that has been conducted by Patil et. al. showed endophytic fungus *A. flavus* exhibit antimicrobial, antioxidant, and antiinflammation activity and the extract from the fungus was reported the presence of flavonoid rutin [27]

RAA4 has black color with cotton white edges on the surface and light yellow at the reverse colony. Microscopic features of RAA4 showed ellipsoidal conidia and round black conidiophore. RAA4 was identified molecularly as *Aspergillus aculeatus* and showed 100% query cover identical to *Aspergillus aculeatus* MN186997. *Aspergillus* sp. is rich in the produce of alkaloids, terpenoids, xanthones, and polyketides. these chemical constituents showed many biological activities such as antibacterial, antifungal, antifouling, and cytotoxic activities [28]. Yodsing et al have been reported some metabolites compounds from this fungus that have various biological activities such as variecolin, ergosterol, and secalonic acid F as antimalarial; ergosterol peroxide as antiviral; and variecoline, variecolactone, ergosterol, ergosterol peroxide, secalonic acid D and F as cytotoxic agent [29]. A new tyrosine-derived metabolite known as Aspergillusol has been isolated from *A. aculeatus* that is associated with sponge *Xestospongia testudinaria*, this compound showed the ability to inhibited α-glucosidase of Saccharomyces cerevisae with an IC_{50} value of 222±8 [30]. Crude extracts from *A.aculeatus* also showed antimycobacterial, antimalarial, antiviral, and antioxidant activity [31]

The fungus SaDa1 has a bluish-green colony surface and flat white edges, the reverse of the colony was yellowish white. This fungus exhibits branched conidiophore and ellipsoidal conidia. The molecular identification result indicated SaDa1 is *Penicillium rubens*. The phylogenetic tree construct with the neighbor-joining method in figure 3, showed SaDa1 close to *Penicillium rubens* MT558923. *Penicillium chrysogenum* and *P. rubens* are phenotypically similar but have differences in extrolite production. *P. chrysogenum* produced secalonic acid F/D or lumpidin related metabolite, while *P. rubens* does not [32]. Extract from marine-derived fungi *P. rubens* that associated with sponge *Agelas oroides* showed antioxidant and cytotoxic activity [33]. Chloctanspirone A is a metabolite compound isolated from *P. rubens* that showed activity against HL-60 and A-549 cell lines [34]. Houbraken et. al. have been analyzed the extrolites produced by *P. rubens* using HPLC diode-array detection method and the analysis revealed that P. rubens produced many extrolites such as andrastin A & B; chrysogine, 2-pyruvoylaminobenzamide, 2-acetyl-quinazolin-4(3H)-one & 2-(2-hydroxypropionylamino)-benzamide; citreoisocoumarin; 7-deacetoxyyanuthone; penicillins; roquefortine C, D & meleagrin; sorbicillins (including bisorbibutenolide, bisorbicillinol, bisvertinoquinol, bisvertinolone,2',3'-dihydrosorbicillin, oxosorbicillinol tautomer, sorhinones A, B, & C, rezishanones A, B, C & D, sorbicillin); xanthocillins; PR-toxin; quinazolone X [35].

Table 1. antibacterial activity of fungal isolate extract against several pathogens

Fungal isolate	Diameter of inhibition zone (mm ± Standard of Deviation)			
	MDRPA	*B. cereus*	*E. coli*	*S. aureus*
RAA 1	23.16 ± 1.39	20.80 ± 0.42	21.87 ± 0.81	26.12 ±0 .21
RAA 2	0	0	0	0
RAA 3	0	0	0	8.05 ± 0.18
RAA 4	12.2 ± 1,11	10.4 ± 0.29	10.31 ± 0.12	14.18 ± 1.00
RAK 1	8.27 ± 0.31	7.92 ± 0.32	0	7.72 ± 0.53
RAK 2	8.65 ± 0.56	6.05 ± 0.00	0	8.40 ± 0.14
RAD 1	0	0	0	0
SADA 1	13.25 ± 0.88	10.65 ± 0.01	14.75 ± 0.01	11.65 ± 0.10
SADA 2	9.04 ± 0,01	10.67 ± 0.37	7.83 ± 0.72	10.48 ± 1.5

The test was performed with three repetitions

Table 2. The antituberculosis activity of fungal isolate extracts in the eighth week

Fungi extracts	**Concentrations**		
	1 %	**3 %**	**5 %**
RAA1	Contaminated	R	R
RAA2	R	S	S
RAA3	Contaminated	S	S
RAA4	Contaminated	S	S
RAD1	Contaminated	R	Contaminated
SADA1	Contaminated	S	S
SADA2	R	S	S
RAK1	Contaminated	Contaminated	Contaminated
RAK2	R	S	S
Negative control	S	S	S
Positive control	R	R	R

R : Resistant to *M. tuberculosis*, S: Sensitive to *M. tuberculosis*;

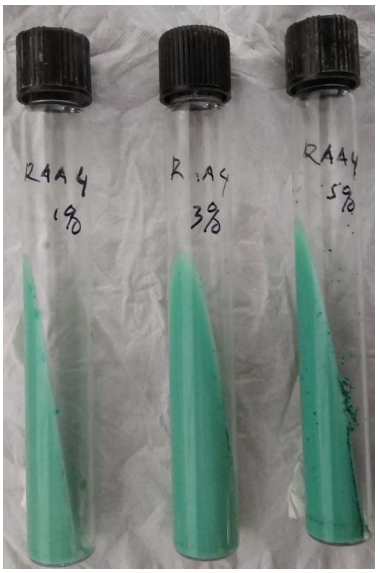

Figure 1. No bacterial growth in the culture medium of *M. tuberculosis* containing RAA4 extract in the eighth week.

Table 3. Phytochemical screening of the potential fungal extract

Fungal code	Alkaloid	Flavonoid	Tanin	Phenolic	Saponin
RAA 1	-	+	-	-	-
RAA 4	-	-	-	-	+
SADA 1	-	+	-	-	-

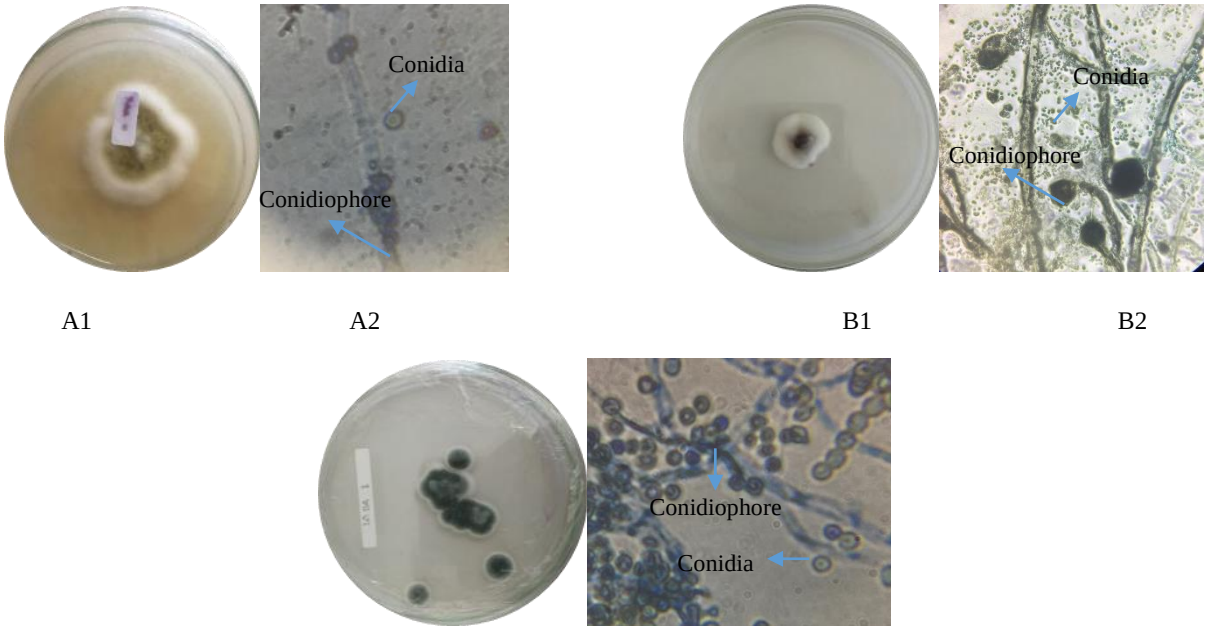

Figure 2. Macroscopic and microscopic identification of RAA1 (A1, A2), RAA4 (B1, B2), and SaDa1 (C1, C2)

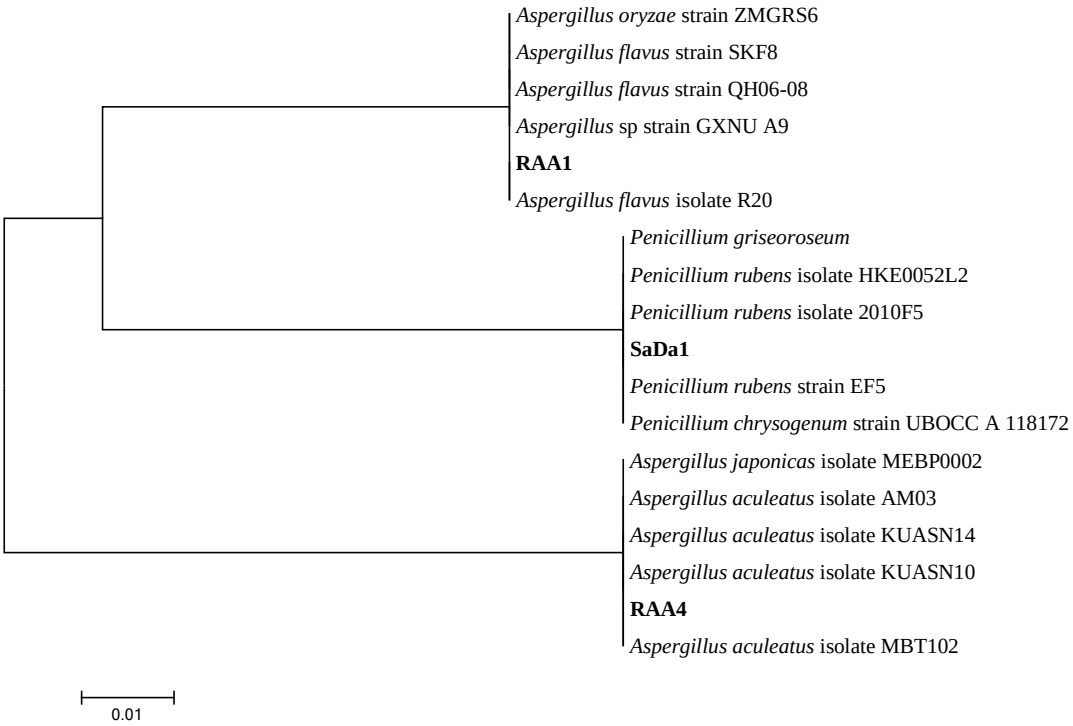

Figure 3. Neighbor-Joining phylogenetic tree of RAA1, RAA4, and SaDa1

4. CONCLUSION

Fungal isolate RAA1, RAA4, and SaDa1 revealed as *A. flavus*, *A. aculeatus*, and *P. rubens* respectively showed the potential of significantly antibacterial activity. *Aspergillus* sp and *Penicillium* sp are the widely studied marine and mangrove fungi. These fungi produce diverse chemical constituents and unique bioactivities. In further research, it is necessary to isolate the bioactive compounds of these fungi to be developed as drug candidates.

AUTHORS' CONTRIBUTIONS

All authors contributed equally.

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