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Research Article

Antimicrobial Activities of *Rhopalaea*-Associated Fungus *Aspergillus flavus* Strain MFABU9

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Conflict of interest

We have no conflict of interest to declare.

Abstract

Background and Objective: *Rhopalaea* is a genus of ascidian belonging to the family Diazonidae. Ascidians provide niches for various microorganisms including fungi. This present study describes the potential new source for natural bioactive compounds from *Rhopalaea*-associated fungi obtained from Bunaken marine park. **Materials and Methods:** As part of an on-going research program to explore the chemical diversity of marine derived fungi, we performed an antimicrobial bioactivity-guided screening of EtOAc extracts of the fungi isolated from ascidian *Rhopalaea* sp.

Results: The study confirms that the ascidian obtained from Bunaken marine park was *Rhopalaea* sp.The fungus isolated from the ascidian was *Aspergillus flavus* which showed antimicrobial activity against bacteria *Escherichia coli*, *Staphylococcus aereus*, *Aeromonas hydrophila*, and antifungal against the human pathogenic fungus *Candida albicans*.

Conclusion: *Aspergillus flavus* isolated from ascidian *Rhopalaea* sp. has the potential as antibactial and antifungal.

Keywords:

ascidian, antimicrobial, *Aspergillus,* bioactive compound, marine derived-fungus, *Rhopalaea*

Introduction

Marine-derived fungi are well recognised as a source of various novel metabolites, many of which possess valuable biological properties [1, 2] and pharmacological properties [3]. Marine-derived fungi whichfound in algae [4], mangrove [5], ascidians [6], and sponges [7] were shown to have antibacterial and anticancer activities.

Ascidians are found abundantly all over the world. They mostly live in shallow water with salinities over 2.5% [8]. However, they also can be found in the depths of the sea. Ascidian is a marine invertebrate animal and a member of the subphylum Tunicata. It productively produce a wide variety of secondary metabolites which are biologically and pharmacologically active [9]. These compounds have properties such as antibacterial [10] and anticancer [11] activities which make them candidates for potential new drugs.

Correlations between ascidian microorganisms and ascidian metabolites are also being investigated, while the structures and synthetic pathways of a growing number of relevant compounds have been identified [12]. Many of these metabolites are produced by the fungi isolated from ascidian [9, 13, 14]. A number of new metabolites have been reported describing the fungal

association with ascidians. As an example, Indonesian ascidian produced *Penicillium verruculosum* which inhibited the activity of PTP 1B [15] as well as *P. albobiverticillium* [16]. Menezes et al. [17] analyzed in detail aboutfungal diversity in *Didemnum* spp.

Some compounds have been isolated from marine ascidians-derived fungi, which are taxonomically similar or identical to terrestrial fungi, such as *Aspergillus* [18]. The genus *Aspergillus*, which includes approximately 200 species, has been well studied and shown to produce many new metabolites [19]. Various fungal strains which predominated by *Aspergillus* and *Cladosporium*were have been isolated from various Australian coral reefs [20]. An ascidian-derived fungus *Aspergillus* sp. KMM 4676 exhibited cytotoxic activity as hormone therapy-sensitive human prostate cancer cells [21]. *Aspergillus candids* isolated from colonial ascidian had a cytotoxic activity against hormone-sensitive line LNCaP [22]. Various kind of marine fungal strains has been reported to produce many kind of novel antimicrobial compounds. These compounds belong to alkaloids, macrolides, terpenoids, peptide derivatives as well as other structure types [23].

Bunaken marine park in North Sulawesi (Indonesia) has been known for its various types of ascidians, among other is *Rhopalaea* sp. This type of ascidian is rarely investigated in association with the fungi and their antibacterial activities. In this present study, *Rhopalaea* sp. from Bunaken marine park was studied to observe the possibility of its associated fungi for antibacterial activity.

Materials and Methods

Sample preparation

Ascidian *Rhopalaea* sp. (Figure 1) was collected at the Bunaken marine park in North Sulawesi, Indonesia, in March 2019. The ascidian was rinsed with sterilized sea water and immersed in ethanol 70% for 1 minute, then kept in the bottle vial and stored in the cool box and transported to the laboratory for further observation [24].

Isolation of ascidian-derived fungi from* Rhopalaea *sp.

A small piece of the sample was cut into cubes, washed with sterilized seawater, and grown on PDA plate (BD, Franklin Lakes, NJ, theUSA). The plate was incubated at 25^{o}C for a week. The different appearing fungal colonies surrounding the samples that have different characteristics were isolated and grown on PDA. The isolation process was conducted for another round to obtain a pure single colony [24]. One pure isolate designated as MFABU9 was chosen and grown on PDA. Afterwards, the isolate was inoculated into a 100-mL Erlenmeyer flask containing 50 mL sterilized sea water and 50 mg sterilized rice as rice medium for 14 days.

EtOAc Extraction of MFABU9 Isolate

The 100 g of rice medium containing the selected growing fungus was extracted with EtOAc for 24 hours. To complete the extraction process, the rice was incubated in 200 ml of EtOAc for 3 days at 25˚C with constant shaking. The solvent was filtered using Whatman No.1 filter paper. The first filtrate was set aside in one clean Erlemeyer, and the remaining debris was soaked in 200 ml of EtOAc for 3 days at 25˚C with constant shaking. The second and third filtrates were obtained using the same process. All of the filtrates were then combined and concentrated using a vaccuum rotary evaporator at 40°C to obtain concentrated extract. The concentrated extract was then evaporated in the incubator to obtain a dry extract. The dry extract was weighted and stored in 20°C until used.

Screening for antimicrobial activity

The indicator pathogens used were bacterial strains *E. coli*, *S. aereus*, *Aeromonas hydrophila*, *Salmonella* sp., *Edwardsiella tarda* and *C. albicans.* Each of the bacterial inoculum *E. coli*, *S. aereus*, and *A. Hydrophila* were cultured in liquid media B1 (peptone, meat extract, NaCl

and aquadest) for 1 x 24 hours, while *Salmonella* sp. and *E. tarda* were cultured in TSA media. Anti-bacterial assay was carried out by the paper disc method using agar diffusion Kirby-Bauer methods, following the guidelines of Clinical and Laboratory Standard Institute (CLSI) [25].

The EtOAc extract of isolate MFABU9 was examined for the inhibitory activities against the indicator pathogenic bacteria and fungus with the concentrations of 20 µg / disk. The pathogenic bacteria were grown in Nutrient Agar (NA), while the indicator fungus was grown in Saboraud Dextrose Agar (SDA). Chloramphenicol was used as positive control for bacteria and Ketoconazole for fungus. For negative control, 40% CH_3OH was used. The plates were incubated at 37°C and the inhibitory activities were measured after 48 hours of incubation. The results were interpreted by measuring the diameter of the inhibition zone using a caliper. The inhibition zone is a measure of the effectiveness of an active compound. Generally, a larger zone of inhibition means that the antimicrobial is more potent.

Molecular identification of isolate MFABU9

DNA extraction of the fungal isolate was carried out using Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, D6005). The ITS (internal transcribed spacer) region was amplified using with primer pair ITS1 (F 5'- TCC GTA GGT GAA CCTGCG G-3') and ITS4 (R 5'- TCC TCC GCT TAT TGA TATGC-3') in the MyTaq HS Red Mix (Bioline, BIO-25047). The following PCR amplification conditions were one cycle of initial denaturation at 95°C for 5 minutes, followed by 35 cycles with a step of denaturation at 95°C for 30 seconds, step annealing at 55°C for 1 minute, and step extension at 72°C for 1 minute, followed by one cycle at 72°C for 6 minutes.

PCR products were purified using Zymoclean™ Gel DNA Recovery Kit (Zymo Research, D4001) and sent to the sequencing service provider. The sequencing result was processed following the procedure performed by Tallei et al. [26] and subjected to BLAST (Basic Local Alignment Search

Tool) search at NCBI (National Center for Biotechnology Information https://www.ncbi.nlm.nih.gov/), ISHAM (International Society for Human & Animal Mycology https://www.isham.org/), and BOLD (Barcode of Life Data System http://www.boldsystems.org/), andMycoBank (http://mycobank.org) for species identification.

Results and Discussion

The fungal extract of isolate MFABU9 only showed antibacterial activity against *E. coli*, *S. aureus*, *A. hydrophila*, and antifungal activity against *C. albicans*as (Table 1). The extract, however, failed to suppress the growth of *E. tarda* and *Salmonella* sp. The isolate MFABU9 was molecularly identified as *Aspergillus flavus*, hence it is called *A. flavus* strain MFABU9. Inhibitory activity could be defined by the diameter of inhibition zones. Inhibitory activity is defined as weak if the diameter is less than 10 mm. Inhibitory activity is defined as intermediate if the diameter ranges between 10 to 15 mm. Meanwhile, inhibitory activity is defined as strong if the diameter is more than 16 mm. According to this, the extract of *A. flavus* strain MFABU9 showed a weak inhibitory activity against *S. aureus* and *C. albicans*, and intermediet inhibitory activity against *E. coli* and *A. hydrophila*.

Only a little information about *A. flavus* isolated from ascidian that has been reported. Marine-derived *A. flavus* produced various kinds of secondary metabolites including mutagenic mycotoxins and other bioactive compounds [27]. Ivanets et al. [21] were able to isolate asperindoles A–D and a p-terphenyl derivative from the ascidian-derived fungus *Aspergillus* sp. KMM 4676 which inhibited the growth of hormone therapy-resistant PC-3 and 22Rv1 and hormone therapy-sensitive human prostate cancer cells.

Some marine algal-derived *A. flavus* has been reported to produce bioactivy compounds. A cerebroside, an antibacterial cerebroside derivate isolated from *A. flavus* had antibacterial activity

against *S. aureus*, methicillin-resistant *S. aureus*, and multidrug-resistant *S. aureus* [28]. Citrinadins A and B had been isolated from *A. flavus* which is associated with a green alga *Enteromorpha tubulosa*. This compound was cytotoxic to several tumor cell lines HL-60, MOLT-4, A-549, and BEL-7402 [29].

Ascidian has become a source of so many types of secondary metabolites. The resulting metabolites are used for physiological functions and specifically for defense mechanism against predators. Secondary metabolites which are synthesized by ascidians are not only synthesized by ascidians themselves, but also can be synthesized by the associated-microorganisms [30]. Fungi are one of the microorganisms associated with ascidians. Most of the fungi can be found in the ascidian's tunic and several others can be found in the inside of the ascidian [31].

Some types of fungi show specific relationships with ascidians. Fungi are involved in the ascidian's synthesis of bioactive secondary metabolites. However, the relationship between most fungi and specific ascidians is unclear. This indicates that the functional interactions between ascidians and fungi, especially in the interaction of *Rhopalaea* sp. and *A. flavus*, are still unclear [32].

Aspergillus is a genus of filamentous fungi. It has been widely used as a source of medicines [32]. Several species of the genus *Aspergillus* produce various types of compounds. Some of the compounds were aminobenzoic peptide secoclavatustide B, clavatoic acid derivative 5-acetyl-2,4-dihydroxy-3-methyl-benzoic acid [33], clavatustide B [34], demethylsiderin, 3,7-dihydroxy-4-methylcoumarin and demethylkotanin [35].

Fungal species of the genus *Aspergillus* which ~~could be~~ found in water and associated with marine organisms, have many metabolites that have been proven to have the antibacterial and antifungal ability [36]. The analysis of *A. flavus* extract showed that this fungus has antibacterial activity against *S. aureus*, *E. coli* and *A. hydrophila* and antifungal against *C. albicans*. This can be caused by the role of secondary metabolites of *A. flavus*. However, these antibacterial and

antifungal activities did not only depend on certain types of synthesized compounds. These activities may be influenced by various metabolite compounds that play a synergistic or antagonistic role that support these activities [37]. This is because the responses showed by the extract not only arises against fungi (*C. albicans*), but also against Gram-positive (*S. aureus*) and Gram-negative bacteria (*E. coli* and *A. hydrophila*).

Besides the synergistic and antagonistic roles, specific types of synthesized compounds may also perform different antibacterial mode of actions to specific types of bacteria. It is because of different types of bacterial cells, may only be affected by the specific mode of action [38]. It can be implied that *A. flavus* may synthesize compounds with antibacterial activity against *S. aureus*, *E. coli* and *A. hydrophila* with specific reaction and mode of action.

Conclusion

The results obtained from the present study confirmed that *A. flavus* strain MFABU9 isolated from ascidian *Rhopalaea* sp. showed weak to intermediete inhibitory activities against selected indicator pathogenic microorganisms. Further studies are needed to be conducted to isolate each of bioactive compounds from this strain and test for their antibacterial and anticancer activities. The data obtained in this study have opened of the importance of a preliminary screening study. The molecular docking can be conducted after bioactive compounds from this strain have been elucidated.

Significance statement

This study discovered that fungus *A. flavus* isolated from ascidian *Rhopalaea* sp. obtained from the Bunaken marine park showed antibacterial activities against *E. coli*, *S. aureus*, and *A.*

hydrophila and antifungal activity against *C. albicans*. However, it is advisable to explore further the bioactive compounds of this fungus which can be used as antimicrobial agents.

Acknowledgment

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Table 1: Antimicrobial assay of *A. flavus* strain MFABU9 extract

Indicator Microbes	Inhibition Zone (mm)
Indicator Bacteria	
Escherichia coli	10.75
Staphylococcus aureus	9
Edwardsiellatarda	-
Salmonella sp.	-
Aeromonashydrophila	11.45
Control + (Chloramphenicol)	25
Control -	-
Indicator Fungus	
Candida albicans	9.5
Control + (Ketoconazole)	25
Control -	-

Figure 1. Ascidian *Rhopalaea* sp. collected at Bunaken marine park in North Sulawesi, Indonesia

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