

Title:

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

Keywords:

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Abstract

Background and Objective: *Rhopalaea* is a genus of ascidian belonging to the family Diazonidae. Ascidiaceans 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 aureus*, *Aeromonas hydrophila*, and antifungal against the human pathogenic fungus *Candida albicans*. **Conclusion:** *Aspergillus flavus* isolated from ascidian *Rhopalaea* sp. has the potential as antibacterial and antifungal.

Authors' contribution

Name of the author and e-mail ID	Types of contribution
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Conflict of interest

We have no conflict of interest to declare.

Significance statement

This study discovered that fungus *Aspergillus flavus* isolated from ascidian *Rhopalaea* sp. obtained from the Bunaken marine park showed antibacterial activities against *Escherichia coli*, *Staphylococcus aureus*, *Aeromonas hydrophila* and antifungal activity against *Candida albicans*. However, it is advisable to explore further the bioactive compounds of this fungus which can be used as antimicrobial agents.

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 properties³. Marine-derived fungi which found in algae⁴, mangrove⁵, ascidians⁶, and sponges⁷ 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 active⁹. These compounds have properties such as antibacterial¹⁰ and anticancer¹¹ 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 identified¹². 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 1B¹⁵ as well as *P. albobiverticillium*¹⁶. Menezes et al¹⁷ analyzed in detail about fungal 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 metabolites¹⁹. Various fungal strains which predominated by *Aspergillus* and *Cladosporium* were isolated from various Australian coral reefs²⁰. An ascidian-derived Fungus *Aspergillus* sp. KMM 4676 exhibited cytotoxic activity as hormone therapy-sensitive human prostate cancer cells²¹. *Aspergillus candidus* isolated from colonial ascidian had a cytotoxic activity toxic against hormone-sensitive line LNCaP²². 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 types²³.

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 sea water was studied to observe the possibility of associated fungi with antibacterial ability.

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 coolbox and transported to the laboratory for further observation²⁴.

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, the USA). The plate was incubated at 25°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 colony²⁵. One pure isolate designated as MFABU9 was chosen and grown on PDA. Afterwards, the isolate was inoculated in 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 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 Erlenmeyer, 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 vacuum 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 strain of *E. coli*, *S. aureus*, *Aeromonas hydrophila*, *Salmonella* sp., *Edwardsiella tarda* and *C. albicans*. Each of the inoculum of *E. coli*, *S. aureus*, 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. Antibacterial 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)²⁶.

The EtOAc extract of isolate MFABU9 was examined for the inhibitory activities against the indicator pathogenic bacteria and yeast with the concentrations 20 µg/disc. The pathogenic bacteria were grown in Nutrient Agar (NA), while the indicator fungus was grown in Sabouraud Dextrose Agar (SDA). Chloramphenicol was used as positive control for bacteria and Ketoconazole for fungus. For negative control, 40% CH₃OH 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 1BASE in Malaysia. The sequencing result was processed following the procedure performed by Tallei et al.²⁷ 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/>), and MycoBank (<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 seen in Table 1. The extract, however, failed to suppress the growth of *E. tarda* and *Salmonella sp.* The MFABU9 isolate 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 resistance if the diameter is less than 10 mm. Inhibitory activity could be defined as intermediate if the diameter is around 10-15 mm. Meanwhile, inhibitory activity is defined as vulnerable 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 intermediate inhibitory activity against *E. coli* and *A. hydrophila*.

Based on the molecular identification, marine-derived fungi strain MFABU9 was identified as *Aspergillus flavus*, a saprotrophic and cosmopolitan fungus. 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 compounds²⁸. 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 bioactive compounds. A cerebroside, an antibacterial cerebroside derivative isolated from *A. flavus* had antibacterial activity against *Staphylococcus aureus*, methicillin-resistant *S. aureus*, and multidrug-resistant to *S. aureus*²⁹. 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-7402³⁰.

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-microorganisms³¹. Fungi is 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 ascidian³².

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 unclear³².

Aspergillus is a genus of filamentous fungi. It has been widely used as a source of medicines³³. Several species of the genus *Aspergillus* could produce various types of compounds. Some of the compounds were aminobenzoic peptide secoclavaturoid B, clavatoic acid derivative 5-acetyl-2,4-dihydroxy-3-methyl-benzoic acid³⁴, clavaturoid B³⁵, demethylsiderin, 3,7-dihydroxy-4-methylcoumarin and demethylkolanin³⁶.

The fungi species of the genus *Aspergillus* which could be found in waters and associated with marine organisms, have many metabolites that have been proven to have the antibacterial and antifungal ability³⁷. 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 could 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 could be influenced by various metabolite compounds that play a synergistic or antagonistic role that support these activities³⁸. This could be seen from the response that not only arise against bacteria and fungi (*C. albicans*), but also in its response to gram-positive (*S. aureus*) and gram-negative bacteria (*E. coli* and *A. hydrophila*).

Besides the synergistic and antagonistic roles, specific types of synthesized compounds could also perform different antibacterial mode of actions to specific types of bacteria. It is because of different types of bacteria cell, which are gram-positive and gram-negative bacteria, could only be affected by the specific mode of action³⁹. It could be implied that *A. flavus* could synthesized compounds with antibacterial activity to *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 intermediate inhibitory activities against selected indicator pathogenic microorganisms. Further studies are needed to be conducted to isolate each of bioactive compounds from this strain and tested for their antibacterial and anticancer activities. The data obtained 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.

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

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



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

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