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Research Article

Antimicrobial Activity Screening of Symbiotic Fungi from Marine Sponge *Petrosia nigrans* Collected from South Coast of West Sumatera, Indonesia

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ABSTRACT

The present study was concerned with the screening of antimicrobial activity from symbiotic fungi associated with marine sponge *Petrosia nigrans* collected from Carocok Coast of West Sumatera, Indonesia. The symbiotic fungi that associated with sponge were cultivated on SDA media. The test of antimicrobial activity was done by using agar diffusion method. The identification of bioactive fungi isolates was done by comparing its macroscopic and microscopic characteristic base on literature. A total of 18 fungi were obtained from marine sponge *P. nigrans*. The antimicrobial activity was studied with all the 18 isolates. Among those isolates shown to have antibacterial activity, namely P_2, P_4, P_6, P_{11}, P_{15} and P_{16} against *S. aureus*. Three of active fungi were identified as *Aspergillus fumigatus* (P_2), *Aspergillus flavus* (P_{11}), and *Candida* sp (P_{15}). This study suggests that symbiotic fungi of *P. nigrans* having antibiotic producing properties and need to be investigated further so that it can be exploited in new drug discovery and used commercially in the future.

Keywords: Antimicrobial activity, Symbiotic fungi, Marine sponge, *Petrosia nigra*

INTRODUCTION

Sponge was known as the largest producer of bioactive compounds among other marine invertebrates, so that it has a great potential of lead compound to be developed. Taylor was reported in the last decade as many as 50 percent of the bioactive compounds found in marine invertebrates derived from sponges. Antibacterial, antifungal, antitumor and antiviral are some potential that have been discovered and developed from sponges[1]. The sponge is also known as a very fertile host for a variety of microorganisms symbionts. Microbial symbionts can be either bacterial or fungal and contribute over 40-60% of the animal biomass. All sponges are filter feeders and have many pores on the surface that allow water to enter and circulate through a series of canals. Through its pores, microorganisms and organic particles are entered, filtered out and eaten. Since the effective sponge as filter feeders, some microorganisms that can resist digestion and the immune system can inhabit sponge. For sponges, symbiont microorganisms have function in aiding the process of acquiring nutrients (especially in the fixation of carbon and nitrogen), stabilizer of sponges from, the process of excretion and took part in the cycle of production of secondary metabolites[2]. Sponge which was associated with fungi has been repeatedly shown as the source of new bioactive secondary metabolites. Unusual fungal metabolites including hortein, a new polyketide from fungi *Hortaea werneckii* isolated from the sponge *Aplysina aerophoba*, anthraquinone and its derivatives butanone and ç-pyrones from *Microsphaeropsis* sp. were isolated also from the sponge *A. aerophoba*, spicifera derivatives of *Drechslera hawaiiensis* derived from sponges *Callyspongia aerizusa*[3] and xestodecalactones is produced by *Penicillium montanense* isolated from sponges *Xestospongia exigua*[4,5]. Screening of bioactive compounds from microorganisms that are symbiotic with sponge seem to be more profitable than isolating it from host (sponge) itself. The relatively slow growth of the sponge is very influential on the limited supply of biomass for extracting secondary metabolites. The use of microbial that associated with the sponge is better because it can be purified and cultured in the laboratory so it does not need to collect them from the wild, can be propagated in a faster time, and relatively easy to manipulate. The results of our previous research on the isolation of the main chemical compounds of non-polar fraction of the marine sponge *P. nigrans* showed antibacterial activity, this compound is believed to epidioxy-5,8-24-ethylcholest-6-en-3-ol. The antibacterial activity of this compound has a value of 50 ppm minimum inhibitory concentration against *Staphylococcus aureus* and 100 ppm for *Escherichia coli* and *Pseudomonas aureginosa.* Due to potentially produce antibacterial compounds, the present study aims to isolate various fungi from marine sponge *P. nigrans* and to evaluate their antimicrobial activity by performing antibacterial studies. This continuous researches were done in order to exploit the bioactive potential of symbiotic microbial with marine sponge derived from West Sumatera[6,7].

MATERIALS AND METHODS

Sample collection

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Table 1: Phytochemicals screening (+ = Indicates the presence, - = Indicates the absence)

Fungi Extract	Test			
	Steroids dan Terpenoids		Phenols	Alkaloid
	Lieberman-Bouchard	Vanilin sulfat	$FeCl_3$	Reagen Mayer
P_1	-	-	-	+
P_2	-	-	+	-
P_3	-	+	-	+
P_4	-	+	-	-
P_5	-	+	-	-
P_6	-	+	-	-
P_7	-	-	-	-
P_8	-	+	-	+
P_9	-	-	-	-
P_{10}	-	-	-	+
P_{11}	-	+	-	+
P_{12}	-	+	-	-
P_{13}	-	-	-	-
P_{14}	-	-	-	+
P_{15}	-	-	+	+
P_{16}	-	+	-	-
P_{17}	-	-	-	-
P_{18}	-	+	-	-

Table 2: Antimicrobial activity of endophytic fungi extracts of *P. nigrans* against human pathogenic (- = Indicates the no zone of Inhibition)

Fungi Extract	Zone of inhibition (mm)		
	Escherichia coli	*Staphylococcus aureus*	*Candida albicans*
P1	-	-	-
P2	-	15	-
P3	-	-	-
P4	-	15	-
P5	-	-	-
P6	-	14	-
P7	-	-	-
P8	-	-	-
P9	-	-	-
P10	-	-	-
P11	-	24	-
P12	-	-	-
P13	-	-	-
P14	-	-	-
P15	-	14	-
P16	-	24	-
P17	-	-	-
P18	-	-	-

P. nigrans was collected from the Carocok Coast, South Coast of West Sumatera, Indonesia, in the depth of ± 5m. The sponge put soon after collection to a sterile plastic bag and stored in the ice box for the isolation of symbiotic fungi and transported to the laboratory.

Isolation of Fungi associated with marine sponge

Isolation of symbiotic fungi begins with a sample surface sterilization. Sponge was rinsed with sterile seawater, then cut into small pieces, taken about 10 grams and put in to erlenmeyer ad 100 ml with sterile seawater. Then it was diluted until its concentration 10^{-4} and inoculated by pour plate method, taken about 1 ml from each dilution to inoculate on (Sabouraud Dextrose Agar) SDA medium in the petri disk $\pm$ 15 ml aseptically then incubated at temperature 37°C for 5-7 days. Colonies that have a different shape to the other colonies can be regarded as different isolates. Then be purified to obtain pure isolates (single fungi).

Purification of Symbiotic Fungi Isolates

Symbiotic fungi that has grown on SDA medium, then gradually purified one by one. Purification aims to separate the colonies of symbiotic different morphologies to be a separate isolates. Morphological observation performed after incubation for 5-7 days, and if still found that colony growth different from the macroscopic must be separated again to obtain pure isolates. Symbiotic fungi incubated at 25 ° C-27 ° C for 5-7 days in accordance with its growth. Each is made of pure isolates on agar slant Duplo. Respectively as stock culture and culture for research.

Cultivation of Pure Fungi Isolates

Pure isolates obtained in the insulating phase and then cultured in media SDB, pure isolates taken one loop, then put in a 10 ml SDB medium and incubated at room temperature for 7 days. Furthermore, isolates were grown on a scale of 10 ml were transferred aseptically similar to the culture medium at a larger scale (100 ml). 100 ml scale fungi cultivation were incubated at room temperature for 3-4 weeks.

Extraction of Secondary Metabolites from Fungi Isolates

Pure isolates that had been grown for 3-4 weeks, then extracted by maceration with ethyl acetate in the ratio of 2:1. Once macerated overnight, the fungal mycelium was then split using a sonicator for 5 minutes.Furthermore, the ethyl acetate extract was separated from the culture medium using a separating funnel. Then carried back to the maceration of the same mold as many as three replications. Maserat ethyl acetate was then evaporated with a rotary evaporator until diperoreh ethyl acetate crude extract. Furthermore, ethyl acetate crude extract produced calculated yield obtained and tested antimicrobial bioactivity.

Extraction of secondary metabolites from fungi isolates

Pure isolates that had been grown for 3-4 weeks, then extracted by maceration with ethyl acetate (EtOAC) in the ratio of 1:1. After macerated overnight, the fungal mycelium was then split using a sonicator for 5 minutes. The cultures were then filtrated by whatman paper. Furthermore, the ethyl acetate extract was separated from the culture medium using a separating funnel. This organic solvent was pooled and then taken to dryness using a vacuum rotary evaporator at 40ºC.

Preliminary qualitative screening of phytochemicals

The EtOAc extracts of symbiotic fungi was tested for the presence of alkaloid, phenols, steroids and terpenoids[8].

Screening for antimicrobial activity

For screening of antimicrobial activity, the EtOAc extract of symbiotic fungi was tested against *Staphylococcus*

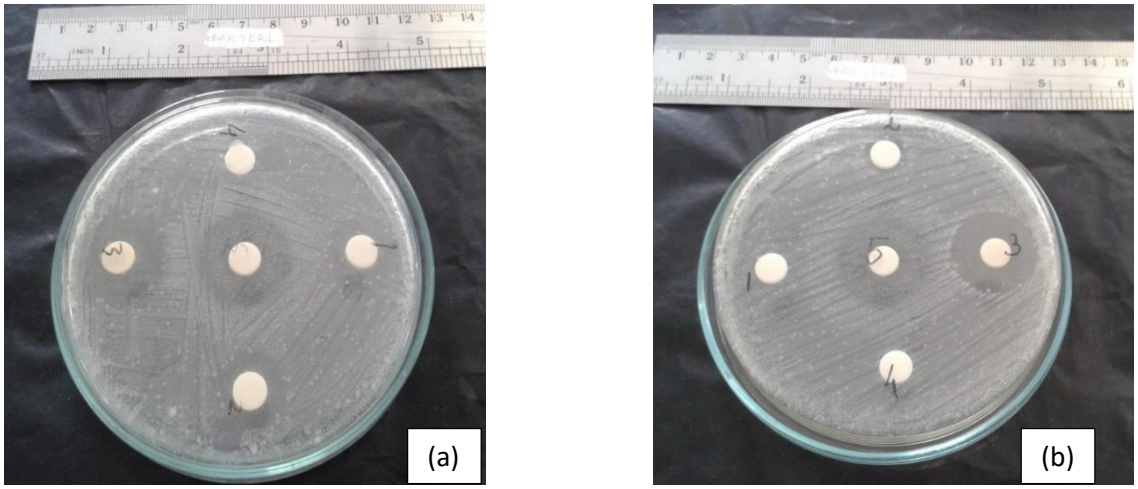

Figure 1: Results of screening of endophytic fungi for antibacterial activity against *S. aureus*. a) Fungi Extract P_{11}; b) Fungi Extract P_{16}
1 : Fungi extract with a concentration of 0.1%, 2 : Fungi extract with a concentration of 0.5%, 3 : Fungi extract with a concentration of 1%, 4 : The negative control (DMSO), 5 : Positive controls (oxytetracycline 1%)

aureus, *Escherichia coli*, and *Candida albican*s using the paper disk method. One pieces of 6 mm sterile paper disk was soaked in each of EtOAc extract (10 mg/ml in DMSO). Paper disks were also inoculated with DMSO (negative control), Oxytetrasiklin (20 $mg.ml^{-1}$ in distilled water) and Clotrimazol (500 $\mu g.ml^{-1}$ in distilled water) as positive controls. The disk was placed on the surface of the medium containing 10^5 cell of bacteria and fungi test strain. The plates were incubated at 37 °C for 24 hours for bacteria and at a temperature of 25 ° C-27 °C for 5-7 days for fungi. The width of inhibition zones was measured. Each treatment consisted of three replicates. The experiment was repeated twice.

RESULTS AND DISCUSSION

A total of 18 symbiotic fungi were isolated and characterized from marine sponge *P. nigrans*. The EtOAc extracts of fungi were tested for its chemical contents with chemical reagents. Based on the results of phytochemicals screening, the extracts contain some secondary metabolites such as alkaloids, phenolic, steroids and terpenoids (Table 1). The symbiosis that produces secondary metabolites can be triggered due to obstruction biotic environment. In the marine microbes, environmental conditions with limited nutrients lead to the use of carbon and cellular metabolites not only used for cell growth but can be used also for production of secondary metabolites[9]. The 18 extracts of each fungi were tested for antimicrobial activity, there were 6 active extracts against bacterial *of S. aureus* isolates, but no activity against the bacteria *E. coli* and the fungus *C. Albicans* (Table 2 and Figure 1). Based on the test results, it can be assumed that the fungal isolates are more sensitive against gram-positive bacteria *(S. aureus)* than gram-negative bacteria *(E. coli).* This most likely occurs because of differences in the thickness of the cell wall structure of each the bacteria. The resistance of gram-negative bacteria and gram positive against antibacterial compound is different. Differences in sensitivity gram positive and gram negative with regard to the structure of the cell wall, such as the amount of peptidoglycan (the presence of the receptor, the pores and lipids), and the crosslinking properties of autolytic enzyme activity. These component is a factor that determines the penetration, binding and activity of antimicrobial compounds. Fungal endophytes were selected for futher characterization and identification based on Brigitte (1980)[10]. Examinations were performed by macroscopic and microscopic of fungi. Macroscopic examination includes a visual observation to the form colony or hyphal, surface and reverse colony color, and colony texture. While on microscopic examination was carried out by observing the characteristic of the spores or conidia, and reproductive structures (sexual and asexual) under a light-field microscope. From the results of the microscopic identification of fungal isolates showed that the isolates of the fungi P_2 was *Aspergillus fumigatus* , fungal isolates P_{11} was *Aspergillus flavus*, and P_{15} was *Candida* sp. Another fungal isolates of P_4, P_6 and P_{16} cannot be identified yet. The literature study shows that the study of some fungus of A. *fumigatus* and *A. flavus* has been done. In the study conducted by Kang et al. (2013)[11] to *A. fumigatus* obtained compounds fumagillin, helvolic acid, and 1,2-dihydrohelvolic acid, the diameter of which produce inhibition against *Malassezia furfur*, a bacteria pathogens causing skin epidermis disorder. Wang et al. (2012)[12] reported in their study that *A. flavus* isolated from mangrove plant produces aflatoxin which is active as an antimicrobial and cytotoxic. Compounds were examined showed diameter inhibitory effect on *E. coli, Bacillus subtilis, and Enterobacter aerogenes*. From the results of our present study, *A. fumigatus* and *A. flavus* have only antagonist activity against *S. aureus* alone. Bioactivity differences produced by the fungus *Aspergillus* may be caused by differences in waters where taking a sponge and different species of host. According to Bell and Barnes (2003)[13], morphology and physiology of the sponge is influenced by factors micro environment where it life. Growth and metabolism as well as symbionts associated with a sponge will also be influenced by these factors. Their research also showed that these factors may cause

differences in morphology and bioactivity significantly on differences in the habitat. Based on the above, it can be seen that the secondary metabolites of fungal isolates are not always active as an antimicrobial in spite of having the same species, but may have other bioactivity such as cytotoxic, anti-tuberculosis, and others[14].

CONCLUSION

From the results of this research, 18 symbiotic fungi from marine sponge *P. nigrans* were isolated. 6 isolates of which can inhibit the growth of *S aureus*. It can also be conclude from the present study that symbiotic fungi might be used as an alternative to produce the antibiotic used in pharmaceutical on the basis inhibition of pathogenic microorganisms. However, further research needs to be done in determining antibiotic compounds produced by those symbiotic fungi and investigation regarding characterization using molecular techniques for their identification.

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