Journal of Applied Pharmaceutical Science Vol. 7 (02), pp. 237-240, February, 2017
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2017.70234
ISSN 2231-3354

Antibacterial and cytotoxic activities of ethyl acetate extract of symbiotic fungi from West Sumatra marine sponge *Acanthrongylophora ingens*

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ARTICLE INFO

Article history:
Received on: 04/10/2016
Accepted on: 26/11/2016
Available online: 27/02/2017

Key words:
Marine sponge, symbiotic fungi, *Acanthostrongylophora ingens*, anticancer activity, antibacterial activity.

ABSTRACT

Screening of anticancer and antibacterial activities of ethylacetate extract of symbiotic fungi from marine sponge *Acanthostrongylophora ingens* was conducted. Sponge *A.ingens* was collected from Mandeh island, West Sumatra. Isolation of symbiotic fungi was done by pour plate method using *sabouraund dextrose agar* (SDA) medium and purified by scratch method. Pure isolates were cultivated in rice medium for 6 week at room temperature. Result of cultivation was extracted with ethyl acetate. Each extract further was screened for cytotoxic activity with BSLT method using shrimp larva of *Artemia salina* Leach and antibacterial activity against *Basillus subtilis*, *Staphylococcus epidemidis*, *Salmonella typosa* and *Escherichia coli* using agar diffusion method. The ethyl acetate extract of fungi strains which showed interesting results on the bioactivity screening was performed a phytochemical test to determine the chemical content. There were 8 isolated symbiotic fungi strains from the sponge. Based on the screening cytotoxic result, LC_{50} value from each extract were IB121 isolate (44.59 ppm), IB131 isolate (11.52 ppm), IB142 isolate (662.2 ppm), IB161 isolate (221.82 ppm), IB102 isolate (172.66 ppm), IB141 isolate (58.56 ppm), IB101 isolate (3.801 ppm) and IB151 isolate (0.53 ppm). Based on the result of antibacterial activity screening against *B. substilis*, *S. epidemidis*, *S. typosa* and *E. coli*, the biggest inhibition growth was resulted by fungi strain of IB141 with diameter inhibition of 14.4 mm, 11.5 mm, 10.25 mm and 14.75 mm (at a concentration of 5%) respectively. The examination of main chemical constituents of ethyl acetate exctract of symbiotic fungi showed that it contains phenolic, terpenoid and steroid compounds. From the study, it can be concluded that fungi assosiated with marine sponge *Acanthostrongylophora ingens* are capable in producing of bioactive compounds, so it has potential as a source of anticancer and antibacterial compounds that are useful mainly in the fields of pharmaceuticals and health.

INTRODUCTION

Marine environment represents half of global biodiversity which is a rich source of structural diversity and biologically active metabolites (Edrada *et al*., 2000; Habbu, *et al.*, 2016). These metabolites are produced specifically by certain organisms to follow the paths of biogenetic concern and also become a sign of the kinship relations between various organisms. Sponge produces bioactive compounds that could paralyze the microbes trapped. The trapped microbes will survive and live symbiotically inside the sponge body (Cetkovic and Bilela, 2003). Most sponges contain microbial symbionts in large numbers that include bacteria, cyanobacteria, algae, archaea and fungi (Rozas *et al*., 2011).

Some studies indicate that the symbiont microbe has a role in the production of bioactive compounds that function in ecological adaptation for sponge (Proksch *et al.*, 2003; Thakur and Muller, 2004; Zheng *et al*., 2005).

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Bioactive compounds from microorganisms associated with sponge has demonstrated activities as anticancer, antibacterial, antifungal, antiviral, antiprotozoal, anthelmintics, anti-inflammatory, neurosuppresive, imunosuppressive, antifouling, and cytotoxicity (Vasanthabharathi dan Jayalakshmi, 2011; Subramani *et al*., 2013; Singh *et al*., 2014). More recently, a preliminary study on the antimicrobial activity of fungi and bacteria associated with a sponge *Haliclona fascigera* of Mandeh Island, West Sumatra has been done (Handayani *et al*., 2015a; 2015b).

Aspergillus fumigatus, *Aspergillus flavus* and *Candida sp.* fungal symbionts of sponge *Petrosian nigrans* were also active as antibacterial (Handayani *et al.*, 2016). In this study, the marine sponge *Acanthostrongylophora ingens* was used as a sample for the isolation of fungi.

Some other studies have reported that the sponge has potential as cytotoxic and antimicrobial activity (Samoylenko *et al*., 2009; El-Desoky *et al.*, 2014; Ibrahim *et al.*, 2015). Therefore, this study aimed to obtain fungal isolates from the sponge symbiont *Acanthostrongylophora ingens*, which is capable of producing cytotoxic and antibacterial compounds.

MATERIALS AND METHODS

Sponge Material

Sponge *A. Ingens* was collected from the Mandeh island, South Coast of West Sumatra, Indonesia, in depth of ± 5-8m. Sponge was transferred into a sterilized plastic bag and stored in the ice box. The samples were transferred to the laboratory and processed immediately for the isolation of symbiotic fungi. The sponge was identified by Dr. Nicole J. De Voogd, Natural Biodiversity Center, Netherlands. A voucher specimen (IB101) has been preserved at the Marine Reference Collection, Laboratory of Sumatran Biota, Andalas University, West Sumatra, Indonesia.

Isolation of Symbiotic Fungi from Marine Sponge

The isolation of fungi was started with sterilization on the surface of the sample. Sponge was rinsed with sterile seawater, and then cut into small pieces. Sponge was taken as much as 10 grams an inserted into Erlenmeyer and add 100 mL of sterile seawater.

Then it was diluted until its concentration 10-6 and inoculated on SDA (*Sabouraud Dextrose Agar*) as medium, and incubated at a temperature of 27-29 °C for 5-7 days. Colonies that have different shapes and colors with other colonies could be regarded as different isolates. Then be purified by the scratch method to obtain pure isolates. Pure isolate than identified based on Brigitte (1980).

Cultivation of Isolated Fungi in Medium of Rice

The pure isolates of symbiotic fungi were cultured in rice as medium and incubated at room temperature for 4-6 weeks until the volume of rice in the Erlenmeyer is overgrown by the fungi (Kjer, *et al.,* 2010).

Extraction of Secondary Metabolites from Fungi Isolates

Fungi isolates that have maximum grown, and then extracted by maceration with ethyl acetate (EtOAc) in the ratio 1: 1 with 3 repetitions. The extract of ethyl acetate was collected and evaporated in *vacuo* using a rotary evaporator. The EtOAc extracts were tested for antibacterial activity and cytotoxic.

Screening of antibacterial activity

For screening of antibacterial activity, the EtOAc extract of symbiotic fungi was tested against *Basillus subtilis*, *Staphylococcus epidemidis*, *Salmonella typosa* and *Escherichia coli* using the paper disk method. One pieces of 6 mm sterile paper disk was soaked in each of EtOAc extract (50 mg/ml in DMSO). Paper disks were also inoculated with DMSO (negative control) and Amicaxin as positive control. Antagonist activity was detected after incubation for 24 h at 30 ^{o}C. The existence of the clear zone in the media was considered as indicator for antibacterial activity. The zone of inhibition was measured and expressed in millimeters. Strain that showed maximum inhibition was selected to phytochemistry test.

Screening of cytotoxic activity

Brine shrimp (*Artemia salina*) eggs were hatched in 500 mL of filtered seawater under constant aeration for 48 h at (27±2) ^{o}C. After hatching, active nauplii free from egg hells were collected and used for the assay. Five hundred, fifteen and five microliters of all fungi isolate were added in well plate at 1000 ppm, 100 ppm and 10 ppm concentration in triplicate. Fiftteen microliters were added 50 µl of DMSO and until 5000 µl of seawater containing ten nauplii, while placed in the respective well and maintained at room temperature for 24 h. Filtered seawater was used as negative control. The LC_{50} value was calculated using curva method based on probit analysis (Meyer *et al.,* 1982).

Phytochemical screening

Phytochemical examinations were carried out for all the ethyl acetat extracts of symbiotic fungi as per the standard methods (Tiwari *et al*., 2011).

Detection of alkaloids

Extracts were dissolved individually in dilute Hydrochloric acid and filtered. Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow coloured precipitate indicates the presence of alkaloids.

Detection of phenolics

Extracts were dissolved individually in dilute Hydrochloric acid and filtered. Filtrates were treated with $FeCl_3$. Formation of a blue coloured precipitate indicates the presence of phenolic.

Detection of Steroid and Terpenoid

Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride and concentrated H_2SO_4. Formation of a blue or purple coloured precipitate indicates the presence of steroid, while a red colored precipitate indicates the presence of terpenoid.

RESULTS AND DISCUSSION

The sponge *A. ingens* forms irregular thick columns with a coarse surface. The oscules were several mm in diameter, on low hummoks or flush. The consistency is firm, incompressible, and crumbly. The color is brown. The skeleton consists of reticulation of thick spicule tracts with average diameter of 75 μm, forming rather squarishly rounded meshes of 200-280 μm in diameter (Hooper *et al*., 2002).

In this study, 8 symbiotic fungi were isolated from the marine sponge *A. ingens*. The EtOAc extract of symbiotic fungi were tested for its bioactivity against bacterial pathogens, two gram-positive bacteria *Basillus subtilis*, and *Staphylococcus epidemidis*, and two gram-negative *Salmonella typosa* and *Escherichia coli* by agar diffution method. Antibacterial activity test results are listed in table 1.

Tabel 1: Antibacterial activity of symbiotic fungi extracts against human pathogenic bacteria.

Fungi Extract	Zone of inhibition (mm)			
	Gram-positive		Gram-negative	
	B. sutilis	*S. epidermidis*	*S. typosa*	*E.coli*
IB101	7.85	-	-	-
IB102	-	-	-	-
IB121	-	7.25	9.15	7.85
IB131	8.7	9.35	8.25	8.05
IB141	14.4	11.5	10.25	14.75
IB142	-	-	-	-
IB151	8.9	9.5	8.3	8.7
IB161	-	-	-	-

Four symbiotic fungi extracts showed inhibitory activity against the Gram-positive bacterium *B.subtilis* and *S. epidermidis* and five fungi extracts displayed inhibitory activity against the Gram-negative bacterium *S. typosa*. The IB141 isolate of symbiotic fungi extract showed the highest activity againts bacterial patogens of *B. subtilis*, *S. epidemidis*, *S. typosa* and *E. coli* (each inhibition zone of 14.4 mm, 11.5 mm, 10.25 mm and 14.75 mm). Three extracts obtained from IB102, 1B161 and IB142 isolates were inactive against *B.subtilis*, *S. epidermidis, S. typosa* and *E. coli.* All eight symbiotic fungi extracts were showed significant cytotoxicity against brine shrimp larvae as shown in Table 2. The extract was declared active when the LC_{50} value is smaller than 1000 ppm (Meyer *et al.*, 1982). The highest citotoxicity was shown by IB151 with LC_{50} of 0.53 ppm.

Table 2: LC_{50} value of symbiotic fungi extracts.

Fungi extract	LC_{50} (ppm)
IB101	3.80
IB102	172.66
IB121	44.59
IB131	11.52
IB141	58.56
IB142	662.2
IB151	0.53
IB161	221.82

Fungal endophytes spesies were identificated in macroscopic and microscopic. Macroscopic examination included 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, reproductive structures (sexual and asexual) under a light-field microscope. Based on the result of morphologic identification of a total eight isolates of symbiotic fungi that show cytotoxic activity, four of which are the same type of fungi. The IB101, IB102, IB142 and IB161 isolates were *Mucor sp*. Futhermore, IB141 isolate was fungi *Aspergillus flavus*, IB151 isolate was *Aspergillus sp*, IB121 and IB131 isolates were *Aspergillus niger*.

Based on the results of phytochemical constituents, can be known that the ethyl acetate extract of symbiotic fungi containing phenolic, terpenoids and steroid compounds as shown in Table 3. Some literature have reported the presence of phenolic compounds and sesquiterpenes bisablolane produced by symbiotic fungi *Aspergillus* sp. with the sponge *Xestospongia testudinaria* (Sun, *et al*, 2012;. Li, *et al*, 2012).

Table 3: Phytochemical constituents of ethyl acetate extract of symbiotic fungi.

Fungi Extract	Phenolic	Alkaloid	Terpenoid	Steroid
IB101	+	-	+	-
IB102	-	-	-	+
IB121	+	-	+	-
IB131	+	-	+	-
IB141	+	-	+	-
IB142	-	-	+	-
IB151	+	-	+	-
IB161	-	-	-	+

According to some reports, symbiotic fungi with its host (sponge) have a close relationship. Some symbiotic fungi isolated from sponge produce a natural product with identical its host (Proksch, *et al*., 2003a; 2003b). It can also affect the growth and metabolism of the sponge and affect the bioactivity of microbial symbionts associated with the sponge.

CONCLUSIONS

In this study, antibacterial and cytotoxic activity of symbiotic fungi from marine sponge *A. ingens* arises due to the presence of secondary metabolites. The ability of symbiotic fungi in producing bioactive compound, will improve the chances to find a new lead compound that can be used primarily in the field of pharmaceutical and health.

ACKNOWLEDGEMENT

We are grateful to Dr. Nikole J. De Voogd, Natural Biodiversity Center, Netherlands for identification of the marine sponge.

Financial support and sponsorship: This research was supported by Directorate General of Higher Education ministry of Nasional Education, Indonesia, in project "Master Program of Education Leading to Doctoral Degree for Excellent Graduates (PMDSU), No: 324/SP2H/LT/DRPM/IX/2016, September 2016.

Conflict of Interests: There are no conflicts of interest.

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How to cite this article:

Handayani D, Aminah I. Antibacterial and cytotoxic activities of ethyl acetate extract of symbiotic fungi from West Sumatra marine sponge *Acanthrongylophora ingens*. J App Pharm Sci, 2017; 7 (02): 237-240.