Journal of Applied Pharmaceutical Science Vol. 7 (05), pp. 066-069, May, 2017
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2017.70512
ISSN 2231-3354 

Antibacterial and cytotoxic activities screening of symbiotic fungi extract isolated from marine sponge *Neopetrosia chaliniformis* AR-01

Dian Handayani*, Muh. Ade Artasasta

Laboratory of Sumatran Biota, Faculty of Pharmacy, University of Andalas, Kampus Limau Manis 25163, Padang, Indonesia.

ARTICLE INFO

Article history:
Received on: 08/11/2016
Accepted on: 07/01/2017
Available online: 30/05/2017

Key words:
Marine sponge, symbiotic fungi, *Neopetrosia chaliniformis*, cytotoxic activity, antibacterial activity.

ABSTRACT

Research on the potential of ethyl acetate extracts from symbiotic fungi of marine sponge *Neopetrosia chaliniformis* AR-01 as producer of cytotoxic and antibacterial compounds has been conducted**.** Symbiotic fungi of *N. chaliniformis* were isolated by casting method using Sabouraud Dextrose Agar (SDA) and purified by the scratch method. Pure isolated fungi then was cultivated using rice as media at temperature of 25-27 ° C for 4-8 weeks and extracted using ethyl acetate solvent. The ethyl acetate extracts were tested as cytotoxic using Brine Shrimp Lethality Test (BSLT) and tested as an antibacterial against pathogenic bacteria of Gram positive (*Bacillus subtilis* and *Staphylococcus epidermidis*) and Gram negative (*Escherichia coli* and *Salmonella typhosa*) using agar diffusion method. There are 13 symbiotic fungi of marine sponge *N. chaliniformis* that had been isolated. The screening result of the cytotoxic activity showed that 76.92 % or 10 fungi isolates were classified as cytotoxic with LC_{50} <100 ppm, namely NC01, NC02, NC03, NC05, NC06, NC07, NC08, NC09, NC10, and NC11. The results of the antibacterial activity screening showed that 5 isolates namely NC01, NC03, NC04 NC07 and NC10 could inhibit the growth of pathogenic bacteria by diameter of inhibition zone > 10 mm. Based on the screening results, it can be concluded that ethyl acetate extracts of the symbiotic fungi of marine sponge *N. chaliniformis* are a potential source for producing anticancer and antibacterial compounds.

INTRODUCTION

Symbiotic microbes of marine sponge are microbes living in the sponge tissue with mutualism association possibility. There are two ways how the microbe could transmit into the sponge tissue, namely transmitted horizontally as well as vertically. Horizontally, microbes which are located around the environment of sponge transmitted to the sponge tissue due to filter feeder process, while vertically, microbes transmitted to the sponge when the sponge was still in the larval stages (Bright and Bulgheresi, 2010; Hentschel *et al*., 2012). Basically more than 40% by volume of the sponge body contains microorganisms which have a biological effect on the sponge itself. For example, through the process of photosynthesis, symbiotic *cyanobacteria* provide 50 % carbon for energy requirement of specific tropical sponge (Webster and Taylor, 2012; Wilkinson, 1983). In addition, symbiotic microbes of marine sponge could maintain the availability of nutrients for the sponge, stabilize skeleton of sponge, and also produce secondary metabolites against pathogenic microbes (Hentschel *et al*., 2002). Therefore, Proksch *et al*., (2002) hypothesized that the symbiotic microbes of marine sponge are original producer of secondary metabolites. Exploration of symbiotic microbes of marine sponge for isolated their secondary metabolites being concern at this time, because they are producer of potential secondary metabolites against various diseases, especially cancer and pathogenic microbial attack (Vasanthabharathi and Jayalakshmi, 2011; Zhou *et al*., 2014).

In addition, the exploration of symbiotic microbes of marine sponge could suppress exploitation sponge on a large scale and maintaining the ecosystem of the sponge itself. The use of symbiotic microbes of marine sponge is also better because it could be cultured and purified in the laboratory and could be reproduced in a short time and easily manipulated using molecular technology.

Corresponding Author
Dian Handayani, Laboratory of Sumatran Biota, Faculty of Pharmacy, University of Andalas, Kampus Limau Manis 25163, Padang, Indonesia.
Email: dianh_17 @ yahoo.com

Among the types of symbiotic microorganisms, symbiotic fungi of sponge is known as a manufacturer of the most interesting bioactive compounds (Bhadury *et al*., 2006) and Ebel, (2011) explained that the fungi isolated from the marine sponge is one of three main sources to produce new secondary metabolites as anticancer and antimicrobial. Methyl-averantin, isolated compound of *Aspergillus versicolor* was active against tumor cell line (XF498) with LC_{50} of 0.41 µg/mL. Meroterpenoid class of *Alternaria sp.* obtained from *Callyspongia sp.* was tested as an inhibitor of NF-*k*B in RAW264.7 cancer cells with LC_{50} of 39 µM, and dankastin C was tested on lymphocytic leukemia cell (P388) with LC_{50} of 57 µg/mL, isolated of *Gymnascella dankaliensis* obtained from *Homaxinella sp.* (Lee *et al*., 2010; Zhang *et al*., 2013; Amagata *et al*., 2013). Recently, a preliminary studies on the antimicrobial activity of fungi and bacteria associated with a sponge *Haliclona fascigera* and *Petrosia nigrans* of Mandeh Island, West Sumatra have been done (Handayani *et al*., 2015a, 2015b, 2016a, 2016b). In addition, averantin of *Aspergillus versicolor* obtained from *Neopetrosia sp.* had activity as antibacterial against several strains of pathogenic bacteria *Staphylococcus* with its MIC range of 0.78-1.56 µg/mL (Lee *et al*., 2010). The marine sponge *Neopetrosia chaliniformis* is one of endemic sponge in Indonesian marine. But until now there is no literature that describes the secondary metabolites of *N. chaliniformis* including secondary metabolites of symbiotic microbes. However, the *Neopterosia* genus had been a major concern as a producer of secondary metabolites, and also more than 85 secondary metabolites had been isolated from this sponge (Qaralleh *et al*., 2016).

MATERIAL AND METHODS

Sponge Material

N. chaliniformis was collected from the island Mandeh, South Coast, West Sumatra, Indonesia, which was taken at a depth of ± 10 m using scuba diving. Sponge was immediately placed in a sterile plastic bag and stored in an ice box. The sponge was identified by Dr. Nicole J. De Voogd, Natural Biodiversity Center, Netherlands. A voucher specimen (AR-01) 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 and 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

After fungi isolates grow optimally, each fungus was extracted after optimal growth with 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 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 shells 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).

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 Amikacin 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 phytochemical test.

Phytochemical Test

Phytochemical examinations were carried out for all the ethyl acetat extracts of symbiotic fungi as per the standard methods (Tiwari *et al*., 2011). Phenolic, alkaloid, steroid and terpenoid test were performed to know secondary metabolite constituent by this method.

RESULTS AND DISCUSSION

Identification of sponge was confirmed as *N. chaliniformis* based on spicules morphology. In Taxonomy, this sponge including Demospongiae class, and part of the family petroicidae.In this study, 13 symbiotic fungi were isolated from

the sponge. Based on the results of the cytotoxic test screening, 10 fungi isolates or approximately 76.92% of the total fungi isolates were potentially to produce cytotoxic compounds with their cytotoxic activity below 100 ppm. The cytotoxic isolates were NC01, NC02, NC03, NC05, NC06, NC07, NC08, NC09, NC10 and NC11. The most cytotoxic activity of total isolates was NC01 with LC_{50} of 16.79 ppm.

Table 1: LC_{50} value of symbiotic fungi extracts.

No	Fungi extract	LC_{50} (ppm)
1	NC01	16.79
2	NC02	26.04
3	NC03	39.81
4	NC04	371.53
5	NC05	25.58
6	NC06	54.95
7	NC07	60.12
8	NC08	71.38
9	NC09	26.99
10	NC10	94.62
11	NC11	79.48
12	NC12	>1000
13	NC13	969

Antibacterial activity test results are listed in Table 2. In this study, 5 fungi extracts (NC01, NC03, NC04, NC07 and NC10) showed inhibitory activity against pathogenic bacteria test with diameter of inhibition zone more than 10 mm. The highest antibacterial activity result was shown by extract of NC07 against bacterial pathogens of *B. subtilis*, *S. epidermidis*, *S. typhosa* and *E.coli* (each inhibition zone of 20.9 mm, 23.25 mm, 22.85, and 20.9 mm).

Table 2: Antibacterial activity of symbiotic fungi extracts against human pathogenic bacteria.

Fungi Extract	Zone of Inhibition (mm)			
	Gram positive		Gram negative	
	B. subtilis	*S. epidermidis*	*S. typhosa*	*E. coli*
NC01	8.95	7.85	10.25	7.2
NC02	-	-	-	-
NC03	8.75	12.6	15.25	12.85
NC04	10.65	10.5	10.65	8.3
NC05	-	-	-	-
NC06	-	-	-	-
NC07	20.9	23.25	22.85	20.9
NC08	7.2	8.95	8.3	7.45
NC09	8.95	7.85	8.9	8.3
NC10	8.85	11.75	10.25	9.4
NC11	7.85	7.45	7.45	8.3
NC12	-	-	-	-
NC13	-	-	-	-
Amikacin	23	22.7	20	20

Analysis of the chemical reaction of ethyl acetate extract of symbiotic fungi isolates from *N. chaliniformis* was conducted to determine the constituents of secondary metabolites. In this study, phenolic, alkaloid, steroid and terpenoid were tested using appropriate reagents. Based on the results of phytochemical examinations, most of fungi extracts contain terpenoids, but does not contain alkaloids. In addition, some of fungi extracts contain phenolics and terpenoids (isolates NC02, NC08, NC10 and NC12) and other fungi extracts containing steroid (isolates NC01, NC05, NC09 and NC13).

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

Fungi Extract	Chemical constituent			
	Phenolic	Alkaloid	Steroid	Terpenoid
NC01	-	-	+	-
NC02	+	-	-	+
NC03	-	-	-	+
NC04	-	-	-	+
NC05	-	-	+	-
NC06	-	-	-	+
NC07	-	-	-	+
NC08	+	-	-	+
NC09	-	-	+	-
NC10	+	-	-	+
NC11	-	-	-	+
NC12	+	-	-	+
NC13	-	-	+	-

"+" indicates positive reaction; "-" indicates negative reaction.

The results of the screening of cytotoxic and antibacterial activity showed that isolates NC01 most cytotoxic. The results of the macroscopic identification, NC01 had bluish green with white edges. Diameter colonies after 10 days of growth is ± 2.5 cm at 28 ° C. The microscopic identification, NC01 had conidiophore ingreen with a smooth surface and had branched monoverticillate. Hyphae of fungal isolate NC01 was not septape and the phialid was slender clyndrical. From the macroscopic and microscopic observations, NC01 was identified as *Penicillium* sp.

A symbiotic fungus of NC07 is the most active as an antibacterial agent. Based on identification macroscopically, fungal colonies are black. Diameter of colony after 10 days was 5 cm at 28 ° C. Microscopically, NC07 showed black conidiophore with a smooth surface, and also had spherical vesicles. Based on the results of macroscopic and microscopic identification, the fungal NC07 classified as *Aspergillus niger*.

Basically, cytotoxic compounds are toxic compounds which characterized by the death cell after interacting with it. However, to become potential anticancer agents, a cytotoxic compound at least have to show selectivity towards normal and cancer cell, have activity against multidrug-resistent (MDR), and show the cell death mechanism through inhibiting non-apoptotic. In other words, not all cytotoxic compounds could be used as an anticancer agent (Gomes *et al*., 2015).

Secondary metabolites of symbiotic fungi of marine invertebrates repeatedly show their ability to kill cancer cells through non-apoptotic cell death mechanisms (Gomes *et al*., 2015). For example, the ethyl acetate extract of *Aspergillus versicolor* which isolated from *Neopetrosia sp.* had cytotoxic activity with LC_{50} of 32 µg/mL (Lee *et al*., 2010). The moreterpenoid, isolated compound from symbiotic fungi of Altenaria sp. had been reported by Zhang *et al*. (2013). This compound can inhibit the NF-kB in cancer cells of RAW264.7 with LC50 of 39 µM. NF-kB is a marker of most cancer incidence as well as key transcription factors that are involved in the inflammatory reaction, therefore inhibition of the formation of NF-kB will inhibit the growth of tumor cells (Vallabhapurapu and

Karin, 2009). Antibacterial compounds from symbiotic fungi of marine sponge are a potential source and being concern lately (Zhou *et al*. 2014). It has been reported that several strains of symbiotic fungi of marine sponge could produce a potential antibacterial compound. Henriquez *et al*. (2013) reported that the extract of the symbiotic fungi from Antartic marine sponge could inhibit some pathogenic bacteria such as *P. aeruginosa*, *S. aureus* with the inhibition zone diameter above 10 mm. Moreover, the ethyl acetate extract of the *Penicillium sp.* which obtained from marine sponge of South China Sea could inhibit both of Gram positive and Gram-negative pathogenic bacteria. Further investigation showed that *Penicillium sp.* contains penicifuran A which was capable for inhibiting pathogenic bacteria *S. albus* with minimum inhibitory concentration (MIC) of 3,13 μM (Qi *et al*., 2013).

CONCLUSION

Symbiotic fungi of marine sponge *N. chaliniformis* AR-01 with code NC01 (*Penicillium sp.*) and NC07 (*Aspergillus niger*) were the most potential fungi isolates to produce anticancer and antibacterial compounds. The presence of cytotoxic activity and antibacterial activity were suspected due to the presence of secondary metabolites content of steroids and terpenoids.

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 funded by Ministry of Research and Technology, Indonesia in project "Master Program of Education Leading to Doctoral Degree for Excellen Graduate (PMDSU) research, No: 324/SP2H/LT/DRPM/IX/2016".

Conflict of Interests: There are no conflicts of interest.

REFERENCES

Amagata T, Tanaka M, Yamada T, Chen YP, Minoura K, and Numata A. Additional cytotoxic substances isolated from the sponge-derived *Gymnascella dankaliensis*. Tetrahedron Lett, 2013; 54:5960-5962.

Bhadury P, Mohammad BT, and Wright PC. The current status of natural products from marine fungi and their potential as anti-infective agents. J. Ind. Microbiol. Biotechnol, 2006; 33:325–337.

Bright M and Bulgheresi S. A Complex Journey: Transmission of Microbial Symbionts. Nature Reviews (Microbiology), 2010; 8:219-230.

Brigitte G. Kompendium der Medizinschen Mykologi. 1980, Berlin-Hamburg. Verlag Paul Parey.

Gomes NGM, Lefranc F, Kijjoa A and Kiss R. Can Some Marine-Derived Fungal Metabolites Become Actual Anticancer Agents? Marine Drugs, 2015; 13:3950-3991.

Handayani D, Ahdinur RF, Rustini R. Antimicrobial Activity of Endophytic Fungi from Marine Sponge *Haliclona fascigera*. Journal of App Pharm Sci, 2015a; 5: 154-156.

Handayani D, Sandrawaty N, Murniati M, and Regina,R. Screening of Endophytic Bacteria Isolated from Marine Sponge *Haliclona fascigera* for Inhibition against Clinical Isolates of *Methicillin Resistant Staphylococcus aureus* (MRSA). J. App Pharm Sci, 2015b; 5(9): 139-142.

Handayani D, Murniati M and Rustini R. *In Vitro* Inhibitory Activity of Ethyl Acetat Extract of Symbiotic Bacteria Isolated from Marine Sponge *Haliclona fascigera* against Multidrug Resistant Organism (MDRO). J App Pharm Sci, 2016a; 6 (11): 218-222.

Handayani D, Ornando R, ,Rustini. Antimicrobial activity screening of symbiotic fungi from marine sponge *Petrosia nigrans* collected from south coast of West Sumatera, Indonesia. International Journal of Pharmacognosy and Phytochemical Research, 2016b; 8(4): 623-626.

Henriquez M, Vergara K, Norambuena J, Beiza A, Maza F, Ubilla P, Araya I, Chavez R, Martin AS, Darias J, Darias MJ, and Vaca I. Diversity of Cultivable Fungi Associated With Antartic Marine Sponges and Screening for their Antimicrobial, Antitumorial and Antioxidant Potential. World J. Microbiol Biotechnol, 2013; 30: 65-76.

Hentschel U, Hopke J, Horn M, Friederich AB, Wagner M, Hacker J, and Moore BS. Molecular Evidance for a Uniform Microbial Community in Sponges from Diffrent Oceans. Applied and Environmental Microbiology, 2002; 68: 4432-4440.

Hentschel U, Piel J, Degnan SM, and Taylor MW. Genomic Insights Into the Marine Sponge Microbiome. Nature Review Microbiology, 2012; 10(9):61-654.

Kjer J, Debbab A, Aly AH, dan Proksch, P. Methods for isolation of marine-derived endophytic fungi and their bioactive secondary products. Nature Protocols, 2010; 5:479-490.

Lee YM, Li H, Hong J, Cho HY, Bae KS, Kim MA, Kim DK, and Jung JH. Bioactive Metabolites from the Sponge-Derived Fungus *Aspergillus versicolor*. Archives of Pharmacal Research, 2010; 33:231-235.

Meyer BN, Figni NR, Putman JE, Jacobsen DE, Nichols DE, and Mc-Laughlin JL. Brine shrimp: A convient general bioassay for active plant constituents. Planta Medica,1982; 45:31-34.

Proksch P, Edrada RA, and Ebel R. Drugs from the Seas - Current Status and Microbiological Implications. Appl Microbiol Biotechnol, 2002; 59:125-134.

Qaralleh H. Chemical and Bioactive Diversites of Marine Sponge *Neopetrosia*. A Journal of the Bangladesh Pharmacological Society, 2016; 11:433-452.

Qi J, Shao CL, Li ZY, Gan LS, Fu XM, Bian WT, Zhao HY, and Wang CY. Isocoumarine Derivates and Benzofurans from a Sponge-Derived *Pencillium sp*. Fungus. Journal of Natural Products, 2013; 76(4):571-9.

Rateb ME, and Ebel R. Secondary Metabolites of Fungi from Marine Habitats. Nat. Prod. Rep, 2011; 28: 290–344.

Tiwari P, Kumar B, Kaur M, Kaur G., and Kaur H. Phytochemical screening and Extraction: A Review, Internationale Pharmaceutica Sciencia, 2011; **1**(1): 98-106.

Vallabhapurapu S, Karin M. Regulation and Function of NF-kB Transcription Factor in Immune System. Annu. Rev. Immunol, 2009; 27:693-733.

Vasanthabharathi V, and Jayalakshmi S. Bioactive potential of symbiotic bacteria and fungi from marine sponges. African Journal of Biotechnology, 2011; 11:7500-7511.

Webster NS, and Taylor M. Marine Sponges And Their Microbial Symbionts : Love And Other Relationships. Environmental Microbiology, 2012; 14**:**335-346.

Wilkinson CR. Net primary productivity in coral reef sponges. Science, 1983; 219: 410-412.

Zhang G, Wu G, Zhu T, Kurtain T, Mandi A, Jiao J, Li J, Qi X, Gu Q, and Li D. Meroterpenoids with diverse ring systems from the sponge-associated fungus *Alternaria sp*. JJy-32. Journal of Natural Products, 2013; Doi : 10.1021/np4005757.

Zhou Y, Debbab A, Wray V, Lin W, Schulz B, Trepos R, Pile C, Hellio C, Proksch P, and Aly AH. Marine Bacterial Inhibitors from the Sponge-Derived Fungus *Aspergillus sp*. Tetrahedron Letters, 2014; 56:2789-2792.

How to cite this article:

Handayani D, Artasasta MA. Antibacterial and cytotoxic activities screening of symbiotic fungi extract isolated from marine sponge *Neopetrosia chaliniformis* AR-01. J App Pharm Sci, 2017; 7 (05): 066-069.