Antibacterial Activity Ods Fractions Of Marine Sponge *Auletta* sp. Against *Mycobacterium smegmatis*

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ABSTRACT

The marine sponge collected from tropical coral reefs in Manado North Sulawesi Indonesia was screened for antimicrobial activities. In the screening program to search for antituberculotic inhibitors, the result found that the ethanol extract ODS fractions 4 – 6 with concentration 5 mg/mL of an Indonesian marine sponge *Auletta* sp. was exhibited inhibitory activity against nonpathogenic *Mycobacterium smegmatis* at concentration 10, 20, 30µg/disc each fraction respectively. Fractions 4 - 5 with concentration 10µg/disc were most active, the inhibition zone 11, 12 mm, respectively.

Keywords: marine sponge, antimicrobial, antituberculosis, *Mycobacterium smegmatis*

INTRODUCTION

Marine sponge has proven to be an important resource for bioactive natural product discovery. Secondary metabolites isolated from marine sponge often possess unique structural features and show potent biological activities.

Sponges are a rich source of biologically active natural products with unique structures as well as marine ascidian, soft corals (Blunt *et al*. 2015; Faulkner 2002). These marine natural products obtained from marine sponges are thought to be used for health purposes such as prevention from bacterial, virus, and infection (Puglisi *et al*. 2014); important biological activities for larvicide activity (Wantah, *et al*. 2018); addition to the fish feed on growth and non-specific immune response (Manurung et al. 2019). Seven species of crude extract sponges collected from the waters of North Sulawesi were tested their antibacterial activity and showed antibacterial activity on *Escherichia coli* (Rompis *et al,* 2019).

Some of the marine sponges and their chemical derivatives have been examined in the preclinical trials as an anticancer and antibacterial (Butler *et al*. 2014).

The fast-growing bacterium *Mycobacterium smegmatis* is generally considered to be non-pathogenic and provides a readily handled fast-growing model for the basic biochemistry and genetics of *M. tuberculosis*. *M. smegmatis* is a Gram-positive bacterium with a membrane structure similar to other, pathogenic species such as *M. tuberculosis* (WHO. 2018). Because of the slow growth rate and highly infectious nature of *M. tuberculosis*, nonpathogenic *M. smegmatis* has been frequently used as the model system for studying tuberculosis.

During our research on the new anti-mycobacterial substances from Indonesian sponges collected in Manado, we have reported an anti-mycobacterial bisfunctionalized sphingolipid and new bromopyrrole alkaloid from marine sponge *Agelas* sp. (Abdjul *et al*., 2017) and cyclic 3-alkyl pyridinium dimers as anti-mycobacterial alkaloids, from marine sponge *Haliclona* sp. (Maarisit *et al*., 2017) these species inhibited the growth of *Mycobacterium smegmatis*.

This research conducting studies on the organism in North Sulawesi marine environments and reported the

results from screening bioassays of the extracts from sponge collected in 2014. The EtOH extract from sponge collected in Manado North Sulawesi was examined for the inhibitory activities on the growth of *Staphylococcus aureus* (Gram-positive bacterium), *Escherichia coli* (Gram-negative bacterium), *Candida albicans* (yeast), and *Mucor hiemalis* (filamentous fungus) and nonpathogenic *Mycobacterium smegmatis* using disk diffusion method.

MATERIALS AND METHODS

General experimental procedures

Chemicals and organic solvents such as ethanol, methanol, Glycerol, Agar, Potato Dextrose Agar, Peptone, Glucose, Sucrose, Yeast Extract, ODS C-18 were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Fetal bovine serum (FBS) and other culture materials were purchased from Invitrogen (Carlsbad, CA, USA). Middlebrook 7H9 broth, polysorbate 80, and Middlebrook OADC were purchased from BD.

Bacteria

The strain NBRC 3207 obtained from the Biological Resource Center (NBRC), NITE (Chiba, Japan). Inoculum of *M. smegmatis* NBRC 3207 maintained in 20% glycerol at −80 °C prior to use.

The strain bacteria of *Staphylococcus aureus* IAM 12544T (Gram-positive); *Escherichia coli* IAM 12119T (Gram-negative); *Candida albicans* IFM 4954 (Yeast); *Mucor hiemalis* IAM 6088 (Filamentous fungus); were maintained at −80 °C prior to use were obtained from Faculty of Pharmaceutical Sciences, Tohoku Medical and Pharmaceutical University.

Collection of marine sponge

The marine sponge was collected by scuba diving at Bunaken Island in Manado Indonesia, 2014. Identification of marine sponge using Indo Pacific Coral reef Field Guide by Allen *et al.* 2007 and Coral Reefs Animals of the Indo Pacific by Gosliner *et al.* 1996. Based on this identification guide we found sponge similar with *Auletta* sp. The voucher specimen was deposited at the Faculty of Fisheries and Marine Science Unsrat and at Faculty of Pharmaceutical Sciences, Tohoku Japan.

Extraction

Marine sponge was first chopped into small parts and were taken in to extraction bottle containing ethanol and weigh the sponge, then added with ethanol till the sponge was completely dipped in. These extraction bottles were then kept at room temperature for about 3 days. During this period, shaking of the bottle was performed several times. The ethanol soluble compounds were then filtered using double filter paper. Fresh ethanol was added into the used sponge material and the process was repeated three times. The filtered ethanolic solution containing sponge compounds was subjected to the rotary evaporator for drying. The ethanolic solution was taken in the round bottom flask of rotary evaporator and ethanol was isolated from the solution below 40°C under vacuum pressure. The ethanolic solution was evaporated until dried and the crude extracted was weigh to obtained the final weight.

The dried crude extracts were taken into glass vials and weighed to be tested as crude ethanol extract for screening the antimicrobial activity.

Colum

The EtOH extract was evaporated, and the residue (2.3 g) was separated into seven fractions (Frs. 1− 7) by an ODS column (100 g) with the stepwise elution of CH_3OH in H_2O, 10%, 30%, 50%, 70%, 85%, 100%, 100%+TFA, respectively.

The EtOH extracted and seven fractions of marine sponge *Auletta* sp. was tested against bacteria using *M. smegmatis* NBRC 3207 to observed the inhibition zone of marine sponge. The concentrations of samples were arranged from 10, 20, 30 ug/disc.

Antimicrobial Assay

The crude extracted of sponge *Auletta* sp. was screening to examined the inhibitory activities on the growth of *S. aureus* (Gram-positive bacterium), *E. coli* (Gram-negative bacterium), *C. albicans* (yeast), and *M. hiemalis* (filamentous fungus) and *M. smegmatis.* The fractions 1 -7 from ODS column separation were screening to examined the inhibitory activities on the growth of *M. smegmatis.*

Media Culture *E. coli*, *S. aureus, C. albicans and M. hiemalis*

Media B-1 (liquid) for inoculate: *E. coli*, *S. aureus, and C. albicans* were made with Peptone (polypeptone) 0.5 g, meat extract 0.3 g, NaCl 0.3 g, and strains *E. coli*, *S. aureus, and C. albicans* 1 mL respectively, dH_2O 100 mL at 37 °C, then incubate for 1 days. Media for inoculate *M. hiemalis*: Potato 0.5 g, sucrose 0.3 g, and dH_2O 100 mL *at* 25 °C, and strains of *M. hiemalis* and incubate for 2 days.

Media Culture Middlebrook 7H9 for *M. smegmatis*

1. Middlebrook 7H9 Broth dehydrated base
   Middlebook 7H9 broth containing 0.05% polysorbate 80, 0.5% glycerol, and 10% Middlebook OADC
2. Middlebrook OADC (Approximate Formula Per Liter; 10%)
   Sodium Chloride 8.5 g; Bovine Albumin (Fraction V) 50.0 g; Dextrose 20.0 g; and Catalase 0.03 g
3. Culture of strains *M. smegmatis* NBRC 3207
   Inoculum of strains *M. smegmatis* NBRC 3207 was cultured in media containing: 9 mL Middlebrook 7H9 Broth dehydrated base; 1 mL Middlebrook OADC; strains *M. smegmatis* NBRC 3207 at 37 °C and incubate for 2 days and adjusted to 1.0×10^6 CFU/mL.
4. Media Agar Middlebrook 7H9 for antimicrobial assay (anti TB) *M. smegmatis*

In 100 mL H2O: Middle-brook Broth 7H9 0.52 gr; Middlebrook OADC (10%) 10 mL; Agar 1.5 gr; Polysorbate 80 (Tween) 0.05% 50 µL; Glycerol 0.5% 500 µL

Disk Diffusion Method

An antibacterial assay was carried out using *M. smegmatis* NBRC 3207 by the paper disc method (Ericsson, 1960; Bu, *et al*, 2014; Abdjul, *et al.* 2017; Maarisit, *et al*. 2017) Strain NBRC 3207 was obtained from the Biological Resource Center (NBRC) and NITE (Chiba, Japan), and maintained in 20% glycerol at − 80 °C.

The test microorganism was cultured in Middlebrook 7H9 broth containing 0.05% polysorbate 80, 0.5% glycerol and 10% Middlebrook OADC at 37 °C for 6−12 days and adjusted to 1.0×10 CFU ml. The inoculum was spread on the above medium containing 1.5% agar in a square plate. Each sample in CH_3OH was adsorbed to a sterile filter disc with concentration 10, 20, 30 ug/disc. (6 mm; Advantec, Tokyo, Japan), and, after the evaporation of CH_3OH, the disc was placed on an agar plate and incubated for 2 days at 37 °C. *Streptomycin sulfate* and CH_3OH were used as positive and negative controls, respectively.

Measurement of results

Cultures should be read within 2-3 days after inoculation. Growth is occasionally rapid enough at 37°C, so that the edge of the zone of inhibition are suficiently defined to permit measurement of the zone diameter within 48 hours. The diameter of the inhibition zone, measure with calipers for the accuracy of the measurements. The measurement of sample, will compare with positive and negative control (Table 1 - 2).

Generally, the larger the zone of inhibition with the lower concentration of antimicrobial required to inhibit the growth of the organisms. A larger zone of inhibition usually means that the antimicrobial is more potent.

RESULT

Marine sponge *Auletta* sp. was collected in the coral reefs in North Sulawesi, 2014 (Figure 1). The sponge was respectively extracted three times with EtOH, and the extracts were evaporated to remove EtOH. Seven fractions (1− 7) were yielded by an ODS column and used for the screening bioassays.

Collection and extraction of sponge

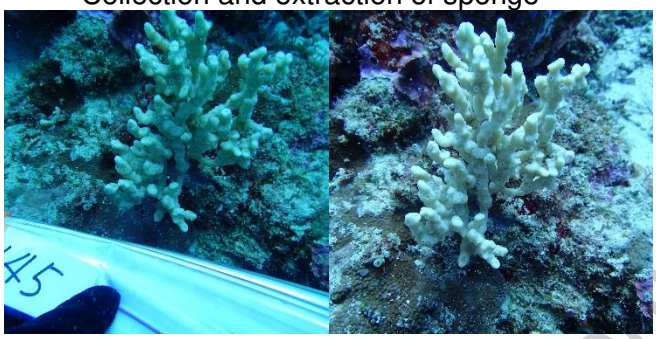

Figure 1. Marine Sponge *Auletta* sp.

Screening bioassays

The EtOH extract of the sponge *Auletta* sp. inhibited the growth of *M. smegmatis* at 50 µg/disc. Therefore, the antimycobacterial activity of fractions 1 – 7 was tested.

The EtOH extract of the marine sponge inhibited the growth of *M. smegmatis* with an inhibition zone of 17 mm at 50 µg/disc and was separated into seven fractions by an ODS column. Fractions 1, 2, and 3 showed no activity at 10, 20, 30µg/disc, respectively, Fractions 4 showed zone inhibition 11, 11, 14 mm at 10, 20, 30µg/disc, respectively, Fractions 5 showed zone inhibition 12, 15, 20 mm at 10, 20, 30µg/disc, respectively, Fractions 6 showed zone inhibition 16, 19 mm at 20, 30µg/disc, respectively, while at concentration 10µg/disc showed no activity. Fractions 7 showed zone inhibition 10, 14 mm at 20, 30µg/disc, respectively, while at concentration 10µg/disc showed no activity. All the tested were compare with control positive and negative (Figure 2 and Table 2).

Gram-positive bacterium *Staphylococcus aureus*, Gram-negative bacterium *Escherichia coli*, *Candida albicans* (yeast), and *Mucor hiemalis* (filamentous fungus), were also examined as screening bioassays for sponge crude extracts (Table 1).

Table 1. Antimycobacterial Assay EtOH Crude Extract

Sponge *Auletta* sp.	**Weight (g)**	**Biological Assay (Inhibition zone; mm; 50 µg/disc)**				
		E. coli	*S. aureus*	*C. albicans*	*M.hiemalis*	*M. smegmatis*
	2.4	-	10	-	8	17
Positive Control		Chloramphenicol (10 µg)	amphotericin B (10 µg)	amphotericin B (10 µg)	amphotericin B (10 µg)	Streptomycin sulfate (2 µg)
Inhibition Zone (mm)		**15**	**15**	**13**	**15**	**25**
Negative Control		-	-	-	-	-

Activities of microbe: inhibition zone (mm): 50 µg/disc; diameter disc: 6 mm; concentration sample: 10 mg/mL;
(-) : no activity.
Staphylococcus aureus IAM 12544T (Gram-positive); *Escherichia coli* IAM 12119T (Gram-negative);

Candida albicans IFM 4954 (Yeast); *Mucor hiemalis* IAM 6088 (Filamentous fungus); *Mycobacterium smegmatis* NBRC 3207 (Gram-positive).

Table 2. Biological Activities of Fractions 1 - 7 Against *Mycobacterium smegmatis*

Fractions	***Mycobacterium smegmatis* (inhibition zone, mm)**		
	Concentration sample in disc		
	10 µg/disc	20 µg/disc	30 µg/disc
Fr.1	**-**	**-**	**-**
Fr.2	**-**	**-**	**-**
Fr.3	**-**	**-**	**-**
Fr.4	**11**	**11**	**14**
Fr.5	**12**	**15**	**20**
Fr.6	**-**	**16**	**19**
Fr.7	**-**	**10**	**14**
Positive Control (*Streptomycin sulfate*)		**25**	
Negative Control (Methanol)		**-**	

Antimicrobial activity: disc diameter: 6 mm; concentration of sample: 5 mg/mL; (-) : no activity

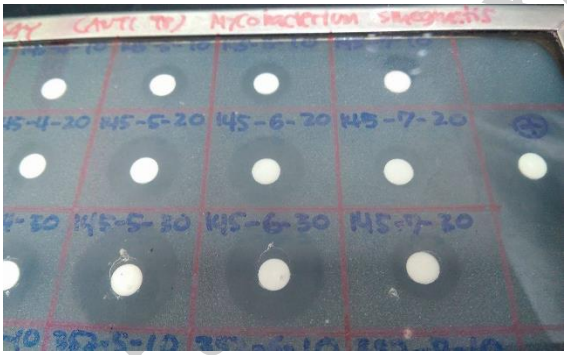

Figure 2. Inhibition zone fractions 1 - 7 against *M. smegmatis*

CONCLUSIONS

The extract sponge *Auletta* sp. collected in North Sulawesi was tested for antimicrobial activities. Crude extract of sponge inhibited the growth of *S. aureus*, *M. hiemalis*, and *M. smegmatis* with inhibition zone 10, 8, 17 mm, respectively. And no activity showed in *E. coli*, and *C. albicans.*

Isolation of sponge with ODS column chromatography method were yielded 7 fractions (1 – 7), and were tested their antimicrobial activities against *M. smegmatis.* The fractions 4 – 7 with concentrations 10, 20 and 30 µg/disc, showed activity antimicrobial active against *M. smegmatis.*

Among seven ODS fractions, fractions 4 - 5 with concentration 10µg/disc were most active (inhibition zone 11, 12 mm, respectively) compared the other fractions and will be an interesting for future study.

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