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Screening antibacterial activity ODS fractions of marine sponges against non-pathogenic bacteria tuberculosis *Mycobacterium smegmatis*

Deiske A. Sumilat

Faculty of Fisheries and Marine Science, Sam Ratulangi University. Campus Unsrat Manado 95115
E-mail: deiske.sumilat@unsrat.ac.id

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Abstract: Nine unidentified marine sponges from Bunaken Island waters in Manado Indonesia were observed on anti-tuberculosis (TB) against non-pathogenic *Mycobacterium smegmatis* NBRC 3207 and it was found that the ethanol crude extracts and ODS fractions of these sponges inhibited the growth of bacteria *M. smegmatis.* Sponge SP.323 is the most inhibiting and showed Intermediate (I) activity with inhibition zones of 9, 11, 11, 8 mm/disk at 10µg/disk., for fraction 3,4,5,6 respectively, and need further experimental analysis to justify the antimicrobial activity, NMR analysis and structure elucidation.

Keywords: sponges; antibacterial; tuberculosis; *Mycobacterium smegmatis*; ODS

INTRODUCTION

Tuberculosis (TB) disease is one of the top 10 causes of death and the leading cause from a single infectious agent (above HIV/AIDS). Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is still one of the main infectious diseases in the the world, including with the human immunodeficiency virus (HIV) and malaria (WHO, 2020). According to World Health Organization (WHO), around 10 million people continue to fall sick with TB each year with the leading cause of *M. tuberculosis.* In 2019, TB caused an estimated 1.2 million deaths (range, 1.1–1.3 million) among HIV-negative people and there were an additional 208.000 deaths from TB (range, 177.000–242.000) among HIV-positive people (WHO, 2020). Because of lack of treatment or lack of adapting treatment, many researchers are interested to find antituberculotic drugs. Urgent action is required to improve the coverage and quality of diagnosis, treatment and care for people with drug-resistant TB.

Marine organisms such as invertebrate are a much sought-after sources of drugs candidate. Sponges are exclusively aquatic animals that dominate in many benthic habitats, are rich source of biologically active natural products with unique structures (Caroll *et al*., 2020). Sponges are thought to be used for pharmaceutical and biotechnological industries and have shown the highest potential for natural product discovery (Mehbub *et al*., 2014). A number of metabolites have been isolated and they were found to have various biological activities, such as antibacterial, antifungal, anti-HIV, anti-inflammatory, anticancer, and as enzyme inhibitory activities (Mehbub *et al*., 2014). Some sponge derived natural and synthetic compounds have been approved for clinical use (Newman and Cragg, 2014; 2020), such as Eribulin (an anticancer agent). Therefore, marine metabolites might be developed as a new recommended source of promising drugs.

For several years our research has focused on antimicrobials with purpose to find new anti-mycobacterial substances from marine sponges and microorganisms collected in North Sulawesi, Indonesia. We found microbe *M. smegmatis* which has often been used in molecular biological experiments and experimental tuberculosis as a substitute of highly pathogenic *M. tuberculosis*.

Mycobacterium smegmatis is used as an alternative microorganism to detect antibacterial activity against tuberculous bacteria. During our group research on sponges, we found anti-mycobacterial activity of a bisfunctionalized sphingolipid from marine sponge *Agelas* sp. collected in Manado (Abdjul *et al.*, 2017), that was

the first study to show that *Agelas* sp. inhibited the growth of *M. smegmatis*. Three new dimeric 3-alkyl pyridinium alkaloids, called haliclocyclamines A–C, were found from Indonesian marine sponge *Haliclona* sp. and showed inhibited antimicrobial activities against *M. smegmatis* (Maarisit *et al*., 2017). On the screening of antimicrobial, it was found that the ethanol extract ODS fractions of an Indonesian marine sponge *Auletta* sp. inhibited the growth of nonpathogenic *M. smegmatis* (Sumilat, 2019).

In the course to continue studies on anti-TB metabolites from Indonesian marine sponges we have tested nine unidentified species of marine sponges against *M. smegmatis* NBRC 3207 and found that the crude extracts and ODS fractions of these nine sponges exhibited prominent activity.

MATERIALS AND METHODS

Experimental Procedures

The chemicals and solvents; Ethanol, Methanol, Glycerol, Agar, Potato Dextrose Agar, Peptone, Glucose, Sucrose, Yeast Extract, Paper disk, ODS C-18 were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Invitrogen (Carlsbad, CA, USA). Middlebrook 7H9 broth, polysorbate 80, and Middlebrook OADC were purchased from BD.

Sample Collection and Extraction

Marine sponges were collected by scuba diving at Bunaken Island in Manado Indonesia, in 2014. The nine-voucher specimens were deposited at the Faculty of Pharmaceutical Sciences, Tohoku Medical and Pharmaceutical University.

Marine sponge was cut into small parts and put in a bottle containing ethanol 500 ml, kept at room temperature for 1x24 h. The ethanol extracted was filtered and subjected to the rotary evaporator for drying to obtain the final weight (Ebada *et al*., 2008).

Microbes

The strain of *M. smegmatis* NBRC 3207 was obtained from the Biological Resource Center (NBRC), NITE (Chiba, Japan) and was maintained in 20% glycerol a t -80 °C.

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

Column Chromatography

The ethanol extract was evaporated, and the residue was separated into seven fractions (Frs. 1− 7) by an ODS (octadecylsilane) column (100 g) with the stepwise elution of CH_3OH in H_2O. (0%, 20%, 40%, 60%, 80%, 100%, 100 %+0.05% TFA).

Antimicrobial Assay

The extracted of sponges were screening to examine the inhibitory activities on the growth of *C. albicans* (yeast), *M. hiemalis* (filamentous fungus), *M. smegmatis*, *S. aureus* (Gram-positive bacterium), *E. coli* (Gram-negative bacterium) (Bu *et al*., 2014; Abdjul *et al*., 2017; Maarisit *et al*., 2017).

ODS column chromatography was performed to separation in 7 fractions and screening to examine the inhibitory activities on the growth of *M. smegmatis.* The concentration of samples was arranged from 10, 20, 30 ug/disk. *M. smegmatis* was cultured in Middlebrook 7H9 broth at 37 °C for 6−12 days and adjusted to 1.0 ×106 CFU/mL. The disk 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. A zone of inhibition test in antibacterial assay was carried out using *M. smegmatis* NBRC 3207 by the paper disk method (CLSI, 2013). The interpretive diffusion disk consists of Susceptible (**S**) ≥ 20 mm, Intermediate (I) 15-19 mm, Resistant (R) ≤ 14 mm, where the sample concentration is 30 µg (CLSI, 2013).

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 of Peptone (polypeptone), meat extract, NaCl (Sodium Chloride), and dH_2O 100 mL. Strains *E. coli, S. aureus, and C. albicans* 1 mL respectively, then incubate at 37 °C, for 1 days. Media B-1 (liquid) for inoculate *M. hiemalis*: Potato, sucrose, and dH_2O 100 mL at 25 °C, and strains of *M. hiemalis* was incubate for 2 days (Bu *et al*., 2014; Abdjul *et al*., 2017; Maarisit *et al*., 2017).

Media Culture Middlebrook 7H9 for *M. smegmatis*

Broth dehydrated base of Middlebrook 7H9; containing 0.05% polysorbate 80, 0.5% glycerol, and 10% Middlebrook OADC. Middlebrook OADC approx. per liter, containing 8.5 g Sodium Chloride; 50.0 g Bovine Albumin (Fraction V); 20.0 g

Table 1. Antimicrobial assay of ethanol crude extracts from marine sponges

Sample Code	Weight (g)	Assay Data (Inhibition Zone; mm; 50 µg/disk) *E. coli*	*S. aureus*	*C. albicans*	*M. hiemalis*	*M. smegmatis*
SP. 102	27.72	-	-	-	-	12
SP. 135	12.40	-	-	-	-	11
SP. 137	22.05	-	-	-	-	8
SP. 205	6.40	-	-	-	-	10
SP. 206	10.50	-	-	-	-	10
SP. 259	0.85	-	-	-	-	7
SP. 323	1.75	7	8	7	-	10
SP. 352	2.25	-	7	-	-	8
SP. 356	0.45	7	7	-	-	10
Positive Control		Chloramphenicol (10 µg)	Chloramphenicol B (10 µg)	Amphotericin B (10 µg)	Amphotericin B (10 µg)	Streptomycin sulfate (2 µg)
		15	15	13	15	25
Negative Control (15 µg)		-	-	-	-	-

Antimicrobial activity: inhibition zone (mm): 50 µg/disk; disk diameter: 6 mm;
concentration of sample: 10 mg/mL
(-): no activity; (EtOH crude extracted from liquid sample 50 mL)

Dextrose; and 0.03 g Catalase. Inoculum of strains *M. smegmatis* NBRC 3207; NBRC 3207 was cultured in media containing: 9 mL Middlebrook 7H9, 1 mL Middlebrook OADC, at 37 °C and incubate for 2 days and adjusted. Medium of the antimicrobial assay (anti TB) *M. smegmatis* for 100 mL H_2O: containing 0.52 gr Middlebrook Broth 7H9; 10 mL (10%) Middlebrook OADC; 1.5 gr Agar; 50 µL Polysorbate 80 (Tween); 500 µL Glycerol.

RESULTS AND DISCUSSION

In this research, we found nine marine sponges collected by scuba diving at Bunaken Island in Manado Indonesia, in 2014. We presented our sample in code number due to their molecular identification is still in progress. The dried crude extract was put into a glass vials, weighed and tested as a crude extract ethanol for screening the antimicrobial activity (Table 1).

ODS Fractions Column Chromatography

The EtOH extract of the nine-sample residue was separated into seven fractions (Fr. 1-7) by an ODS column (100 g) with the stepwise elution of CH_3OH in H_2O. (0%, 20%, 40%, 60%, 80%, 100%, 100 %+0.05% TFA) (Table 2) and were tested against bacteria *M. smegmatis* NBRC 3207 to observe the inhibition zone of marine sponges. The concentration of samples was arranged from 10, 20, 30 µg/disk (Table 3).

Screening Bioassays Antimicrobial

Antimicrobial screening of EtOH crude

Table 2. Weight of ODS fractions of marine sponges

Sample Code	Weight of Sample (g)	Weight of Fractions (mg); CH_3OH in dH_2O (500 mL) 0%	20%	40%	60%	80%	100%	+0.05% TFA
		1	2	3	4	5	6	7
SP. 102	16.76	4380.00	3710.00	3760.00	1780.00	2320.00	624.14	2700.00
SP. 135	11.00	7320.00	675.79	540.36	682.48	1310	315.22	474.39
SP. 137	11.80	7090.00	805.78	552.19	1040	414.91	448.02	325.70
SP. 205	3.10	673.84	221.14	73.00	64.97	209.00	134.70	12.95
SP. 206	10.50	6010.00	298.21	106.29	220.36	133.83	843.83	223.40
SP. 259	0.60	266.05	107.36	11.64	5.32	8.88	31.68	111.51
SP. 323	1.50	897.15	119.52	108.45	75.60	106.77	72.91	183.12
SP. 352	1.75	944.91	219.91	123.02	59.59	42.60	81.40	255.42
SP. 356	0.40	133.41	56.28	23.24	17.28	31.84	75.82	91.61

Table 3. Antimicrobial assay of marine sponges ODS fractions against *Mycobacterium*

Sample Code	Concen-trations (µg/disk)	(Inhibition Zone-mm; µg/mL) Fractions						
		1	2	3	4	5	6	7
SP. 102	10	-	-	-	-	-	-	-
	20	-	-	-	-	-	-	-
	30	-	-	-	-	-	-	-
SP. 135	10	-	-	-	-	-	-	-
	20	-	-	-	-	-	-	-
	30	-	-	-	-	-	-	-
SP. 137	10	-	-	-	-	-	-	-
	20	-	-	-	-	-	-	-
	30	-	-	-	-	-	-	-
SP. 205	10	-	-	10	8	8	8	8
	20	-	-	13	11	8	11	11
	30	-	-	15	11	10	11	11
SP. 206	10	-	-	-	-	-	-	-
	20	-	-	-	10	10	10	8
	30	-	-	-	10	9	9	9
SP. 259	10	-	-	-	-	-	-	-
	20	-	-	-	-	-	-	-
	30	-	-	-	-	-	-	-
SP. 323	10	-	-	9	11	11	8	-
	20	-	-	-	16	14	19	14
	30	-	-	-	21	21	21	16
SP. 352	10	-	-	-	-	-	-	-
	20	-	-	-	-	-	-	-
	30	-	-	-	-	-	-	-
SP. 356	10	-	-	-	8	10	8	-
	20	-	-	-	11	11	9	8
	30	-	-	-	8	13	13	9

Positive Control - Streptomycin sulfate (2 µg): 25
Negative Control - MeOH (20 µg): -
Antimicrobial activity: (-): no activity

extracts of the nine sponges are shown in Table 1. All marine sponges extract inhibited the growth of *M. smegmatis*, but only SP.352, SP.356 inhibited *S. aereus*, while SP.356 inhibited *E. coli*.

The ODS fraction for all specimens were screened against *M. smegmatis* (based on their activities in ethanol crude extracted) and the result shown in Table 3 and Figure 1. Marine sponges SP.205, SP.206, SP.323 and SP.356 inhibited the growth of *M. smegmatis.* ODS fraction of SP.205 inhibited all the concentrations of the sample (at 10, 20, 30 µg/disk) from Fr. 3-7. Fractions of SP.206, inhibited *M. smegmatis* at the Fractions 4-7, at concentration 20 and 30 µg/disk, Fractions of SP.323 inhibited *M. smegmatis* at the Fractions 4-7, at concentration at 10, 20, 30 µg/disk, while SP.356 inhibited the non-pathogen bacteria *M smegmatis* at fractions 4-7. The results showed that SP.323 revealed the highest inhibiting activity against the growth of *M. smegmatis.* It is therefore have a high prospect to continue for the next other purification and NMR analysis.

Based on interpretive diffusion disk (CLSI, 2013), which shown in Table 3 and Figure 1, it can be concluded that marine sponges SP.205, SP.206 and SP.356 were categorized as Resistant (R) with diameter of the inhibition zone range ≤ 14 mm, while SP.323 showed Intermediate (I) 15-19 mm on fractions 3-6 at sample concentration 10 µg/disk. Sample concentration 20 and 30 µg of SP.323 were categorized Susceptible (S) ≥ 20 mm.

Maarisit *et al.* (2017) obtained haliclocyclamines A-C and cyclostellettamines A-C, E-F from *Haliclona* sp. collected in Manado and found that these compounds have antimicrobial activity against

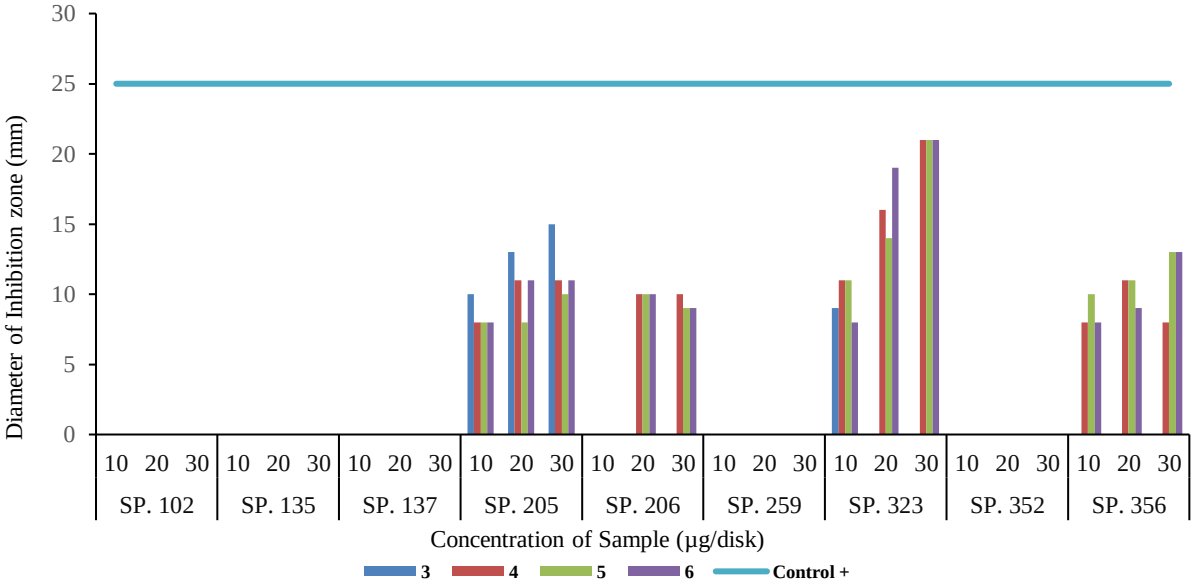

Figure 1. Antimicrobial assay of marine sponges ODS fractions against *Mycobacterium smegmatis*

M. smegmatis with inhibition zone of 17, 10, 13, 14, 8, 8, 12, and 12 mm, respectively at 10 µg/disk, Maarisit *et al.* (2017) found that *Haliclona* sp.have Resistant (R) and Intermediate (I) to *M. smegmatis* with diameter of the inhibition zone range ≤ 14 mm and 15-19 mm, respectively. Indonesian marine sponge, *Agelas* sp., was observed by Abdjul *et al.* (2017) and obtained two compunds 5-bromophakelline and leucettamol A, leucettamol A exhibited moderate anti mycobacterial activity, with inhibition zones of 7, 9, 12 mm/disk at 10, 20, 50/µg/disk. Similar to the results observed by Abdjul *et al*. (2016), for antimycobacterial activities of Okinawan sponge *Halichondria panicea* against *M. smegmatis*, the results showed inhibition zones range from 7-16 mm at 10/µg/disk. The difference in the inhibition of each extract against bacteria can be influenced by the structure and composition of bacteria cell, the density/concentration of bacterial cells and the composition of the active compound contained in the extract.

The standard concentration according to CLSI (2013) is 30 µg per disk, and when compared in this study, which the concentrations range between 10, 20, 30 µg, it is necessary to do more research to produce accurate data with the aim of obtaining standard categories Resistant (R), Intermediate (I) and Susceptible (S).

In general, the best concentration required for antimicrobial activity is the lowest concentration while A larger zone of inhibition usually means that the antimicrobial is more potent.

Based on the interpretive diffusion disk, marine sponges SP.323 as an inhibitor and more potent to inhibit the growth of non-pathogen bacteria *M. smegmatis*. This sponge SP.323, might be recommended for detailed research for structure elucidation and other synthesis experiments, to develop as a new promising antibacterial drug.

CONCLUSION

Marine sponges collected in Bunaken Island in this research were found to have inhibiting activities against the growth of nonpathogenic *M. smegmatis.* Among nine marine sponges, it can be concluded that, marine sponges SP.323 can be used for further experimental analysis as an inhibitor. Its antimicrobial activity needs to be justified and purified with NMR analysis for structure elucidation.

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REFERENCES

ABDJUL, D.B., YAMAZAKI, H., KANNO, S., TOMIZAWA, A., WEWENGKANG, D.S., ROTINSULU, H., SUMILAT, D.A., UKAI, K., KAPOJOS, M.M. and NAMIKOSHI, M. (2017) An Anti-mycobacterial Bisfunctionalized Sphingolipid and New Bromopyrrol Alkaloid from the Indonesian Marine Sponge *Agelas* sp. *Journal of Natural Medicines*, 71 (3), pp. 531-536.

ABDJUL, D.B., YAMAZAKI, H., KANNO, S., TAKAHASHI, O., KIRIKOSHI, R., UKAI, K., and NAMIKOSHI, M. (2016) Haliclonadiamine derivatives and 6-*epi*-Monanchorin from the marine sponge *Halichondria panicea* collected at Iriomote island. *Journal of Natural Products*, 79 (4), pp. 1149-1154.

BU, Y.Y., YAMAZAKI, H., UKAI, K. and NAMIKOSHI, M. (2014) Anti-mycobacterial nucleoside antibiotics from a marine-derived *Streptomyces* sp. TPU1236A. *Marine Drugs*, 12 (12), pp. 6102-6112.

CARROLL, A.R., COPP, B.R., DAVIS, R.H., KEYZERS, R.A. and PRINSEP, M.R. (2020) Marine natural products. *Natural Product Reports*, 37, pp. 175-223.

CLSI (2013) *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Third Informational Supplement M100-S23*; Clinical and Laboratory Standards Institute: Wayne, PA, USA. p. 205.

EBADA, S.S., EDRADA, R.U., LIN, W. and PROKSCH, P. (2008) Methods for isolation, purification and structural elucidation of bioactive secondary metabolites from marine invertebrates. *Nature Protocols*, 3 (12), pp. 1820-1831.

MAARISIT, W., YAMAZAKI, H., ABDJUL, D.B., KATO, H., ROTINSULU, H., WEWENG-KANG, D.S., SUMILAT, D.A., KAPOJOS, M.M., UKAI, K. and NAMIKOSHI, M. (2017) Anti-Mycobacterial Alkaloids, Cyclic 3-Alkyl Pyridinium Dimers, from the Indonesian Marine Sponge *Haliclona* sp. *Bioorganic & Medicinal Chemistry Letters*, 27 (15), pp. 3503-3506.

MEHBUB, M.F., LEI, J., FRANCO, C. and ZHANG, W. (2014) Marine Sponge Derived Natural Products between 2001 and 2010: Trends and Opportunities for Discovery of Bioactives. *Marine Drugs*, 12, pp. 4539-4577.

NEWMAN, D.J. and CRAGG, G.M. (2014) Marine-sourced anti-cancer and cancer pain control agents in clinical and late preclinical development. *Marine Drugs*, 12 (1), pp. 255-278.

NEWMAN, D.J. and CRAGG, G.M. (2020) Natural Products as Sources of New Drugs over the Nearly Four Decades from 01/1981 to 09/2019. *Journal of Natural Products*, 83 (3), pp. 770-803.

SUMILAT, D.A. (2019) Antibacterial Activity ODS Fractions of Marine Sponge *Auletta* sp. against *Mycobacterium smegmatis*. *Jurnal Ilmiah Platax*, 7 (1), pp. 329-334.

WHO (2020) *Global Tuberculosis Report 2020*. Geneva: World Health Organization. p. 232. Licence: CC BY-NC-SA 3.0 IGO. ISBN 978-92-4-001313-1.