Extraction and Characterization of Bioactive Compounds from Cultured and Natural Sponge, *Haliclona molitba* and *Stylotella aurantium* Origin of Indonesia

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***Abstract*—Soft-body invertebrates, such as sponges are physically-weak organisms; however they mostly survive for thousands of years. This is due to the fact that they are capable of defending themselves from predators by releasing toxic substances as their secondary metabolites against the predators. These compounds are highly potent for medicine such as anti-microbial, anti-cancer, and anti-inflammatory activities. A large quantity of sponges are needed to produce bioactive compounds as their secondary metabolites only could get in small amount. This could provoke the sea-sponges overexploitation. Cultured sponges could be the one of the alternatives to prevent excessive natural sea-sponge exploitation. However, it is still unclear whether the cultured sponge has similar bioactivity compare to the natural sponge as well. The purposes of this research were to extract secondary metabolites from natural and cultured sea-sponges (Haliclona molitba and Stylotella aurantium), and to evaluate their antimicrobial, hemolysis, and hemagglutination activities with differentiation (?) on cultured and natural sponges. Aquadest and methanol were used as solvents on this research. Methods were used to determine the antimicrobial activity using the round disc diffusion. Hemolysis and hemagglutination assays utilized red blood from Deutsch Democratic Yokohama (DDY) mice. Hemagglutination and hemolysis results were measured with a microplate reader. The extraction yield of natural sponge was not significantly different from cultured one. The aqueous extract resulted in higher yield than methanolic extract. As antimicrobial activity, Bacillus cereus was the most sensitive against the crude extracts either from the natural and cultured sponges among all tested bacteria. Although both types of sponges did not show hemolysis activity, they showed hemogglutination activity.**

***Index Terms*—Antimicrobial, cultured sponge, *Haliclona molitba*, hemagglutination, hemolysys, and *Stylotella aurantium*.**

I. Introduction

As the biggest archipelago country, Indonesia exhibits high marine biodiversity, particularly the coral reef ecosystem. Due to high competition in the ecosystem, physically weak organisms need to develop a biological defense system in order to be able to protect themselves. Sponges, for example, need to develop a secondary metabolism system which is primarily used for self-defense. The toxic compounds produced from the sponge's secondary metabolism are highly potential to be used as medicine.

Nowadays 850 sponge species have been discovered in Indonesia and some of them are found to contain secondary metabolites. These compounds have shown to have anti tumor and anti-inflammatory activities. Some examples of secondary metabolites found in sponges are terpenes, alkaloids, and polyphenols. Specific compounds which have shown promising activities are among others: calyculins from *Dicodermia calyx*, lantruculins from *Lantrunculia magnifica*, and spongistatins from *Spongia* sp., and *Spirastrella* sp. [1]-[3]. In addition to having bioactive compounds, some marine sponges, such as suberitin (which is derived from *Suberites douncula*) and *heolysin* (which is derived from *Tetchya lincurum*), also showed hemolytic and hemagglutination activities [3]. The fact that sponges are potentially useful as ingredients for medicine could threaten the sustainability of these sponges because a massive amount of sample is needed to obtain secondary metabolites from sea sponges. To produce one gram of anticancer compound, for example, one ton of *Ecteinascidia turbinate* sponge is needed [4]. Therefore, an alternative source of raw materials should be found.

One possible alternative for acquiring sufficient amount of secondary metabolites is by cultivating sponges. Sponge and other reef organism cultivation has much been developed lately, especially through transplantation method. Coral transplantation method is relatively easy to be carried out and its production can be customized in terms of quantity and time.

The use of cultured sponges as alternative materials, however, requires careful content analysis because natural sponges and cultured sponges could produce different substances. Sponges which belong to the same species may have different secondary metabolites as they live in different places [3]. Natural *Mycale hentscheli*, for example, has the ability to produce secondary metabolites. Yet, secondary metabolites are not produced by cultured *Mycale hentscheli* [5]. In addition, the extracted secondary metabolites also depend on the solvent used during the extraction process [6].

This study aims to extract and to characterize the secondary metabolite from sponges originated from Indonesia (*Haliclona molitba* and *Stylotella aurantium*) based on antimicrobial, hemolytic, and hemagglutination activities by comparing natural and cultured sponges and types of solvent used.

II. Materials and Methods

A. Materials

Natural and cultured sponges Stylotella aurantium and

Manuscript received June 21, 2013; revised September 23, 2013. This work was supported by Atma Jaya Catholic University of Indonesia and PT. Dinar Daru Lestari.

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Haliclona molitba were used in this study. The natural sponges were collected from the water around the Thousand Islands, Indonesia, while the cultured ones were collected from Pramuka Island, which part of the Thousand Islands clusters.

B. Bioactive Compounds Extraction

Bioactive compounds were extracted following Bakus and Green method [7] with some modifications. Extraction was conducted by using methanol and distilled water as solvents. When methanol was used for extraction, the sponge was dried via lyophilization for two days, and then the sponge tissue was grounded until it became powder and then soaked in methanol for five hours.

TABLE I: YIELD PERCENTAGE AND INHIBITION ZONE OF *STYLOTELLA AURANTIUM* AND *HALICLONA MOLITBA* CRUDE EXTRACT

Source	Solvent	Yield (%)	Inhibition zone (mm)			
			Bacillus cereus	*Escherichia coli*	*Staphylococcus aureus*	*Salmonella typhi*
Stylotella aurantium						
Natural	Aqueous	16.05 ±2.27	0.3 ±0.3	0.0 ±0	0.3 ±0.3	0.0 ±0
	Methanol	11.04 ±0.24	7.3 ±1.2	2.3 ±1.5	2.3 ±1.5	2.7 ±1.2
Cultured	Aqueous	18.06 ±2.53	2.0 ±1.5	0.0 ±0	1.0 ±1.0	0.0 ±0
	Methanol	8.86 ±2.01	2.7 ±1.8	1.3 ±1.3	1.3 ±0.9	1.7 ±1.7
Haliclona molitba						
Natural	Aqueous	25.59 ±10.64	1.0 ±0.0	1.0 ±1.0	0.0 ±0.0	0.0 ±0.0
	Methanol	22.22 ±10.29	0.67 ±0.33	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
Cultured	Aqueous	13.20 ±2.16	2.33 ±0.88	0.0 ±0.0	3.67 ±1.86	0.0 ±0.0
	Methanol	7.70 ±1.79	3.67 ±0.33	0.0 ±0.0	5.0 ±2.52	0.0 ±0.0

The amount of methanol used was 20 times as much as the weight of the sponge (5% w/v). The solution was then filtered through Whatman Filter Paper No. 3. Filtered solution was air dried then stored in a refrigerator (4 ℃) for further use.

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When the distilled water was used for extraction, the sponge was boiled for 5 minutes in the boiling water. The solution from this process was then filtered with Whatman Filter Paper No. 3. Filtered solution was evaporated in a vacuum oven (50 ℃) until it was dry and then the extract was stored in a refrigerator (4 ℃) for further use.

C. Antimicrobial Activity Assay

Antimicrobial activity assay was conducted using the disc diffusion method. Petri dishes with nutrient agar were inoculated with four species of bacteria, i.e. *Bacillus cereus*, *Escherichia coli*, *Salmonella typhi*, and *Staphylococcus aureus*. Those bacterial cultures were obtained from the Microbiology Laboratory at Atma Jaya Catholic University of Indonesia. Sponge extract was dissolved in distilled water to achieve the concentration of 30 mg/ml. Sponge extract solution was sterilized by passing it on GV 0.22 µm Millipore Filter. 0.01 ml of the extract solution from each bacteria species was dropped into each paper discs (6 mm diameter) and placed in the middle of inoculated petri dish. Petri dishes were incubated overnight at the temperature of 37^{o}C, and then the inhibition zone was measured.

D. Hemolytic Assay

Hemolytic assay was conducted using the method used in the study by Sepcic *et al.* [3] with some modifications. Fresh blood from Deutsch Democratic Yokohama (ddY) mice was used for hemolytic assay. The erythrocyte was prepared by centrifugating the blood at 2000 rpm for 4 minutes at the temperature of 4 ℃. Clear and colorless supernatant was discarded, while the pellet, which was the erythrocyte, was washed with physiological saline. The mass of physiological saline needed was 9 times of as much as the amount of pellets obtained. This step was repeated three times. Pellet which had been washed was then diluted with buffer containing 0.13M NaCl in 0.02 M pH 7.4 Tris-HCl until the concentration was 0.5% w/v.

Hemolytic activity was assayed with Micro plate Reader (BioRad), Model 680 and U-bottom microplate. Each well was filled with 20 µL sponge extract which had been dissolved in buffer to produce erythrocyte suspension with the concentration of 10 mg/ml. Subsequently, 100 µl of erythrocyte suspension was added for hemolysis assay. Positive control used was 1% Triton X-100 and negative control was a buffer and added with erythrocyte suspension. The absorbance value was measured with a microplate reader at the wavelength of 655 nm on 0; 5; 10; 15; 20 minute time span. During the measurement interval, the microplate was agitated at moderate speed at 25^{o}C.

E. Antimicrobial Activity Assay

Hemagglutination assay was conducted following Sepcic *et al.* method [3] with some modifications. Erythrocyte suspension which was made for hemolytic assay was added to U-bottom microplate. 100 µl of erythrocyte suspension was added for each well. 20 µl of sponge extract which had the concentration of 30 mg/µl was then added to each well. Hemagglutination activity could be observed visually after 45 minute incubation at the temperatue of 25 ℃. Before the microplate could be seen visually, it was agitated at a medium speed for 1 minute.

III. RESULTS

The yield percentages of cultured sponge and natural sponge were not significantly different ($p = 0.961$); however, the yield percentages were influenced by the type of solvents used. The yield percentages of both S. aurantium and H. molitba extracts in methanol showed significantly higher activities ($p = 0.007$) rather than in water (Table I).

In antimicrobial assay, all extracts were able to inhibit *B. cereus* growth and the greatest inhibitory activity (7.3 ± 1.2) was shown by the extract of *S. aurantium* using methanol as solvent. In fact, *S. aurantium* extracts showed inhibitory activity on all bacteria tested. However, extracts with

aqueous solvent generally showed weaker inhibitory activity. Extracts from the sponge *H. molitba* could only inhibit *B. cereus* and *S. aureus*.

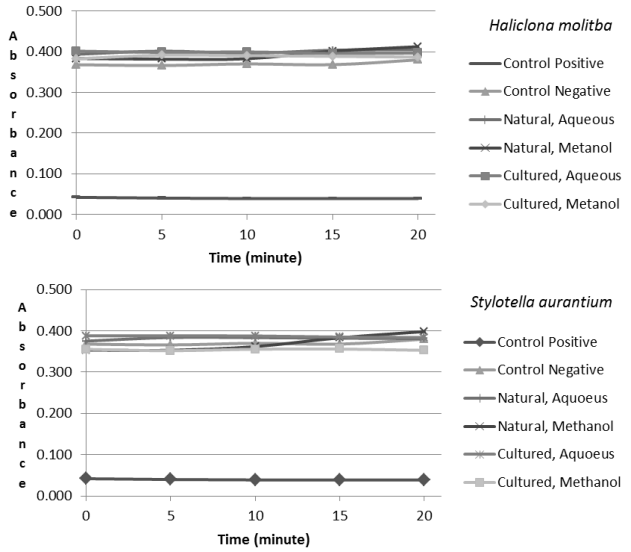

Fig. 1. *Stylotella aurantium* and *Haliclona molitba* extract hemolysis assay result based on absorbance value on 655 nm wavelength.

For hemolysis assay, all extracts from both *S. aurantium* and *H. molitba*, either natural or cultured, either using aqueous solvent or methanol solvent showed no hemolytic activity (Fig. 1). All samples did not show any absorbance reduction while the absorbance value of positive control was far below the absorbance value of all samples.

TABLE II: HEMAGGLUTINATION ASSAY RESULT
(+ : POSITIVE HEMAGGLUTINATION, - : NEGATIVE HEMAGGLUTINATION)

Source	Solvent	Hemagglutination
Stylotella aurantium		
Natural	Aquadest	+
		+
		+
	Methanol	+
		-
		-
Cultured	Aquadest	+
		+
		+
	Methanol	-
		+
		-
Haliclona molitba		
Natural	Aquadest	+
		+
		+
	Methanol	-
		+
		-
Cultured	Aquadest	+
		+
		-
	Methanol	+
		+
		-

In terms of hemagglutination assay, 8 out of 12 samples of *S. aurantium* and 8 out of 12 samples of *H. molitba* showed hemagglutination activity (Fig. 2). Hemagglutination activity was shown by the formation of red sediment on the bottom of the microplate well. The extracts from aqueous solvent showed more hemagglutination activity than the one from methanol solvent (Table II). In terms of the type of sponge, the natural and cultured sponge did not show any difference in the hemmaglutination activity. As for *S. aurantium*, all extracts showed hemagglutination activity when aqueous solvent was used, while only two out of 6 samples of those extracts indicated these activities when methanol solvent was used. As for *H. molitba*, 5 out of 6 samples using aqueous solvent showed hemagglutination activity and 3 out of 6 samples with methanol solvent showed hemagglutination activity.

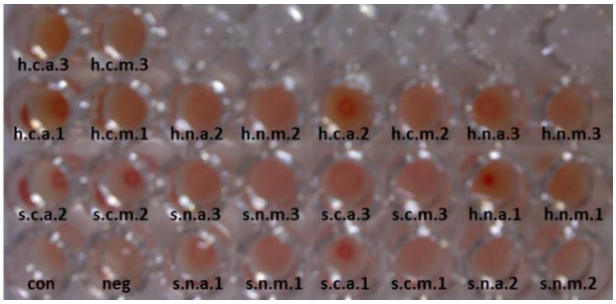

Fig. 2. Hemagglutination assay on 96 well U-bottom microplate. (con: control, neg: control negative, s: S. *aurantium*, h: H. molitba, n: natural, c: cultured, a: aqueous, m: methanol, #: number of replicates).

IV. DISCUSSION

As mentioned in previous section, neither the natural sponge nor the cultured sponge influenced the yield percentage in the research. It was the type of solvent that determines the yield percentage; aqueous solvent produced higher yield percentage than the methanol solvent. Similar results were found in Kumar *et al.* [8] on *Spongosorites halichondriodes* sponge. In contrast, Boobathy *et al.* [6] showed that *Callyspongia diffusa* with methanol solvent performed greater yield percentage. One possible cause to such differences was the type of sponge used. Different solvent also have different polarity. The polarity of the bioactive compound and that of the solvent determine the extractive yield. The polarity of water is higher than that of methanol and thus, the yield percentage of water was also higher. The higher polarity of water might result more yield than methanol which has lower polarity. The average yield percentage found in this study (15.34%) was greater than that of the studies conducted by Boobathy *et al.* [6] (6.05%) and Kumar *et al.* [8] (12.5%).

In regard to the bacteria inhibitory activity assay, crude extracts from sponge samples were most effective in inhibiting the growth of *B. cereus* and *S. aureus*, but less effective in inhibiting the growth of *E. coli* and *S. typhi*. Unlike the two other bacteria, *B. cereus* and *S. aureus* belonged to gram positive grove that had a thick peptidoglycan layer, which were superficial to the cell membrane. Inhibition against the two bacteria might have been related to the structure of this cell membrane. In terms of the solvent, the crude extract using methanol solvent had a higher inhibitory activity than the crude extract from aqueous

solvent. This suggested that bioactive compounds which inhibit the growth of bacteria were mostly semi-polar, and thus, could be easily extracted by methanol which was also semi polar. Antimicrobial compounds produced by sponge might play an effective role in improving the efficiency of getting food which was in the form of bacteria [9].

Both types of sponge extracts did not show any hemolysis activity. This indicates that no cytotoxic activity could be a candidate of whether antineoplastic or anticancer compounds [10].

Another activity tested using these extracts was hemagglutination activity. Both types of samples were proven to have hemagglutination activity. Better hemagglutination activity was observed in extracts using aqueous solvent. Hemagglutination activity is generated by protein and the protein found in sponges which usually shows hemmaglutination activity is lectin. It should however be pointed out that the size of such a protein varies and that some types of lectin may not trigger hemagglutination activity. Lectin protein with size of 60 kDa also showed antimicrobial activity against pathogenic bacteria and fungi [11]. Several types of lectin show the activity which binds tumor cells [12]. Therefore, the finding of hemagglutination activity resulted in the possibility of the presence of lectin, which potentially had antimicrobial activity or antitumor activity.

In general, our study indicated that cultured sponges and natural sponges did not show any difference in both the yield percentage and activity assays. The opposite result was found in Pelorusida A. The compound was produced by the natural sponge *Mycale hentscheli*, but not by the cultured one [5]. However, Duckworth and Battershill [13] reported that the production of chemical compounds in cultured sponge may be equal to or even greater than its natural condition. After all, the production of bioactive compounds in sponge is determined by their environmental factors, namely the presence of predators, season, temperature, sea depth, microbial infection, type of food, and the appearance of wounds on the body of the sponge [14].

The fact that the yield percentage result, inhibition assay, hemolysis, and hemagglutination assay from natural and cultured sponges were similar in this experiments might have been related to the environmental conditions. Indeed, the cultured site (Pramuka Island) is close as it is one of the islands of Thousand Island cluster from where the natural sponge originated. One advantage of this findings is that the cultured sponges *H. molitba* and *S. aurantium* can be used as an alternative feedstock to substitute for the wild one. Therefore, excessive exploitation of natural sponge can be avoided. Another advantage of the cultured sponge is related to stock management. Stock procurement of cultured sponge is fully customizable to the needs, both in terms of quantity and time.

V. Conclusion

The yield percentage of native Indonesian sponge was relatively high (on average 15.34%) and the yield percentage using aqueous solvent was greater than that using methanol solvent. Bioactive compounds extracted from sponges were capable of inhibiting *B. cereus* and *S. aureus* growth. The sponges extracts did not show any hemolytic activity but all of them, especially those using aqueous solvent, exhibited haemagglutination activity. There were no any differences between natural and cultured sponges in terms of extract yield percentage, bacteria inhibition, dan hemagglutination activities. The cultured sponges could then be used as alternative raw material to replace the natural ones.

Acknowledgment

We express our gratitute to Atma Jaya Catholic University of Indonesia for funding this research as well as providing facilities to conduct this research. We are also thankful to PT. Dinar Darum Lestari for supplying samples needed in this research.

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