IN VITRO ANTAGONISTIC ACTIVITIES OF INDONESIAN MARINE SPONGE *AAPTOS AAPTOS* AND *CALLYSPONGIA PSEUDORETICULATA* EXTRACTS AND THEIR TOXICITY AGAINST *Vibrio* spp.

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(*Received 22 June 2011; Accepted 14 September 2011*)

ABSTRACT

Vibriosis is one of diseases which often results in mass mortality of *Penaeus monodon* larval rearing systems. It attacks shrimp of all stages in zoea, mysis and shrimp postlarva stage. This disease is caused by *Vibrio* spp, particularly *Vibrio harveyi* (a luminescent bacterium). Several kinds of antibiotics and chemical material have been used to overcome the disease but they have side effects to environment and human. The searching of bioactive compounds as an alternative treatment has been done for multi purposes. In this study diethyl eter, butanol and aqueous extract of Indonesian sponges *Aaptos aaptos* and *Callyspongia pseudoreticulata* were tested for *in vitro* activity against *Vibrio* spp. and *Vibrio harveyi* by using disc diffusion method. The result showed that all extracts of *Aaptos aaptos* gave a positive antibacterial activity towards those pathogenic bacteria. Meanwhile, only butanol extract of *Callyspongia pseudoreticulata* obtained to exhibit an antibacterial activity on those pathogenic bacteria. The strong anti-vibrio activity were shown by butanol and aqueous extract of *Aaptos aaptos* with the minimum inhibitory concentration (MIC) value of 0.313 and 0.625 mg/mL, respectively. Whilst, the butanol extract of *Callyspongia pseudoreticulata* indicated a low antibacterial activity with the MIC value of 10 mg/mL. Toxicity of those active extracts was evaluated by Brine Shrimp Lethality Test (BST). Interestingly, butanol and aqueous extracts of *Aaptos aaptos* did not show any toxic effect in *Artemia salina* larvae up to 8 x MIC (2.504 mg/mL and 5.000 mg/mL). It is the first report for the anti-vibrio activity of both *Aaptos aaptos* and *Callyspongia pseudoreticulata.* This results suggest that *Aaptos aaptos* has a potential to be used as a source of alternative compound to vibriosis prevention for mariculture.

KEYWORDS: sponge, *Aaptos aaptos*, *Callyspongia pseudoreticulata*, *Vibrio* spp., and *V. harveyi*

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INTRODUCTION

Aquaculture is the fastest growing food sector globally and found to be high protein resource. However, the biggest problem faced by farmers in aquaculture is high post-larvae mortality caused by vibriosis (Lavilla-Pitogo *et al*., 1998). Among the causative agents of vibriosis is *Vibrio harveyi*, a luminescent bacterium which often results in mass mortality of *Penaeus monodon* larval rearing systems (Mariyono *et al*., 2002). The bacterium infects almost all cultured marine animals such as crustacean, mollusc and fish (Isnansetyo *et al*., 2009). Crustacean, including shrimp, crab, lobsters and artemia are very susceptible to this opportunistic pathogenic bacterium (Jivaranichpaisal *et al*., 1994; Karunasagar *et al*., 1994; Liu *et al*.,1996; Robertson *et al*., 1998; Diggles *et al*., 2000; Soto-Rodriguez *et al*., 2003; Borne *et al*.,2007). Cases of this disease is apparently typical for the tropics (Sunaryanto & Maryam, 1986; Lavilla-Pitogo *et al*.,1992).

Efforts to overcome these problems have been done by using chemical material such as formalin, malachite green and antibiotics such as chloramphenicol, oxytetracycline, furazolidone and streptomycin (Moriarty, 1999). The use of these treatments in vibriosis disease prevention brings harmful effects as a result from the accumulation of the harmful residues in the shrimp. These chemical materials also may upset the chemical balance in the pond by affecting useful organisms like nitrifying bacteria and kill food organisms in the pond. Since these antibiotics may cause a side effect, these treatments have become less effective. Beside that the presence antibiotics residue could cause shrimp being rejected by importer countries. Based on these problems, some researchers have attempted other alternative methods to control the diseases by using safe natural antibiotics (Tendencia *et al*., 2004). Some of mangrove plants, such as *Rhizopora* sp and *Achantus* sp have been proven capable of reducing the bacteria population in brackish water ponds (Ahmad & Manganpa, 2000). Another plant species, *Isotoma longiflora* could inhibit the growth of *Aeromonas* sp and *Pseudomonas* sp on fish (Suryati, 1993; Suryati & Hala, 1993).

Marine sponges have been considered as a gold mine during the past 50 years, with respect to the diversity of their secondary metabolites which pronounced pharmacological activities (Astuti *et al*., 2005). Most bioactive compounds from sponges can be classified as antiinflammatory, antitumor, immunosuppressive or neurosuppressive, antiviral, antimalarial, antibiotics and antifouling (Sipkema *et al*., 2005). It is suspected that there are about 10,000 species of sponges and it is estimated that 200 species live in coral reef ecosystem of Southeast Asia (Dahuri, 2003). The number of sponge species in Indonesia was estimated about 830 species, nevertheless it has not been used optimally particularly in disease prevention on fishery culture (Sujatmiko, 2000),

In our searching of antibacterial compounds and compounds which are able to increase the expression of scavenger receptor B-1 against pathogen bacteria and atherosclerosis, we screened 13 methanolic extracts of Indonesian and Malaysian sponge. The result showed that the methanolic extract of Indonesian *Aaptos aaptos* and Malaysian *Aaptos* as well as *Callyspongia pseudoreticulata* gave positive antibacterial activity towards several species of pathogenic bacteria (Rosmiati *et al*., 2011). The present study, We wish reported the *in vitro* antibacterial activity of Indonesian *Aaptos aaptos* (Figure 1) and *Callyspongia pseudoreticulata* (Figure 2) extracts against *Vibrio* spp and *Vibrio harveyi.*

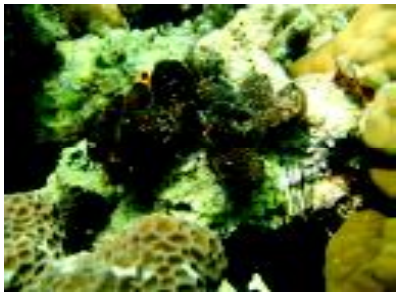


Figure 1. *Aaptos aaptos*

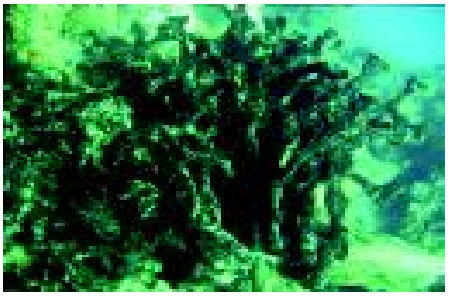


Figure 2. *Callyspongia pseudoreticulata*

MATERIALS AND METHODS

Sponges *Aaptos aaptos* and *Callyspongia pseudoreticulata* were collected from Barrang Lompo Islands, South Sulawesi by SCUBA diving at the depth of 10-12 m (Figure 3). Sponge was placed in cool box added with ice pack and brought to Biotechnology Laboratory of Research Institute for Coastal Aquaculture (RICA), Maros. The samples were kept in sealed plastic bags then preserved in a freezer at -20°C. Bioindicator used for testing of antibacterial activity were *Vibrio harveyi* (1b) and *Vibrio* spp (2WT) isolated from diseased shrimp collected from Banyuwangi (East Java) and Negara (Bali).

Extraction

A hundred gram of fresh sponge was cut in small size, dried below 40°C and smashed by grinder and repeatedly extracted with methanol by using a forma orbital shaker with the temperature of 37°C till the residue was colourless. The methanolic extracts was filtered by using a buchener and collected for concentrated under reduce pressure by a rotary evaporator to yield a dark gummy solid (21.4 g for *Aaptos aaptos* and 10.0 g for *Callyspongia pseudoreticulta*).

Removal of salt and lipid/waxes

HP-20 resin was activated with methanol and transferred to short glass column with the length of 6.5 cm and diameter of 1.5 cm. Distilled water was passed through the resin with twice volume of column length. The dried crude methanol extracts was then re-dissolved in distilled water and load onto the resin and eluted by distilled water. The extracts bound by resin were kept out by using methanol. The extracts were concentrated by a rotary evaporator at 37°C to get 2 mL of dried salt free crude methanolic extracts. Two mL of the salt free methanolic extracts were directly passed through discovery DSC-18 6 mL tube activated with methanol before removing lipid/fat. The extracts were then eluted with methanol to get interference material free extract metha-

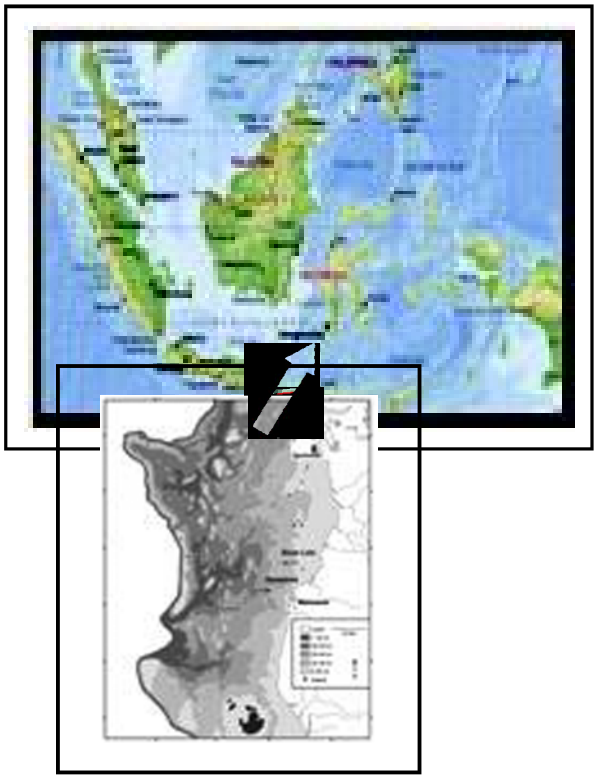


Figure 3. Sample collection sites

nol. After concentrating the solvent, interference material free crude extracts were obtained and used for the bioassay and partitioning.

Partitioning of methanolic extract

The interference material free methanolic extract added distilled water and partitioned with diethyl ether (DE) and 1-butanol (BuOH) to give diethyl ether, butanol and aqueous extract. All the extracts were assayed against *Vibrio* spp. (2WT) and *Vibrio harveyi* (1b) as described below. The extracts exhibiting an antibacterial activity were again evaluated to determine their minimum inhibitory concentration (MIC) and toxicity.

Antibacterial activity

The antibacterial activity was tested by using disc diffusion method. Prior to testing, a loopful of *Vibrio* spp and *Vibrio harveyi* inoculums was zig-zag streaked on TCBSA (*Thiosulfat Citrate Bile Sucrose Agar)* and incubated for 24 hours at 28°C. One colony of the bacteria growth was sub-cultured in 50 mL of nutrient broth medium (Sigma, FRG) and incubated for 4 hours using a shaker at 150 rpm and 28°C to produce the density of 10^7 CFU/mL (Khodria *et al*., 2011). 100 µL of bacteria inoculum were spread on Mueller Hinton agar. The filter paper discs (6 mm in diameter) were individually impregnated with 20 µL of the extracts (10 mg/mL), dried in a laminar air flow and then placed onto the agar plates previously inoculated with the tested microorganisms. The plates were incubated at 30°C for 24 h. The diameters of the inhibition zones were measured in millimetres. All the tests were performed in triplicate. Streptomycin was served as positive controls. The antibacterial activity is interpreted as follow; The diameter of inhibition zone 8 15.0 mm-strong;10,0 to 14.5mm-moderate and <10 mm-weak (Habsah *et al*., 2007).

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of the active fractions against *Vibrio* spp and *V. harveyi* was tested by using disc diffusion method as mentioned above. Two-fold dilution of butanol and aqueous extract of *Aaptos* aaptos and butanol extract of *Calyspongia pseudoreticulata* from 10 mg/mL were be used to evaluate their minimum inhibitory concentration (MIC). The final concentrations were 5, 2.5, 1.25, 0.625, 0.313, 0.156, and 0.078 mg/mL.

Toxicity testing against the brine shrimp

Toxicity (bioactive) of the active extract was monitored by the brine shrimp lethality test (Meyer *et al*., 1982). The active extracts were dissolved in sterilized seawater. Ten of *Artemia* nauplii hatched for 48 hours were exposed with butanol and aqueous extract of *Aaptos aaptos* and butanol extract of *Callypongia pseudoreticulta* at the concentration of 0 (control treatment), 1, 2, 4 and 8 x MIC in triplicate in 20 mL disposable scintillation vials. The vials were then examined and the number of dead larvae in each bottle after 24 hours was counted and recorded. The mean percentage mortality was counted by equation:

$$\text{Percentage mortality (\%)} = \frac{\text{The number of dead larvae}}{\text{The number of total larvae}} \times 100\%$$

Thin Layer Chromatography (TLC) profiling

All of the active extracts were run on normal phase TLC Silica gel 60 F_{254} Plastic sheets, Merck as described by Habsah *et al*. (2007). Mobile phase ratio used was chloroform : methanol (8 : 2). Plates were visualized under UV 366 nm and stained with dragendorf and anisaldehyde-sulfuric acid reagent.

RESULTS AND DISCUSSION

Results

The anti-vibrio activity of the extracts of sponge

The methanolic extracts of *Aaptos aaptos* and *Callyspongia pseudoreticulata* were chosen to partitioned based on their antibacterial activity (Rosmiati *et al*., 2011). Removal of salt and lipid on those methanol extract before being tested for their activity need to be done is due to they came from a saline environment. The extracts resulted in the partitioning were tested for their antibacterial activity against *Vibrio* spp and *Vibrio harveyi* with the concentration of 10 mg/mL (200 µg/disc) each. The anti-vibrio activity was shown in Table 1.

Table 1. Inhibition zones (mm) of the active fractions as measured by the disc diffusion method

Sponge species	Extract	Inhibition zone (mm)	
		***Vibrio* spp (2WT)**	***Vibrio harveyi* (1b)**
Callyspongia pseudoreticulata	Methanol	15 ± 0.1	17 ± 0.5
	Diethyl eter	-	-
	Butanol	11 ± 0.2	11 ± 0.1
	Aqueous	-	-
Aaptos aaptos	Methanol	45 ± 0.5	34 ± 0.1
	Diethyl eter	10 ± 0.3	9 ± 0.1
	Butanol	23 ± 0.1	23 ± 0.1
	Aqueous	21 ± 0.1	22 ± 0.1
Control	Streptomycine	28 ± 0.1	30 ± 0.1

(-) : No inhibition

In general, methanol extract of *Aaptos aaptos* and *Callyspongia pseudoreticulata* showed a strong antibacterial activity against *Vibrio* spp. (2WT) and *Vibrio harveyi* (1b) with the inhibition zone 15-45 mm. Partitioning on methanol extract of *Aaptos aaptos* exhibited that all extracts of this sponge were able to inhibit the growth of *Vibrio* sp (2WT) and *Vibrio harveyi* (1b). The strong antibacterial activity was shown by the butanol and aqueous extract of this sponge with the inhibition zone of 21-23 mm. Meanwhile that on *Callyspongia pseudoreticulata* only butanol extract was obtained to give an antibacterial activity towards *Vibrio* spp. (2WT) and *V. harveyi* (1b). The antibacterial activity exhibited by butanol extract of *Callyspongia pseudoreticulata* was similar to that of diethyl eter of *Aaptos aaptos*. They only were able to give a moderate anti-bacterial activity on both *Vibrio* spp. (2WT) and *Vibrio harveyi* (1b).

Determination of minimum inhibitory concentration of the active extracts of sponge

The minimum inhibitory concentration of the active sponge was determined. In this study, it was obtained the minimum inhibitor concentration of the active extracts of the two sponge used (Figure 4, 5 and 6).

From the figure 4, 5 and 6, it can be seen that the butanol and aqueous of *Aaptos aaptos* had MIC values of 0.313 and 0.625 mg/mL, whilst that for the butanol extract of *Callyspongia pseudoreticulata* was 10 mg/mL. Although all the extracts of *Aaptos aaptos* had anti-*Vibrio* activities, the butanol and aqueous

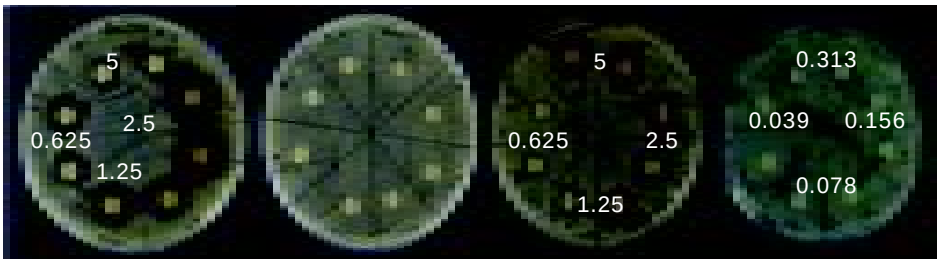
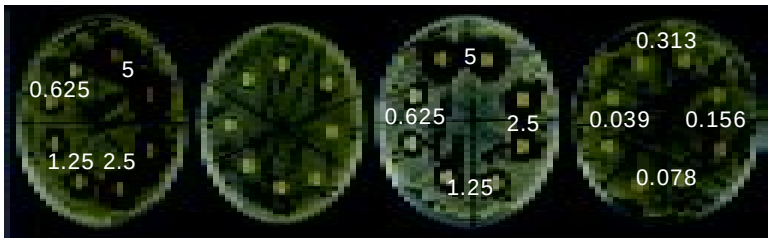
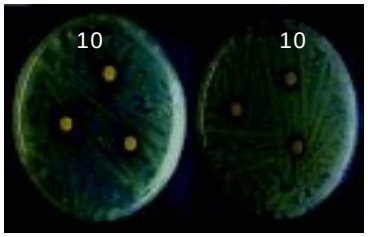


Figure 4. Minimum inhibitory concentration of butanol extract of *Aaptos aaptos* against *Vibrio harveyi* (1b) and *Vibrio* sp (2WT)



1b 2WT

Figure 5. Minimum inhibitory concentration of aqueous extract of *Aaptos aaptos* against *Vibrio harveyi* (1b) and *Vibrio* sp. (2WT)



1b 2WT

Figure 6. Minimum inhibitory concentration of butanol extract of *Callyspongia pseudoreticulata* against *Vibrio harveyi* (1b) and *Vibrio* sp (2WT)

fraction was more active based on their MIC value. The MIC value showed by butanol extract of *Callyspongia pseudoreticulata* was the similar to diethyl eter extract of *Aaptos aaptos*. This study has shown that the active extracts of the two sponges have antagonistic activities against *Vibrio* species, although the methanol extract appears to be more active.

Toxicity assay using the brine shrimp lethality test (BST)

The brine shrimp lethality test was conducted on butanol and aqueous extract of *Aaptos aaptos* and butanol extract of *Callyspongia pseudoreticulata*. The toxicity of the three extracts indicated by the percentage of mortality of Artemia larvae was shown in Table 2.

In this study, butanol and aqueous extract of *Aaptos aaptos* did not show any mortality up to the concentration of 2.5 mg/mL (8MIC) and 5 mg/mL (8MIC), respectively. Meanwhile, butanol extract of *Callyspongia pseudoreticulata* was obtained that there was 30% mortality of larvae at the concentration of 20 mg/mL (2MIC).

Thin Layer Chromatography (TLC) profiling of the active extracts of sponge

The TLC profiling of the compounds was shown by Figure 7. The TLC were visualized using UV366 nm, dragendorf and anisaldehyde-sulfuric acid reagent.

The TLC profiling (Figure 8) showed the presence of alkaloids and other nitrogen containing compounds in the active butanol extract of *Aaptos aaptos* indicated by orange spots. In contrast there was no those compounds detected in diethyl eter extact of *Aaptos aaptos* and butanol extract of *Callyspongia pseudoreticulata*. Nevertheless, the existence of other compounds can be detected by UV 366 nm and anisaldehide reagent in both diethyl eter extract of *Aaptos aaptos* and butanol extract of *C. pseudoreticulata*.

Discussion

This study suggested that sponge *Aaptos aaptos* has a potential to be used as a source of anti-vibrio compound for vibriosis in mariculture. It was supported by the strong antibacterial activity shown by the active extracts

Table 2. Percentage of mortality of *Artemia salina* exposed with butanol and aqueous extract of *Aaptos aaptos* and butanol extract of *Callyspongia pseudoreticulata* after 24 hours

Extract	Concentration (mg/mL)	Percentage of mortality (%)
Control	0 (0MIC)	0
BUOH	0.313 (1MIC)	0
	0.626 (2MIC)	0
	1.252 (4MIC)	0
	2.504 (8MIC)	0
Control	0 (0MIC)	0
HOEI	0.625 (1MIC)	0
	1.250 (2MIC)	0
	2.500 (4MIC)	0
	5.000 (8MIC)	0
Control	0 (0MIC)	0
BUEL	10.000 (1MIC)	0
	20.000 (2MIC)	30 + 0.20
	40.000 (4MIC)	100 + 0.0
	80.000 (8MIC)	100 + 0.0

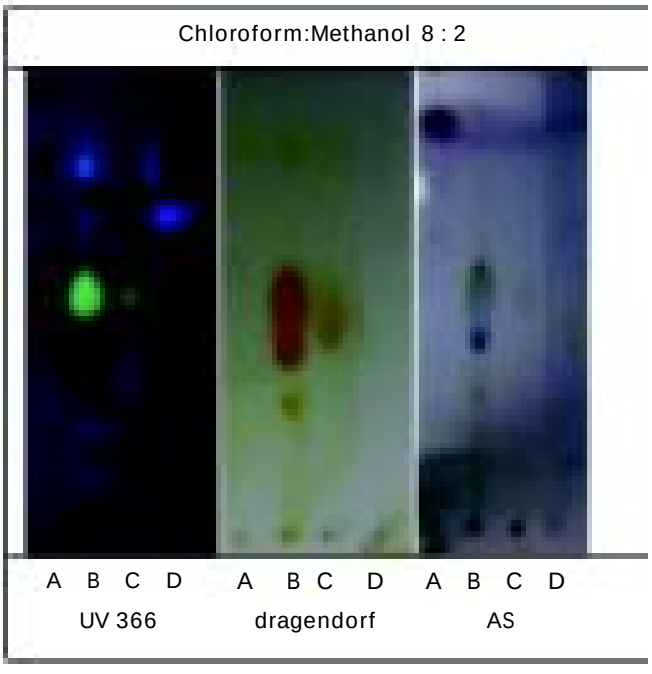


Figure 7. TLC profiling of diethyl eter (A), butanol (B) and aqueous (C) extract of *Aaptos aaptos* and butanol extract (D) of *Callyspongia pseudoreticulata* visualized by UV 366, dragendorf and Anisaldehide-sulfuric acid (AS)

of sponge with the low minimum inhibitory concentration (below 1.6 mg/mL). According to Aligiannis *et al.* (2001), MICs above 1.6 mg/ml indicate weak activity. In addition the active extract of this sponge did not show any toxic on *Artemia salina* larvae up to 8 x MIC (0.313 mg/mL for butanol extract and 0.625 mg/mL for aqueous extract). Moshi *et al*., 2010 reported that extract or compound having LC_{50} > 100 µg/mL is nontoxic.

Another sponge, *Callyspongia pseudoreticulata* is a promising candidate to be developed as a raw material source for vibriosis prevention. This may be due to its antagonistic activity against both *Vibrio* spp (2WT) and *V.harveyi* (1b).

The anti-vibrio activity shown by butanol and aqueous extract of *Aaptos aaptos* was suspected due to the presence of alkaloid compounds and other nitrogen containing compounds. According to previous study, marine sponge of the genus Aaptos produces aaptamine which is a α-adrenoceptor blocking compound and is higly toxic to fish. This compound also exhibited an antibacterial activity against Gram-positive and Gram-negative bacteria (Utkina *et al*., 2009). Nevertheless, aaptamine is not the only one compound which gives the contribution on the antibacterial activity against the target bacteria. The similar case is also obtained in *Callyspongia pseudoreticulata*. This sponge was reported to yield (3S,18S,4E,16E)-eicosa-l,19-diyne-3,18-diol-4,16-diene which was found to be toxic in the brine shrimp assay (Braekmam *et al*., 2003; Sonia *et al*., 2009). In this study, extract of butanol from *Callyspongia pseudoreticulata* was found to be not toxic to brine shrimp. The strong antibacterial and low/no toxicity to brine shrimp actually of the extracts are good starting points for further research that can lead to the isolation, purification and characterization of active compounds for new anti-*vibrio* drug development purposes.

CONCLUSSION

Indonesian *Aaptos aaptos* and *Callyspongia pseudoreticulata* have a potential to be developed for new bioactive compounds for disease prevention in mariculture particularly to vibriosis. It is due to their strong anti-vibrio activity showed by the low minimum inhibitory concentration of 0.313 and 0.625 mg/mL. Furthermore it is nontoxic against *Artemia salina* with the LC_{50}>30 mg/mL.

ACKNOWLEDGEMENT

The authors thanked the Government of South Sulawesi Province, Indonesia for providing the scholarship and the Ministry of Science, Technology and Innovation (MOSTI), Malaysia for funding research through a Grant no: 53051 and 59161.

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