MARINE SPONGE *Aaptos suberitoides*, IT'S POTENTIAL SOURCE OF NATURAL ANTIBACTERIAL FOR CONTROLLING *Vibrio harveyi* ON TIGER SHRIMP (*Penaeus monodon*) CULTURE

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ABSTRACT

The study aims to isolate and identify the natural antibacterial compounds potential from *Aaptos suberitoides* for *Vibrio harveyi* control on tiger shrimp (*Penaeus monodon*) culture. The agar diffusion method using paper discs was used to determine the antibacterial activity of extracts (diethyl ether (DEE), butanol (BUE) and aqueous (HOE) and compounds successfully isolated against *Vibrio harveyi*. Findings showed that the antibacterial activity was concentrated in BUE with the inhibition zone of 17.2±0.1 mm. Meanwhile, two other extracts (DEE and HOE) did not exhibit any antibacterial activity against *V. harveyi*. From the active BUE, it was successfully isolated two compounds giving a strong anti-vibrio activity with the inhibition zone of 22±0.1 mm. The IR, ^{1}H, ^{13}C, COSY, HMQC, HMBC, and MS spectrum analysis indicated that both active compounds identified as aaptamine (1) and 9-demethyloxyaaptamine (2). The study suggested that marine sponge *A. suberitoides* may have potential compounds source for controlling of *V. harveyi* on tiger shrimp culture.

KEYWORDS: ***Aaptos suberitoides*, aaptamine, 9-demethylaaptamine, *Vibrio harveyi*, *Penaeus monodon***

INTRODUCTION

Tiger shrimp *Penaeus monodon* culture gives a big contribution to the foreign exchange revenue in Indonesia. Furthermore, tiger shrimp cultivation also got a high attention due to its unique taste, high nutrition, and persistent demand in the world market (Shailender *et al*., 2013). Nevertheless, the tiger shrimp farm still facing problem on diseases that caused low productivity. Vibriosis is one of bacterial diseases causing the declining of shrimp production (Sarjito *et al*., 2012).

To control the growth of *Vibrio harveyi* to be not reach quorum, farmers have been routinely used use antibiotics such as chloramphenicol and oxytetracycline. However, widespread antibiotics applications could result in antibiotic resistant pathogens (Karunasagar *et al*., 1994; Tendencia & de la Pena, 2001), the presence of antibiotic-resistant bacteria (Sengupta *et al*., 2003), antibiotics residue which may accumulate in the shrimp, and concerns of antibiotic resistance to human pathogens (Salyers, 1995). Since antibiotics application resulted in the side effects, the use of antibiotics have been no more recommended or prohibited.

In the search of anti-vibrio from sponge, it was obtained that methanol extract of marine sponge *Aaptos suberitoides* (Figure 1) inhibited the growth of *Vibrio harveyi* in vitro (Rosmiati *et al*., 2011). Nevertheless, bioactive compounds of this sponge as bactericide have not been identified. Therefore, in the present paper, it was reported potential, isolation, and identification of bioactive compounds from *A. suberitoides* for controlling of *Vibrio harveyi*. Bioactive

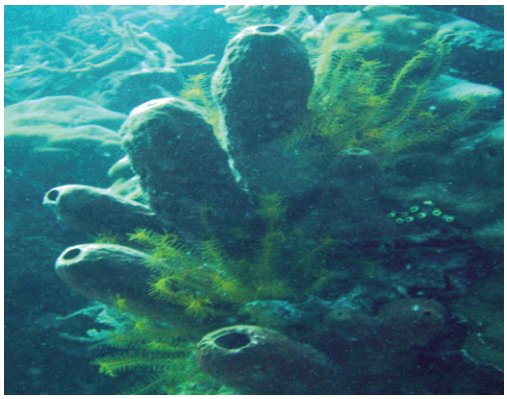

Figure 1. *Aaptos suberitoides*

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compounds isolated from *Aaptos suberitoides* hopely can be used to control bacterial diseases such as *V. harveyi*.

MATERIALS AND METHODS

Animal Material

Marine sponge *Aaptos suberitoides* were collected in August 2010 from Barrang Lompo Island Waters, South Sulawesi by SCUBA diving at a depth of 10 m and identified by Ridwan (Station Marine of Hasanuddin University). Sponge was placed in a cool box added with ice pack and brought to Biotechnology Laboratory of Research and Development Institute for Coastal Aquaculture (RICA), Maros. The sample were kept in the sealed plastic bags then preserved in a freezer at -20°C and *Vibrio harveyi* (MRF 275) collection of RICA was use as a bio-indicator.

Extraction and Removing of Salt and Wax/Lipid

Sample of fresh sponge (1 kg) was air-dried to become 250 g, then directly extracted with methanol at room temperature using forma orbital shaker with air temperature of 37°C for three days. Extract was then filtered, combined and concentrated under reduced pressure to obtaine 15 g of methanolic crude extract. The salt and wax/lipid extract was removed by using HP-20 and C-18, respectively with the method as described by Rosmiati *et al*. (2011) in order to get interference material-free methanol crude extract (MCE).

Liquid-Liquid Extraction (Solvent Partitioning)

Liquid-liquid extraction was used for further separation the active compound that accumulates in the two phases of different solvents polarity. Dry MCE was reconstitued in diethyl ether, transferred into a separation funnel and added up with distilled water in 2:1 ratio of diethyl ether to water respectively. The separation funnel was then shaken up vigorously before the active compounds were allowed to settle down according to its polarity. The partitioning process was repeated for at least three times or until the diethyl ether phase turned to colorless. In order to obtain the polar compound from the separation, n-butanol was added to the separation funnel in exchange of diethyl ether and was shook vigorously together with the distilled water used in non-polar extraction beforehand. The three extracts (diethyl ether (DEE), n-butanol (BUE), and aqueous (HOE)) were dried completely using rotary evaporator and freeze drier. Bioassay-guided fractionation of these three extracts retained a maximum of activity in the BUE extract.

Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) was used to profile fractions resulted from dry vacuum column chromatography (DVCC) and column chromatography (CC) before being combined. TLC was also performed to determine the best solvent system used for the separation of the active compounds with column chromatography (CC). TLC was performed by spotting one drop of samples on a precoated plates with Si-gel F_{254} (layer thickness 0.2 mm, Merck, Darmstadt, Germany) as stationary phase as described by Habsah *et al*. (2007). Liquid mobile phases were semipolar (CH_2Cl_2 : MeOH; 9:1, 5:1, v/v), (CH_2Cl_2 : Aceton; 1:1 v/v), and ($CHCl_3$: MeOH; 9:1, 8:2, 7:3, v/v). A one-dimensional ascending development technique was used to detect the constituents of an extract on TLC plate. Visual detection was done in daylight and under UV light at a wave length of 254 and 344 nm depending on the group of compounds investigated. The compounds were also detected by spraying with dragendorff and anisaldehyde-sulfuric acid reagent.

Fractionation

The active BUE was continued to fractionate its active compounds by using a Dry Vacuum Column Chromatography (DVCC) method. Approximately 10.0 g of this BUE extract was fractionated by subjecting to a dry vacuum column chromatography of silica gel and gradual eluting with n-Hexane, n-Hexane /DCM, DCM, DCM/MeOH, and MeOH. A total of 17 fractions of 300 mL each were collected and coded A-G. Bioassay-guided fractionation was obtained fraction of E and F active on *Vibrio harveyi*. These two fractions were combined and coded as (EF) and further separated its compounds.

Isolation and Purification

Approximately 1,000 mg of the active EF was subjected to Sephadex-LH20® column chromatography and eluted with Chloroform : methanol with the ratio of 1:1 to give three fractions. About 10 mg of the second fraction was further separated using reversed-phase HPLC (CSC-Inertsil® ODS2 column, 2 mL/min. flow rate, dual wave length 210 nm and 254 nm UV detection) eluted with an isocratic mixture of 20% MeCN-80% H_2O (1% TFA) as a solvent system. Two major peaks were observed at 15 min. and 19 min., corresponding to aaptamine (1) and 9-demethyl-aaptamine (2), respectively. The HPLC fractions were air dried in the fume hood prior to IR, NMR, and MS analysis. Both compounds isolated were assayed their antibacterial activity against *V. harveyi*.

Determination of Compounds Structure

Pure isolates successfully separated from the HPLC were identified their structure by using IR, 1D NMR (^{1}H NMR, ^{13}C NMR, and DEPT), and 2D NMR (HMQC, HMBC, dan COSY) as well as MS. IR was used to determine the functional group of compound. Spectra were recorded with Perkin Elmer FTIR (model 1725X) spectrophotometer using KBr discs. The absorption bands were measured in cm^{-1}. MS was used to determine molecular weight and formula of the compounds. Mass spectra were recorded by Direct Induction Probe (DIP) using a Shimadzu GCMS-QP5050 spectrometer with ionization induced by electron impact at 70 eV. ^{1}H- and ^{13}C-Nuclear Magnetic Resonance Spectroscopy (NMR) data was used to determine number of proton and thirteen-carbon, respectively, COSY data was used to determine proton spin systems, HSQC data was used to determine proton-carbon attachments whilst, HMBC data was used to provide information about long range proton-carbon connectivity and the assignments of any quaternary carbon resonances. Nuclear Magnetic Resonance (^{1}H- NMR) spectra were recorded on Bruker ARX 400 and DMX 600 NMR spectrometer with tetramethylsilane (TMS) as internal standard. Chemical shifts are quoted in d units and the signals were described in terms of chemical shift, multiplicity, coupling constant (*J*) where applicable and number of protons. The abreviations *s* (singlet), *d* (doublet), *t* (triplet), *q* (quartet), *m* (multiplet), *dd* (doublet of doublets), *ddd* (doublet of doublets of doublets), *dddd* (doublet of doublets of doublets of doublets), *br s* (broad of singlet) have been used to express multiplicities. The solvent used was deuterated methanol (MeOD).

Antibacterial Assay

Culture Preparation

Prior to testing, one loopful of *Vibrio harveyi* and *Vibrio* sp. from TSA stock was streaped zig-zag on TCBSA medium and incubated without agitation at 28°C for 24 hours. Few growth colonies (3-4) of the microorganisms were sub-cultured in 9 mL of nutrient broth (NB) medium (Sigma, FRG) to achieve density of 10^7 colony forming units (cfu/mL).

Antibacterial Activity Assay

A hundred µL of bacteria sub culture were spread on Mueller Hinton agar medium. The filter paper discs (6 mm in diameter) were individually impregnated with 20 µL of the extracts (DEE, BUE, and HOE) and compounds (1 and 2) with the concentration of 10 and 1 mg/mL, respectively, dried in a laminar air flow and then placed onto the agar plates previously inoculated with the tested microorganisms. The plates were incubated at 28°C for 24 h. The diameter of the inhibition zones indicated by the clear zone around paper discs was measured in millimetres. All the tests were performed in triplicate. Streptomycine was served as positive control.

RESULTS AND DISCUSSIONS

In vitro antibacterial activity of diethyl ether (DEE), butanol (BUE), and aqueous (HOE) extracts of marine sponge *Aaptos suberitoides* at the concentration of 10 mg/mL was assayed against *Vibrio harveyi*. The antibacterial activity of these three extracts indicated by clear inhibition zone surrounding the discs is shown in Table 1.

From the Table 1, it can be seen that butanol extract of *Aaptos suberitoides* was able to strongly inhibit the growth of *V. harveyi* with the inhibition zone of 17.20±0.10 mm. Meanwhile, two other (diethyl ether and aqueous) extracts did not display any antibacterial activity against *V. harveyi*. It means that bioactive materials of *A. suberitoides* are semi-polar organic substances. The antibacterial activity shown by its butanol extract was higher than that displayed by its methanol extract giving an inhibition zone of 13.00±0.30 in the preliminary study (Rosmiati *et al*., 2011). It was indicated by bioactive substances of *A. suberitoides* giving the antagonistic effect in inhibiting the growth of *V. harveyi*. Compared to the antibacterial activity of streptomycine as a positive control in this study, the antibacterial displayed by the

Table 1. The antibacterial activity of diethyl ether, butanol, and aqueous extract against *V. harveyi* indicated by their inhibition zone

Extract	Diameter of inhibition zone (mm)	Standard deviation (± mm)
Diethyl ether	-	-
Butanol	17.20	0.10
Aqueous	-	-
Streptomycin	30.00	0.35

Note: (-) No inhibition zone

active butanol extract of *A. suberitoides* was lower. The result suggested that the method used to determine the antibacterial level of sponge extracts against *V. harveyi* was reliable. The antibacterial activity of *A. suberitoides* toward *V. harveyi* has been reported for the first time in this study. Previously, it was obtained to give the cytotoxicity effect against HeLa cells and proteasome inhibitor (Tsukamoto *et al*., 2005).

Further separation on the active butanol extract of *A. suberitoides* by fractionation method using dry vacuum column chromatography (DVCC) resulted in seven fractions combined based on the similarity of their spots. The antibacterial test carried out on the all fractions against *Vibrio harveyi* and their anti-vibrio activity was shown in Table 2.

Table 2 indicated that two fractions (E and F) out of seven fractions of *A. suberitoides* butanol (BUE) extract gave the antibacterial activity on *V. harveyi* with the inhibition zone of 18-19 mm. It indicated that the bioactive compounds were presented by the two active fractions (E and F). The thin layer chromatography (TLC) profiling of both active fractions is presented in Figure 2.

The TLC profiling of the active fractions (E and F) (Figure 2) shows the presence of alkaloid compounds indicated by orange spots after being sprayed with dragendorf reagent (specific reagent to detect the existence of alkaloid compounds) (LAdlera & Wink, 2001) with the best system solvent of dichloromethane and methanol with the ratio of 8:2.

Further isolation on the combination of fraction E and F using sephadex LH-20 column chromatography gave three subfractions (EF1, EF2, and EF3). The antibacterial activity assay carried out on the three fractions obtained exhibited only subfraction EF2 which was active to inhibit the growth of *V. harveyi* with the inhibition zone of 20 mm as shown in Table

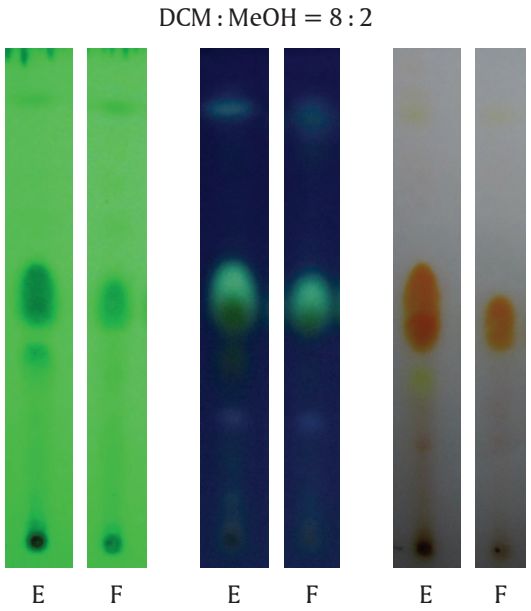


Figure 2. TLC profiling of the active fraction of E and F visualized with UV_{254}, UV_{366}, and dragendorf

3. Purification of the subfraction EF2 using HPLC let to the isolation of two compounds namely compound (1) and compound (2). Both compounds produced the strong antibacterial activity against *V. harveyi* with the inhibition zone of 21-23 mm as shown in Table 3 and Figure 3.

The active compound (1) was isolated as bright yellow crystal, 110 mg, R_f = 0.28 (DCM/MeOH 8:2). It showed an orange spot after being sprayed with dragendorf reagent indicating an alkaloid compound (Figure 6). The IR spectrum (Figure 4) showed the existence of N-H stretching at 3420 cm^{-1} and sp^3 C-H stretching indicated by the absorption at 2949 and 2867 cm^{-1}. In addition, the presence of carbon-carbon double bond, C-O stretching, and bending vibration of aromatic group was assigned to the peak at 1626 cm^{-1}, 1248 cm^{-1}, and range 966-776 cm^{-1}, respec-

Table 2. The antibacterial activity of fractions isolated from the *A. suberitoides* active butanol extract

Fraction	Diameter of inhibition zone (mm)	Standard deviation (± mm)
A	-	-
B	-	-
C	-	-
D	-	-
E	18	0.38
F	19	0.35
G	-	-
Streptomycin	30	0.35

Table 3. The antibacterial activity of subfractions of the active fraction EF and compounds isolated against *V. harveyi* indicated by the inhibition zone

Subfraction/ compound	Diameter of inhibition zone (mm)	Standard deviation (± mm)
Subfraction		
- EF1	-	-
- EF2	20	0.11
- EF3	-	-
Compound		
- **1**	21	0.25
- **2**	23	0.28
Streptomycin	30	0.35

Note: (-) No inhibition zone

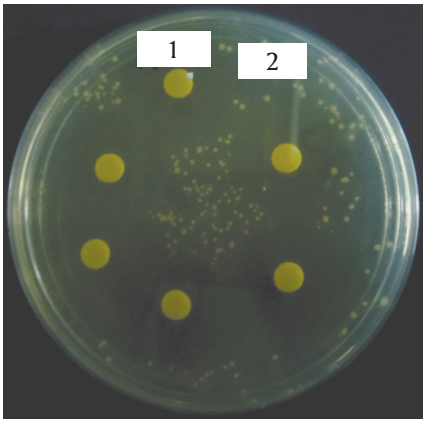
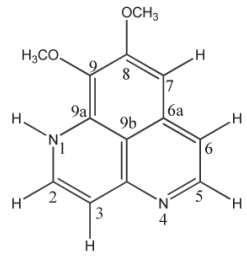


Figure 3. The antibacterial activity of compound (1) and (2) against *V. harveyi*

tively. The EIMS spectrum (Figure 5) indicated the presence of molecular ion peak at *m/z* 228, which is consistent with the molecular formula of $C_{13}H_{12}N_2O_2$. Other fragment ions were observed at *m/z* 213 $[M\text{-}CH_3]^+$, 183 $[M\text{-}CH_3\text{-}OCH_3]^+$, and 167 $[C_{11}H_7N_2]^+$. The ^{1}H NMR spectral data of compound (1) showed eleven protons observed as two sets of doublet and three singlet signals. Five protons consisted of two sets of doublet were observed at δ_H 7.78 (H-2) and 6.35 (H-3) and 7.27 (H-5) and 6.88 (H-6) and a singlet at δ_H 7.07 (H-7) in the aromatic region and six protons occurred two singlet signals were observed at δ_H 4.06 (8-OCH_3) and 3.95 (9-OCH_3) in up field region. In addition, one proton belongs to NH could not be detected due to exchangeable with proton MeOD as

(1)



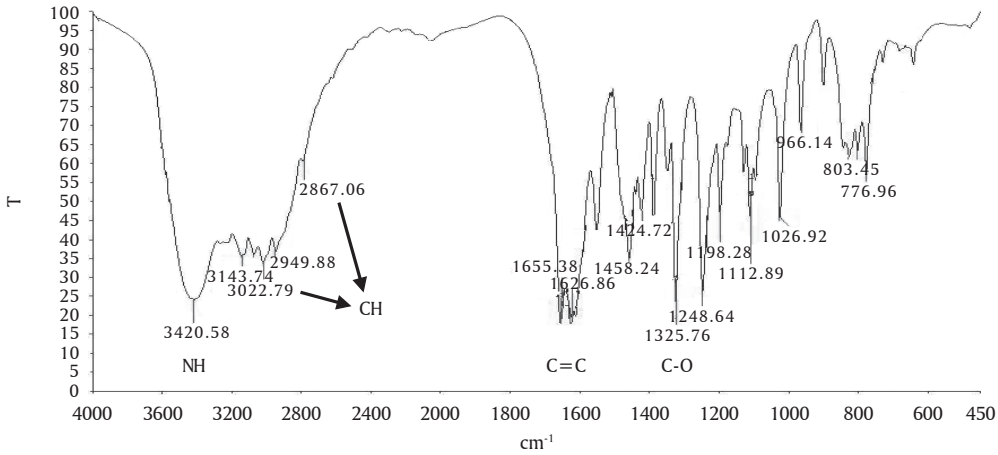


Figure 4. The IR spectrum of compound (1)

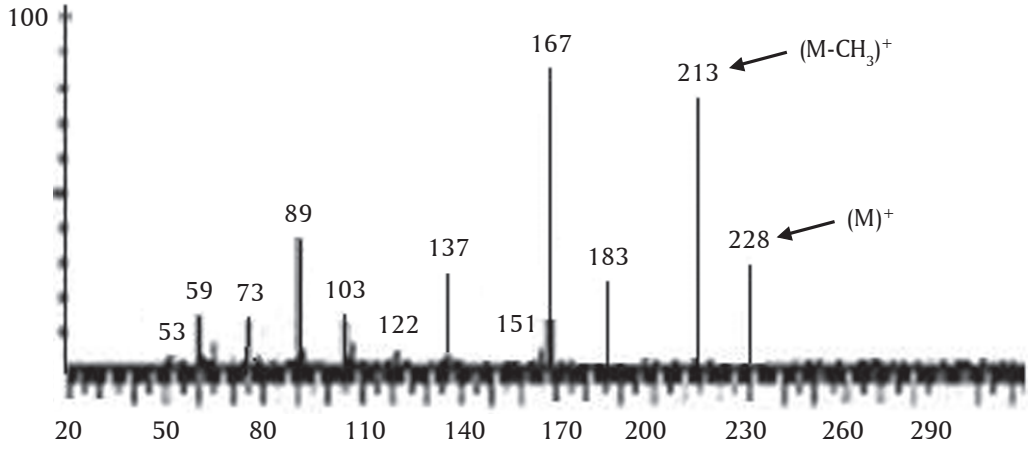


Figure 5. The EIMS spectrum of compound (1)

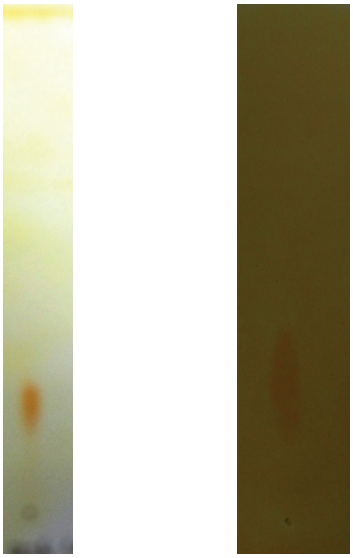
the solvent. The ^{13}C NMR spectral data of compound (1) showed the presence of 13 carbon atoms observed at δ_C 55.6 (8-OCH_3), 59.7 (9-OCH_3), 97.7 (C-3), 100.8 (C-7), 113.0 (C-6), 116.7 (C-9b), 129.1 (C-5), 132.0 (C-9), 132.9 (C-6a), 133.9 (C-9a), 141.1 (C-2), 150.4 (C-3a), and 157.5 (C-8). The HMBC spectral data exhibited the existence of the long C-H correlation between H-2 and C-3, C-9a and C-3a, H-3 and C-9b and C-2, H-5 and C-6, C-6a and C-3a, H-6 and C-7, C-9b and C-5, H-7 and C-6, C-9b, C-9, and C-8, 8-OCH_3 and C-8, and 9-OCH_3 and C-9, respectively. Meanwhile, the HMQC spectral data allowed the assignments of all protonated carbons at C-2, C-3, C-5, C-6, C-7, 8-OCH_3, and 9-OCH_3. The existence of ^1H-^1H correlation between H-2 and H-3 and H-3 and H-2 as well as H-5 and H-6 and H-6 and H-5 further supported that these protons were neighbouring protons. All 1D and 2D NMR spectral data were summarized in Table 4. Detail examination of spectroscopic data (IR, MS, 1D, and 2D NMR) of compound (1) readily established indentity with aaptamine (1) previously described from the Indonesian *Xestospongia* sp. (Calcul *et al*., 2003) and *Aaptos aaptos* (Rosmiati, 2013).

Compound (2) was isolated as yellow powder, 15.0 mg, R_f = 0.26 (ACN/MeOH, 2:8, C_{18} TLC Plate). Spraying with Dragendorff reagent showed an orange spot indicating that this compound was from alkaloid (Figure 6). The IR spectral data of this compound exhibited the existence of the overlapping medium absorption at 3280 and 3100 cm^{-1} indicating the presence of N-H and O-H stretching. The weak absorption observed at 2948 cm^{-1} showed the occurrence of C-H stretching. Moreover, the absorption at 1661, 1322, and 1194 cm^{-1} indicated the presence of C=C aromatic double bond, C-O stretching, and C-N stretching in the molecule, respectively. The EIMS spectral data showed the presence of molecular ion peak at *m/z* 214 which suggested the molecular formula of

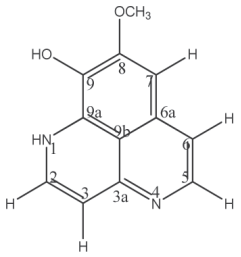
Table 4. 1D and 2D NMR spectral data for compound (1)

Position C/H	δ^1H (m, *J* in Hz) 400 MHz in MeOD	δ^{13}C	HMBC	HMQC	COSY
2	7.78 (*d*, 7.2)	141.1	3, 9a, 3a	2	3
3	6.35 (*d*, 7.2)	97.7	9b, 2	3	2
3a	-	150.4			
5	7.27 (*d*, 7.6)	129.1	6, 6a, 3a	5	6
6	6.88 (*d*, 7.6)	113.0	7, 9b, 5	6	5
6a	-	132.9			
7	7.07 (*s*)	100.8	6, 9b, 9, 8	7	
8	-	157.5		8-OCH_3	
9	-	132.0		9-OCH_3	
9a	-	133.9			
9b	-	116.7			
8-OCH_3	4.06 (*s*)	55.6	8		
9-OCH_3	3.95 (*s*)	59.7	9		
1-NH					

DCM : MeOH = 8 : 2 ACN : MeOH = 2 : 8



1 Dragendorff 2 Dragendorff



(2)

Figure 6. TLC profiling of compound (1) and (2)

Table 5. 1D and 2D NMR spectral data for compound (2)

Position C/H	δ^1H (m, *J* in Hz) 400 MHz in MeOD	δ^{13}C	HMBC	HMQC	COSY
1-NH					
2	7.71 (*d*, 6.8)	141.3	3, 9a, 3a	2	3
3	6.23 (*d*, 6.8)	96.8	9b, 2	3	2
3a		150.6			
5	7.11 (*d*, 7.2)	126.9	6, 6a, 3a	5	6
6	6.82 (*d*, 7.2)	113.3	7, 9b, 5	6	5
6a		130.4			
7	7.06 (*s*)	100.3	6, 9b, 9, 8	7	
8		128.7		8-OCH_3	
9		152.3		9-OCH_3	
9a		128.5			
9b		117.0			
8-OCH_3	4.06 (*s*)	55.7	8		
9-OH			9		

$C_{12}H_{10}N_2O_2$ and *m/z* 183 due to the loss of OCH_3 group in the molecule. The ^{1}H-NMR spectrum of compound (2) only showed one methoxy (OCH_3) group in the up field region observed as a singlet peak at δ_H 4.06. Meanwhile, five resonance in the aromatic region consisted of two sets of doublet observed at 7.71 (H-2) and 6.23 (H-3) and 7.11 (H-5) and 6.82 (H-6) and a singlet observed at 7.06 indicated the presence of a 1,6-napthpyridine (aaptaminoid) skeleton (Walz & Sundberg; Sugino *et al*., 1999; Into, 1998). The ^{13}C-NMR spectral data (Table 5) exhibited 12 carbon atoms. The COSY, HMQC, and HMBC spectral data were similar to compound (1). The all NMR spectral data are summarized in Table 5. Based on all spectral data, compound (2) very agreed to 9-demethylaaptamine. This compound was previously isolated from the Okinawan *Aaptos aaptos* (Nakamura *et al*., 1987) and Indonesian *Aaptos suberitoides* (Pedpradab, 2005).

CONCLUSIONS

Aaptos suberitoides is the potential source of natural antibacterial for controlling of *Vibrio harveyi* in fishery culture as indicated by its the active methanol and butanol extract against *V. harveyi* with the inhibition zone of 13.00 and 17.20 mm, respectively. Further isolation and purification of the active butanol

extract of this sponge let to the identification of two bioactive compounds, aaptamine (1) and 9-demethyl-aaptamine (2).

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