Study on the potential of *Aaptos aaptos* aqueous extract as an antibacterial agent against *Vibrio harveyi* in tiger shrimp (*Penaeus monodon* Fabricius) post larvae

Rosmiati and Muliani
Research Institute for Coastal Aquaculture and Fisheries Extension, Maros, Indonesia

Email: emirosmiati@yahoo.co

Abstract. The sponge *Aaptos aaptos* is known to contain bioactive compounds with bactericidal properties. These compounds could be utilized in shrimp aquaculture to inhibit the growth of *Vibrio harveyi*, the causative agent of vibriosis. This study aimed to evaluate the antibacterial activity of an aqueous extract of *Aaptos aaptos* on *V. harveyi* isolated from diseased shrimp post larvae and identify bioactive compounds active against *V. harveyi*. An aqueous extract was obtained through solvent partitioning of active *Aaptos aaptos* extract using methanol with n-hexane and butanol. The bioactive compound was isolated from the aqueous extract through repeated column chromatography with silica gel as stationary phase and a mixture of dichloromethane and methanol as mobile phase. The antibacterial activity of the aqueous extract was tested using a 96 microwell plate with extract concentrations of 10,000, 1,000, 100, 10, 1, and 0 mg/L. An *in vitro* aqueous extract antibacterial activity challenge test against *V. harveyi* was conducted using reaction tubes with extract concentrations of 5,000, 500, 50, 5 and 0 mg/L. Antibacterial activity of isolated bioactive compounds was determined through an agar diffusion assay using paper discs with a concentration of 20 µg/disc. Results showed that the *Aaptos aaptos* aqueous extract displayed strong antibacterial activity with a minimum inhibitory concentration (MIC) of 10 mg/L. *V. harveyi* colonies treated with aqueous extract at concentrations of 500 and 5,000 mg/L had decreased drastically (5.50 x 10^3 and 0.83 x 10^2 CFU/mL respectively) 4 hours post treatment. The orange spot exhibited when sprayed with Dragendorff reagent indicated that one of the compounds successfully isolated belonged to the alkaloid group, with an Rf value of 0.2 (acetonitrile/methanol, 2:8, C18 TLC Plate). This bioactive compound was one of those responsible for the *Aaptos aaptos* aqueous extract activity, with an inhibition zone of 22 ± 0.1 mm.

1. Introduction
Luminescent vibriosis is a disease caused by *Vibrio* species. This disease has been the main causative agent of mass mortality in shrimp culture [1,2,3]. Luminescent vibriosis can attack all stages of shrimp reared from seed in the hatchery until grow out in the pond. Mortality of *Penaeus monodon* larvae associated with luminescence has been observed in hatcheries in Indonesia [4], Thailand [5] and the Philippine [6].

Prevention and control efforts with respect to *V. harveyi* have been carried out by maintaining adequate water quality with low bacterial biomass, sterilizing or filtering re-circulated water, together with routine monitoring of shrimp and ponds for early diagnosis of problems [7]. The use of chemicals (e.g. formalin and malachite green) and antibiotics (e.g. chloramphenicol and oxytetracyclin) have been commonly use in attempts to solve this disease problem [8]. However, these treatments have several side effects such as the accumulation of residues in water causing a decrease in shrimp quality; they can be dangerous to consumers [9] as well as decreasing the quality of the aquatic environment. Therefore, the other alternatives to solve this problem need to be sought.
One of approach which can be applied is the use of natural bioactive compounds with a broad spectrum without dangerous side effects. Several species of sponge such as Geodia sp. [10], Denrillanigra [11], Gracilaria verrucosa [12], and Aaptos aaptos [13] are reported to contain bioactive compounds which are able to inhibit the growth of V. harveyi. Previously, the antibacterial activity of Aaptos aaptos butanol extract against V. harveyi was investigated. This research aims to determine the antibacterial activity of Aaptos aaptos aqueous extract against V. harveyi isolated from diseased shrimp and its potential for the prevention of vibriosis on shrimp, as well as to determine the bioactive compounds responsible on the antibacterial activity of this sponge and the effective concentration for inhibiting of the growth of V. harveyi.

2. Method

2.1. Preparation of Aqueous Extract of Aaptos aaptos

Aqueous extract preparation began with maceration of the sponge using methanol as a solvent to obtain a methanol extract at room temperature. Extraction with methanol was repeated for 3 x 24 hours or until a colourless filtrate was produced. The extracts were then combined and filtered through a Whatmann filter paper no. 42 using a Buchner glass. The methanol extract filtrate obtained was evaporated using a rotary evaporator to obtain dried methanol extract. Water was added to the dried methanol extract which was then partitioned with n-hexane and butanol in a separation funnel to obtain n-hexane, butanol and aqueous extracts.

2.2. Antibacterial Test

Prior to the antibacterial test, the n-hexane, butanol and aqueous extracts were loaded onto HP-20 and C18 columns to remove their salt and fat content. This was necessary as the samples were collected from sea water. The extracts were tested for their antibacterial activity against Vibrio harveyi using a 96 microwell plate at extract concentrations of 10,000 mg/L in 10 % DMSO. The bioassay test was carried out after preparing Vibrio harveyi at a density of 10^6 CFU/mL. Approximately 40 µL of each extract was entered to each well, filled with 40 µL Nutrient Broth (NB) medium, and homogenized. Finally, 20 µL of Vibrio harveyi bacterial suspension was added to each well to obtain a final volume of 100 µL. The microwell plates were then incubated at a temperature of 28°C for 24 hours, after which 10 µL of cell growth indicator (MTT) was added. A colour change to blue/purple indicated the presence of bacterial growth, and thus indicated the absence of antibacterial activity of the extracts tested.

2.3. Determination of minimum inhibitory concentration (MIC).

The minimum inhibitory concentration (MIC) of the aqueous extract was determined based on the results of bioassay tests against Vibrio harveyi. The MIC test was carried out by preparing solutions of the aqueous extract at the concentrations of 1,000, 100, 10, 1, and 0 mg/L in 10 % DMSO. The antibacterial activity of each aqueous extract concentration was evaluated using the antibacterial test method described above.

2.4. Determination of the effective aqueous extract concentration in inhibiting of V. harveyi growth

Determination of the effective aqueous extract concentration for inhibiting V. harveyi was carried out using 10 mL reaction tubes. The active aqueous extract was prepared at concentrations of 5,000, 500, 50, 5 and 0 mg/L. One mL of diluted extract was separately mixed in the tube with 9 mL of V. harveyi at a density of 10^5 CFU/mL and incubated by using a shaker at a temperature of 28°C, 150 rpm/minute. The V. harveyi population was counted at 4, 8, 12,16,20, and 24 hours by taking 100 µL of bacterial suspension from each concentration and growing it on a TCBSA medium. After incubation at 28°C for 24 hours the V. harveyi present were counted.

2.5. Antibacterial activity challenge test of the active aqueous extract in vitro

2.5.1. Preparation of medium and Vibrio harveyi. To carry out the challenge test in vitro, Vibrio harveyi was sub-cultured in 10 mL Nutrient Broth medium in an Erlenmeyer glass. TCBSA medium in petri dishes was used to observe V. harveyi growth resulting from the challenge test. One hundred µL
of sub-cultured *V. harveyi* was cultured in 10 mL nutrient broth (NB) and incubated at 28°C on an orbital shaker (forma type) at 150 rpm for 24 hours. After 24 hours incubation, one mL of starter culture was transferred to 100 mL NB and incubated on the shaker at 150 rpm, 28°C for 4 hours to obtain a bacterial density of 10^8 CFU/mL.

**2.5.2. Extract preparation.** Aqueous extract was prepared at a concentration of 10,000 mg/L in 10% DMSO. A series of concentrations was prepared through dilution of the aqueous extract in 10% DMSO to obtain final concentrations of 5,000 mg/L, 500, 50 mg/L, 5, and 0 mg/L. One mL of the extract at each concentration was separately transferred into 9 mL NB with three replicates per concentration.

**2.5.3. Challenge test with *V. harveyi*.** A volume of 100 µL *V. harveyi* at a density of 10^8 CFU/mL was added to the tubes filled with aqueous extract at the concentrations above, in order to provide an initial bacterial population of 10^6 CFU/mL, and then gently homogenized using a vortex. The extracts challenged with *V. harveyi* at concentrations of 5, 50, 500, and 5,000 mg/L were incubated at room temperature using a shaker for 24 hours. The *V. harveyi* population was calculated every 4 hours and post treatment for 24 hours. The *V. harveyi* population was counted using the total plate count (TPC) with spread plate method [14, 15]. The *V. harveyi* count was done using a 1 mL sample taken from each treatment and diluted in 9 mL of sterile saline solution (0.85% NaCl) which was assigned as the original solution or dilution factor (10^3). Serial dilutions of the original culture tube were prepared by transferring 1 mL of culture into 9-mL of sterile saline solution, mixing and removing from this solution 1 mL to be transferred to another 9-mL sterile saline solution to obtain the dilution factor of 10^3 and continued until a dilution factor of 10^-7 of the original bacterial suspension was reached. One hundred µL (0.1 mL) of each dilution was dispensed onto two plates of 100 ppm rifampicin containing TCBS Agar and spread over each duplicate plate with the same bent glass rod. The plates were then inverted and incubated at 28°C for 24 hours. The *V. harveyi* population was counted by using the following equation:

\[
N(CFU/mL) = \frac{T}{Q} \times [1/S] \times 1/V
\]

where:

- **N** = total colony density (CFU/mL)
- **T** = total bacterial colonies on the plate with the same dilution factor
- **Q** = total plate
- **S** = dilution factor
- **V** = sample volume (0.1 mL).

**2.6. Isolation of bioactive compounds**

An aliquot of approximately 5.0 g of the active aqueous extract was fractionated by subjecting the sample to dry vacuum column chromatography on silica gel followed by gradual elution with n-hexane, n-hexane/DCM, DCM, DCM/MeOH, and MeOH. A total of 8 fractions were collected, each with a volume of 200 mL, and were coded with the numbers 1-8. Bioassay-guided fractionation showed that fractions 6 and 7 were active against *Vibrio harveyi*. These two fractions were combined and further re-chromatographed over a silica gel column and eluted with CHCl3/MeOH, in a ratio of 8:2, to yield a yellow powder mixture from subfraction 5 after drying at room temperature. This yellow powder mixture was diluted with methanol and loaded onto a C18 sephadex cartridge to obtain a pure compound which was coded as HOA.

**2.7. Thin Layer Chromatography profiling**

Thin Layer Chromatography (TLC) was performed on a plate pre-coated with Si-gel F254 (layer thickness 0.2 mm, Merck, Darmstadt, Germany) as stationary phase. Liquid mobile phases were semipolar (CH2Cl2:MeOH in the ratio 8:2 v/v). A one-dimensional ascending development technique was used to detect the constituents of the aqueous fraction extract on the TLC plate. Visual detection was performed in daylight and under UV light at wave lengths of 254 and 344 nm. The compounds were also detected by spraying with Dragendorff reagent.
2.8. Antibacterial test of isolated compound
Prior to testing, a V. harveyi culture was prepared to obtain a bacterium density of 10^6 CFU/mL, using the method described above. The antibacterial test was performed using an agar diffusion method with paper discs. An aliquot of 100 µL of bacteria was spread on Mueller Hinton agar medium. The filter paper discs (6 mm in diameter) were individually impregnated with 20 µL (20 µg/disc) of isolated compound (HOA), dried in a laminar air flow cupboard, and then placed onto the agar plate previously inoculated with the tested bacterium. The plates were incubated at 28 °C for 24 h. The diameter of the inhibition zones was measured in millimetres. Chloramphenicol was used as a positive control.

3. Result and discussion

3.1. Antibacterial activity of Aaptos aaptos extracts
Testing of the antibacterial activity of the Aaptos aaptos extracts for activity against Vibrio harveyi by using the 96 microwell plate gave positive results. Qualitatively, all extracts (methanol, n-hexane, butanol, and aqueous extracts) were able to inhibit the growth of V. harveyi at a concentration of 10,000 mg/L (Table 1).

| Extract fraction | Antibacterial activity against Vibrio harveyi |
|------------------|---------------------------------------------|
| Methanol         | Positive                                    |
| n-Hexane         | Positive                                    |
| Butanol          | Positive                                    |
| Aqueous          | Positive                                    |

Quantitatively, based on inhibition zone diameter, the inhibition response of V. harveyi differed between the various Aaptos aaptos extract fractions (Table 2). Based on the inhibition zone category classification of Davis and Stout (1971), the inhibition level of three out of the four Aaptos aaptos extracts (methanol, butanol and aqueous) was in the strong activity category, with an inhibition zone diameter greater than 10.00 mm. Antibacterial activity can be influenced by several factors such as extract concentration, the presence of antibacterial compounds in the extract, extract diffusion ability, and the inhibited bacteria species [16]. The similar antibacterial activity levels shown by butanol and aqueous extract at the concentration of 10,000 mg/L indicate the possible presence of similar antibacterial compounds in the two extracts, possibly belonging to the alkaloid group. The presence of alkaloids in Aaptos aaptos is known to be able to disturb the formation of peptidoglicant by bacterial cells, causing lysis of these cells [17].

Table 2. Antibacterial activity of methanol, n-hexane, butanol and aqueous extract of sponge Aaptos aaptos against Vibrio harveyi using an agar diffusion method with paper discs

| Extract fraction | Diameter of inhibition zone (mm) |
|------------------|----------------------------------|
| Methanol         | 13.73 ± 0.3                      |
| n-Hexane         | -                                |
| Butanol          | 10.30 ± 0.1                      |
| Aqueous          | 10.35 ± 0.1                      |
| Chloramphenicol  | 10.48 ± 0.2                      |

3.2. Minimum inhibitory concentration (MIC) of the active extracts
The minimum inhibitory concentration (MIC) of the active extracts was determined in order to identify the Aaptos aaptos extract with the strongest activity. The MIC values of n-hexane, butanol and aqueous extract on Vibrio harveyi using a 96 microwell plate show that the aqueous extract had the lowest MIC value (10 mg/L), indicating that this extract had the strongest antibacterial activity, followed by butanol, while the n-hexane extract had the highest MIC value (Table 3).
Table 3. Minimum inhibitory concentration (MIC) of n-hexane, butanol and aqueous extracts of *Aaptos aaptos* against *Vibrio harveyi*

| Extract     | MIC value (mg/L) |
|-------------|------------------|
| Aqueous     | 10               |
| Butanol     | 100              |
| n-Hexane    | 10,000           |

3.3. Challenge test of aqueous extract antibacterial activity

The aqueous extract which showed the lowest MIC value was further studied to determine the ability of this extract to inhibit the growth of *V. harveyi* in vitro. *Vibrio harveyi* populations treated with aqueous extract at different concentrations showed different population trends over time (Figure 1).

![Figure 1. Effect of the aqueous extract on *V. harveyi* populations treated in vitro at different concentrations for 24 hours](image)

At 4 hours post treatment, all treated populations of *Vibrio harveyi* were lower than the control. Populations of *V. harveyi* treated with aqueous extract at concentrations of 500 and 5,000 mg/L produced a sharp decrease to $5.50 \times 10^3$ and $0.83 \times 10^2$ CFU/mL, respectively. The *V. harveyi* populations treated with aqueous extract at these concentrations were quickly reduced to 50-60% of the initial population size, and populations under the 5,000 mg/L treatment were reduced below the detection level. However over the 24 hour study period the populations treated with aqueous extract at the lower concentrations (5 and 50 mg/L) had similar *V. harveyi* populations compared to the control, and times even increased above the control level ($2.69 \times 10^8$ and $6.61 \times 10^7$ CFU/mL, respectively).

3.4. Thin Layer Chromatography (TLC) profiling of water extract compounds

The thin layer chromatography (TLC) profiling of aqueous extract, the active fractions and isolated compounds of *Aaptos aaptos* (Figure 2) showed the presence of alkaloid derivatives indicated by the presence of orange spots after the aqueous extracts were sprayed with Dragendorff reagent. These compounds were present in the raw extract; however, after fractionation, alkaloid compounds were only present in fractions 6 and 7. One alkaloid compound (HOA) was successfully isolated from the combination of fractions 6 and 7. The pure compound (HOA) was isolated as a yellow powder with a rate of flow (Rf) of 0.2 (Acetonitrile/Methanol, 2:8, C18 TLC Plate). A mixture of dichloromethane and methanol with the ratio of 8:2 was found to be an appropriate solvent to separate the compounds of *Aaptos aaptos* aqueous extract.
Figure 2. Thin layer chromatography (TLC) profiling of aqueous extract, fractions 6 and 7 of the aqueous extract, and the pure compound (HOA)

4. Conclusion
An aqueous extract of *Aaptos aaptos* was able to inhibit the growth of *Vibrio harveyi* with the minimum inhibitory concentration (MIC) value of 10 mg/L. The aqueous extract also greatly decreased *V. harveyi* populations (from $10^8$ CFU/mL to $5.50 \times 10^3$ and $0.83 \times 10^2$ CFU/mL) at concentrations of 500 and 5,000 mg/L. One of bioactive compounds responsible for the antibacterial activity of the aqueous extract was isolated and identified as an alkaloid derivative. Based on the results of this study, *Aaptos aaptos* aqueous extract may have potential for further development as an antibacterial agent for the prevention and control of vibriosis.

References
[1] Chen FR, Liu P C and Lee K K 2000 Lethal attribute of serine protease secreted by *Vibrio alginolyticus* strains in Kurama Prawn *Penaeus japonicas* Zool. Naturforsch. 55 94–99
[2] Soundarapandian P and Babu R 2010 Effect of probiotics on the hatchery seed production of Black Tiger Shrimp *Penaeus monodon* (Fabricius) Int. J. Animal Veterinary Advances. 2 9-15
[3] Traifalgar R F M, Corre V L and Serrano A E 2013 Efficacy of dietary immunostimulants to enhance the immunological responses and Vibriosis resistance of juvenile *Penaeus monodon J. Fish. Aqua. Sci.* 8 340- 354
[4] Kadariah I A K 2012 Development of Rapid Detection Methods of Vibriosis Diseases in Penaeid Shrimp Aquaculture (Bogor: Ph.D Thesis Institute Pertanian Bogor)
[5] Pasharawipas 2011 Inducible viral receptor,a possible concept to induce viral protection in primitive immune animals *Virol. J.* 8 326
[6] Rattanachuay R 2010 Inhibition of shrimp pathogenic vibrios by extracellular compounds from a proteolytic bacterium *Pseudomonas* sp. W3. *Electronic Journal of Biotechnology* 13 1-12
[7] Prajitno A 2006 Prevention of *Vibrio harveyi* by Seaweed (Halimedaapuntia) on Tiger shrimp (*Penaeus monodon* Fab.)(Malang: Ph.D Thesis Brawijaya University)
[8] Raffi S M and Suresh T V 2011 Screening of chloramphenicol in wild and cultured shrimp *Penaeus monodon* by competitive enzyme linked immunosorbent assay (International
Conference Chemical Biology and Environmental Science)

[9] Defoirdt T, Boon N, Sorgeloos P, Verstraete W and Bossier P 2007 Alternatives to antibiotics to control bacterial infections: Luminescent Vibriosis in aquaculture as an example Trends. Biotechnol. 25 472-479

[10] Isnansetyo A, Trijoko, Setyowati E P and Anshory H H 2009 In vitro antibacterial activity of methanol extract of a sponge, Geodia sp. against oxytetracyclin-resistant Vibrio harveyi and its toxicity J. Biol. Sci. 9 224-230 (http://dx.doi.org/10.3923/jbs.2009.224.230)

[11] Selvin J and Lipton A P 2004 Dendrillanigra, a marine sponge, as potential source of antibacterial substances for managing shrimp diseases Aquaculture 236 277-283

[12] Maftuch, Toban M H and Risjani Y 2012 Administration of marine algae (Gracillaria verrucosa) immunostimulant enhances some innate immune parameters in black tiger shrimp (Penaeus monodon Fabricus) against Vibrio harveyi infection J. Appl. Sci. Res. 8 1052-1058 (http://www.aensiweb.com)

[13] Rosmiati, Habsah M and Muhammad T S T 2011 Biological activities of methanolic extracts of several sponge species (Malaysia:10th Int. Ann. Symp. Universiti Malaysia Terengganu)

[14] Feng P 2007 Bacteriological Analytic Manual (BAM) Salmonella (http://www.fda.gov/food/FoodScienceResearch/LaboratoryMethods/ucm070149.htm)

[15] Osawa 2008 Osawa Sensei’s Vibrio cholera: Isolations Protocol for Environmental Samples Seafood and River or Melted Ice Water (Japan: KOBE University)

[16] Jawetz E, Melnick J L and Adelberg E A 1996 Microbiology of Medical (Jakarta: Medical Book Publisher)

[17] Saxena M, Saxena J, Nem R, Singh D and Gupta A 2013 Phytochemistry of medicinal plants J. Pharmacognosy and Phytochemistry 1 168-182