THE EFFECT OF MARINE SPONGE *Aaptos aaptos* EXTRACT IN VIBRIOSIS TREATMENT OF BLACK TIGER SHRIMP *Penaeus monodon* LARVAE

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ABSTRACT

Black tiger shrimp *Penaeus monodon* post larvae were challenged with *Vibrio harveyi* and butanol extract of selected marine sponge *Aaptos aaptos* to determine its antibacterial bioactive potential in vibriosis treatment. Based on the preliminary toxicity study, the *A. aaptos* butanol extract with concentrations of 31.25, 62.5, and 125 mg/L were selected in the study. Black tiger shrimp post larvae were challenged with *V. harveyi* at 10^7 cfu/mL and immersed *A. aaptos* butanol extract with the concentration of 125 mg/L showed significantly in ($P<0.05$) decrease mortality of the post larvae treated. Besides at this concentration, *V. harveyi* population in the rearing water and the post larvae treated decreased compared to control (untreated post larvae). Histological observation indicated that there was no changing on hepatopancreas of the black tiger shrimp post larvae. Based on this result, it is suggested that the butanol extract of *A. aaptos* is a potential bioactive compounds source in the treatment of vibriosis which may replaced the current antibiotics application.

KEYWORDS: ***Aaptos aaptos*, marine sponge, *Penaeus monodon*, vibriosis**

INTRODUCTION

Vibriosis luminescence is one of bacterial diseases caused by *Vibrio* species. This disease has been the main causative agent of mass mortality in shrimp culture. Vibriosis has resulted in mass mortality of larva and postlarval stages of *Penaeus monodon* rearing systems (Sarjito *et al*., 2012). Mortalities of *P. monodon* larvae associated with luminescence have been observed in hatcheries in Indonesia (Kadriah, 2012), Thailand (Pasharawipas, 2011) and Philippines (Traifalgar *et al*., 2013). Among the causative agents of Vibriosis, *Vibrio harveyi* (luminescent bacterium) often results in mass mortality of *P. monodon* larval rearing systems (Ramesh *et al*., 2014). The pres-

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ence of *V. harveyi* at density of 10^7 cfu/mL in the rearing water was reported to cause mortality in black tiger shrimp *P. monodon* post larvae 14 days (14 days post larvae) (Kadriah, 2012).

Current treatments of vibriosis are largely based on the use of potent antibiotics, but currently their efficacy is low, very poor. The use of antibiotics has been less effective and resulting in severe side effects in the rearing environment, shrimp and human, and even death in shrimp (Raffi & Suresh, 2011). Several disadvantages of antibiotics are the presence of antibiotic-resistant bacteria (Kumar *et al*., 2014), antibiotics residue which may accumulate in the shrimp, upsetting the chemical balance in the ponds by affecting useful organisms like nitrifying bacteria and killing food organisms, and concerns of antibiotic resistance to human pathogens (Hettiarachchi *et al*., 2005).

Marine organisms are a rich source of biological diversity and potential for discovery of novel drugs (Ninawe, 2007). Among marine organisms, sponge is the richest of both biologically active secondary metabolites and chemical diversity. Several species of sponges were described to have antibacterial properties against vibriosis treatment such as *Geodia* sp. (Isnansetyo *et al*., 2009) and *Denrillanigra* (Selvin & Lipton, 2004). This study aims to determine the potentiality of *Aaptos aaptos* butanol extract as bioactive compounds source in the treatment of vibriosis due to *Vibrio harveyi* in tiger shrimp post larvae.

MATERIALS AND METHODS

Acclimatization of Post Larvae

Seven days post larvae (PL-7) of black tiger shrimp, *Penaeus monodon* were collected from a commercial hatchery in Bone Regency, South Sulawesi, Indonesia. They were stocked in fibre glass tank (500 L) in the wet laboratory of Research and Development Institute for Coastal Aquaculture (RICA), Maros, Indonesia. The shrimp post larvae were acclimatized in the temperature-controlled wet laboratory and reared for seven days prior to starting of experiment (PL-14). During the rearing period, they were fed with commercial feed at 10% of body weight twice a day (06.00 and 18.00). The post larvae which were free of white spot (based on PCR test) and around 1 cm length size were selected.

Extract Preparation

Sponge *Aaptos aaptos* was collected from Barrang Lompo Islands, South Sulawesi latitude (-5.047 degrees) 5°2'49" South of the Equator and longitude (119.329 degrees) 119°19'44" East of the Prime Meridian on the Map of the world by SCUBA diving at the depth of 8-12 m on 16th August 2010. Sponges were kept in sealed plastic bag and placed in cool box on fresh condition and brought to Biotechnology Laboratory of RICA, Maros. The fresh sponge was cut into small pieces and oven dried at 37°C for three days. It was then grinded into powder and extracted with methanol using a forma orbital shaker at 37°C till the residue became colourless. The methanolic extracts was filtered through Whatmann No. 1 filter paper fitted in a Buchner funnel using suction and collected for concentrated under reduce pressure by a rotary evaporator (Buchi-type) to yield a dark gummy solid. Later, salt was removed from methanol extract using HP-20 while lipid/waxes were removed using C-18 catridge as described by Rosmia *et al*. (2011) to get interference material free methanolic extract.

Partitioning of Methanolic Extract

The interference material free methanolic extract above was added with distilled water and partitioned with diethyl eter and 1-butanol to give diethyl eter, butanol, and aqueous extract. The butanol extract obtained was diluted in distilled and sterilized seawater (30 g/L) to get a concentration of 32.25, 62.5, and 125 mg/L based on the preliminary toxicity experiment.

Challenge Test Activity

Preparation of *Vibrio harveyi*

Vibrio harveyi culture (MR 275 Rif) being used was a collection of RICA, Maros, Indonesia with the density of 10^7 cfu/mL. Preparation of *V. harveyi* was done as described by Kadriah *et al*. (2012). One hundred µL of glycerol : nutrient broth (1:1) containing the bacterium isolate was cultured in 10 mL nutrient broth (NB) and incubated at 28°C on orbital shaker (forma type), 150 rpm for 24 hours. One mL of starter culture was transferred to 400 mL NB and incubated using shaker at 150 rpm, 28°C for four hours to obtain the density of bacterium of 10^8 cfu/mL. The bacterium was diluted in Nutrient Broth to get the density of 10^7 cfu/mL before used.

Challenge Test

Soaking method as described by Kadriah (2012) with some modifications was used for challenge test. The larval rearing units were consisted of conical shaped aquaria containing 1 L filtered and chlorinated seawater at 30 g/L. The density of 14 days-old post larvae was 20 larvae/L for each unit. In this study, Completely Randomized Design being used were five treatments with three repetitions. The five treatments were consisted of: (A) positive control PL + *V. harveyi*; (B) PL + *V. harveyi* + 31.25 mg/L extract; (C) PL + *V. harveyi* + 62.5 mg/L extract, (D) PL + *V. harveyi* + 125 mg/L extract, and (E) negative control (PL without *V. harveyi* and extract). During this study, all rearing units were provided with gentle, continuous aeration, and without water exchange. In addition, the post larvae were fed with commercial feed at 10% of body weight twice a day (06.00 and 18.00).

Data Collection

The mortality or survivability of post larvae in the water rearing was monitored at 1, 3, 6, 9, 12, 24, 36, 48, 72, and 96 hours post-treatment. The enumeration of *V. harveyi* in the rearing water was calculated in the initial, one and every three hours for the first 24 hours, continued in every 24 hours up to four days whilst in the post-larvae was calculated in 6, 12, and 24 hours, continued in every 24 hours post-treatment up to four days. The clinical signs were also observed during the rearing. In addition, water quality parameters such as, pH, temperature, and dissolved oxygen were also measured in the initial, mid, and the end of this study as supporting data. At the initial of six hour and the end of experiment, three larvae were harvested and fixed in 10% buffered formalin solution and then were subjected to standard histology processing as described by Sarma & Devi (2012) with some modification.

The enumeration of *V. harveyi* population in post larvae and rearing water was done using total plate count (TPC) with spread plate method. The enumeration of *V. harveyi* in rearing water was done taking out 1 mL rearing water of every treatment and diluted in 9 mL sterile saline solution (0.85% NaCl) and assigned as the original solution or dilution factor (10^0). Meanwhile, in the post larvae was done by harvesting two post larvae from every treatment, cleaning of the surface with alcohol containing cotton, grinding, and diluting in 10 mL sterile saline solution (10^0). Serial dilutions of the original culture tube were prepared by transferring 1 mL of culture into a 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^{-1} and continued until the dilution factor 10^{-7} of the original bacterial suspension. One hundred µL (0.1 mL) of each dilution was dispensed into two plates of 100 mg/L rifamphycine containing TCBS Agar and spread it in duplicate with the same bent glass rod. The plates then were inverted and incubated at 28°C for 24 hours. Those duplicate plates which had 30 to 300 colonies were counted individually. The mortality percentage was counted by the following equation.

$$\text{Mortality } (\%) = \frac{Nt}{No} \times 100\,\%$$

where:
Nt = Number of dead post larvae
No = Total number of the initial post larvae

The enumeration of *V. harveyi* population (*Vh*P) was counted by equation as followed:

$$VhP \text{ (CFU/mL)} = A \times \frac{1}{V} \times \frac{1}{P}$$

where:
A = Total of colony (CFUs)
V = Volume of bacterium which was spread on agar (mL)
P = Dilution factor

Data Analysis

Analysis of mortality/survivability data was performed by one way analysis of variance (ANOVA). When differences were found among the treatments, Tukey's test was used to compare means by SPSS Version 15.0 software packages (SPSS® Inc., USA). Differences of results were considered statistically significant difference if the *P* value were ≤ 0.05. Whilst, clinical signs, enumeration of *V. harveyi* in water rearing and post-larvae and water quality were analyzed descriptively. The analysis of any adverse effect in tissue was observed via histological examination.

Results and Discussion

The mortality rate of tiger shrimp, *P. monodon* post larvae (Table 1) showed that there

Table 1. Mortality of black tiger shrimp, *P. monodon* post larvae challenged with *V. harveyi* and different concentrations of *Aaptos aaptos* butanol extract during the rearing period

Concentrations (mg/L)	Mortality (%), n = 3										
	T1	T3	T6	T9	T12	T24	T36	T48	T72	T96	Total
0 (Control)	0	13.3	26.7	6.7	6.7	3.3	3.3	10	3.3	6.7	80
31.25	0	6.7	13.3*	3.3	10	6.7	10	6.7	6.7	6.7	70
62.5	0	0*	6.7*	6.7	10	13.3	0	10	10	10	66.7
125	0	0*	0*	0	0*	0	10	10	10	13.3	43.3*

Percentage of mortality with (*) are significantly different (P<0.05) as compared to control

was no difference among all the concentrations of *Aaptos aaptos* butanol extract used at 1, 9, 24, 36, 48, 72, and 96 hours post treatment compared to control. Nevertheless, the mortality rate significantly decreased at 3, 6, and 12 hours post treatment in which the post larvae treated with the extract at the concentration of 62.5 and 125 mg/L exhibited a significantly different mortality and all the concentrations were significantly difference on 3 and 6 hours post treatment, respectively. Meanwhile, at 12 hours post treatment only the post larvae being treated with the extract with the concentration of 125 mg/L gave a significantly difference compared to the control. The highest mortality rate (80%) in the end of this experiment was obtained in the untreated post larvae (control), followed by 31.25, 62.5, and 125 mg/L with the mortality rate of 70%, 66.7%, and 43.3%, respectively with the significant difference was obtained on the treatment of 125 mg/L and two other concentrations gave a similar mortality effect compared to control. The efficacy shown by this *Aaptos aaptos* butanol extract concentration in decreasing the tiger shrimp post larvae mortality at 12 hours post treatment was the same result as antibiotic oxytetracyline treatment with the concentration of 40-50 mg/L (Mariyono *et al*., 2002). Nevertheless, after 96 hours of the treatment its efficacy in the total decreasing of mortality was less lower than probiotic BL 542 bacteria isolate application which gave mortality of 42% (Muliani *et al*., 2005 *in* Agung, 2007). *Penaeus monodon* larvae infected by vibriosis commonly suffered mortalities within 48 hours of immersion challenged with strains of *V. harveyi* and *V. splendidus* whilst, the mass mortality of larvae was occurred in the period of 1-3 days since the clinical signs of vibriosis observed (Kadriah, 2012).

Population of *V. harveyi* also decreased in the rearing water of the post larvae treated with *Aaptos aaptos* butanol extract of all the concentrations during the rearing period (Figure 1). Population of *V. harveyi* in the rearing water treated with butanol extract at the concentration of 31.25 and 62.5 mg/L after one hour infection gave a maximum growth namely, 7.5×10^7 and 5.3×10^7 cfu/mL. Meanwhile, control showed the highest bacteria growth of 1.91×10^8 cfu/mL after three hours post infection. In contrast, a drastic decreasing of *V. harveyi* population (9.3×10^5 cfu/mL) was exhibited by butanol extract treatment with the concentration of 125 mg/L. In general, population of *V. harveyi* in the rearing water was seemed to decrease drastically after six hours post infection for all the post larvae treated.

Population of *V. harveyi* in black tiger shrimp post larvae after treatment of *Aaptos aaptos* butanol extract was also lower than control (Figure 2). The maximum growth of *V. harveyi* on all treatments of *A. aaptos* butanol extract was found six hours after challenged with *V. harveyi* and continued to decrease until the end of study. The lowest *Vibrio harveyi* population was obtained on the treatment of *A. aaptos* butanol extract with the concentration of 125 mg/L (1.77×10^3 cfu/mL), followed by 62.5 mg/L (4.13×10^4 cfu/mL) and 31.25 mg/L (6.13×10^4 cfu/mL). In contrast, the highest was showed by control (without butanol extract) namely; 1.51×10^6 cfu/mL. The decreasing of *V. harveyi* population in post larvae and rearing water showed that butanol extract was able to inhibit the growth of *V. harveyi* population which caused increasing immune system of black tiger shrimp post larva to defend against bacterial infection. *A. aaptos* butanol extract at the concentration of 31.25 and 62.5

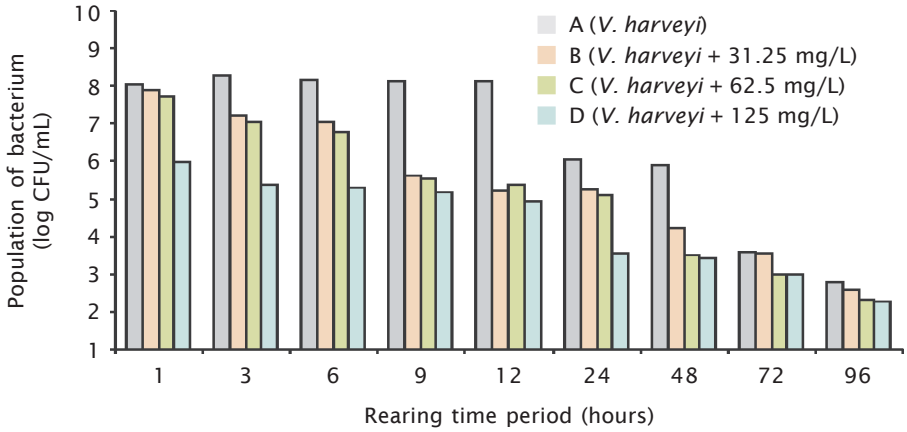


Figure 1. Population of *V. harveyi* in the rearing water treated with different concentration of *Aaptos aaptos* butanol extract during the rearing time period

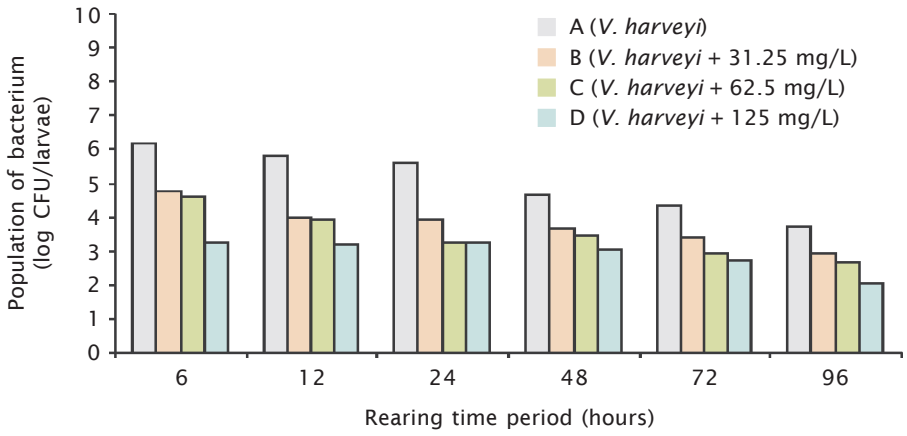


Figure 2. Population of *V. harveyi* in the post larvae treated with different concentration of *Aaptos aaptos* butanol extract during the rearing time period

mg/L also decreased the population of *V. harveyi*. However, they were not able to prevent the infection of this bacterium due to the low concentration. The weak antibacterial shown by *A. aaptos* butanol extract at the concentration of 32.25 and 62.5 mg/L caused the high mortality of post larvae. The antibacterial shown by butanol extract of *A. aaptos* was caused by the presence of alkaloid compounds. As it was well known that majority of *A. aaptos* compounds is alkaloid. Guittat *et al.* (2003) demonstrated that alkaloid fight against bacteria by inhibiting certain vital processes of bacterial cells or metabolism.

Clinical signs observation on tiger shrimp post larvae during challenge test with *V. harveyi* showed behavior and morphology change. The behavior change on the post larvae treated with *Aaptos aaptos* butanol extract at the concentration of 31.25 and 62.5 mg/L was indicated by decreasing to low appetitive and erratic swimming. The same signs were also exhibited by positive control. Meanwhile, the post larvae treated with *Aaptos aaptos* butanol extract at the concentration of 125 mg/L did not show any abnormal behavior as displayed by negative control, except on the fourth day, the post larvae showed a low appetitive which was suspected due to molting. As reported by Ramesh *et al.* (2014) post larvae suffering vibriosis are anorexia, lethargy, jumping to the surface, passive, slow swimming, more feed resi-

due, red feces, red or brown hepatopancreas. The morphology change observed six hours post treatment and it was indicated by red discoloration body on the positive control and the treatment of 125 mg/L while it became dull and dirty on the treatment of 31.25 and 62.5 mg/L. After 96 and 168 hour of *Aaptos aaptos* butanol extract treatment, the post larvae treated with 125 mg/L showed behavior and morphology back to normal. Meanwhile, the post larvae treated with the concentration of 32.25 and 62.5 mg/L were still dull and dirty. Except for the post larvae treated with 125 mg/L, all the post larvae had died 168 hours post treatment.

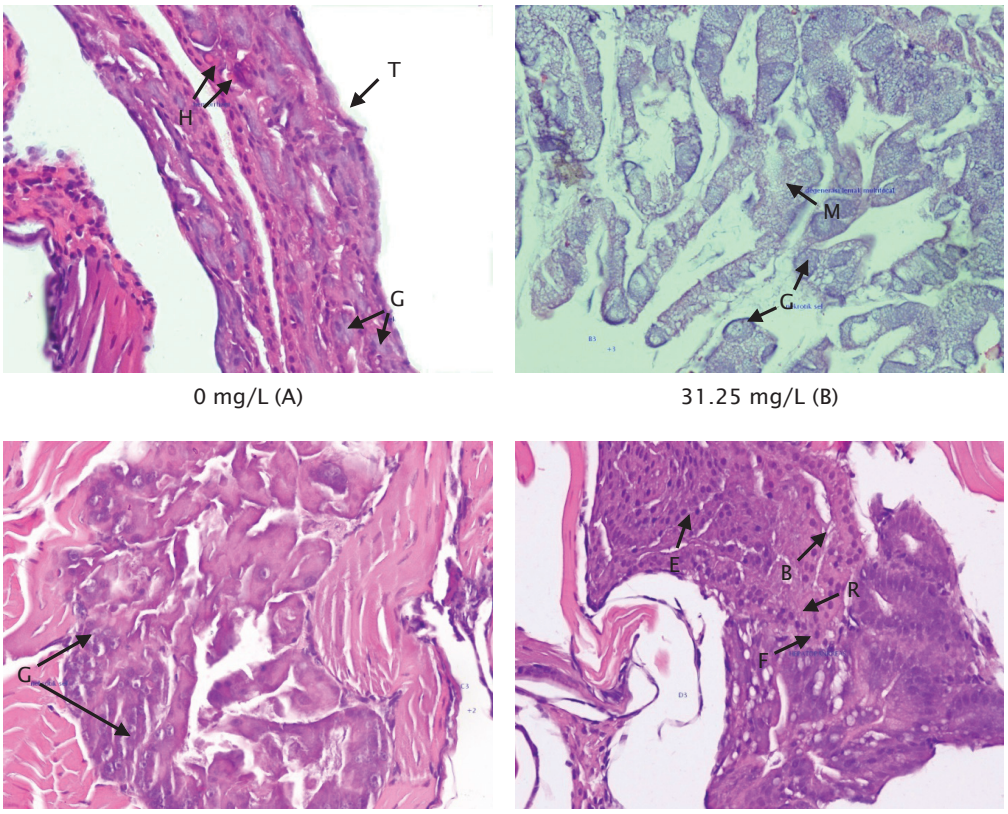
The observation of water quality during challenge test period (Table 2) was suitable for the growth of tiger shrimp post larvae. Soundarapandian *et al*. (2009) reported that for optimum growth of *Penaeus monodon*, the optimum temperature was between 25°C-31°C and the optimum dissolved oxygen was between 4-8 mg/L. Meanwhile, Parado-estepa *et al*. (1996) reported that the optimum pH for *P. monodon* is 7.5 to 8.5.

Results of the histopathological examination on hepatopancreas of black tiger shrimp post larvae challenged with *Vibrio harveyi* and treated with different concentration of *Aaptos aaptos* butanol extract six hours post treatment in this study (Figure 3) showed the changes in cells and tissue morphology due to pathogen infection. The changes in cells and tissue morphology observed on control (A) were the presence of necrosis cells and the existence of haemocytic nodule formation (hemorrhage) as well as atrophy of the hepatopancreas. The cells necrosis were also exhibited by the treatment of *Aaptos aaptos* butanol extract with the concentration of 32.5 mg/L (B) and 62.5 mg/L (C). Nevertheless, more necrosis cells were observed on treatment B compared to C. Meanwhile, fatty degeneration observed in treatment B was not caused by the pathogenity of *Vibrio harveyi* but it was due to malnutrition. According to Ambipillai *et al*. (2003), severe hepatopancreatic pathology typical of enteric vibriosis are cells necrosis, haemocytic nodule formation (hemorhage), and atropy. In contrast, the observation on treatment D showed a normal hepatopancreas i.e. lumen containing a granular material and the lumen-facing surface on the tubule was covered with a microvillus border. Besides that, the tubular apex was contained undifferentiated embryonic cells (E cells). R (Restzellen), B (Blasenzellen), and F (Fibrillazellen) cells were also observed while F cells nuclei were larger than those of R cells which characteristically contained numerous of nuclei.

The similar histological observation was also showed after 96 hours treatment (Figure 4), control displayed not only cells necrosis, haemorrhagy, and atropy but also lysis on hepatopancreas. Cells necrosis were also observed on treatment C whilst, on treatment B haemorrhagy were occurred besides cells necrosis. In contrast, we observed a normal hepatopancreas on treatment D. According to Jiravanichpaisal *et al*. (1994) histophatological observation of shrimps/post larva suffering vibriosis shows systemic vibriosis typically results in the formation of septic haemocytic nodules in the lymphoid organ, heart and connective tissues of the gills, hepatopancreas, antennal gland, nerve cord, telson, and muscle. Infected hepatopancreas may appear poorly vacuolated indicating low lipid and glycogen reserves and atrophy of multifocal hepatopancreas with necrosis and inflammation. Vibriosis in *Penaeus monodon* is associated with the formation of "spheroids" in the lymphoid organ (Nash *et al*., 1992).

Table 2. The water quality observation of tiger shrimp post larvae challenged with *V. harveyi* and different concentration of butanol extract for four continues days

Extract concentration (mg/L)	pH	Temperature (°C)	Dissolved oxygen (mg/L)
0 (Control)	7.19-7.99	26.3-26.4	7.77-7.80
31.25	7.54-8.07	26.2-26.4	6.89-7.74
62.5	7.45-7.90	26.3-26.4	7.07-7.23
125	7.80-8.08	26.2-26.3	7.06-7.22



0 mg/L (A) 31.25 mg/L (B)

62.5 mg/L (C) 125 mg/L (D)

Figure 3. Histological observation of *Aaptos aaptos* butanol extract treated and *V. harveyi* challenged post larvae hepatopancreas on six hours post treatment. E = Embryonic cell; B = Blasenzellen cell; R = Restzellen cell; F = Fibrillazellen cell; G = Necrosis cell; M = Fatty degeneration; H = Haemorrhage; T = Atrophy. Haematoxylin and Eosin staining, magnification = 40x

Findings of the present study suggested that *Aaptos aaptos* butanol extract could be a potential alternative bioactive compounds source for the development of tiger shrimp, *Penaeus monodon* post larvae in vibriosis disease therapy. As reported that marine sponge *Aaptos aaptos* contains alkaloid compounds such as aaptamine, isoaaptamine, and demethyloxyaaptamine (Shaari *et al*., 2009). Those compounds were found to be inhibit the growth of Gram-positive and Gram-negative bacteria (Rasyid, 2009).

CONCLUSION

Aaptos aaptos butanol extract at the concentration of 125 mg/L was significantly able to inhibit the growth of *Vibrio harveyi* in rearing water and post larvae. This *Aaptos aaptos* butanol extract treatment also showed lower mortality (43.3%) than control (80%). At this concentration, no changing on post larvae hepatopancreas tissue was observed. Based on this study, *Aaptos aaptos* butanol extract has the potential as bioactive compounds source for treatment of vibriosis.

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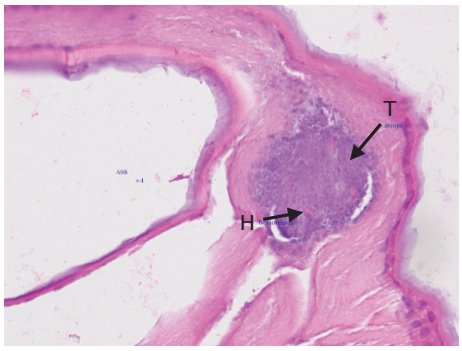
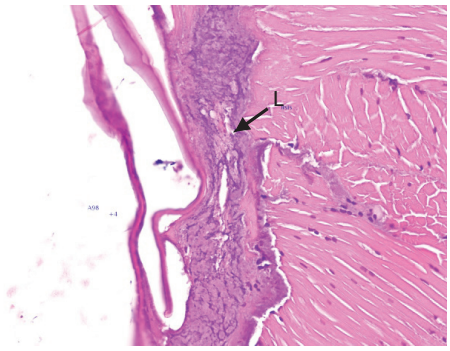
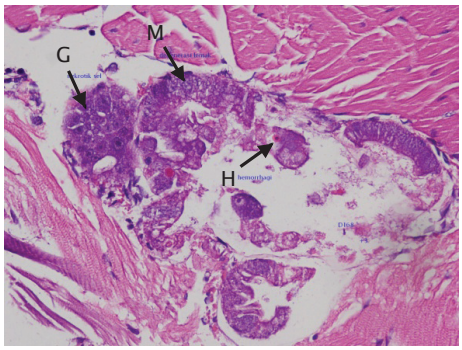


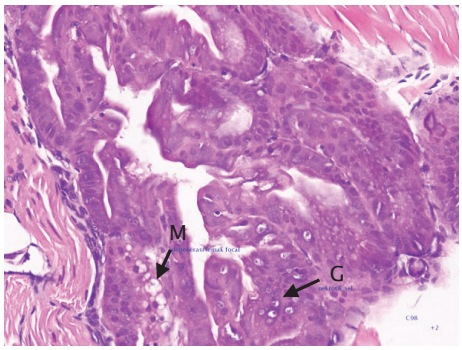
0 mg/L (A)



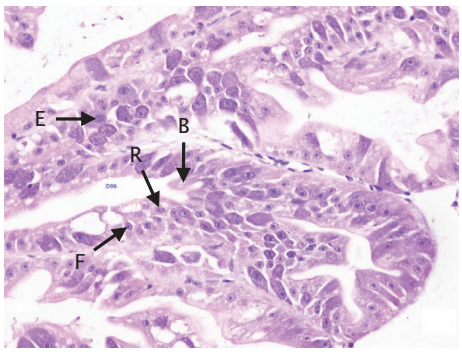
0 mg/L (A)



31.25 mg/L (B)



62.5 mg/L (C)



125 mg/L (D)

Figure 4. Histological observation of *Aaptos aaptos* butanol extract treated and *V. harveyi* challenged post larvae hepatopancreas 96 hours post treatment. E = Embryonic cell; B = Blasenzellen cell; R = Restzellen cell; F = Fibrillazellen cell; G = Necrosis cell; M = Fatty degeneration; H = Haemorrhage; T = Atrophy; L = Lysis. Haematoxylin and Eosin staining, magnification = 40x

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