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The Effectiveness of Ascidian *Didemnum molle* Extracts as Antibactery *Vibrio harveyi* on Tiger Shrimp (*Penaeus monodon*)

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Abstract

Christin Jelita, Eri Bachtiar, and Evi Liviawaty. 2014. The Effectiveness of Ascidian *Didemnum molle* Extracts as Antibactery *Vibrio harveyi* on Tiger Shrimp *(Penaeus monodon). Aquacultura Indonesiana, 15 (2) : 51-56.* This research studied the effectiveness of Ascidian *Didemnum molle* extracts as curative for bacteria *Vibrio harveyi* infection in tiger shrimp (*Penaeus monodon*). The purpose of the research was to find out the effective concentration of antibacterial compound Ascidians *Didemnum molle* to curative the infection of bacteria *Vibrio harveyi* in tiger shrimp. The Ascidians *Didemnum molle* which were used in this research was originated from Kepulauan Seribu waters. Ascidians *Didemnum molle* collected by 3.5 kg with an average size of 5-15 cm per individual. The research was conducted from September to December 2013 at the Biotechnology Laboratory and Aquaculture Laboratory of the Faculty of Fisheries and Marine Sciences, Padjadjaran University. The method which was used in this research was a laboratory experimental method such as phytochemical test, extraction, anti-*Vibrio harveyi* activity test, LC_{50} test and chalenge test. Descriptive analysis was used in this research. Based on the anti-*Vibrio harveyi* activity test, it could be concluded that Ascidian *Didemnum molle* extract had potential as an antibacterial for *Vibrio harveyi*, which indicated by inhibition zones as 11.1 mm at a concentration of 1,000 mg/L. The result of EPA Probit analysis on LC_{50} test, with 48-hours of observation, determine 387 mg/L as a safe extract concentration for tiger shrimp. The chalenge test showed that treatment D with concentration of 290.25 mg/L was effective to be used as treatment for tiger shrimp that were infected by *Vibrio harveyi* bacteria solution of density 10^6 CFU/ mL, with the highest survival rate was 51.7%.

Keywords : Antibacterial; Ascidian *Didemnum molle;* Tiger Shrimp; *Vibrio harveyi*

Introduction

Tiger shrimp is one of the commercial marine product that have high economic value. Tiger shrimp production is targeted for 199.000 thousand tons (KKP, 2013). However, the main issue in the cultivation of shrimp is a matter of parasitic disease of shrimp larvae, one of which is caused by bacteria. The main pathogenic bacteria that causes illness in tiger shrimp is *Vibrio harveyi* (Lavilla-Pitogo *et al.,* 1990). The attacks bacteria caused high levels of mortality for tiger shrimp larvae for a short time. Consequently tiger shrimp farmers suffered losses.

Countermeasures on cultivation of tiger shrimp disease is usually done with synthetic antibiotics such as Chloramphenicol and Nitrofurant. The actual disease control efforts should not use it, because it has a negative effect on humans and causes bacteria to become resistant (Johnny *et al.,* 2002).

Didemnum molle is a species of Ascidians, generally found in shallow tropical waters (Monniot *et al*., 1991; Abrar and Manuputty, 2008). *Didemnum molle* is a species from the family of Didemnidae that are known contained secondary metabolites compounds like alkaloids, flavonoids and steroids. Availability of *Didemnum molle* in tropical waters such as Indonesia considerable abundant because this species has high adaptability (Abrar and Manuputty, 2008).

Arlyza in 2008 exposed the Ascidians *Didemnum molle* contains secondary metabolites compounds were alkaloids and terpenoids that extracted with organic solvents. These metabolites compounds were antibacterial against gram-negative bacteria *Pesudomonas aeruginosa*. Antibacterial compounds contained in the Ascidian *Didemnum molle* is bacteriostatic which inhibits the growth of bacteria but not until die.

Study on utilization of Ascidians especially in Indonesia still needs to be developed. The need for active compounds from natural marine products continues to increase. To that end, the application of biotechnology are expected to develop and harness the potential of

Ascidians especially *Didemnum molle* as antibacterial in treating diseases of tiger shrimp caused by the *Vibrio harveyi* bacteria.

Materials and Methods

Preparation of The Sample Ascidians Didemnum molle

Samples were collected and weighed wet weight, cleaned and cut into thin then dried outdoor for 4-5 days. Dried Ascidian *Didemnum molle* pulverized using a blender into a powder and weighed.

Anti-Vibrio harveyi Activity Test

Natrium Agar medium, petri dish prepared and sterilized. Natrium agar was poured into petri dish. *Vibrio harveyi* isolate as much as 0.1 mL at a density of 10^7 CFU/mL was taken from a pure culture that has been diluted in 10 mL of sea water and spread evenly on agar surface using L glass. Paper discs that have been soaked in the extract with a concentration of 10 mg/L, 100 mg/L, 1000 mg/L, 10,000 mg/L, 100,000 mg/L, distilled water, and 30 mg/L chloramphenicol placed on the surface of the agar medium using tweezers. The bacteria were incubated in an incubator with a temperature of 30°C and observed after 24 hours. Inhibition zone diameter was measured with caliper.

$LC5_0$ Test

LC_{50} test conducted by soaking tiger shrimp in jar of ascidian *Didemnum molle* extracts on 5 concentrations of 0, 10 mg/L, 100 mg/L, 1000 mg/L, and 10,000 mg/L with 2 replications. 6 tiger shrimp was put into each jar. The number of dead shrimp and clinical symptoms for 48 hour were observed (Maryani, 2003). The results of mortality data calculated using the EPA Probit software to find a safe concentration limits which used in chalenge test.

Chalenge Test

The chalenge test was used an experimental laboratory with the number of treatment were 5 treatments and 2 replications. Tiger shrimp PL 11 acclimatized for 5 days. The number of shrimp each treatment was 30 shrimp. After that shrimp infected by *Vibrio harveyi* soaking through a bacteria solution of density 10^6 CFU/mL for 15 minutes. Shrimp have already infected were returned to the aquarium maintenance then observed clinical symptoms include the changes in behavior and morphology. If there was a clinical symptoms, shrimp soaked in the ascidian *Didemnum molle* extract for 15 minutes with 5 treatments and 2 replications. Observed for 7 days. There were the treatment :

A = without soaked extract.
B = soaked extract concentration of 25% of the LC50 test results .
C = soaked extract concentration of 50% of the LC50 test results .
D = soaked extract concentration of 75% of the LC50 test results .
E = soaked extract concentration of 100% of the LC50 test results.

Data Analysis

Data of anti-*Vibrio harveyi* activity test, clinical symptoms, survival rates obtained will be analyzed descriptively in the form of images, tables and graphs. Data of LC_{50} test results will be analyzed using EPA probit analysis to obtain a safe concentration of ascidian *Didemnum molle* extract.

Results and Discussion

Weight of dried ascidian *Didemnum molle* was 247 g. Results extraction of ascidian *Didemnum molle* powder with methanol solvent obtained dark green thick paste extract of 26.5 g. The green color derived from symbiotic microalgae in ascidian *Didemnum molle* tissues caused by *Prochloron*. According to Hirose in 2004 *Prochloron* is a blue green algae or Cyanophyta which have chlorophyll a and B. ascidian *Didemnum molle* from Kepulauan Seribu waters was positive contains alkaloids, steroids and saponins.

Antibacterial activity test of ascidian *Didemnum molle* extracts against *Vibrio harveyi* showed that the extract has antibacterial activity. It shown by the formation of inhibition zones around the paper discs treatment 10 mg/L, 100 mg/L, 1,000 mg/L, 10,000 mg/L and 100,000 whereas the positive control treatment was not formed inhibition zones. Table 1 shows that the average diameter of inhibition zone ranged from 8 - 11.3 mm. Referring to Davis and Stout (1971), the antibacterial activity of the secondary metabolites contained in *Didemnum molle* have medium to strong activity because the inhibition zone diameter range of 5-10 mm and 10-20 mm.

Visually inhibition zone formed at a concentration of 10 mg/L, 100 mg/L, 1,000 mg/L and 10,000 mg/L were not clear or there were several colonies of bacteria. Inhibition zone of ascdian *Didemnum molle* extract was clearly evident at a concentration of 100,000 mg/L and positive controls where no visible colonies of bacteria growing in it. Inhibition zone which occured clean was called the radical inhibition zone where there is absolutely no presence of bacterial growth, inhibition zone while covered with a bacteria called iradikal inhibition zone because the area is inhibited bacterial growth but not until die (Sulistyawati *et al.*, 2009).

Antibacterial classified into bacteriostatic and bactericidal. Bacteriostatic is only inhibits the growth of bacteria while killing bacteria are bactericidal. Because almost all of the inhibition zones formed a iradikal zone it can be concluded that the antibacterial activity of ascidian *Didemnum molle* extracts is bacteriostatic or only inhibit the growth of bacteria.

Antibacterial activity due to the natural products in the extract. Phytochemical test results, ascidian *Didemnum molle* contains secondary metabolites were steroids, alkaloids and saponins. Alkaloids can inhibit microbial growth due to its ability to inhibit the synthesis of cell wall and DNA. Saponins works as an antibacterial to disrupt the stability of the bacterial cell membrane, causing bacterial cell lysis and the release of various important components of the cell such as proteins, nucleic acids and nucleotides (Ganiswarna, 1995).

LC_{50} Test of *Didemnum molle* Extracts Against Tiger Shrimp

LC_{50} test observations were carried out for 48 hours gave the different results of survival and mortality of tiger shrimp larvae. This occured because higher concentration of the extract, then the content of active compounds even higher. High content of active compounds might became a toxic for tiger shrimp. Ascidian *Didemnum molle* secondary metabolites is known contains saponins. Saponins in high concentrations became a toxic to cold-blooded animals. It was shown at the time of testing, the concentration of 1,000 mg/L and 10,000 mg/L form a foam when aerated.

The analysis results of LC_{50} test by using EPA Probit 1.5 program gave the concentration of 387 mg/L with concentration below the threshold of 180.124 mg/L and concentrations above the threshold of 810.578 mg/L. This indicated that the ascidian *Didemnum molle* extract concentration of 387 mg/L caused the death of tiger shrimp larvae as much as 50% within 48 hours. At the end, the concentration of ascidian *Didemnum molle* extracts which safe to use for tiger shrimp were under 387 mg/L concentration.

Table 1. Diameter of Inhibition Zone on Anti-*Vibrio harveyi* Activity Test

Concentration (mg/L)	Diameter of Inhibition Zone (mm)			Average (mm)
	I	II	III	
*Control -	0	0	0	0
*Control +	17.1	15.6	15.3	16
10	7.7	8.4	8.1	8.0
100	9.5	7.5	8.6	8.5
1000	10.1	10.1	13.1	11.1
10000	10.1	10.3	12.6	11.0
100000	10.8	11.8	11.4	11.3

Chalenge Test of *Vibrio harveyi* Bacteria Infected Tiger Shrimp

The survival rate of tiger shrimp treated with soaking in ascidian *Didemnum molle* extracts higher than controls (Table 2). Graph the average of survival rate has increased to the limit of treatment D (290.25 mg/L), but there was a decreasing in the E treatment (387 mg/L).

In addition to survival rate observation, clinical symptoms observations conducted to see the effect of Ascidian *Didemnum molle* extract on the healing tiger shrimp. Observations were conducted on morphological changes of shrimp (body color observations, hepatopancreas, necrosis) and behavior (movement and response to feed). Observations on the movement of shrimp were presented in Table 3 and the response to feed of shrimp were presented in Table 4.

Table 2. Mortality and Survival Rate of Tiger Shrimp After Treatment With Extract Ascidians *Didemnum molle*

Treatment	The count of shrimp in early	Mortality of Tiger Shrimp of Day- (tail)							The count of shrimp in end	Survival Rate (%)	Average (%)
		1	2	3	4	5	6	7			
A1	30	12	10	2	1	0	0	0	5	16.7	21.7
A2	30	8	12	1	1	0	0	0	8	26.7	
B1	30	6	15	2	2	0	0	0	5	16.7	33.4
B2	30	2	10	1	2	0	0	0	15	50	
C1	30	3	9	2	0	0	0	0	16	53.3	41.7
C2	30	7	11	1	2	0	0	0	9	30	
D1	30	2	7	3	1	0	0	0	17	56.7	51.7
D2	30	4	9	3	0	0	0	0	14	46.7	
E1	30	4	3	6	3	0	0	0	14	46.7	35
E2	30	10	10	3	0	0	0	0	7	23.3	

Table 3. Observation of Tiger Shrimp Larvae Movement on Chalenge Test of Ascidians *Didemnum molle* Extract

Day-	Concentration of Ascidian *Didemnum molle* Extract				
	A Control	B 96.75 mg/L	C 193.50 mg/L	D 290.25 mg/L	E 387.00 mg/L
1-2	+	+	+	+	+
3-5	+	++	++	+++	++
6-7	++	++	+++	+++	+++

\+ : abnormal
++ : passive at the bottom
+++ : active

Table 4. Observations of Tiger Shrimp Larvae Feeding Response on Chalenge Test of Ascidians *Didemnum molle* Extract

Day	Concentration of Ascidian *Didemnum molle* Extract				
	A Control	B 96.75 mg/L	C 193.50 mg/L	D 290.25 mg/L	E 387.00 mg/L
1-2	+	+	+	+	+
3-5	+	+	++	+++	++
6-7	++	++	+++	+++	+++

\+ : no responsive
++ : less responsive
+++ : responsive

In treatment A (control) or without soaking extracts, their survival rate were very low at 21.7%. Tiger shrimp movements in the control treatment were passive and less responsive to the given feed. *Vibrio harveyi* is known attack the digestive tract of shrimp, this is what causes the low response of shrimp to feed.

Tiger shrimp in the control treatment was able to survive despite the low survival rate. Tiger shrimp back to normal circumstances on the 6th day observation period. In agreement with the statement of Maftuch *et al.* 2009, shrimp were stricken with the disease still able to survive and grow normally. Tiger shrimp naturally have non-specific immune system that is run by hemocytes. However, the immune system of shrimp needs to be activated by immunostimulant in order to fight pathogens. In addition, the immune of tiger shrimp in stadia larvae were still weak. That causes the recovery process on a shrimp long enough and caused a high mortality on larvae.

Different with the shrimp which soaked with Ascidians *Didemnum molle* extract. It could be seen from the graph, the survival rate were increasing. Treatment D (290.25 mg/L) gave the best results with a survival rate of 51.7%. Clinical symptoms changed to normaly condition and faster than other treatments. Shrimp on treatment B (96.75 mg/L), C (193.5 mg/L) and E (387 mg/L) started to look much active and responsive the given feed on day 5th of observation. Shrimp on treatment D has been actively moving in the water column and responsive to feed on the 3rd day of observation. It shows that the most effectiveness treatment of the ascidian *Didemnum molle* extract was at the level of concentration 290.25 mg/L.

In treatment E (387 mg/L), the extract was able to cure *Vibrio harveyi* bacteria infections, but it seemed that on that concentration the active compounds was too high so that it became a toxic for tiger shrimp. It indicated by the formation of foam at a concentration of 387 mg/L and according to Harborne 1987, the presence of saponins in the extracts shown by the formation of foam. High content of saponins were toxic to cold-blooded animals. In addition to the result of *Vibrio harveyi* bacteria infections, the high mortality of tiger shrimp larvae in treatment E also caused by high concentration of ascidian *Didemnum molle* extract.

Effectiveness of ascidian *Didemnum molle* extracts in treating *Vibrio harveyi* bacteria infected tiger shrimp influenced by the concentration of extract. Because the concentration related to quantity of secondary metabolites content that become active antibacterial compound in the extract. The lower concentration of extract caused treatment process less effective. However, when the extract concentration is too high, secondary metabolites which contained are toxic to the bioassay.

Secondary metabolites contained in ascidian *Didemnum molle* extracts acts directly against *Vibrio harveyi*. According to Rheinheimer 1985 in Hidayat 2011 one due to infection of *Vibrio harveyi* is damaged cuticle layers containing chitin because of chitinase enzyme. Vibrio known to have chitinase enzyme, lipase and protease. Alkaloid contained in the extract of ascidian *Didemnum molle* acts in disrupting the metabolic processes in bacteria. When metabolism is disrupted bacterial cells can inhibit the synthesis of the enzyme. As a result, bacteria can not infect tiger shrimp more.

Saponins in the extract may interfere the stability of bacterial cell membranes so that damaged bacterial cell membranes. When the cell membrane is damaged, bacteria can undergo cell lysis (Ganiswarna, 1995). When more bacterial cells that undergo cell lysis will reduce the density of bacteria in the shrimp body. As a result, bacterial pathogenicity is reduced.

Conclusion

Ascidian *Didemnum molle* extracts derived from Kepulauan Seribu waters contained secondary metabolites were alkaloids, saponins and steroids. Chalenge test of ascidian *Didemnum molle* extract at a concentration of 290.25 mg/L is most effectively used to cure *Vibrio harveyi* infection in tiger shrimp with survival rate of 51.7%.

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