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Use of Lempuyang Extract (*Zingiber zerumbet*) to Prevent *Aerococcus viridans* Bacterial Infection in Freshwater Lobster (*Cherax quadricarinatus*)

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Abstract

Esti Handayani Hardi, Henny Pagoray and Ningsi Juni Kristiani Harefa. 2021. Lempuyang (*Zingiber zerumbet*) extract is an extract of the rhizome plant which has benefits as antibacterials and imonostimulant in fish. Aquacultura Indonesiana. 22(2):20-28. The study aims to was evaluated the efficacy of the lempuyang extract in freshwater lobster (*Cherax quadricarinatus*) to prevent the infection of aerobic bacteria in freshwater crayfish (*C. Quadricarinatus*). The lobsters were obtained 7-8 cm from Makroman village. The cencentrations were used 0 ppm, 500 ppm, 600 ppm and 700 ppm. The lobsters were immersed in all concentrations of extract for 30 minutes. The challenge test was performed through intraperitonial injection method with *Aerococcus viridans* in 24 hours after immersion with extract as much as 0.1 mL. The lobster was observed for 5 days after challenge test. The parameters were observed the clinical sign of lobster, percentage of mortality, mean-time death, total haemocyte count (THC), and differential haemocyte count (DHC). The results were showed that the infected lobster has clinical sign likely erosion of tail and the onset of red spots, loose and sunken eyes, loose circumcision, loose and pale claws. The result of effective concentration to inhibit *A. viridans* was 500 ppm.in freshwater lobster (*Cherax quadricarinatus*).

Key words: Antibacterial, *Aerococcus viridans, Cherax quadricarinatus,* Plant extract.

Introduction

Red claw freshwater lobster (*Cherax quadricarinatus*) is one of the potential fishery commodities in Indonesia (Wijayanto and Hartono 2006). Freshwater lobster is relatively easy to be cultivated in large quantity compared to sea lobster. It also can be place in aquarium because of its color. The price of *C. quadricarinatus* is quite expensive especially for consumption and export because it contains high nutrition and delicious taste (Mukti 2009). In 2002-2003, freshwater lobster cultivation has been developed and in demand for consumption commodity in Indonesia. Although *C. quadricarinatus* has a positive impact on the economy value, there are various problems, one of them is bacterial disease.

According to Kelly and Evans (1974), one of the commonly pathogenic bacteria found in *C. quadricarinatus* is *Aerococcus viridans*.

A. viridans is one of the lobster growth problems troubling in international lobster trade, and it is proved that this disease has been found in Indonesia through the import of *Homarus americanus* (Stebbing *et al*. 2012). Controlling of progressing disease in general can be done by field monitoring and hygienic business sanitation. The use of chemicals and antibiotics often cause bacterial resistance or can endanger consumers for eating shrimp (Sabiladiyni 2016). Recommendation for taking care of bacterial infection is with bioactive compounds derived from local plants growing in the surrounding environment (Saptiani *et al*. 2012)

Lempuyang extract (*Zingiber zerumbet*) contains of levamisole, flavonoids, steroids, carbohydrates and can inhibit the growth of pathogenic bacteria of *Aeromonas hydrophila* and *Pseudomonas* sp. infecting in tilapia (Hardi et al. 2016; Hardi et al. 2017). This research was evaluated the use of lempuyang extract (*Z. Zerumbet*) as natural antibacterial against *A. viridans* in freshwater lobster (*C. quadricarinatus*)

Materials and Methods

Experimental material

The experimental materials were used extact of *Zingiber zerumbet* and *Cherax quadricarinatus*. The extract of *Z. zerumbet* was obtained from the collection of Laboratory of Aquatic Microbiology, Faculty of Fisheries and marine Sciences, Mulawarman University, Samarinda, East Kalimantan, Indonesia. The concentrations of *Z. zerumbet* were used 0, 500, 600, and 700 ppm. *C. quadricarinatus* (7-8 cm) were obtained in Samarinda, East Kalimantan. Before used for experiment, the lobster were acclimated into maintenance container for 4 days (as much as 3 lobster each container). The shelter tank was 2 cm x 1 cm x 1 cmand the containers were used 12.000 cm^3. After cleaned, the container box is filled with ± 5 L of water with aeration for 24 hours before the lobster get inside into container.

Bacterial Culture

A. Viridans was obtained from the Laboratory of Aquatic Microbiology, Faculty of Fisheries and Marine Sciences, Mulawarman University. The bacteria were cultured into Brain-heart Infusion (BHI) medium (DIFCO®) and incubated at 30ºC for 24 hours. The concentration of bacteria (10^{12} CFU mL^{-1}) was prepared with a serial dilution. The bacteria were rinsed with sterile phosphate buffer saline (PBS) twice before used for research.

Challenged test

Lobster were divided into four groups and three replicate with 3 Ind $container^{-1}$. As much as 35 lobster were immersed in lempuyang extract for 30 minutes. Four treatments were applied in the extract of

lempuyang with concentrations of 0 ppm, 500 ppm, 600 ppm and 700 ppm. During treatment with extract, the lobster was not fed to avoid stress. After immersed, the lobster were transferred into keeping container. After treatment with extract lempuyang, the lobsters were challenged with *A. viridans* using injection in third segment abdomen. . Lobsters were observed for 5 days according to pathology anatomy of external conditions, percentage of mortality, mean-time death (MTD), total haemocyte count (THC), and differential haemocyte count (DHC).

Pathology of external anatomy

The pathology of external anatomy was observed behavior of lobsters after challenged. The pathology observed was through changes in body color, completeness of the antenna organs, the completeness and color of the eyes, the completeness and condition of the claws, the condition of the tail.

Percentage of mortality

The percentage of mortality was observed and counted after challenged with bacteria. The calculating of mortality was used Effendi method (1995):

$$Mortality\ (\%) = \frac{number\ of\ mortality\ at\ the\ end\ of\ the\ study}{number\ of\ fish\ during\ research} \times 100\%$$

Mean-time death (MTD)

The mean-time death(MTD) was aimed to calculate time of the average required by *A. viridans* infectedlobster. MTD calculation are performed after 5 days of observation have been completed using the following formula (Hubert 1980) as follows:

$$MTD = \frac{\sum_{i=1}^{N} aibi}{\sum_{i-1}^{n} bi}$$

ai : time of death on day-i (hours)
bi : number of dead lobsters on day-i (fish)

Total Haemocyte Count (THC)

Sampling of Hemolim and calculation of the total number of hemocytes (THC) is done by the procedure Campa-Courdova *et al.* (2002).

$$THC = \left(\frac{number\ of\ cells\ counted}{calculated\ volume}\right) \times dilution \times 10^{6}$$

Differential Haemocyte Count (DHC)

Calculation on Hemocyte differential is done at 120th hours which is modified according to Jussila (1997)

$$DHC = \left(\frac{number\ of\ each\ type\ of\ haemocyte}{total\ haemocyte}\right) \times 100\%$$

Data Analysis

All the data obtained in the form of numbers were tabulated with Windows Microsoft Excel 2013. The pathology anatomy of external conditions were presented descriptively. The data of percentage mortality, MeanTime Death (MTD), Total Haemocyte Count (THC), and Differential Haemocyte Count (DHC) calculated with formula and presented descriptively.

Results

The external pathology anatomi of infected lobster was showed in Figure 1. The abnormal of infected lobster started to show at 12 h after challenge test. The pathological changes after infected such us white spot and pale in the body, hemorrhagic, chela and eyes detached from lobster. The lobster after immersed with extract and challenged with *A. viridans* were showed fewer anatomical pathological changes of external organs compared to infected lobster.

The percentage mortality of infected lobster and after treatment with extract *Z. zerumbet* was showed in Table 1. The highest mortality was 100% in concentration extract of 600 ppm, while the lowest was 66.67% in concentration extract of 500 ppm, respectively. The mortality of infected lobster without treatment with extract *Z. zerumbet* was 77.78%. The mean-time death of infected lobster and after treatment with extract *Z. zerumbet* was showed in Table 2. The MTD of infected lobster without extract *Z. zerumbet* treatment was occured in 2.7 or 3 days after challenge with *A. viridans.* The fastest MTD of infected lobster with extract *Z. zerumbet* treatment was occured in 2.1 or 2 days (600 and 700 ppm of extract *Z. zerumbet*), while the slowest in 3.5 or 4 days after treatment with 500 ppm. According to the data, the MTD of lobster after immersed with 500 ppm of extract *Z. zerumbet* can slow the occurrence of death compared to infected lobster (0 ppm of extract *Z. zerumbet*) after challenge with *A. viridans*.

The THC and DHC of infected lobster and after treatment with extract *Z. zerumbet* were showed in Table 3. The THC and DHC of lobster was taken once at the end of the study. The THC of infected lobster without treatment with *Z. zerumbet* extract were 14×10^6 sel mL^{-1}. The highest of THC of infected lobster treatment with 500 ppm extract *Z. zerumbet* was 26.4×10^6 sel mL^{-1} and the lowest was 13.6×10^6 sel mL^{-1}. The DHC of infected lobster and after treatment with extract *Z. zerumbet* consist of granula, agranula, and hyalin. The granula, agranula, and hyalin of infected lobster after treatment with 500 ppm extract *Z. zerumbet* were 31%, 28%, and 41%, respectively. At the same time, the granula, agranula, and hyalin of infected lobster after treatment with 700 ppm

extract *Z. zerumbet* were 27%, 34%, and 29%, respectively. Based on the Tabel 3, the infected lobster after treatment with extract *Z. zerumbet* have a higher number of hyalin.

Discussion

The symptoms of infected lobster with *A. viridans* can be characterized with the pale color, the loosened antennae, the body is weak and then the appetite disappears. The symptopms of infected lobsters with *A. viridans*such as look weak, lying down one side by removing one or both of their claws, there are also symptoms in the form of pink on the lower tail (red tail) and in laboratory conditions the lobster stops eating (Stebbing *et al*. 2012). Based on the anatomical pathology of lobster that infected with *A. viridans*, it can be concluded that it is because of the infection of *A. viridans*.

The lobster that treatment with *Z. zerumbet* extract then challenged with *A. viridans* has lower mortality. That was indicated *Z. zerumbet* extract has the ability as an antibacterial activity and immunostimulant. *Z. zerumbet* contains essential oils namely levamisol, flavonoid, and steroid able to inhibit the growth of bacteria pathogen (Hardi *et al*. 2017). The mortality of lobster given *Z. Zerumbet* lempuyang extract solution with different dosage and infected with *A. viridans* has the same mortality rate (*Z. Zerumbet* 0 ppm) above 50%. It can be suspected that *Z. Zerumbet* extract with high concentration level does not really affect the immune system of the lobster so that the immune system of the lobster unable increased and resistant to the attack of *A. viridans*. *Z. Zerumbet* Lempuyang extract has bioactive ability for freshwater crayfish, one of which is to be antibacterial, but it occurs when the concentration is reduced or brought 500 ppm, because of the results obtained that the concentration of 500 ppm extract provides the best protection even though only 11.11%.

The hemocytes of lobster play an important role in humoral immunity through the role of phagocytosis (Bowden 2017). There are three classes of hemocytes in crustaceans, such us hyalinocytes, semigranular cells and granular cells (Johansson 2000). Hyaline cells play a role in the system shrimp defense. These hyaline cells are activated by Opsonin Factor resulting from its active proPO to PO in granular cells, so that can phagocytize foreign material include bacteria (Clark 2014). The THC value in healthy lobsters is approximately 5.6×10^6 cells mL^{-1} (Jussila *et al.* 1997). The highest lobster hemocytes are above the normal THC value lobster, and the lowest THC value is thought to produce hemocytes in large numbers as a body's defense mechanism so that the lowest THC value is above the normal lobster THC value.

Bowden (2017) stated that hemocytes store immune reactive (such as peroxinectin, antibacterial peptide and clotting components) in the body of shrimp, so that the increase in the total number of hemocytes (THC) is one indicator of increased shrimp endurance.

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Z. Zerumbet extract solution given to lobsters takes a long time to increase the immune system in lobster. The high value of hemocytes is due to the efforts of lobsters to defend the body.The increase in total hemocytes in lobsters must be followed by increasing granular cells in lobster hemocytes.Granular cells play a role in phagocyte-causing agents of infection while agranular cells do not (Taqwa, 2011). Ridho and Pramesti (2009) stated that immunostimulants are dose dependent, giving concentrations of doses below the minimum value for the occurrence of immune responses will not have an effect on increasing the number of hemocytes, whereas at too high doses it also cannot effect or behave as an inhibitor.

The use of lempuyang extract to freshwater lobster can prevent bacterial infection *A. viridans* is characterized by a reduction in the pathology of the anatomy of the external organs that occur in lobsters treated with *Z. zerumbet* compared to controls, then the time of death of infected lobsters is longer and increases the number of freshwater lobster hemocytes infected with *A. viridans* bacteria. The best concentration to prevent *A. viridans* infection is 500 ppm of extract *Z. zerumbet.*

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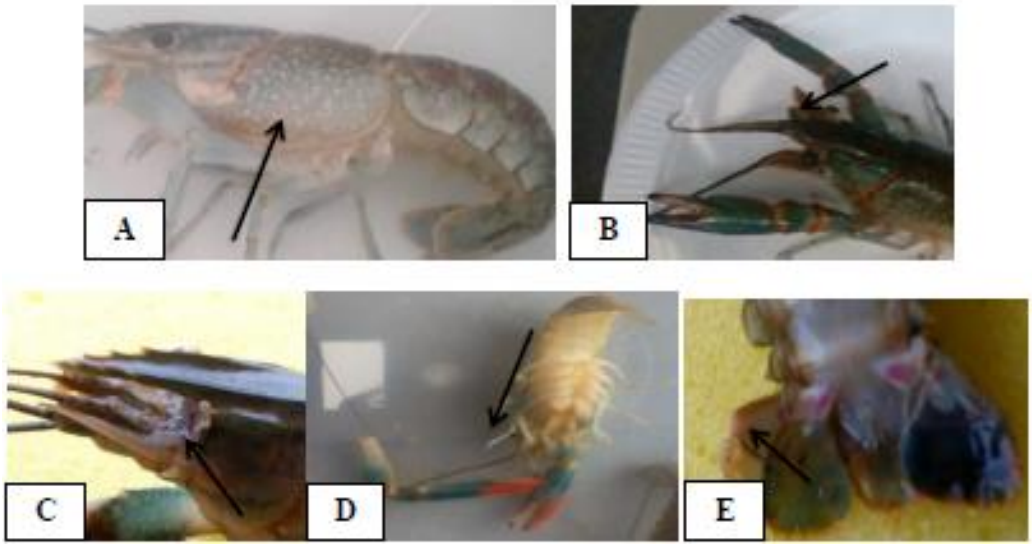

Figure 1. Clinical sign of lobsters after exposured with *A. viridans*. White spot and pale in the body (A) chela and eyes detached from lobster (B and C), and hemorrhagic (D and E)

Table 1. The percentage of Mortality of lobster after challenge with *A. viridans*

Concentration of *Z. Zerumbet* (ppm)	Number of mortality	Mortalitas (%)
500	6	66.67
600	9	100.00
700	7	77.78
0	7	77.78

Table 2. The mean-time death of lobster after challenge with *A. viridans*

Concentration of *Z. Zerumbet* (ppm)	Time (hours)	Days
500	84	3,5
600	51	2,1
700	51	2,1
0	65	2,7

Table 3. The Total Haemocyte count (THC) and Differential Haemocyte Count (DHC) of lobster after challenge with *A. viridans*

Concentration of *Z. Zerumbet* (ppm)	THC ($\times 10^6$ sel mL^{-1})	% Differential Haemocyte Count (DHC)		
		Granula	Agranula	Hyalin
500	26.4	31	28	41
700	13.6	27	34	39
0	14.0	34	28	39