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Immunostimulant Activity of *Gracilaria* sp. and *Padina* sp. on Immune System of Vannamei Shrimp (*Litopenaeus vannamei*) Against *Vibrio harveyi*

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Abstract

The pathogenic bacterial infection is one of the problems in the cultivation of vannamei shrimp (*Litopenaeus vannamei*), causing a high mortality rate of cultured shrimp. The use of antibiotics or chemicals with inappropriate concentrations can harm the aquatic environment, cause resistance, and endanger consumer health because the residues from the chemicals used will periodically accumulate in the body of shrimp. One way to control and prevent shrimp disease is to increase the shrimp immune system by using immunostimulants from seaweed. This study aims to analyze the immunostimulant activity of seaweed extract (*Gracilaria* sp. and *Padina* sp.) against vannamei shrimp (*L. vannamei*) infected with *Vibrio harveyi* by observing the nonspecific immune system based on its hematological features, namely by counting the number of hemocytes and phagocytic activity. The research was conducted at the Hatchery Unit, Brackish Water Cultivation Development Center (BPAP) Situbondo, East Java. Seaweed sample *Gracilaria* sp. and *Padina* sp. purchased from seaweed farmers in Jepara, Central Java. The result of this study shows that supplementation of *Gracilaria* sp. and *Padina* sp. at a dose of 10 g/kg of feed can increase the total number of hemocytes and phagocytosis activity of *L. vannamei* shrimp. The best treatment is *Gracilaria* sp.

INTRODUCTION

The pathogenic bacterial infection is one of the problems in the cultivation of vannamei shrimp (*Litopenaeus vannamei*), causing a high mortality rate of cultured shrimp. Pathogenic bacteria that commonly attack in shrimp culture are Vibrio bacteria (Nitimulyo *et al*., 2005). Vibrio harveyi is the most popular bacteria that causes vibriosis disease that worries shrimp farmers because it can cause shrimp mortality up to 80% within a few days (Israngkura and Sae-Hae, 2002). This disease can also cause mass mortality in both hatchery and the enlargement of vannamei shrimp because of its virulent nature (Soonthornchai *et al*., 2010). The clinical symptoms of shrimp infected with vibriosis show a reddish black color, and several external organs appear red, especially on the gills and limbs (Saptiani *et al*., 2012).

Shrimp farmers have done treatment with antibiotics and other drugs without a veterinary prescription (Lim *et al*., 2020). However, the use of antibiotics or chemicals with inappropriate concentrations can harm the aquatic environment, cause resistance, and endanger consumer health because the residues from the chemicals used will periodically accumulate in the body of shrimp (Defoirdt *et al*., 2007; Kraemer *et al*., 2019). One way to control and prevent shrimp disease is to increase the shrimp immune system by using immunostimulants, vitamins, and hormones (Johnny *et al*., 2005; Mahasri *et al*., 2018).

Seaweed is a multicellular alga containing immunologically active substances. Several studies have shown that seaweed has prospects that are still open for development in the field of disease control. Seaweed extract can increase macrophage chemotaxis activity, stimulates oxygen radical secretion activity, and phagocytosis in peritoneal and splenic murine macrophages (Hou and Chen, 2005). Secondary metabolites such as phenolic compounds from some seaweed can increase shrimp's immunostimulatory activity (Hou and Chen, 2005; Selvin *et al*., 2004; Klongklaew *et al*., 2020).

METHODOLOGY

Place and Time

The research was conducted in July 2020 at the Hatchery Unit, Brackish Water Cultivation Development Center (BPAP) Situbondo, East Java. Seaweed sample *Gracilaria* sp. and Padina sp. purchased from seaweed farmers in Jepara, Central Java.

Research Design

This study aims to analyze the immunostimulant activity of seaweed extract (*Gracilaria* sp. And *Padina* sp.) against vannamei shrimp (*L. vannamei*) infected with *Vibrio harveyi* by observing the nonspecific immune system based on its hematological features, namely by counting the number of hemocytes and phagocytic activity.

Work Procedure

Seaweed Extract Preparation

Seaweed samples were cleaned then dried by shade drying (avoid direct sunlight). The *L. vannamei* shrimp were obtained from the cultivation of BPAP-Situbondo with a weight of ± 15 grams.

The dried seaweed is powdered according to published articles (Kilawati and Islamy, 2019), i.e., the seaweed is cleaned using freshwater then shade-drying for four days. Dried seaweed then ground until seaweed powder obtained. Total of 500 g seaweed powder put into a jar, then macerated using methanol 1: 3 (w/v) for 3x24 hours at room temperature, in three times replication. The maceration solution is then filtered using filter paper (Whatman no. 41), then filtrate and residue obtained. The filtrate then evaporated using a rotary evaporator vacuum at 40°C until a concentrated extract was obtained and calculated the percentage of yield.

A total of 500 grams of seaweed powder is boiled with 2 liters of distilled water for 2 hours, then filtered. The dregs are boiled again, then filtered, and the filtrate is combined with the first extraction results. The extracted solution is evaporated using a rotary evaporator until dry.

Shrimp Feed Preparation

The Commercial shrimp feed (composition: 40% crude protein, 11% water, and 3% fiber) (Ridlo and Pramesti, 2009) is crushed and then added with seaweed extract as much as 10 g/kg of feed, mixed until homogeneous and done pellets used for the test. The shrimp test was acclimatized in a tub equipped with an aeration system and water circulation for 15 days as well as commercial pellet feeding ad libitum (until full), each aquarium containing 15 shrimp (Rodríguez *et al*., 2004). Feeding during

the treatment was carried out 4 times a day, that is, in the morning (05.30), afternoon (11.30), evening (17.30), and evening (23.00) as much as 5% of the bodyweight of the shrimp based on the published method (Esteban *et al*., 2001; Rodríguez *et al*., 2004; Haliman, 2005).

Bacterial Preparation

The isolate of *V. harveyi* was purchased from Jepara Brackishwater Aquaculture Center. Preparation procedure according to the published article (Islamy, 2019). These bacteria were kept in Trypticase Soy Agar (TSA) media at 4°C and sub-cultured Trypticase Soy Broth (TSB) overnight at room temperature before use. There is no added salt in TSB during the research.

Data Analysis

Shrimp hemolymph samples were collected at the base of the pleopod in the abdominal segment, near the genital opening, using a 1 mL syringe moistened with an anticoagulant solution (EDTA 10%). Hemolymph was taken at intervals of 4 days for 12 days, then placed in a sterile microtube and preserved in a cool box. According to a published procedure, determine total hemocyte count (THC) using a hemocytometer (Campa-Córdova *et al*., 2002).

$$\text{Total hemocyte} = \frac{\text{cell counted}}{\text{volume counted}} \text{ x dilution x } 10^6$$

Determination of Phagocytic activity according to the published method (Isnansetyo, 2007). The *V. harveyi* bacterial cells were mixed with formalin solution until all the suspension precipitates were immersed for 24 hours, centrifuged at 1000 rpm for 3 minutes, and the filtrate was separated. A total of 250 μL of hemocytes were mixed with 500 μL of the killed bacterial suspension, then incubated at room temperature for 60 minutes. Phagocytic activity was observed using a 1000 magnification microscope.

$$\text{Phagocytic activity (\%)} = \frac{\text{Number of active phagocytic cells}}{\text{The observed number of phagocytic cells}} \text{ x 100 \%}$$

RESULTS AND DISCUSSION

The immune response of *L. vannamei* shrimp against *V. harveyi* bacterial infection and the effect of immunostimulants application from seaweed can be indicated through the hematological features, namely the total number of hemocytes and phagocytic activity. Hemocytes are a form of cellular defense for the body. Hemocytes can kill infectious agents through the synthesis and exocytosis of microbicidal protein bioactive molecules (Smith *et al*., 2003).

Table 1. The effect of seaweed extract administration on Total Hemocyte Count (THC) of shrimp *L. vannamei.*

Treatment	Total Hemocyte (x 10^7 cell/L)			
	day 0	day 4	day 8	day 12
without immunostimulant	0.323 ± 0.068	0.462 ± 0.151	0.398 ± 0.100	0.547 ± 0.063
Gracilaria sp.	0.541 ± 0.160	0.858 ± 0.321	1.127 ± 0.260	0.311 ± 0.038
Padina sp.	0.561 ± 0.055	0.756 ± 0.203	1.091 ± 0.533	0.352 ± 0.100

Table 1. Phagocytic activity during the study.

Treatment	Phagocytic Activity (%)			
	day 0	day 4	day 8	day 12
without immunostimulant	78.601 ± 2.10	81.216 ± 1.69	90.216 ± 2.16	95.346 ± 1.14
Gracilaria sp.	87.008 ± 3.29	101.447 ± 46.66	85.862 ± 3.64	53.258 ± 1.89
Padina sp.	86.654 ± 3.30	101.308 ± 60.38	89.256 ± 129	63.474 ± 2.14

The results showed that the number of hemocytes and phagocytic activity varied, depending on the treatment time length. The administration of seaweed

extract tends to increase the total number of hemocytes and phagocytic activity up to day 8. The increase in the number of hemocytes is a measure of a substance's ability to stimulate the shrimp immune system. The increased phagocytic activity was characterized by a significant increase in the percentage of phagocytic cells (Pope *et al*., 2011). However, on day 12 the total number of hemocytes and phagocytosis activity decreased faster than the control. On the 8th day, *Gracilaria* sp. shows the highest value compared to *Padina* sp. and control. In contrast, on the 12th day, the extract of *Sargassum* sp. and *Padina* sp. reduced hemocyte and phagocytic activity compared to controls.

Shrimp hematocytes play an important role in immune response, including through recognition, phagocytosis, melanization, cytotoxicity and communication between cells (Johansson *et al*., 2000). It shows that the administration of seaweed extract can increase the total hemocytes and phagocytosis activity of *L. vannamei* shrimp. We assume that the decrease in the number of hemocytes and phagocytosis activity on day 12 is due to the shrimp's immune system having gradually completed its fight against *V. harveyi* bacterial infection. The administration of certain plant extracts containing active compounds from the alkaloid class will increase the resistance of the fish body so that the hematological value of the fish returns to the normal range (Nurjannah *et al*., 2013).

Phagocytosis activity is one of the most important ways of controlling and destroying foreign particles. Through phagocytosis, the immune process is divided into several processes, namely: chemotaxis, recognition, and internalization (Bachère *et al.*, 1995). Hemocytes perform inflammatory-type reactions such as phagocytosis, hemocyte clumping, production of oxygen-reactive metabolites, and microbicidal proteins release (Smith *et al*. 2003).

CONCLUSION

Supplementation of *Gracilaria* sp. and *Padina* sp. at a dose of 10 g/kg of feed can increase the total number of hemocytes and phagocytosis activity of *L. vannamei* shrimp. That treatment was increasing simultaneously depending on the dose and time. The best treatment is *Gracilaria* sp.

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