Effect of *Ulva reticulata* Extract in Increasing Hemocyte Count and Phagocytosis Activity in Tiger Shrimp (*Penaeus monodon*)

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Abstract- Tiger shrimp is one of Indonesia's foreign export commodities. Tiger shrimp has decreased production due to various diseases. *Ulva reticulata* seaweed is known to have polysaccharide sulfate and secondary metabolites that can enhance immunity in shrimp. This study was conducted to analyze the potential of *Ulva reticulata* extract in increasing the immune response in tiger shrimp. The study design used a Completely Randomized Design (CRD) with 4 treatments with 3 replications. As a treatment, mixing *Ulva reticulata* extract on commercial feed with a dose of 0, 0.5, 1.0 and 1.5 g kg^{-1} feed for 14 days. The observation parameters consisted of hemocyte counts and phagocytic activity**.** The results showed the highest hemocytes and the highest phagocytic activity was shown in the treatment of 1.5 g kg^{-1} with total hemocytes of 3.0 x 105 cells/ml and the percentage of phagocytic activity was 77%.

Index Terms- Ulva reticulata, hemocyte count, phagocytosis activity, tiger shrimp

I. INTRODUCTION

Tiger shrimp is one of Indonesia's foreign export commodities. The disease is one of the main causes of the failure of tiger shrimp production. The shrimp immune system depends on non-specific defense processes as a defense against infection (Lee et al., 2004). The first defense against disease in shrimp was carried out by hemocytes through phagocytosis, encapsulation and nodule formation. The activity of phagocytosis can be increased by activating the contradictory oxidase (Pro-PO) system an in semigranular and granular hemocytes (Selvin et al., 2004). One effort to prevent shrimp disease is through enhancing the body's defense system using immunostimulants, vitamins, and hormones (Johny et al., 2005).

Immunostimulants are chemical compounds, drugs or other ingredients that can improve specific and non-specific response mechanisms of fish (Anderson, 1992). The administration of immunostimulants is carried out with the intention of activating non-specific immune systems such as macrophages invertebrates and hemocytes in invertebrates (Dugger and Jory, 1999). One of the natural ingredients that can be used for immunostimulants is seaweed. (Ridlo, 2009). Seaweed is a multicellular alga that contains immunologically active substances. The utilization of seaweed so far is still limited to carrageenan products and agar. The potential for seaweed in the field of disease control is still not widely explored and exploited. Some studies show that seaweed has a prospect that is still open to its development in the field of disease control. Seaweed extract has been known to have acted as an antitumor, increase the activity of chemotaxis macrophage, stimulate oxygen radical secretion activity and phagocytosis peritoneal and splenic murine macrophage (Castro et al., 2004). Secondary metabolites from Halimeda macroloba have anti-fungal bioactive compounds (Widiastuti, 2003). Seaweed *Ulva* sp., Dendrilla sp., Spirulina sp., Enteromorpha sp., Dictyota sp., And Porphira sp. has been shown to increase shrimp immunostimulatory activity (Castro et al., 2004; Selvin et al., 2004).

Ulva reticulata is one type of seaweed that includes green algae. *Ulva reticulata* has natural bioactive compounds which contain the sulfate polysaccharides (Abd El-Baky et al., 2008). Selvin et al., (2004) found that the active compound in the *Ulva* fasciatacould enhance the immunostimulant activity of shrimp. This was indicated by the enhancement of the Total Haemocyte Count (THC) when the *Ulva* fasciata extract was administrated. In addition, Selvin et al. (2011) also reported that the extract of the *Ulva fasciata* could increase the survival rate of tiger prawn against the vibrio infection and the THC. Some studies were also revealed that the composition of polysaccharide from the *Ulva*'s extract could increase the macrophage cell, phagocytic cell, and respiratory burst (RB) of shrimp through the immersion (Castro et al., 2006; Tabarsa et al., 2012).

Hemocytes are a very important factor in the cellular defense system that is non -specific. Increased shrimp body resistance can be seen from the increased phagocytic activity of hemocyte cells. Phagocytosis is a non-specific defense mechanism that can generally protect against pathogenic attacks (Fontaine and Lightner, 1974).

This study was to analyze the potential of *Ulva reticulata* extract in increasing the total number of hemocytes and phagocytic activity in tiger shrimp.

II. THE RESEARCH METHOD

Collection Sample

Samples of *Ulva reticulata* were collected during the low tide along the Punaga coast, Takalar, South Sulawesi Indonesia on 13 December 2018. The algae were washed with cleaned sea water and put into plastic bags before kept in a cool box to prevent photolysis and thermal degradation during transportation.

Sample Preparation

Seaweed samples that have been taken are put into plastic then put into the coolbox and taken to the laboratory for the extraction process. The samples were washed with sea water and sterile freshwater and aquadest. Wet algae were then cleaned from sediments and associated organisms. After the washing process, the sample is drained until it is completely drained and weighed as wet weight. Seaweed samples were sun-dried carefully under shade for 4-6 days. Dry samples of seaweed made powder to increase extraction effectiveness for the extraction process were then weighed again as dry weight.

Extraction of Algae

Extraction of algal materials was conducted as described previously (Kursia., 2013). 100 g of finely powdered algal material was extracted with 200 mL ethanol in a 1 L capacity round bottom flask (1:2, w/v). The extraction was run for 24 h under room temperature. The extracts were filtered through a Whatman no. 1 filter paper then evaporated until 5-10 mL volume.

Experimental animals and acclimation period

The test organisms used in this study were tiger shrimp obtained from the Politehnik Pertanian Negeri Pangkep. The tiger shrimp used was 9 ± 3.3 with 20 shrimp/aquarium. Firstly, tiger shrimp are adapted for three days and maintained in controlled conditions. Shrimp are fed three times a day with a feeding rate (FR) of 4−5% of biomass/day. Water, containers, and maintenance equipment are disinfected first.

Preparation of medicated feeds

Ulva reticulata extract was weighed first based on the dose and dissolved in 100 mL of water. *Ulva reticulata* extract solution is mixed into commercial shrimp feed, then coated with 2.5% egg white and dried again at room temperature, in an open room, protected from direct sunlight (Nurdiansah, 2013). The prepared food is put in a plastic container and stored in the refrigerator until used.

Treatment Schedule

The method used in this study was a completely randomized design (CRD) method with 4 treatments and 3 replications. The treatment given is based on the Declarador., et al. Method. (2014) black tiger shrimp fed with a mixture of *Ulva reticulata* extract of K: 0 g kg-1,(A) 0.5 g kg^{-1}, (B) 1.0 g kg^{-1} and (C) 1.5 g kg^{-1} feed shrimp, all treatments and controls were fed three times a day with FR of 4-5% biomass/day. The provision of treatment lasts for 14 days. Measurement of hemocytes and phagocytosis activity was carried out on the 15th day after treatment.

Total Hemocyte Count

The shrimps' hemolymph was conducted at the pleopod at the abdominal segment near the genital hole by using syringe 1 mL (Xian et al., 2009). Before the hemolymph collection, the syringe was loaded with 0.1 mL of Na-citrate 10% used as an anticoagulant (Vargas-Albores et al., 1993). The calculation of the total quantity of hemocyte (THC) was conducted by using hemocytometer (Abdollahi-Arpanahi et al., 2018).

Phagocytosis Activity

Black shrimp hemolymph is added as much as 0.1 mL into Eppendorf and mixed evenly with 25 µL Staphylococcus sp bacteria and incubated for 20 minutes. Then as many as 5 µL were crushed on a glass object and made a screw preparation. Then fixed with 100% methanol for 5 minutes, then stained with Giemsa (10%) for 15 minutes Preparations are rinsed with running water and dried. Phagocytosis activity is measured based on the percentage of phagocytic cells that show the process of phagocytosis (Anderson dan Siwicki, 1993). The phagocytic activity is calculated by a formula :

$$\mathrm{AP} = \frac{the\ quantity\ of\ phagocyte\ cell}{the\ quantity\ of\ observed\ phagocyte\ cell}\ x\ 100$$

Data analysis

Analysis of Total Haemocyte Count (THC) data and Phagocytosis Activity on shrimp were analyzed by ANOVA, and if there were differences between treatments followed by the Tuckey Test.

III. RESULTS

The body defense system of tiger shrimp (Penaeus monodon) on the administration of seaweed extract *Ulva* retuculata is indicated by the number of hemocytes and on the phagocytic activity. Hemocytes are one form of cellular defense. Hemocytes play a role in the process of phagocytosis, encapsulation, degranulation, and nodular aggregation of foreign pathogens and particles as well as the production and release of prophenoloxidase (proPO) in the immune system of crustaceans (Sahoo et al., 2008). The hemocytes counts in crustaceans are very important in maintaining resistance to pathogens. Immunoreactive factors such as peroxinextin, antibacterial peptides, and clotting components are stored in the hemocytes, so an increase in the number of hemocytes is a measure of the ability of a substance to stimulate the body's defense system of shrimp.

The results showed that the number of hemocytes given *Ulva reticulata* extract gave higher results than control. The results of the variance analysis and further testing of total hemocytes differed significantly ($P < 0.05$) between treatments and controls.

The administration of *Ulva reticulata* Extract 1.5 g kg^{-1} showed a higher total hemocyte value compared to other Johansson et al. (2000), shrimp hemocytes play an important role in immune responses including recognition, phagocytosis, melanization, cytotoxicity, and inter-cell communication. The results of the above research are in accordance with the research of Selvin et al. (2004) which states that *Ulva* sp extract can

increase the total hemocytes of tiger shrimp. Similar research that has been done shows that the shrimp immune response can be improved by the application of sulfate polysaccharides contained in *Ulva* sp. Sakai (1999) Sakai (1999) states that the ability of immunostimulants to enhance the immune response and develop protection against pathogenic infections is influenced by the dose of the application. Giving immunostimulants at concentrations below the minimum value for the occurrence of an immune response does not have an effect on increasing the number of hemocytes.

Brown (2000), states that increasing immunity can be known from the increase in phagocyte cell activity from hemocytes, namely the ability of immune response cells to phagocytosis of disease agents that enter the body.

The results showed that the phagocytosis activity given *Ulva reticulata* extract gave higher results than controls. The results of the variance analysis showed that the addition of *Ulva reticulata* extract had a significant effect (P <0.05) on the phagocytosis activity of tiger shrimp.

The administration of *Ulva reticulata* extract in feed at a dose of 1.5 g kg^{-1} showed the best results with a phagocytosis activity of 77% compared to other treatments.

Increasing the phagocytosis activity can be indicated that the addition of *Ulva reticulata* extract in feed can stimulate or enhance the immune system in shrimp. According to Suleman et al (2018) stated that the content of sulfate polysaccharides contained in extracts from *Ulva* sp was able to increase the phagocytic activity by 53% in vannamei shrimp.

Table 1. Total Haemocytes Count of Tiger Shrimp After Feeding Treatment with *Ulva reticulata* Extract

Treatment	Mean ($x10^5$sel/ml)±SD
K (0 g kg^{-1})	1,3±0,15[a]
A (0,5 g kg^{-1})	1,9±0,82[a]
B (1,0 g kg^{-1})	2,0±0,57[a]
C (1,5 g kg^{-1})	3,0±0,75[b]

Data (mean ± SD) at the same time of observation with different letters show significant differences in results (p <0.05)

Tabel 2. Percentage of phagocytosis Activity of Wind Shrimp After Treatment of Feeding with *Ulva Reticulata* Extract

Treatment	Mean(%)±SD
K (0 g kg^{-1})	43±8,1[a]
A (0,5 g kg^{-1})	48±10,7[a]
B (1,0 g kg^{-1})	70±8,5[b]
C (1,5 g kg^{-1})	77±6,0[b]

Data (mean ± SD) at the same time of observation with different letters show significant differences in results (p <0.05)

Phagocytosis activity is a very important way of controlling and destroying foreign particles. The defense process through phagocytosis is divided into several processes, chemotaxis, recognition, and internalization (Bachere, 1995). According to Smith et al. (2003), hemocytes perform an Inflammatory-type reaction such as phagocytes, hemocyte clumping, production of oxygen-reactive metabolites and microbicidal release of proteins.

The process of phagocytosis begins with attachment (ingestion) and ingestion (ingestion) of microbial particles into phagocytic cells. Phagocytes cells then form digestive vacuole (digestive vacuole) called phagosomes. Lysosomes (granules in phagocyte cytoplasm) then fuse with phagosomes to form phagolysosomes. Subsequent microorganisms are destroyed and microbial debris is excreted from the cell through the egestion process Destruction of phagocytic microbial particles involves the release of enzymes into phagosomes and the production of ROI (reactive oxygen intermediate) now called respiratory burst (Rodriquez and Le Moullac, 2000 in Manoppo, 2011

Dugger and Jory (1999) said phagocytes of hemocytes are one of the nonimmune systems specific to shrimp. These cells recognize stimulants depending on the type of surface of the molecules and carbohydrates found on the surface of pathogens and how these molecular types differ from the surface of the host cell. Cell recognition itself includes a number of complex structures on the surface of the host cell where the hemocytes can recognize and interpret.

IV. CONCLUSION

Based on the results of research feeding with the addition of *Ulva reticulata* extract, the cellular immune response increased in the form of total hemocytes and phagocytosis activity in tiger shrimp. The best results for the number of hemocytes and

phagocytosis indices are indicated by the addition of *Ulva reticulata* extract at a dose of 1.5 g kg^{-1} feed.

V. SUGGESTION

Further research to obtain a pure extract of Ulva reticulata as immunostimulants in shrimp farming.

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