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Immunomodulatory Potential of *Eucheuma serra* as Haemocyte Cell Production Enhancer on *Litopenaeus vannamei*

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ABSTRACT

Litopenaeus vannamei is one of the most well-known fishery products and has high market value. In contrast, production of vannamei shrimp is facing threats from several diseases caused by bacteria, virus, and even parasites that attack the shrimp immune system. The purpose of this study is to identify the immunomodulatory effect of *Eucheuma serra* extract to enhance haemocyte cells production in *Litopenaeus vannamei* as the non-specific internal immune substance. The research method used was a complete randomised design with 5 concentrations of injected *Eucheuma serra* extract treatments and one placebo as control. *Eucheuma serra* extract administered on shrimp abdomen and the haemocyte cell samples collected from shrimp on day 0 and day 6. Treatment using 8 ppm injected *Eucheuma serra* extract showed the highest haemocyte cell amount increase about 15.90 million cells/ml in 6 days after injection. Statistical calculation using ANOVA test showed a significant difference in the amount of haemocyte cell at before and after treatment.

Keywords: Eucheuma serra, haemocyte cell, immunomodulator, *Litopenaeus vannamei*

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INTRODUCTION

Crustacean aquaculture represents a major industry in tropical developing countries (Vazquez et al., 2009). *Litopennaeus vannamei* (white leg shrimp) is an important aquaculture species (Sirirustananun et al., 2011). In Indonesia, *L. vannamei* has a major market. Its production volume increased 99.27% from 206.6 tonnes in 2010 to 411.7 tonnes in 2014.

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Shrimp farming has also led to prevalence of disease. Crustaceans are affected by opportunistic pathogens causing huge economic losses (Vazquez et al., 2009). Shrimp farming has suffered from problems linked to deteriorating pond environments, subsequently resulting in stress-induced disease incidences mainly of viral and bacterial aetiologies (Sirirustananun et al., 2011). Commercial shrimp farming has faced disease outbreaks, especially white shrimp *L. vannamei*, such as *Vibrio alginolyticus*, *Vibrio damsela*, *Vibrio harveyi*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*, infectious Myonecrosis Virus (IMNV), Monodon Baculovirus (MBV), White Spot Syndrome Virus (WSSV), Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV), Yellow Head Virus (YHV) and Taura Syndrome Virus (TSV). In Taiwan, the total farmed production of *L. vannamei* shrimp declined almost 90%, from 82,598 tonnes to 8,878 tonne, in the space of 15 years (Cheng, Liu, Yeh, & Chen, 2004).

There is also evidence of *L. vannamei* infected with WSSV, TSV, IHHNV and IMNV in several regions in Indonesia. Lampung, West Java, East Java and South Sulawesi are the regions which are exposed to various types of infection. The IMNV has led to death of 60% of the shrimps (Sugama, Novita, & Koesharyani, 2006). *V. alginolyticus* bacterium isolated from infected *L. vannamei* is known to cause high mortality rate among shrimp in stressful environments (Wang & Chen, 2005). The WSSV is considered to be an extremely virulent pathogen and may cause death within a few days post infection (Chou, Huang, Wang, Chiang, & Lo, 1995).

Physical barriers are the biggest challenge to prevent pathogenic microorganisms in most invertebrates (Vazquez et al., 2009). Invertebrates do not possess a specific, adaptive immune system such as found in vertebrates. In the absence of lymphocytes and functional antibody, they have been traditionally thought to rely instead entirely on innate or non-specific immunity for internal defence against parasites and pathogens (Rowley & Powell, 2007; Wei et al., 2012). Haemocyte cell in shrimp has similar function with leukocyte in vertebrate as non-self matter recognition and elimination (Sritunyalucksana & Soderhall, 2000). Circulating haemocytes are generally classified into three types: hyalinocytes, semi-granulocytes and haemocytes with refractile granules. Haemocyte common defence mechanisms include phagocytosis and encapsulation of extrinsic fungal spore, yeast and organisms (Cheng et al., 2004; Vazquez et al., 2009). Other defence mechanisms of haemocytes are activated depending on the pathogen characteristics, such as prophenoloxidase and lectin as sugar-binding properties of crustacean proteins (Vazquez et al., 2009).

Extensive use of human antibiotic drugs in shrimp farming as preventive purpose is potentially damaging and the residues can still enter contaminate the environment and enter the human food chain (Cabello, 2006). Moreover, the European countries have strict regulations on residues of antibiotics

in shrimp product (Food and Agricultural Organization of the United Nations, 2006). Therefore, proper immune modulators for shrimp are important (Pope et al., 2011).

MATERIALS AND METHODS

Materials

Materials used were acetone (Merck, Darmstadt), ammonium sulphate (Merck. Darmstadt), ethanol (Merck, Darmstadt), phosphate buffer saline pH 7.4 / PBS (Brataco Chemica, Jakarta), sea water (Surabaya, Indonesia) and sodium ethylenediaminetetraacetic (Merck, Darmstadt).

Animal and Plant Materials

Litopenaeus vannamei shrimp was obtained from a shrimp farmer in Gresik, Indonesia. *Eucheuma serra* was collected in Pandawa Beach, Bali, Indonesia.

Eucheuma serra Extraction

E. serra was dried inside a closed and clean room. Dry *E. serra* was later powdered and weighed at 30 g. Phosphate buffer saline pH 7.4 solution was dissolved into the dried *E. serra* powder with a ratio 2:1. The solution was stirred using homogeniser for 2 hours at 4°C and then centrifuged at 3200×g for 15 minutes. Supernatant was collected then added with acetone 1:1. The solution was centrifuged at 3200×g in 15 minutes. The pellet was collected as crude extract of *E. serra.* The yield of extract is 10 g.

Lectin concentration in *E. serra* crude extract is determined using spectrophotometry method for protein analysis at wavelength λ 540 nm. Protein standard solution was used for quantitative analysis as external standard. The lectin concentration in *E. serra* crude extract is 5.60% or 2.58 ppm.

Litopenaeus vannamei Treatment

L. vannamei shrimp was acclimatised in aquarium with controlled salinity water at 15 ppt and temperature at 29°C for 48 hours. During acclimatisation period and treatment process, the subject was fed using commercial shrimp food three times a day. Probiotic was given only once in three days. The treatment aquarium was cleaned every day to reduce stress impact on shrimp and avoid contamination of ammonia from shrimp waste.

The treatment was conducted using completely randomised design consisting of five treatment groups and a control group (Table 1). Each group was replicated four times. Each replication contained four *L. vannamei*.

Haemolymph Collection and Total Haemocyte Count

Haemolymph was collected (0.50 ml) at fourth segment of the shrimp abdomen using disposable syringe and later stored inside an Eppendorf tube which already contained Na-EDTA in cold temperature storage. Haemocytometer was used for Total

Table 1
Prerequisite of dosage injection on treatment subject

Group	Treatment
Control (G0)	0.50 ml injection of PBS
Treatment 1 (G1)	0.50 ml injection of 2 µg/ml extract solution
Treatment 2 (G2)	0.50 ml injection of 4 µg/ml extract solution
Treatment 3 (G3)	0.50 ml injection of 6 µg/ml extract solution
Treatment 4 (G4)	0.50 ml injection of 8 µg/ml extract solution
Treatment 5 (G5)	0.50 ml injection of 10 µg/ml extract solution

Haemocyte Count (THC) of haemolymph of each group. Blank haemocyte (H0) was collected from haemolymph before lectin was injected. Final haemocyte result for THC was collected from haemolymph after six days (H6).

Survival Rate Count

Survival rate was counted as percentage of shrimps still available until the end of treatment compared with the amount before the treatment. Survival rate count was conducted in every treatment and control group.

Statistical Analysis

Analysis of Variance (ANOVA) statistical test was conducted to test the differences between the results.

RESULTS AND DISCUSSION

Total haemocyte count (THC) measures innate immunity and non-specific immune activity of invertebrates (Cheng et al., 2004). THC before treatment was calculated as average from all group. While THC after treatment was calculated as average haemocyte on a shrimp in each treatment group. According to THC result (Figure 1), shrimp in Group 4 (G4) via treatment with 8 µg/ml extract injection, had the highest haemocyte cell increase compared with another treatment group, including control group (G0). Haemocyte cell amount in G4 increased 15.29 million cells/ml from 3.16×10^6 cells/ml at H0 to 18.45×10^6 cells/ml at H6. Other treatment groups also had various increase in haemocyte cell. The lowest increase was group 3 (G3), which saw an increase of 9.88 million cells/ml from H0.

Control group also showed a slight increase in haemocyte after six days, even though the subject did not get any lectin treatment injection. The haemocyte number increase in control group was caused by the influence of substances and nutrition from feed and probiotic which was given to all of subject (Olmos, Ochoa, Michael, & Contreras, 2011). ANOVA test give the result of F calculation (F_{calc}) as 19.04 and F table 0,01 ($F_{tab\ 0.01}$) as 4.25. The higher value of F_{calc} compared with $F_{tab\ 0.01}$ shows the significant difference of *L. vannamei* haemocyte amount at before and after treatment using lectin extract solution via

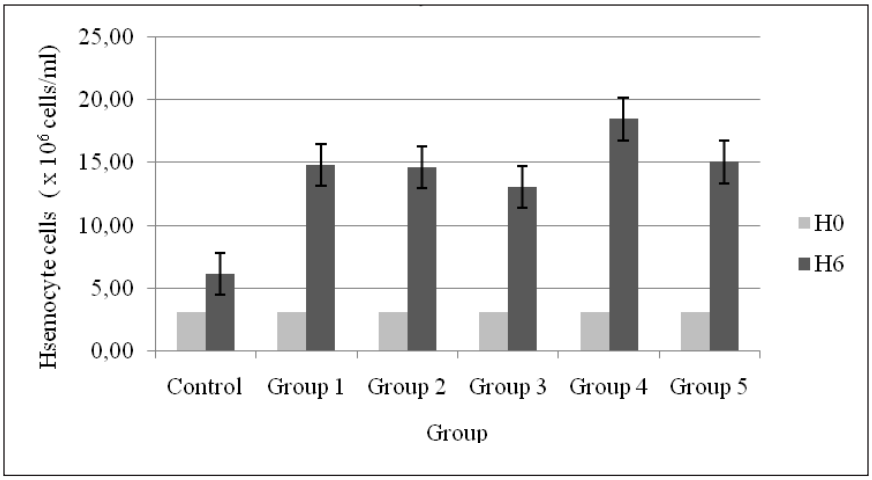

Figure 1. THC comparison of each treatment at H0 and H6

injection method. The group with the highest survival rate is G0 (87.50%) in control group and the lowest is G5 with 56.25%.

Based on the THC result of treatment groups, *E. serra* extract could be considered as the novel treatment as immunomodulator agent for *L. vannamei*. The increase in haemocyte cell means the innate immunity system has become stronger to encounter infectious pathogens, such as virus and bacteria. *E. serra* extract is more acceptable than using antibiotic to deter microorganism growth in pond.

E. serra gives high yields of isolectins - ESA–1 and ESA–2. The yield could be up to 1% from powdered algae with 0.1% as ESA–1 and 0.9% as ESA–2. *E. serra* could be a valuable source of lectin molecules that work even at relatively high temperatures and over a wide pH range that have preferential affinity for glycoproteins bearing high mannose–type N–glycans (Hori et al., 2007; Kawakubo, Makino, Ohnishi, Hirohara, & Hori, 1997). Lectin is defined as carbohydrate-binding proteins of non-immune origin that agglutinate cells or as carbohydrate-binding proteins other than antibodies or enzymes. It is a group of non-immunogenic proteins possessing at least one noncatalytic domain that binds reversibly to specific carbohydrates (Janeway & Medzhitov, 2002; Teixeira et al., 2012). Lectin has been regarded as primary candidates for pattern recognition receptors in animal innate immunity due to its ability to bind to specific carbohydrates on the surfaces of microorganisms (Zhao et al., 2009). Lectin can specifically recognise the carbohydrates from the membrane or surface of cell. Moreover, it is able to induce agglutination of these cell or can lead to diverse cellular events, such as phagocytosis (Marques & Barracco, 2000).

The carbohydrate-binding profile of ESA–2 was examined as 45 different complexes. N-acetyl glucosamine (GlcNAc) is related in 35 different complexes and N-acetyl galactosamine (GalNAc) takes

part in 4 different complexes (Hori et al., 2007). Lectin with structural characteristics and specificity has been identified in *L. vannamei*, known as C-type lectin (Ma, Tin, He, & Chan, 2007; Vazquez et al., 2009). C–type lectins has ligand binding specificities for carbohydrates, such as GlcNAc, GalNAc, sialic acid and lipopolysaccharide, indicate antimicrobial activity against several bacteria and fungi, also in response to WSSV (Vazquez et al., 2009; Zhao et al., 2009). The similarity of ligand binding carbohydrates of ESA–2 and C–type lectin is necessary for increase the immunity on *L. vannamei.*

Furthermore, immune memory in invertebrates that is referred as immune priming or specific immune priming, appears to be passed from brood stock to offspring (Pope et al., 2011). *L. vannamei* subjected to treatment and has an increase of haemocyte cell can be maintained as particular brood stock called Specific Pathogen Free (SPF). *L. vannamei* SPF can produce progeny that have can resist pathogens (Sugama et al., 2006).

CONCLUSION

L. vannamei is vulnerable to infection by pathogen microorganisms, such as virus and bacteria. *E. serra* extract contains lectin which serves as immunomodulator for *L. vannamei* to provide an innate immunity. *E. serra* extract as injection treatment for *L. vannamei* leads to a significant increase in haemocyte. Treatment of 8 µg/ml extract injection enhances haemocyte production as much as 15.29 million cells/ml in six days. Lectin from *E. serra* is a novel solution to enhance immunity of *L. vannamei* against pathogens.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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