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Administration of Hot Water Extract Diatomae *Caetoceros Ceratosporum* via Injection Enhances the Immune Resistance of White Shrimp *Litopenaeus vannamei* against Infectious Myonecrosis Virus

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Abstract

Immune resistance of white shrimp against disease can be enhanced through the administration of immunostimulant. Some of sea microalgae extracts has been proved trough examination to be able to stimulate immune system of shrimp. The objective of this study is to know immune response of *Litopenaeus vannamei* injected with hot water extract *Chaetoceros ceratosporum* (HWEC) doses of 5, 10, 15 and 20 $\mu g \cdot g^{-1}$ body weight (BW) against Infectious Myonecrosis (IMNV) virus infection. The findings show that *C. ceratosphorum* administration can enhance immune response of *L. vannamei* infected by Infectious Myonecrosis virus, indicated by the increasing number of hemocytes (THC), total

protein plasma (TPP), phenolokxidase (PO) activity, and respiratory burst (RB) activity. Highest immune response is achieved by injection dose of 15 $\mu g \cdot g^{-1}$ shrimp body weight. The increase of shrimp immune response leads to the increase of shrimp immune system against IMNV infection, so the survival rate of *L. vannamei* infected by IMNV is higher than positive control and similar to those of negative control.

Keywords: Shrimp immune response, Extract *Chaetoceros ceratosphorum*, *Infectious Myonecrosis*, *Litopenaeus vannamei*

1. Background

Main problem to the shrimp culture failure is disease attack caused by parasites, viruses and bacteria. One of the efforts done for disease control in the shrimps culture is by disease prevention by means of increasing shrimp immune system.

Increasing shrimp immune system against disease could be done through immunostimulant administration (Treves-Brown, 2000), one of them using polysaccharide compound. Exopolysaccharide sulfate is a polysaccharide produced by microalgae and applied as anti virus agent, healthy food, antioxidant, anti inflammation and as part of immunomodulatory system (Raposo *et al*., 2013).

Microalgae diatomae contains polysaccharides with a wide structural rate and can be explored as an active biological content (Skjermo *et al*., 2006). Polysaccharides extract from *C. mulleri* could help enhance immune system of *Gadus morhua* L. (Skjermo *et al*., 2006). Polysaccharides extract *A. orientalis* could help increasing immune response and survival rate of shrimps against infection of White spot syndrome virus (Manilal *et al.*, 2009). Polysaccharides produced by Arthrospira plantesis and Porphyridium purpureum resists anti virus strain activity of *V. virus* dan *E. virus* (Radonic *et al.*, 2010).

Chaetoceros sp. diatomae was known to have a high antioxidant activity, more than 90% (Natrah, 2007), and produce Hexadecatrienoic-6,9,12-acid and Hexadecadienoic-9,12-acid (Wang, 1999). Whole cell *C. ceratosporum* in woof can indirectly increase immune response of tiger shrimp, *C. mulleri* contains β-D-(1-3)-glucan while *C. debilis* contains β-D-(1-3,1-6)-glucan. β-glucan is an imunostimulative polysaccharide derivative. How the injection of hot water extract *C. ceratosporum* (HWEC) influences immune response of *L. vannamei* infected by IMNV is not yet known. Therefore, the objective of this research is to know immune response change of *L. vannamei* injected with HWEC against IMNV infection, covering change in THC (total haemocyte count), TPP (total protein plasma), phenoloxidase (PO) activity, respiratory burst (RB) activity and shrimp survival rate (SR).

2. Research Metododology

2.1 Preparation for C. ceratosporum Extraction

Pure isolate of microalgae *C. ceratosporum* is obtained from Center for Aquaculture Research and Development, Gondol. Mass culture was done in the Natural Woof Laboratory of Brackish Water Aquaculture Development Center Situbondo by referring to the method of Haryanti *et al*., (1992). *C. ceratosporum* extraction followed a procedure introduced by Hayashi *et al.,* (1993). *C. ceratosporum* starch is dissolved in deionized hot water (1:5) and allowed to stand for 1-3 hours, then filtered. Filtrate obtained is centrifuged at 800 g for 10 minutes or vacuum evaporated and dried. Extract obtained is known as Hot Water Extract *C. ceratosporum* (HWEC). HWEC extract obtained is re-hydrated with phosphate buffer saline (PBS) liquid, saved in the bottle and kept at 4 ºC.

2.2 Preparation for IMNV Extract

IMNV extraction for artificial transmission is carried out by referring to the method by

Nuraini (2008) which is modified version of Hason *et al.,* (1995) and Anonymous (2003). *L. vannamei* shrimp known to have been positively infected by IMNV (result analysis with RT-PCR), is crushed with hammer/grinder then added with 2 parts of TN buffer, then centrifuged with 300 rpm speed for 10 minutes at 4 ℃. Supernatant obtained is taken then re-centrifuged with speed of 14.000 rpm for 30 minutes at 4 ℃. Supernatant is taken then filtered with miliophore 0,45 µm, followed by miliophore 0,20 µm. Extraction resulted is ready to use or keep in deep freezer – 80 ℃.

2.3 Research Design

White shrimp *L. vannamei* used is obtained from a pond of Brackish Water Aquaculture Development Center Situbondo and have passed Specific Pathogen Free (SPF) test, with 11,08 ± 1,53 g weight. Shrimps are kept in the 18 plastic containers of 60 L capacity filled with 40 L of sterile sea water with salinity range 32 - 34 pro mil and aerated, each container contains 16 shrimps. Before treatment, shrimps is acclimatised for 7 days and given commercial woof four times a day as much as 3% of biomass weight.

After acclimatisation, each shrimp is injected with *C. ceratosporum* extract in the second segment of ventral abdomen with dose of 5, 10, 15, and 20 $\mu g \cdot g^{-1}$ body weight. Positive control is used as control without extract administration with challenge test and negative control without extract administration and without challenge test so that there will be 6 treatments, each of them is performed three times. 7 days after injection of extract, shrimps are intramuscularly infected with extract IMNV as much as 0,01 $ml \cdot g^{-1}$ body weight as performed by Nuraini (2008).

Parameter observation of immune response is performed 6 days after HWEC injection and seven days after infection of IMNV. Shrimps are continued to be kept till 30 days of age. In the end, it is done observation on shrimp survival rate.

2.4 Measurement Parameter of Immune Response

Calculation of THC is performed by referring to a method by Van de Braak (1996) by taking shrimp hemolymph as much as 50 µl then colored Trypan blue solution as much as 50 µl. Number of hemocyte is calculated by using hemocytometer supported with a light microscope.

TPP calculation performed with reference to a method of Van de Braak (1996) by measuring the absorption of plasma protein added to reagent $CuSO_4$ and folin lowry using spectrophotometer at a 660 nm wavelength and converting it with standard liquid bovin albumin serum (BSA).

Calculation of phenoloxidase activity followed the procedure introduced by Hernandez-Lopez *et al*. (1996). Phenoloxidase activity is expressed as a formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) in 50 µl hemolymph in spectrophotometry at a wavelentgh of 490 nm

Anion superoxide test is performed to observe respiratory burst (RB) activity. Measurement of RB activity using nitro blue tetrazolium (NBT) reduction method follows a procedure of

Citarasu *et al*. (2006).

Survival rate indicator is calculated in the end of observation phase by calculating number of survived shrimps in each of plastic container compared to the shrimp number in the beginning of observation phase which is stated in percentage (%).

3. Result and Discussion

3.1 Total of Hemocytes

Total of shrimp hemocyte *L. vannamei* is presented in histogram on Figure 1.

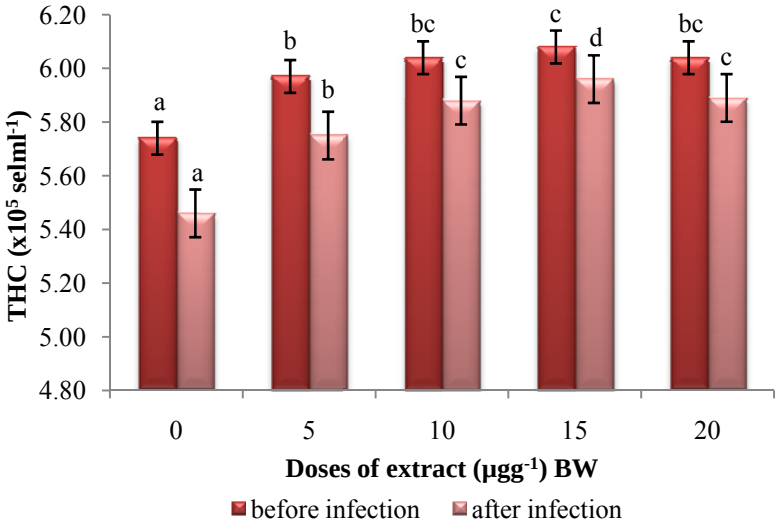

Figure 1. Histogram of average THC of shrimps *L. vannamei* injected with HWEC by doses of 5, 10, 15 and 20 µg·g^{-1} BW before and after IMNV injection

The findings show increase in total of hemocytes of shrimp injected with *C. ceratosporum* extracts as for the extract contains polysaccharides. Extracts containing polysaccharides fraction of some brown algae species has an efficient ability to increase immune response or resistance against disease in the aquatic animal culture (Chotigeat *et al.*, 2004). Polysaccharides from sea microalgae like *Porphyridium*, *Phaeodactylum*, and *C. stigmatophora*, showed pharmacological activity such as anti inflamation activity and an activity as an imunomodulator (Raposo, et al., 2013).

Increasing total of hemocyte indicated increase in the immune response of shrimp caused by polysaccharides extract *C. ceratosporum* administration. Injection of foreign substances like alginate, carrageenin, or hot water extracts of seaweed polysaccharides can increase total hemocytes of shrimps *L. vannamei* (Fu, *et al*. 2007; Cheng, *et al*., 2004; Yeh & Cheng, 2008). An imunostimulant from sea algae extracts *Gracilaria verrucosa* increased total hemocyte of tiger shrimp *P. monodon* compared to control (Maftuch, *et al*., 2012).

Hemocytes are cells in hemolymph. The cells have an important role in shrimp immune

response against disease (Van de Braak, 2002; Citarasue *et al.*, 2006). Hemocyte is a main component in invertebrate immune system circulated in hemolymph, taking a part as guardian soldier which always be ready to respond pathogenic attack or wounded tissue (Battinson *et al*., 2003; Aladaileh *et al*., 2007)). Total of hemocytes could influence the ability of host cells to fight against foreign materials and varied responses toward infection (Johanssons *et al*., 2000).

3.2 Total of Plasma Protein

Total of plasma protein is presented in histogram as seen on Figure 2.

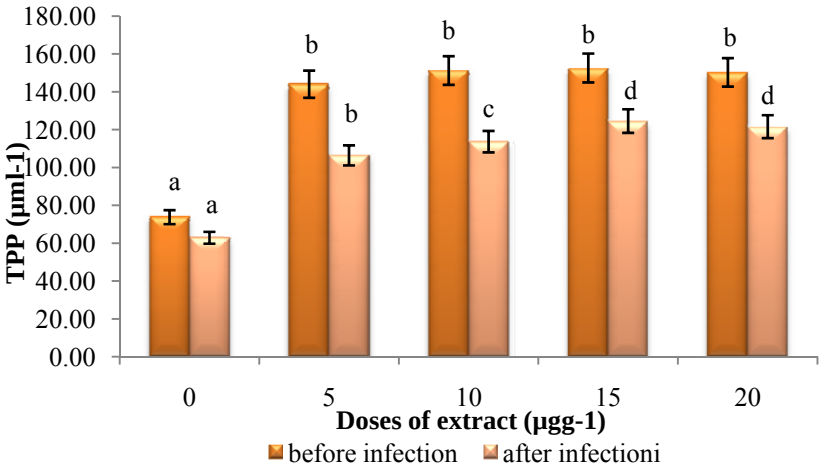

Figure 2. Histogram of average TPP of shrimp *L. vannamei* injected with HWEC by doses of 5, 10, 15 and 20 $\mu g \cdot g^{-1}$ BW before and after IMNV injection

The finding shows that HWEC administration can increase TPP of shrimps *L. vannamei* before and after infection of IMNV. This is consistent with the finding of research by Ekawati (2011), administration of whole cell *C. ceratosporum* in woof increased TPP of tiger shrimp *P. monodon* compared to control, and showed a decrease in TPP after infected by *V. harveyi*. Total of shrimp plasma protein soaked in *G. tenuistipitata* extract restored to the initial condition faster compared to control, before and after temperature change stress treatment and infection of *V. alginolyticus* (Yeh *et al*. 2010). Total of hemocyte and TPP in shrimps fed with β-glucan is lower than 30-50% compared to control in the day-7 after WSSV infection, but restored in 14 days (Ghaednia *et al*. 2012).

3.3 Phenoloxydase Activity

Phenoloxydase Activity of shrimp *L.vannamei* injected with HWEC of varied dose served on Figure 3.

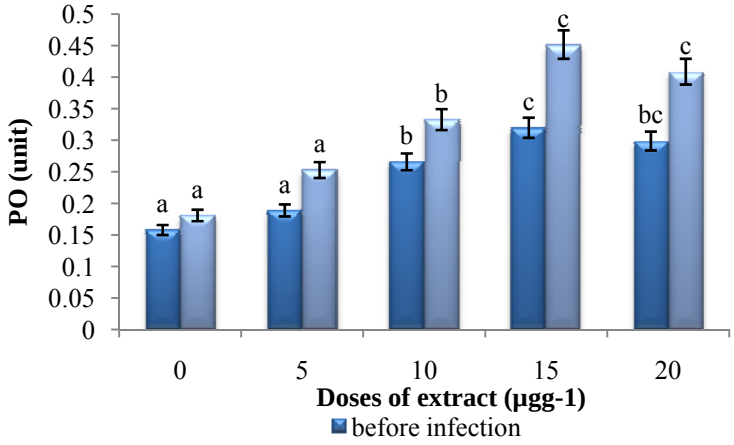

Figure 3. Histogram of average PO activity of shrimp *L. vannamei* injected with HWEC by doses of 5, 10, 15 and 20 µgg^{-1} BW before and after IMNV injection

The findings show that HWEC can stimulate hemocytic degranulation and activates proPO to become PO. PO increase will lead to the increase of shrimp ability to recognize strange materials as proPO plays an important role in the introductory and safety system toward strange materials. Increase of PO activity after HWEC and IMNV injection because of hemocytic proliferation to phagocyte pathogen. Injection of foreign particles like alginate, carageenin, or hot water extract of seaweed polysaccharides significantly increased total of hemocytes, PO and RB activity in white shrimp *L. vannamei* (Fu *et al*., 2007; Cheng *et al*., 2004; Yeh and Cheng, 2008).

3.4 Respiratory Burst (RB) Activity

RB activity with HWEC administration via injection can increase RB activity of shrimp *L. vannamei* compared to control without HWEC as seen on Figure 4.

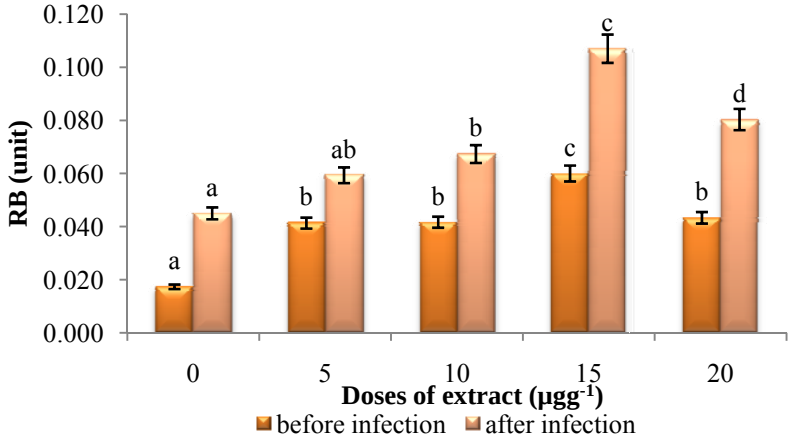

Figure 4 Histogram of average RB activity of shrimp *L. vannamei* injected with HWEC by doses of 5, 10, 15 and 20 µgg^{-1} BW before and after IMNV injection

HWEC administration intra muscularly increases activity of RB in shrimp *L. vannamei*. *L. vannamei* administered with *S. duplicatum* extract by means of immersion or injection both increased THC, PO and RB activity (Yeh *et al*., 2006). Increase in RB indicated activation of phagocytosis system (Yeh & Chen, 2009).

3.5 Survival Rate of L. vannamei

Difference in dose administration of *C. ceratosporum* extract through intra muscular method significantly influences the survival rate of shrimp *L. vannamei* after infected with IMNV ($p<0,05$). As shown on Figure 5.

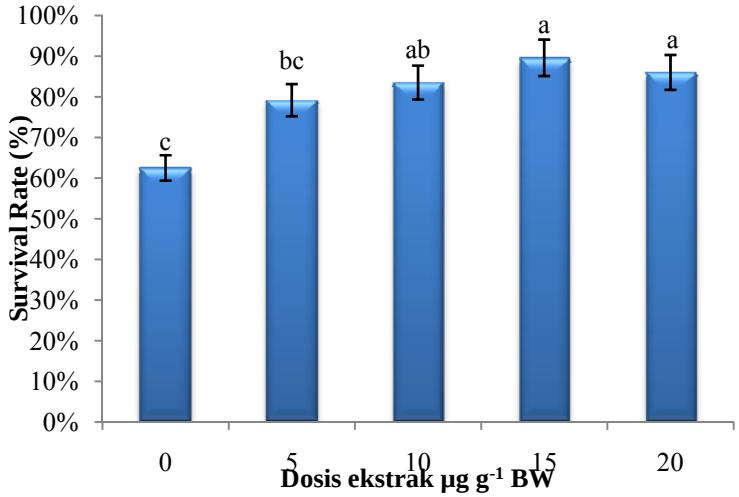

Figure 5. Histogram of average survival rate of *L. vannamei* in the end of research

Research findings show that injection of *C. ceratosphorum* extracts can enhance immune response of *L. vannamei.* Hot water extracts of some algae species are known to be able to increase immune in fish and shrimp against pathogenic infection. Administration of hot water extracts from some species of red algae or brown algae was reported potential to enhance resistance of *C. carpio* and *S. quinqueradiata* against *E. tarda* and *Streptococcus* sp. (Fujiki *et al*., 1992); *L. vannamei* immersed or injected with hot water extract *G. tenuistipitata* (Hou *et al*., 2005) or *S. duplicatum* (Yeh *et al., 2006)* experienced enhancement in their immune system and resistance against infection of *V. alginolyticus. L. vannamei* immersed in sea water containing hot water extract *G. tenuistipitata* could accelerate immue recovery of *L. vannamei* after injected with *V. alginolyticus* (Yeh *et al*., 2009).

Hot water extract *G. tenuistipitata* contained 30% of glucose with galactose as a main component (Chao *et al*., 1999) which is on of polysaccharide derivatives. Polysaccharide compound or its derivatives could be produced from extraction process by utilizing water (Yeh *et al., 2006;* Hou *et al*., 2005), HCl (Manilal *et al.*, 2009) and so on. Storseth *et al*. (2004, 2005) has proved the existence of β-D-(1,3)-glucan structure in *C. mulleri*, while Storseth *et al*., (2006), found β-D-(1,3; 1,6)-glucan stucture in *C. debilis.*

As already known, some of above researches found that polysaccharide resulted from extraction using water or other solvents could significantly enhance immune response and resistance of shrimp against pathogen. Administration of sodium alginate from brown algae extract *M. pyrifera* and *L. nigrescens* could enhance resistance of *L. vannamei* against infection *V. alginolyticus (*Cheng *et al., 2004,* and Cheng*, et al.,2005). C. mulleri* extract could help increase survival rate and growth of Cod fish flyblow (Skjermo, *et al*., 2006). *C. calcitrans* enriched with 20:5(n-3) and 20:4(n-6) had a positive impact on *total haemocyte count* (THC), granulocyte percentage, fagocytosis rate and oxydative activity in hemocyte of scallop species *C. gigas* and *R. phillipinarum* compared to the use of *Isochrysis* sp. and *T. suecica* (Delaporte *et al*., 2003).

β-glucan has a positive impact in enhancing resistance of farming animal against harmful disease WSSV. Optimum oral administration of β-glucan at the level of 10 g kg-1 feeding for effective time of 20 days stimulated immune system and enhanced survival rate of *F. Indicus* infected by WSSV (Ghaednia *et al*., 2012). The findings of a research by Hemtanon *et al.,* (2005) showed that *S. plantesis* effectively prevented infection of WSSV and *V. harveyi* in the larvae and juvenile of *P. monodon*. *S. plantesis* extract produces novel isolation of polysaccharide sulfate called *calsium spirulan* (Ca-SP) as an antiviral.

Sea polysaccharide could resist replication of virus by some ways, by disrupting life cycle of virus of different phases or by increasing antiviral immune response of host to accelerate eradication process of virus (Wang *et al*., 2012). By resisting virus replication, it will automatically non-activate virion before virus infection.

3. Conclusion

The finding shows that *C. ceratosphorum* extract administration can enhance immune response of *L. vannamei* infected by Infectious Myonecrosis virus, indicated by the increasing number of hemocytes (THC), total protein plasma (TPP), phenoloksidase (PO) activity, and respiratory burst (RB) activity. Highest immune response is achieved by injection dose of 15 µg.g-1 shrimp body weight. The increase of shrimp immune response leads to the increase of shrimp immune system against IMNV infection, so the survival rate of *L. vannamei* infected by IMNV is higher than positive control and similar to those of negative control.

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