

Ulvan extract acts as immunostimulant against white spot syndrome virus (WSSV) in juvenile black tiger shrimp *Penaeus monodon*

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Abstract. Sixty *Penaeus monodon* shrimps (2.39 ± 0.38 g) were divided into fifteen 250-L fibreglass tanks, fed with diets containing 0.0 (control diet), 500, 1,000 or 1,500 mg purified ulvan kg^{-1} diet for 14 days and were subjected to a white spot syndrome virus (WSSV) challenge test, total hemocyte count (THC), respiratory burst activity (RBA) and phenoloxidase assay (PO). In the challenge test, viral strain displayed strong virulence which induced 100% mortality after 3 days. Prolonged survival was observed in shrimps fed with diet containing 1,000 mg purified ulvan kg^{-1} diet. Shrimp fed diets containing purified ulvan exhibited 77% higher THC than in those fed the control diet. Highest THC was obtained in shrimps fed the diet containing 1,500 mg ulvan kg^{-1} diet and lowest in those fed the control diet. This suggested that ulvan promoted the activation of cellular immunity in shrimps. Respiratory burst activity of shrimps in all diets containing the purified ulvan was significantly higher than of those fed the control diet. Phenoloxidase activity only became significantly high when shrimps were fed a diet containing purified ulvan at levels of 1000 mg purified ulvan kg^{-1} diet or more (i.e. 1500 mg kg^{-1} diet). No significant difference was observed in shrimp fed diet containing 500 mg purified ulvan kg^{-1} diet and those fed with the control diet. It suggested that adding ulvan at 1000 to 1500 mg kg^{-1} diet could stimulate hemocytic degradation and activate prophenoloxidase to become phenoloxidase.

Key Words: ulvan, *Enteromorpha intestinalis*, total hemocyte count, phenoloxidase assay, respiratory burst activity, *Penaeus monodon* juvenile.

Introduction. In recent years, problems with diseases have emerged as constraints to the growth of shrimp aquaculture. Increased disease occurrences and in particular the uncontrolled movement of live aquatic animals (transboundary diseases) have resulted in the transfer of pathogenic organic organisms (like white spot syndrome virus - WSSV) among countries. WSSV is caused by genus *Whispovirus*, family Nimaviridae (100-400 x 270-420 nm), which is a rod shaped enveloped virus, which infects all stages of *Penaeus monodon*, *P. indicus*, *P. japonicas*, *P. vannamei*, *P. merguensis*, and freshwater prawns (*Macrobrachium* spp.) (Lio-Po 2010). It is considered as an economically significant shrimp disease and by far the most devastating disease, which causes high mortalities and severe damages to shrimp cultures. Clinical signs include white spots in the exoskeleton and epidermis (Citarasu et al 2006), reduction in food consumption (Lio-Po 2010), reddish discoloration, external fouling and mortality (Magbanua et al 2000). This virus can cause 100% mortalities within 3-10 days of the onset of the above symptoms (Citarasu et al 2006).

Immunostimulants are powerful tools to control fish and shrimp diseases. Attempts to control or prevent such devastating outbreaks using conventional antimicrobials and other chemotherapeutants have been generally unsuccessful. The uncontrolled and repeated use of antibiotics to treat bacterial infections has in some cases led to the development of antibiotic resistant pathogens. Considering the potential threat of diseases on one hand and the potential human and animal health issues associated with misuse of antibiotics on the other hand, disease management aspects should concentrate on environment-friendly and preventative methods such as adding

potent plant extracts to the culture water (e.g. Ikhwanudin et al 2014) or to the feed itself i.e. immunostimulants (Jadhav et al 2006; Caipang & Fagutao 2013).

An immunostimulant is a naturally occurring compound that modulates the immune system by increasing the host's resistance against diseases that in most circumstances are caused by pathogens (Bricknell et al 2005). Immunostimulants can be divided into several groups depending on their sources: bacterial, algae-derived, animal derived, nutritional factors as immunostimulants, and hormones/cytokines (Sakai 1999). Recently, polysaccharides from seaweeds have been tested as immunostimulants for shrimps. Ulvans are water soluble polysaccharides from *Enteromorpha* sp. and *Ulva* sp. which are complex sulphated heteropolysaccharides and represent the major polymeric fraction of the algal cell wall (Lahaye et al 2007). Effects of ulvan supplementation in the diet of *P. monodon* on both its growth and immunostimulation have never been evaluated previously. Thus, the present study aims to evaluate the effects of ulvan extract on the immune parameters and resistance upon actual viral challenge of *P. monodon* juvenile.

Material and Method

Experimental animals and acclimation period. *P. monodon* post-larvae (PL 19 i.e. 19 days post-hatched post-larvae) were obtained from the Southeast Asian Development Center-Aquaculture Department (SEAFDEC-AQD) in Tigbauan Iloilo, Philippines. They were maintained and reared in a 5-ton-capacity tank with continuous aeration at the University of the Philippines Visayas Multi-Species Hatchery, Miag-ao Iloilo. The shrimps were acclimated and fed with commercial shrimp diet for 2 weeks. Prior to the experiment, the shrimps were randomly screened by PCR for WSSV infection.

Immune response and disease resistance trial. Two hundred forty shrimps (2.39 ± 0.38 g; 60 shrimps diet⁻¹) were reared in 250-L fibreglass tanks were fed with diets containing 0.0 mg, 500 mg, 1,000mg or 1,500mg purified ulvan kg⁻¹ diet (Table 1) for 14 days (February to March 2014) and the shrimps were subjected to WSSV challenge test, total hemocyte count (THC), respiratory burst activity (RBA) and phenoloxidase assay (PO).

Table 1
Composition of test diets containing various concentration of purified ulvan (%)

Ingredients	Diet A (0 mg ulvan)	Diet B (500 mg ulvan)	Diet C (1,000 mg ulvan)	Diet D (1,500 mg ulvan)
Peruvian fish meal	20.00	20.00	20.00	20.00
Shrimp meal	34.00	34.00	34.00	34.00
Soybean meal	21.00	21.00	21.00	21.00
CMC	3.48	3.43	3.38	3.33
Vitamin mix	1.00	1.00	1.00	1.00
Mineral mix	1.00	1.00	1.00	1.00
BHT	0.02	0.02	0.02	0.02
Lecithin	0.50	0.50	0.50	0.50
Cod liver oil	4.00	4.00	4.00	4.00
Starch	15.00	15.00	15.00	15.00
Purified ulvan	0.00	0.05	0.10	0.15
Total	100.00	100.00	100.00	100.00

WSSV infection challenge. Shrimps were placed in a static water set up (comprised by 30 L plastic containers) with continuous adequate aeration isolated inside a challenge room (Figure 1). The test animals were challenged by feeding infected tissue at the rate of 8% body weight. Infected shrimps were minced thoroughly and were fed at 08:00, 12:00, and 16:00 h. A blank control (unchallenged control) was included in the test. Survival was monitored daily until 100% mortality in one of the treatment was achieved; the dead shrimp were removed promptly. Mortality by WSSV infection was confirmed by physical gross signs and by one-step polymerase chain reaction (PCR) assay in Southeast Asian Fisheries Development Center-Aquaculture Department (SEAFDEC-AQD). Proper disposal of the infected water was also implemented.



Figure 1. Static set-up for white spot syndrome virus (WSSV) challenge.

Extraction of haemolymph. Anticoagulant for haemolymph extraction was prepared by adding 10 nM EDTA-Na₂ salt to a salt solution (450 mM NaCl, 10 nM HEPES, pH 7.3, 850 mOsm kg⁻¹) (Hernandez-Lopez et al 1996). Haemolymph was collected from the pleopod at the first abdominal segment near the genital pore, using a 1-mL syringe (26 gauge) hypodermic needle rinsed thoroughly with pre-cooled anticoagulant. Haemolymph was collected from randomly selected shrimps in each treatment for total hemocyte count (THC), superoxide anion assay and phenoloxidase assay.

Shrimp immune assay

Total hemocyte count. A 100 µL haemolymph was mixed gently with anticoagulant and transferred to a sterile 1.5 mL eppendorf tubes. Hemolymph was fixed with an equal volume of 10% buffered formalin in using the following ratio: 1 part haemolymph, 3 parts anticoagulant, 5 parts formalin. A 20 µL aliquot was stained using 1.2% Rose Bengal in 50% ethanol and allowed to stain for 20 min. Total hemocyte was counted using a Neubauer haemocytometer under a compound microscope at 40x magnification. Obtained values were expressed as THC mL⁻¹ hemolymph (Joseph & Phillip 2007) using the following formula:

$$\text{Total hemocyte count (THC)} = (A \times dcf) / (B \times (4 \times 10^{-6}))$$

$$\text{Dilution correction factor (dcf)} = V_h / (V_h + V_{ac})$$

Where:

A = total number of cell counted;

B = total number of squares counted;

V_h = volume of hemolymph;

V_{ac} = volume of anticoagulant.

Superoxide anion (NBT reduction) assay. Respiratory burst activity of hemocytes was determined by the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anion production (Munoz et al 2000).

One hundred μL hemocyte sample was placed in each well of a 96-well microtitre plate and was incubated at room temperature for 2h. The supernatant was discarded and was replaced with 50 μL MHBSS (Modified Hank's Balanced Salt Solution) medium, 50 μL PMA (phorbol myristate acetate) working solution, 50 μL of 0.3% NBT stock solution and was incubated further for 30 min. Supernatant was removed and haemocytes were fixed by adding 200 μL absolute methanol for 10 min. The well was washed with 200 μL 70% methanol, and air dried. The formazan deposit was solubilized by the addition of 120 μL of 2M KOH and 140 μL of DMSO (dimethyl sulfoxide) in each well. Intensity of the turquoise blue color was read by a microplate reader at 620 nm and respiratory burst activity was expressed as optical density $100 \mu\text{L}^{-1}$ haemolymph. Blank control reactions were performed using 120 μL of KOH and 140 μL of DMSO, the optical density of which was subtracted from corresponding values of non-blank tests.

Phenoloxidase activity assay. Phenoloxidase (PO) activity was assayed spectrophotometrically by measuring the amount of dopachrome formed from *L*-dihydroxyphenylalanine (*L*-DOPA, Hernandez-Lopez et al 1996).

Anticoagulant-free haemolymph was placed in sterile 1.5 mL microcentrifuge tubes and was subjected to a freeze thaw cycle 5 times to induce cell lysis and degranulation. The lysed hemolymph was vortexed and centrifuged at 3,144 $\times g$ for 15 min at 4°C and the supernatant was collected. A 25 μL haemocyte supernatant was then placed in 96-well microtitre plates and incubated for 30 min with 25 μL of 0.1% trypsin in SSS (Shrimp Salt Solution) at room temperature. A 25 μL of 0.3% *L*-DOPA (*L*-3, 4-dihydroxyphenylalanine) was added and incubated for another 3 mins. Optical density of the solution was read at 490 nm by a microplate reader. Enzyme activity was expressed as the change in absorbance $\text{min}^{-1} 100 \mu\text{L}^{-1}$ haemolymph (Joseph & Phillip 2007).

Statistical analysis. Data obtained from immune assays were tested for normality of distribution using Shapiro-Wilk test and homogeneity of variance using Levene's test. Data were analyzed by one-way analysis of variance (ANOVA) where significant difference detected treatments means were compared using orthogonal contrast performed using SPSS 16.0 software. All probability values were set at a significance level of 0.05.

Results and Discussion

WSSV challenge. To test the protective effects of purified ulvan, shrimps were challenge against WSSV. Surviving shrimps were recorded up to the 5th day post infection. All unchallenged shrimp survive. Viral strain displayed strong virulence which induced 100% mortality after 3 days. Prolonged survival was observed in shrimps fed with diet containing 1,000 mg purified ulvan kg^{-1} diet (Figure 2).

Gross examination of the shrimps revealed that all mortalities were caused by WSSV infection and further confirmed with one-step PCR (data not shown). Although there was prolonged survival in shrimps fed the diet containing 1000 mg ulvan kg^{-1} diet, shrimps receiving the highest supplementation of purified ulvan succumb death after 3 days. This indicated that the immunostimulant was not directly dose-dependent and high doses did not enhance but rather inhibited the immune response as is the observation of Jadhav et al (2006). In their study, the death of shrimp receiving the highest purified ulvan supplement, according to them, is due to the overreaction of the immune system of shrimp when given an immunostimulants beyond the required dose. This condition is similar to "immune fatigue" as described by Chang et al (2000) where they find a reduced immunity of *P. monodon* after continuous feeding of glucan at a concentration of 2 g kg^{-1} feed for 40 days.

Citarasu et al (2006) observe that immunostimulants increase survival of *P. monodon* against WSSV after 20 days post-challenged. Similarly, Chotigeat et al (2004)

reveal that after 10 days of infection, the maximum survival rates of shrimps of 5-8 and 12-15 grams were 46% and 93% respectively. Control groups in the two mentioned previous studies died after 7 days and 9 days, respectively while it took only 3 days in the present study. This indicated that the virus used in the previous studies were less virulent than that in the present study. Laramore et al (2009) point out that a measurable virulence difference exists among WSSV isolates which may correspond to geographical region.

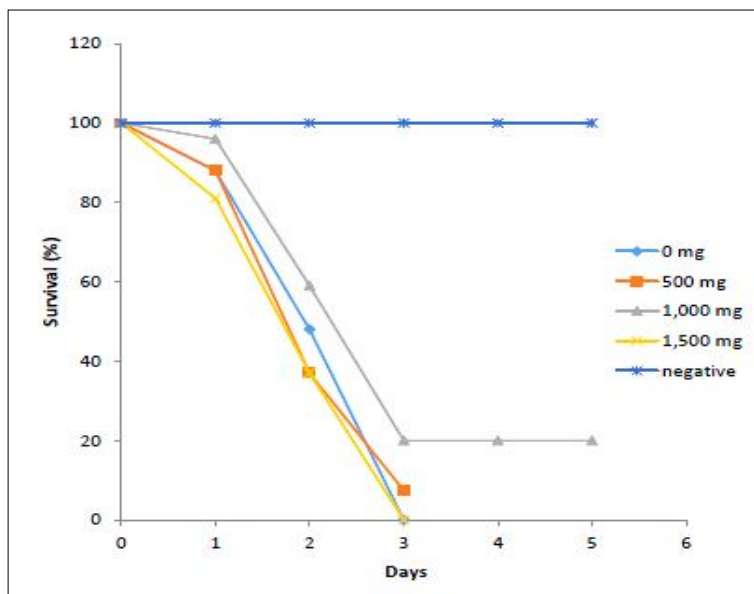


Figure 2. Survival (%) shrimp fed with diets containing different levels of purified ulvan after WSSV challenge.

Shrimp immune response. Immunostimulants efficacy is usually measured by changes in parameters such as phenoloxidase activity, phagocytic index or level of superoxide dismutase (Campa-Cordova et al 2002), encapsulation, melanization, and blood coagulation (Johansson et al 2000), and THC (Citarasu et al 2006). In the present study, shrimp fed with purified ulvan exhibited 77% higher THC than those fed the control diet (Figure 3). Highest THC was obtained in shrimps fed the diet containing 1,500mg ulvan kg^{-1} diet) and lowest in those fed the control diet. This suggested that the incorporation of purified ulvan in shrimp diets promoted activation of cellular immunity. The measured total hemocyte count in the present study was within the range of values obtained by Saraswati et al (2013) upon injecting *Chaetoceros ceratosporum polysaccharides* on *Litopenaeus vannamei* and Cheng et al (2005a) upon injecting different levels of dopamine on *L. vannamei*. In contrast, the values in the present study were much higher than those reported by Manilal et al (2009) on *in vivo* antiviral activity of polysaccharide extracted from green algae *Ascrosiphonia orientalis* against WSSV.

THC measures cellular immune response of organisms, a potential indicator of their health status (Sritunyalucksana & Soderhall 2000). Molting, development of an organ, reproductive status, nutritional condition, and occurrence of infection and even season have shown to influence hemocyte abundance (Cheng & Chen 2001). In healthy *P. monodon*, TCH range from 10^4 to 10^5 cells cubic mm^{-1} while those maintained in an unfavorable environment or with bacterial infection, shows a marked drop in hemocyte count (Supamattaya et al 2005). Citarasu et al (2006) have evaluated the effects of various Indian herbs on the immune response of *P. monodon* against WSSV and have observed a significant decrease in THC on the onset of an infection. Van de Braak (2002) observes that a decrease of THC is caused by pathogenic infection because hemocytes are target for pathogen. However, when immunostimulation happens, gradual increases

in THC is observed until the count becomes normal indicating the immune system copes with infection.

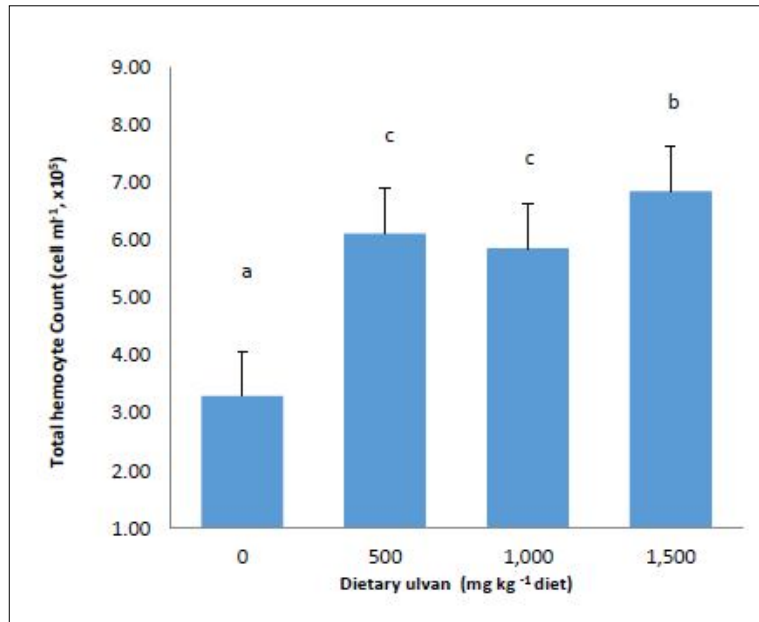


Figure 3. Total hemocyte count of shrimp fed with purified ulvan supplemented diets for 14 days. Values are expressed as Mean±SEM of 4 replicates (n=10).

Respiratory burst activity of shrimps in all diets containing the purified ulvan was significantly higher than of those fed the control diet (Figure 4). Nitroblue tetrazolium (NBT) stain has been used for quantitative analysis of superoxide anion generated by hemocytes. This metabolite is capable of eliminating invading agents particularly when the superoxide level increases as phagocytosis of microbes or foreign bodies takes place (Muñoz et al 2000).

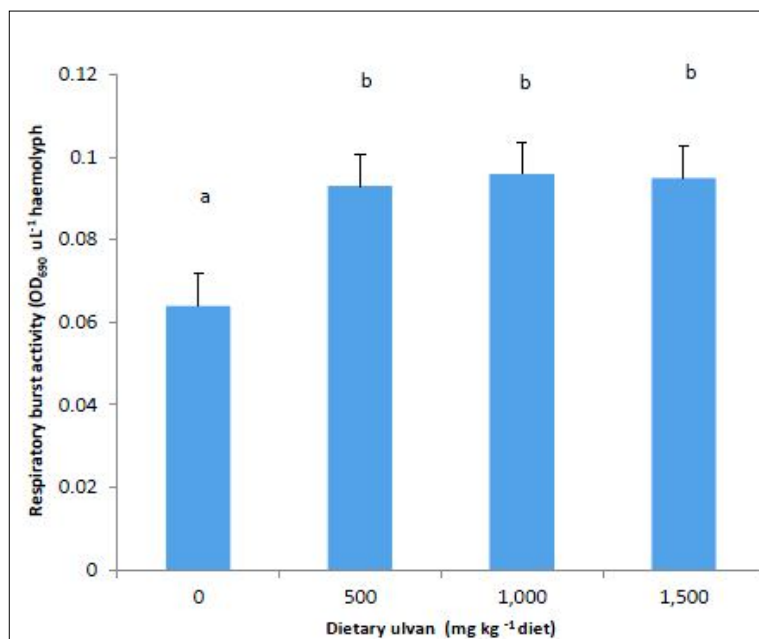


Figure 4. Respiratory burst activity of shrimp fed with purified ulvan supplemented diets for 14 days. Values are expressed as Mean±SEM of 3 replicates (n=5).

Phagocytotic activity and anion superoxide content are proportional with the number of hemocytes i.e. the increase in hemocyte number will be followed by increase of phagocytotic activity and anion superoxide content (Nindarwi et al 2013). In the present study, the superoxide anion values in shrimps fed diets with purified ulvan at all levels were significantly higher than those fed control diet. Furthermore, superoxide anion production showed a similar trend with that of the THC.

Phenoloxidase activity only became significantly high when shrimps were fed a diet containing purified ulvan levels of 1000 mg purified ulvan kg^{-1} diet or more (i.e. 1500 mg kg^{-1} diet). No significant difference was observed in shrimp fed diet containing 500 mg kg^{-1} purified ulvan kg^{-1} diet and those fed with the control diet (Figure 5).

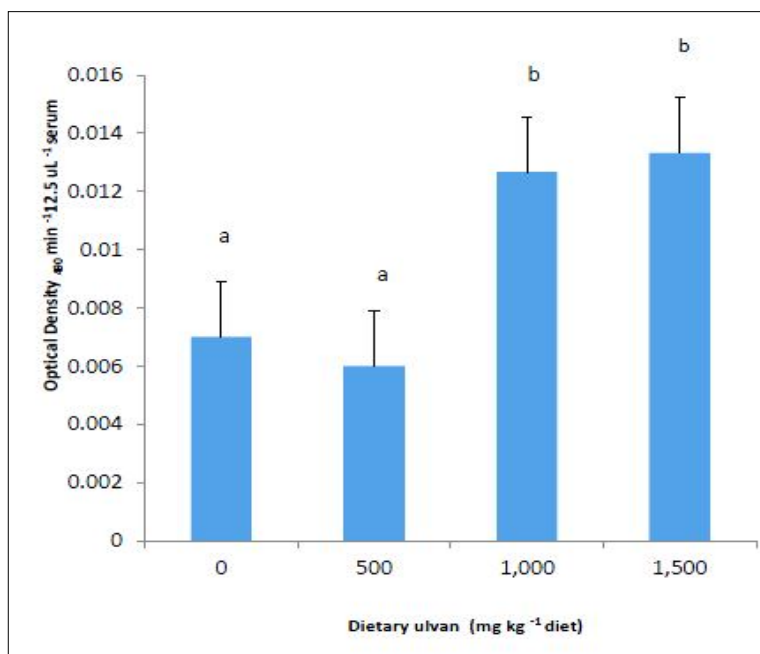


Figure 5. Phenoloxidase activity of shrimp fed with purified ulvan supplemented diets for 14 days. Values are expressed as Mean \pm SEM of 3 replicates (N=5) on each treatment diets.

P. monodon possesses 3 different types of blood cells: hyaline cells, small and no cytoplasmic granules. There are also two types of granular hemocytes: semi-granular hemocytes and large granular hemocytes. The granules in the granular hemocytes consist of precursors to prophenoloxidase (Supamattaya et al 2006). The activation of the prophenoloxidase cascade is exerted by extremely low quantities of microbial cell wall components (lipopolysaccharides LPS, beta-1,3 glucans of peptidoglycans PG) (Sritunyalucksana et al 2000). In the present study, the increased PO activity in shrimps fed diets containing 1000 and 1,500 mg kg^{-1} purified ulvan kg^{-1} diet indicated that this level of supplementation could stimulate hemocytic degranulation and activate proPO to become PO. Similar observation was made by Cheng et al (2005b) where phenoloxidase activity increased significantly in shrimps fed diets containing sodium alginates at 1.0 or 2.0 g kg^{-1} . Furthermore, this increase will lead to the ability of shrimp to recognize foreign materials (Saraswati et al 2013).

Conclusions. Prolonged survival was observed in shrimps fed with diet containing 1,000 mg kg^{-1} purified ulvan kg^{-1} diet. Shrimps receiving the highest supplementation of purified ulvan succumb death after 3 days. This indicated that the immunostimulant was not directly dose-dependent and high doses did not enhance but rather could have inhibited the immune response. Shrimp fed with purified ulvan exhibited 77% higher THC than those fed the control diet. Highest THC was obtained in shrimps fed the diet containing 1,500 mg kg^{-1} purified ulvan kg^{-1} diet and lowest in those fed the control diet. This suggested that the

incorporation of purified ulvan in shrimp diets promoted activation of cellular immunity. The superoxide anion values (i.e. respiratory burst activity) in shrimps fed diets with purified ulvan at all levels were significantly higher than those fed control diet. Furthermore, superoxide anion production showed a similar trend with that of the THC. Phenoloxidase activity only became significantly high when shrimps were fed a diet containing purified ulvan at levels of 1000 mg or more (i.e. 1500 mg kg⁻¹ diet). No significant difference was observed in shrimp fed diet containing 500 mg purified ulvan kg⁻¹ diet and those fed with the control diet.

Acknowledgements. The authors are grateful to the Philippine Department of Science and Technology (DOST)-Accelerated Science and Technology Human Resource Development Program (ASTHRDP) for providing the scholarship to Ms. Declarador and to DOST-Philippine Council for Agriculture, Aquatic and Natural Resources Development (PCAARD) for the research funding and to the Office of the Vice Chancellor for Research and Extension of the University of the Philippines Visayas for the additional research funding and publication support.

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Received: 04 June 2014. Accepted: 19 June 2014. Published online: 20 June 2014.

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How to cite this article:

Declarador R. S., Serrano Jr. A. E., Corre Jr. V. L., 2014 Ulvan extract acts as immunostimulant against white spot syndrome virus (WSSV) in juvenile black tiger shrimp *Penaeus monodon*. *AAFL Bioflux* 7(3):153-161.