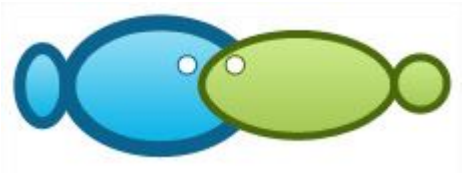

Immune response of *Macrobrachium rosenbergii* immersed in aqueous extract of *Gracilaria edulis* challenged with white spot syndrome virus

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Abstract. *Macrobrachium rosenbergii* is preferential in freshwater aquaculture because it is the largest species in its genus. Its size and hardy nature is an asset that contributes to its economic value as a food source. Crustaceans depend solely on their innate defense systems however; it is not enough to combat the prevailing threats brought by bacteria and viruses, particularly the white spot syndrome virus (WSSV). WSSV is a virus that caused massive financial loss and 100% mortality in shrimp aquaculture in the Philippines and all over the world. We report the efficiency of aqueous extracts of *Gracilaria edulis* as an immunostimulant for the shrimp species, *M. rosenbergii*, against WSSV using immersion tests. Total haemocyte count (THC) and phenoloxidase activity (PO) were examined after the shrimps had been immersed in *G. edulis* extracts at 1% and 3% concentrations. Shrimps treated with 1% concentration of *G. edulis* extract before and after infection exhibited significantly improved THC and PO levels 7 days post-infection. The improved response is attributed to the shrimps having a more conditioned immune system even during WSSV infection, indicating boosted humoral immune response. Furthermore, shrimps immersed in 1% concentration increased at least 40% survival rates compared to the control. The utilization of *G. edulis* as an immunostimulant will provide alternative low-cost and hassle-free options for shrimp farmers to safeguard their shrimp farms from massive economic losses caused by WSSV.
Key Words: immunostimulation, WSSV, total hemocyte count, phenoloxidase activity, β-1,3-glucan.

Introduction. *Macrobrachium rosenbergii* is a decapod belonging to the family *Palaemonidae* (Myers 2013). It has a brown carapace with darker brown rings and its abdomen has a brown mottled pattern. There are bright red markings on each side of its pleural condyle (Wowor & Ng 2007) and its average size is 320 mm for males and 250 mm for females (Cowles 1914). *M. rosenbergii* is a shrimp species that can be found in freshwater bodies in many countries including the Philippines. This species is of economic importance as a food source due to its availability and size. It is commonly found in stalls in local markets and exports (Rosario & Tayamen 2004).

Studies have been done on *M. rosenbergii* with the intent of increasing the diversity of commodities in the business due to it being a hardy and fast-growing species. The species has so much potential as it can yield a better profit due to its high-value in the world and local market. It was not until 2001 when the Philippine Government, through the Bureau of Fisheries and Aquatic Resources-National Integrated Fisheries Technology Development Center (BFAR-NIFTDC) in Dagupan City and the Bureau of Fisheries and Aquatic Resources- National Freshwater Fisheries Technology Center (BFAR-NFFTC) in Muñoz City, embarked on a semi-commercial production of *M. rosenbergii* (Rosario & Tayamen 2004). Freshwater prawn cultures are still at an infancy stage here in the Philippines. The industry itself needs more support from locals before its potential can be seen. Even before the development has progressed, problems have already risen

concerning culturing prawns and its susceptibility to white spot syndrome virus (WSSV).

WSSV is one of the major causes of mortality and loss in today's aquaculture. Belonging to the genus *Whispovirus* under the family *Nimaviridae*, this virus can kill infected shrimp in a short span of 7-10 days after contamination (Corre et al 2012). WSSV infection is not limited to prawns; it can also infect other crustaceans such as crayfishes and crabs. The first recorded occurrence of WSSV in the Philippines was in 2000 when Magbanua et al (2000) reported that all samples obtained from farms in Agusan del Norte, Cebu, Bulacan, and Quezon tested positive for WSSV. The case referred to shrimp populations reported by farms were experiencing mortalities due to unknown causes. The gross manifestations ranged from lack of appetite to red discoloration and external fouling. There were no 'white spots' visible on a macroscopic scale when sampling was done. It is also noted that at least one farm in Agusan del Norte that tested positive for WSSV obtained their prawn larva from a hatchery which also tested positive for WSSV. Another incident back in 2001 was recorded when the virus caused heavy annual loses in pond cultures of *P. monodon* (Corre et al 2012).

WSSV infects almost all crustaceans and the virus may spread from pond to pond via vectors and the natural flow of water. Due to the host residing in ponds and brackish water, there is no cure for WSSV that can be applied to such conditions. Vaccines are usually developed to counteract the mechanism of a virus, but traditional vaccines can only be effective if the animal has an adaptive immune system, which can produce antibodies after vaccination. Shrimp cannot produce their own antibodies since they do not have an adaptive immune system (Crockford 2008). As a preventive measure, disinfection of the sediment where vectors reside has been done and is proven to lessen the risks of outbreaks (Corre et al 2012). Aside from having disinfectants, there is still no doubt that there is a need to develop an antiviral substitute in the form of an immunostimulant.

Immunostimulation is the resulting enhancement of an immune reaction of a specimen due to modulation in the immune system. Modulation of response may involve induction, repression or amplification of an immune response. It is the modification of a specimen's immune system via the activation or suppression via agents. Plant extracts are known to have immunostimulant factors. Different parts of the plants such as fruits, leaves, and roots may be used. Immunostimulants are not limited to terrestrial plants as even marine species, especially the macroalgae, are capable of such prowess (Kumar et al 2011).

The genus *Gracilaria* is a potential source for medicine synthesis. It is known for harboring many active metabolites that contribute to the pharmaceutical industry. Studies have concluded that compounds extracted from *Gracilaria* can inhibit the growth of bacteria however; the more threatening diseases of our time are transmitted by viruses (de Almeida et al 2011). There is very little research regarding the antiviral activities of *Gracilaria*.

Gracilaria edulis belongs to *Rhodophyta* under the genus *Gracilaria* and the family *Gracilariacea*. It is a marine species with thin, spiny appendages and is also differentiated by colors such as red, green, and brown to dark brown. It is known to have antibacterial properties particularly on *Vibrio alginolyticus* (Maningas et al 2013).

Knowing that *G. edulis* is capable of anti-bacterial properties, this study aims to test the immune response of WSSV-challenged *M. rosenbergii* immersed in *G. edulis* extract.

Material and Method

Preparation of G. edulis extract. *G. edulis* was collected along the coast of Calatagan, Batangas. Algal fronds were washed thoroughly with deionized water and air dried at room temperature (25^{o}C) for three days until weight was constant. The extraction was done based on a method by Fujiki et al (1992) with the exclusion of the lyophilizing process. The dried algal fronds were milled using Thomas Wiley® Mill Grinder. Three hundred milliliters (300 mL) of deionized water and 10 g milled algal fronds were boiled for three hours - the mixture was then filtered using cheesecloth. The filtrate was

collected and stored in a cool, dry place. The stored extract was diluted with distilled water to produce 1% and 3% concentrations based on a modified method from Sivasankari et al (2006).

Preparation of viral stock. The gills and somites of WSSV-infected shrimp were dissected and transferred to microcentrifuge tubes containing 9 mL of PBS for every 1 g of tissue. The tissues were then homogenized in ice and subjected to centrifugation under the following conditions: 8,000 rpm, 10 minutes and 4^{o}C. The supernatant was then collected and centrifuged again. The final supernatant was filtered using Whatman ^{TM} 0.45 µm Filter Device. Two concentrations (10^{-2} and 10^{-3}) were tested to determine the median lethal dose (LD_{50}) and 10^{-3} yielded 50% mortality after 7 days.

Experimental design of the immersion test. 200 juvenile, *M. rosenbergii* (mean weight 1±0.4 g) obtained from the Southeast Asian Fisheries Development Center (SEAFDEC), in Binangonan, Rizal. The shrimps were acclimated for three days in 60 L capacity tanks at room temperature. A total of 6 tanks were prepared for the experiment.

Forty-five shrimps were utilized for the therapeutic set-up in which 3 tanks with 15 shrimps each were used for 28 days. Shrimps were first infected before being immersed in their respective tanks and the shrimps were left for five hours to allow the infection to transmit throughout their body. Triplicate sampling was employed on 5 sampling points (day 0, 1, 7, 14 and 28). The set-ups include: (i) Control 1 – WSSV infected shrimps not immersed in extracts; (ii) Thera1 – WSSV- infected shrimps immersed in 1% *G. edulis* extract; and (iii) Thera 3 - WSSV infected shrimps immersed in 3% concentrations of the extract. Therapeutic set-ups were designed to determine the immune response of WSSV-infected shrimps immersed in two different concentrations of *Gracilaria* extracts lasting 28 days and to prove the efficacy of extracts as treatment for infected shrimps.

A total of 90 shrimps were needed for prophylactic set-up in which 3 tanks with 30 shrimps each were used for 56 days. Sampling days were done on day 0, 1, 7, 14 and 28 of pre-infection and another 28 days for post-infection period, using the same sampling days, to test if immersion before infection can enhance the immune response of shrimps upon infection. This set-up was designed to determine the immune response of uninfected shrimps immersed in two different concentrations of extracts (1% and 3%) for 28 days and to test the immunostimulatory effect of *G. edulis* extracts 28 days after infection. Shrimps of the prophylactic setup were immersed in *G. edulis* extract for a total of 56 days. The set-ups include: (i) Control 0 –shrimps not immersed in extracts before and after infection; (ii) Pro1 – shrimps immersed in 1% extract before and after infection; (iii) Pro 3 - shrimps immersed in 3% extract before and after infection.

The shrimps were infected via intra-muscular (IM) injection of 100 µL of WSSV diluted in PBS (Phosphate Buffered Saline) (10^{-2}) in the third dorsal abdominal segment. The shrimps were examined for gross signs of disease on different sampling days. The number of deaths was recorded and the percentage of mortality was also calculated.

Measurement of immune parameters. 300 µL hemolymph samples from each shrimp (3 shrimps per sampling day) were extracted from the ventral sinus using a 3-mL sterile syringe (23 gauges) containing 2700 µL anticoagulant. The haemolymph-anticoagulant mixture was then subjected to total haemocyte count (THC) and phenoloxidase activity (PO).

Total haemocyte count. 300 µL of the haemolymph-anticoagulant mixture was stained with 14 µL of Trypan Blue stain. 10 µL of the stained haemolymph-anticoagulant mixture was then dropped onto a Neubauer haemocytometer and viewed under an Olympus Optical light microscope. Unstained cells are considered viable while those which have absorbed the dark blue stain are non-viable; and should not be counted. The viable cells in the four corner squares were then counted and the cell count per mL was determined by determining the mean viable cell count per corner square and multiplying it to a dilution factor of 1 and to 10^{4}.

Phenoloxidase activity. Phenoloxidase activity was measured using a protocol taken from using the method of Hernandez-Lopez et al (1996). 500 µL of haemolymph-anticoagulant mixture was centrifuged at 8000 rpm at 4^{o}C for 3 minutes. The supernatant was discarded. The pellet was rinsed and re-suspended in 500 µL cacodylate-citrate buffer before being centrifuged. The supernatant was discarded once again. The pellet was then resuspended in 100 µL of cacodylate buffer. 50 µL of the cell suspension was then moved to a clean microcentrifuge tube, which was measured for PO Activity after 10 minute-incubation. To the 500 µL cell suspension, 25 µL of Zymosan was added and after 5 minutes as the elicitor, 25 µL of L - dihydroxyphenylalanine (L-DOPA) was introduced followed by 400 µL of cacodylate buffer. Phenoloxidase activity was then measured upon subjection of the mixture to a wavelength of 490 nm using ELx800 Absorbance Microplate Reader.

Statistical analysis. Results were statistically analyzed using the GraphPad Prism v.5 Demo software. The data from the measurement of THC and PO activity were subjected to one-tailed unpaired t-test to determine the significant differences ($p<0.05$) among treatments.

Results and Discussion

Shrimps have two known defense mechanisms namely cellular and humoral responses. Cellular responses use haemocytes to be able to combat against bacteria and other foreign particles by phagocytosis, encapsulation and formation of nodules (Martinez 2007). The humoral response, on the other hand, use haemolyph that inhibit and eliminate foreign bodies and pathogens which include the anticoagulant proteins, agglutinins, antimicrobial enzymes, free radicals and the phenoloxidase system.

Total haemocyte count. The prophylactic setup determines the ability of the extract to protect and build-up the shrimps immune system prior to infection. For the prophylactic setup (Figure 1), a decline in the total haemocyte count was observed in uninfected shrimps not immersed in *G. edulis* extracts (Control 0). The shrimps were subjected to stress and had difficulty adapting to their experimental environment because of the difference in water conditions between communal and experimental tanks. For shrimps immersed in the 1% extract (Pro 1), a steady increase in THC on days 0 and 1, followed by a decline in day 7 was recorded. On day 14, THC recovered but suffered a decline once again on day 28 upon infection. The loss was quickly replaced by an increase in total haemocyte count by day 29 (Day 1 post infection). On day 35, THC increased drastically marking an excessive activity of phenoloxidase working on counteracting the virus effects. On days 42 and 56, THC drifts back down. Shrimps immersed in 3% extracts before and after infection for 56 days (Pro 3) displayed fluctuating results. It reached its peak THC on the 1st sampling day. A decline was observed on sampling days 7 and 14, which were followed by an increase on day 28. Upon infection on the 28th day, Pro 3 shrimps exhibited lessened THC compared to that of Pro 1. There was a continuous drop in THC upon infection. On day 35, an increase was recorded to confirm cellular response in Pro 3 but it was not as high as the THC of Pro 1 on day 35. It continued to increase up to day 56.

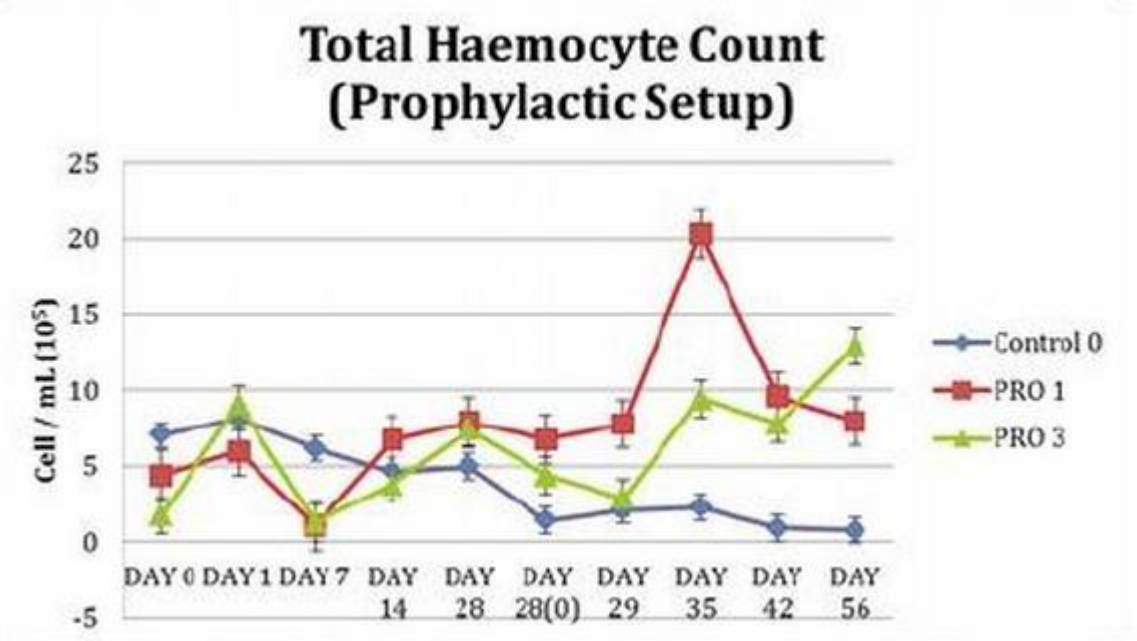

Figure 1. Total haemocyte count for prophylactic set-ups for 56 days pre and post infection- (i) C0 – shrimps that were not immersed in extracts before and after infection; (ii) Pro 1 – shrimps that were immersed in 1% *G. edulis* extract before and after infection; (iii) Pro 3 - shrimps that were immersed in 3% *G. edulis* extract before and after infection.

There was a significant increase in THC ($p<0.05$) of *M. rosenbergii* immersed in 1% and 3% extracts of *G. edulis* before and after infection for 56 days (Pro 1 and 3) in comparison to *M. rosenbergii* that were not immersed in extracts before and after infection for 56 days.

The therapeutic setup was designed to evaluate the extracts effect on the shrimps immune response post-infection. Significant increase in THC ($p<0.05$) for the WSSV-infected shrimps immersed in 1% (Thera 1) and 3% (Thera 3) extract of *G. edulis* were observed after 28 days compared with the control group (control 1) which were the infected shrimps not immersed in extracts (Figure 2). Between day 0 and day 1, the haemocyte count was observed to have varied levels, which can be attributed to stress and due to infection. This is a result of their innate immune response system wherein cellular responses of the haemocytes present in the shrimp are activated to cope with any foreign bodies or rapid change in their environment. Haemocyte count in Control 1 continued to exhibit consistent cell count; however, its total haemocyte count showed very low levels in contrast to Thera 1 and Thera 3. Thera 1 resulted in a steady level of total haemocyte count, because of its continued cellular response against WSSV. Thera 3, however, showed immediate maximum cellular response as evident in its significant increase during day 0 and day 1. THC of Thera 3 then decreased significantly, exhibiting a saturation point through days 1 and 7. Thera1, through days 7 and 28, showed gradual increase in haemocyte count. Thera 3 exhibited lowered levels of haemocyte as it was subjected to saturation beforehand. Shrimps immersed in 1% extract in the prophylactic setup exhibited parallel immune response to those subjected to the similar concentration in the therapeutic setup.

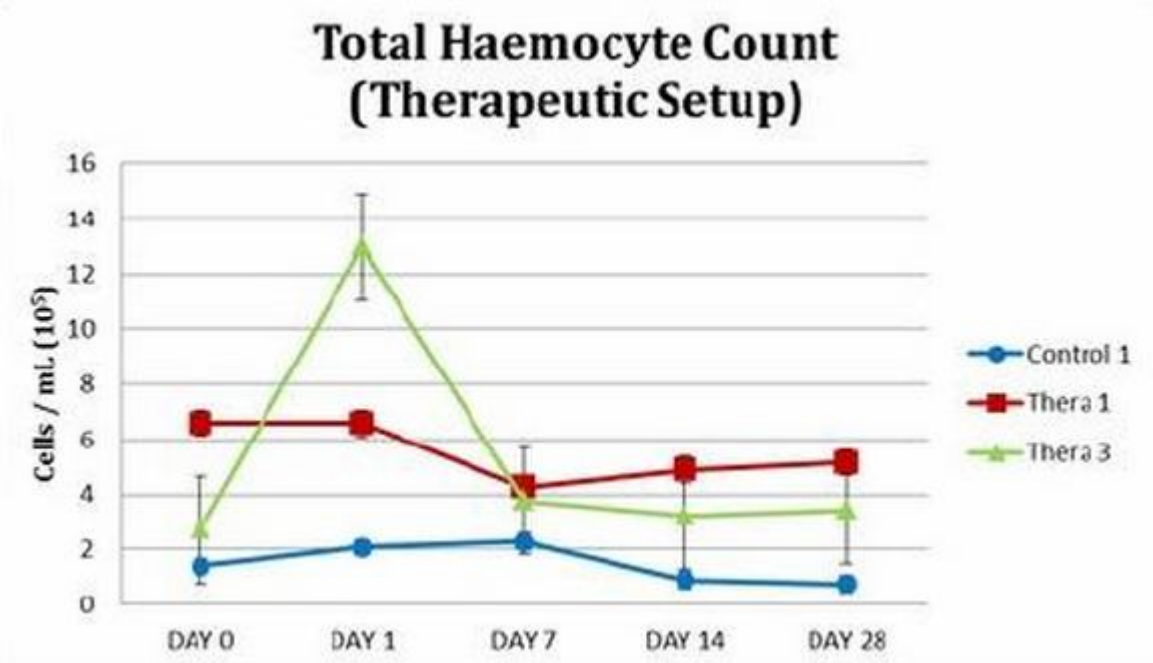

Figure 2. Total haemocyte count of Thera 1, Thera 3 and Control 1 for 28 days - (i) Control 1 – WSSV – infected shrimps not immersed in *G. edulis* extracts; (ii) Thera 1 – WSSV – infected shrimps immersed in 1% *G. edulis* extract; (iii) Thera 3 – WSSV – infected shrimps immersed in 3% *G. edulis* extract.

Cellular response, responsible for phagocytosis, melanization, encapsulation, and coagulation, is activated when lipopolysaccharides, peptidoglycans, and β-1,3-glucan molecules are present – commonly found in bacteria and fungi (Vargas-Albores & Yepiz-Plascencia 2000). β-glucan properties present in *G. edulis* reacts with the β-glucan binding proteins (βGBP) resulting in the degranulation of haemocytes. This immune response would result in the increase in total haemocyte count. However, a slight decrease in total haemocyte count could also be expected and indicate factors such as stress level on the shrimp, fresh infections by bacteria, fungi or virus, or population density present in its environment. 1% and 3% concentrations revealed the different responses of the innate immune system. The 1% set-up showed gradual cellular response, which is desirable since the immune system of the shrimp is not an adaptive immune system. 3% concentration showed the weakness of the shrimp's innate immune system, wherein a high immediate cellular response would eventually saturate the shrimp's βGBP resulting in a detrimental effect on its survival that is linked to the production of free radicals in the haemocytes, which are by-products of the shrimp's phenoloxidase system capable of harming both self and pathogenic cells (Nappi et al 2004). In contrast, the 1% concentration encourages βGBP and β-1,3-glucan interaction gradually – in effect the survivability of the shrimp is not compromised, even upon infection.

Phenoloxidase test. Phenoloxidase (PO) activity in the prophylactic set-up (Figure 3) was observed to have varied activity pre-infection. Shrimps that were not immersed in extract before and after infection for 56 days (Control 0) showed an immediate increase in PO activity through days 0 and 1. It then decreased and plateaued from day 7 to day 14, and lastly showed an increase in activity after 14 days. Pro 1 showed little change in its PO activity through the first 14 days. However, it showed an increase in cellular response from day 14 to day 28. In contrast, Pro 3 showed immediate PO activity until showing its first saturation point at day 7 to day 14. It then exhibited another increase in PO activity 14 days later.

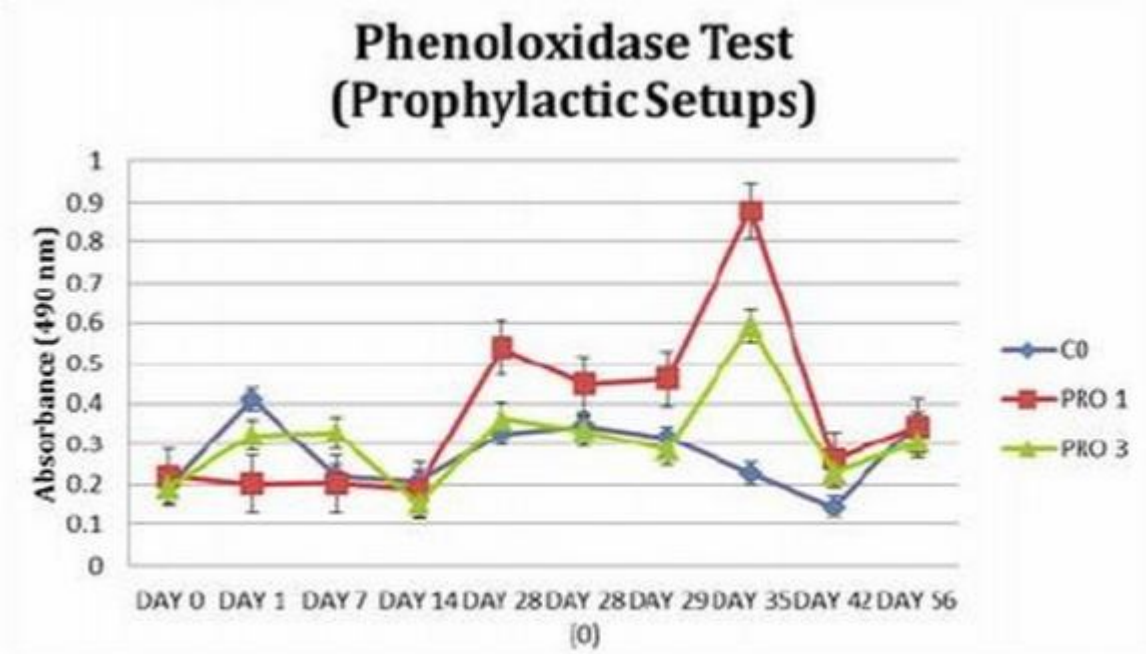

Figure 3. Phenoloxidase test for prophylactic set-ups for 56 days pre and post infection- (i) C0 – shrimps that were not immersed in extracts before and after infection; (ii) Pro 1 – shrimps that were immersed in 1% *G. edulis* extract before and after infection; (iii) Pro 3 - shrimps that were immersed in 3% *G. edulis* extract before and after infection.

After 28 days, control 0 showed a gradual decrease in PO activity because of the introduction of the WSSV. On day 56, however, control 0 shrimps exhibited an increase in PO activity – possibly indicating their innate immune system's effectiveness against WSSV. Pro 1, post – infection, showed little change in PO activity after one day of infection (Figure 3). But, on day 35 or 7 days after infection, a significant increase of PO activity was seen. This is because of the shrimp's reaction to the virus, as it is expected to affect the shrimp at this time based on a study by Corre et al (2012). An immediate drop of PO activity for Pro 1 was observed on day 42, possibly showing the PO activity's normalization to its pre-infection state before slowly escalating on day 56. Pro 3 first exhibited a drop of PO activity from five hours post infection to day 29. PO activity then reached its second saturation point at day 35 as it significantly decreased on day 42 followed by a slight increase on day 56, similar to that of Pro 1.

There was a significant increase in PO Activity ($p<0.05$) for *M. rosenbergii* immersed in 1% and 3% extracts of *G. edulis* for 56 days before and after infection in comparison to *M. rosenbergii* that were not immersed in extracts before and after infection.

For the therapeutic setup, the PO activity of Thera 1 increased moderately while Thera 3 increased drastically on day 0 to 1 (Figure 4). The response of Thera 3 to the infection of WSSV is immediate and maximal while Thera 1 is gradual. The same pattern for Thera 1 and Thera 3 is observed in the THC of the therapeutic setups. The PO activity of Control 1 declines as it is infected with WSSV and absent of any treatment. On day 7, Thera 3 lost its efficiency as reflected by the sudden decrease in PO activity whilst Thera 1 constantly increases indicating its response to the infection. The PO readings at Day 14 for Thera 1 decreased marking its attempt to stabilize the system of the shrimp after reducing the effects of infection. The PO activity at day 14 of Thera 3 displayed very minimal increase after it has reached its peak of saturation. On Day 28, the PO activity of Thera 1 rises again in response to viral activity. In comparison to Thera 3, Thera 1 proves to be more efficient as its gradual increase in PO activity results to an increase in survival rates of shrimps infected with WSSV.

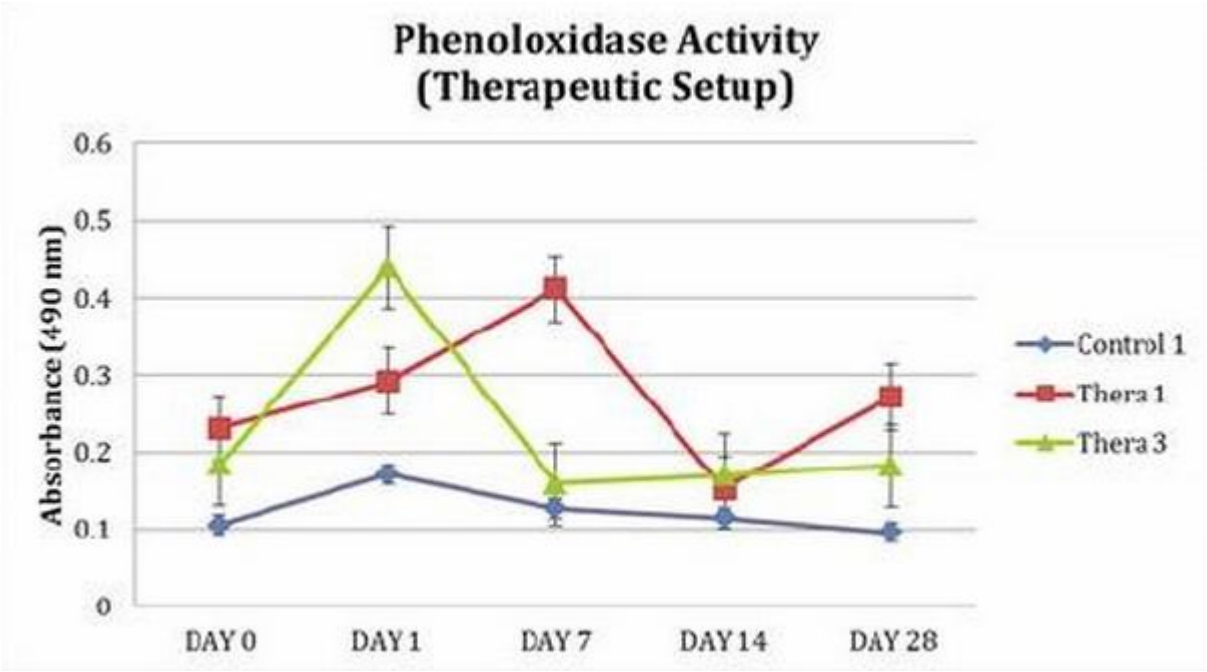

Figure 4. Phenoloxidase test for Thera 1 and Thera 3 vs C1- (i) C1 – Shrimps infected with WSSV not immersed in extracts; (ii) Thera 1 – Shrimps infected with WSSV and immersed in 1% *G. edulis* extract; (iii) Thera 3 – Shrimps infected with WSSV and immersed in 3% *G. edulis* extract.

There was a significant increase in PO activity ($p<0.05$) for WSSV-infected *M. rosenbergii* immersed in 1% and 3% extracts of *G. edulis* for 28 days in comparison with the infected shrimps not immersed in extracts.

In the innate immune system of the shrimp, proPO-activating system serves a key role in non-self recognition or non-specific recognition system in responding to foreign bodies in the shrimp. Haemocytes attract and induce phagocytosis, melanization, cytotoxic reactant production, partical encapsulation, and the formation of nodules when the non-specific recognition system of the crustacean detects viruses or bacterial invaders (Amparyup et al 2012).

The recognition of microbial pathogen-associated molecular patterns (PAMPs) such lipopolysaccharides and β-1,3-glucan as by pathogen-recognition receptors (PRPs) leads to the activation of serine proteinases. Serine proteinase activation leads to the final clip-domain serine proteinase designated as the proPO activating enzyme, and inactive proPO zymogen is converted into the active form of phenoloxidase by serine proteinase. This conversion is caused by the proPO-activating enzyme to produce quinones, which are important in cross-linking neighboring molecules to form melanin around the invading microorganism (Amparyup et al 2012). Melanin synthesis requires multiple steps, both enzymatic and non-enzymatic reactions so that the immune system can be effective against invading microorganisms. Undergoing a rate-limiting step in melanin formation catalyzes the key enzyme, phenoloxidase. Upon catalyzing the oxidation of phenols to quinones, melanin will result from non-enzymatical polymerization. Both mono- and diphenols, when oxidized by phenoloxidase, produce intermediary compounds. These intermediary compounds and melanin alike are toxic to microorganisms (Söderhäll et al 1996) and are also able to assist in wound healing (Hellio et al 2007). Similarly, Vargas-Albores et al (1998) agree that the phenoloxidase system is one of the most important processes in the shrimp immune system because of the process of melanization. Phenoloxidase promotes the hydroxylation of phenols and oxidation of *o*-phenols to quinones. The quinones in this cascade are then converted into important melanin molecules by non-enzymatic reactions. Furthermore, the phenoloxidase system is also activated by β-1,3-glucan and lipopolysaccharides, concluding that this kind of system is a recognition system.

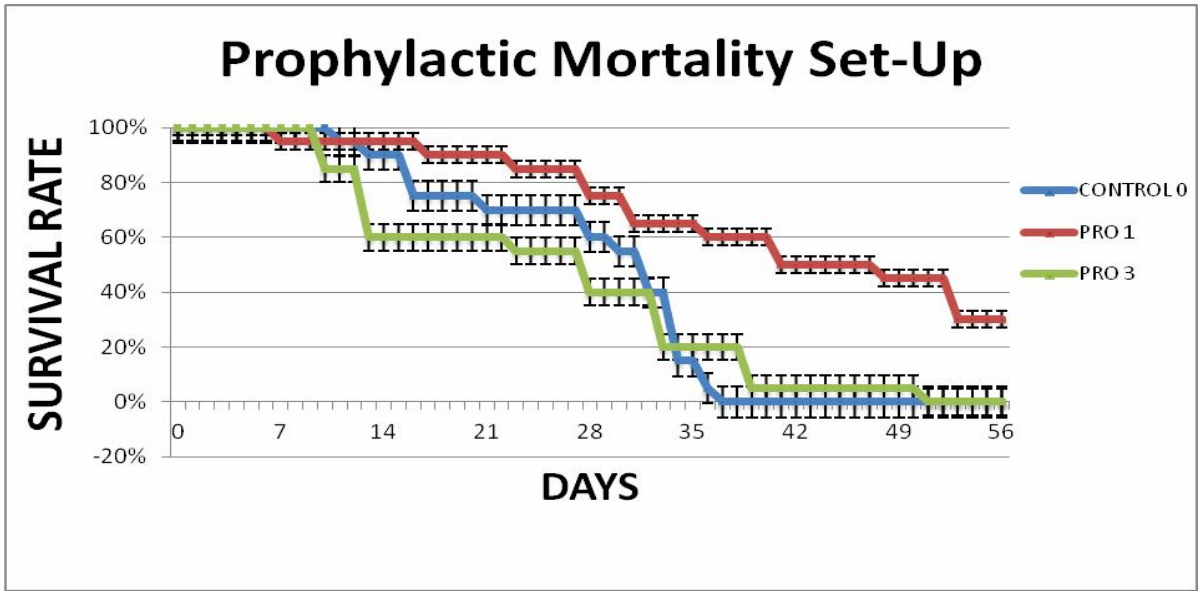

Figure 5. Mortality test for prophylactic set-ups for 56 days pre and post infection- (i) C0 – shrimps that were not immersed in extracts before and after infection; (ii) Pro 1 – shrimps that were immersed in 1% *G. edulis* extract before and after infection; (iii) Pro 3 - shrimps that were immersed in 3% *G. edulis* extract before and after infection.

Shrimps immersed in concentrations of *G. edulis* extract are provided with immunostimulants, in such a way that the β-1,3-glucan and lipopolyssacharides stimulate humoral response. By anticipating the compounds present in microbial and fungal walls, the experimental setups mimicked the conditions of infection through the extract of *G. edulis* being similar to molecular components of non-self organisms. The innate immune system of the shrimps is not capable of recognizing or remembering non-self particles and so there is a great dependence on pathogen-molecular pattern recognition (Lee & Söderhäll 2002).

β-glucan binding protein (βGBP) induces degranulation and the activation of the prophenoloxidase enzyme, both of which are essential in the innate immune system of the shrimp. Data from 1% concentration of *G. edulis* has yielded promising results for the algae to be an alternative source of immunostimulatory substance for the shrimp. Through unpaired t-test, it has been proven that there is a significant increase in total haemocyte count and phenoloxidase activity from both 1% and 3% against control 0 and control 1 from prophylactic and therapeutic set-ups, respectively-supporting the data from Amparyup et al (2012) and Yeh & Chen (2009) wherein β-1,3-glucan molecules propagate βGBP inducing activity for higher cellular and humoral response. Moreover, subjecting the mortality data to one-way ANOVA strengthens the possibility of *G. edulis* to be deployed to shrimp farmers.

Mortality. Testing the survival rate of each set-up showed that there was a significant difference between prophylactic and therapeutic set-up wherein the aqueous extract of *G. edulis* is an effective preventive solution to the growing problem of WSSV. Moreover, testing between concentrations, 1% aqueous extract of *G. edulis* exhibited gradual and consistent cellular and humoral response as proven by the levels of both total haemocyte count and phenoloxidase activity. This is strengthened by the significant difference of 1% against 3% concentrations in the survivability of shrimps when subjected to the treatments, both in prophylactic and therapeutic set-ups. Eliciting an immune response to the shrimp could also have detrimental effects to the shrimps survivability. Prophylactic and therapeutic data show that there is a significant drop of shrimp survival rate when exposed to a higher dose of *G. edulis* this is in contrast to 1% concentration survival rate wherein the decline in shrimp population is gradual and not significant. Sreejamole & Greeshma (2013) conducted a study wherein higher concentration of *Gracilaria* extract proved to be cytotoxic to brine shrimp. Eahamban & Antonisamy (2012) concluded that

flavonoids, triterpenes, steroids, tannins, alkaloids, phenols and glycosides may be responsible for biological activities including cytotoxic activities. This suggests that the best concentration for the shrimp to stimulate an immune response and have the chance of survival is at 1% concentration.

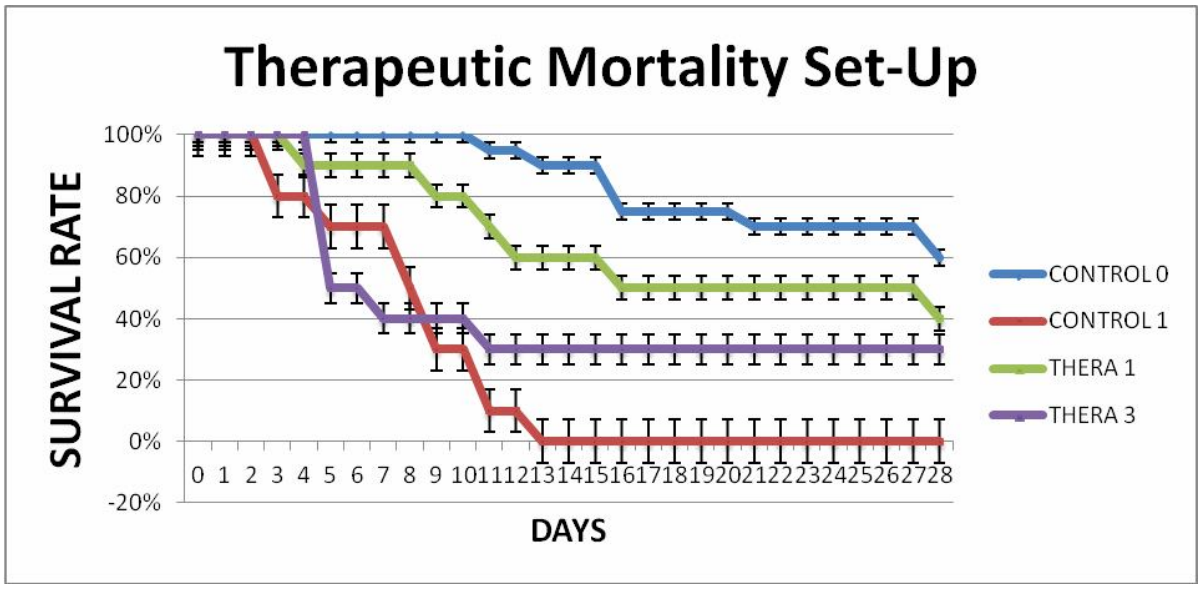

Figure 6. Mortality test for Thera 1 and Thera 3 vs Control 1 and Control 0 - (i) Control 1 – Shrimps infected with WSSV not immersed in extracts; (ii) Control 0 – Shrimps uninfected not immersed in extracts. (iii) Thera 1 – Shrimps infected with WSSV and immersed in 1% *G. edulis* extract; (iv) Thera 3 – Shrimps infected with WSSV and immersed in 3% *G. edulis* extract.

Conclusions. The present study reported that a boost in the immune response of shrimps infected with WSSV immersed in 1% *G. edulis* extract was reflected by the increase of haemocyte count and phenoloxidase activity. Therefore, the aqueous extract of *G. edulis* at 1% can also be used as an immunostimulant for the freshwater shrimp *M. rosenbergii* against WSSV as it was able to reduce mortality drastically. On the other hand, shrimps immersed in 3% *G. edulis* extracts also showed increased immune response but it was not as efficient as that of 1% because concentrations higher than 1% of the extract could be detrimental to the shrimps because of high dose of β-1,3-glucan, leading to immune suppression. The results demonstrated that exploring the species of *Gracilaria* in the Philippines as immunostimulant can significantly contribute to the improvement of the shrimp aquaculture industry. The study also provides a means of producing eco-friendly formulations, cost–effective and hassle-free strategies without sacrificing efficiency in combating shrimp viral diseases.

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