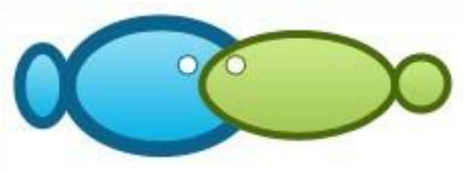

Immunomodulatory response of AHPND-infected *Litopenaeus vannamei* using *Premna odorata*

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Abstract. Shrimp aquaculture is unequivocal a major food source of the growing seafood industry. Production however has been affected by several devastating diseases. One of the obstacles crippling the shrimp industry is the existence of pathogens such as Acute Hepatopancreatic Necrosis Disease (AHPND). Several biotechnological tools have been implemented to prevent the spread of this pathogen. Ecologically sustainable approaches such as immuno-modulation are essential in mitigating disease infection. This study aims to evaluate immunomodulatory responses of shrimps using the extract of *Premna odorata.* Locally known as 'Alagaw'. Immunity and resistance of *Litopenaeus vannamei* infected via immersion with AHNPD-caused by *Vibrio parahaemolyticus* were examined*.* Immune parameters monitored were total haemocyte count (THC), phenoloxidase activity (PO) and superoxide dismutase assay (SOD). Qualitative phytochemical screening and free-radical scavenging activity were also performed. Results of the study indicated that the feeds with *P. odorata* extract can be an effective immunomodulator against Acute Hepatopancreatic Necrosis Disease (AHPND).

Key Words: Acute Hepatopancreatic Necrosis Disease (AHPND), immunomodulator, *Vibrio parahaemolyticus.*

Introduction. Shrimp aquaculture is a major source of income in several countries, including the Philippines. In fact, the Philippines used to be number three in shrimp production in the early 1990's, now we are 7th. Current annual production in the country is around 54,000 metric tons. Dramatic decline was observed in the late 1990s (Mojica 2008).

Litopenaeus vannamei, also known as white leg shrimp, is considered to be the most widely cultured shrimp in the world and is raised in almost 27 countries. AHPND, also known as early mortality syndrome (EMS) is one of the emerging diseases found in the shrimp aquaculture industry. It was first reported in southern China in the year 2010 and subsequently in Vietnam, Thailand, Malaysia, and the Philippines (Dabu et al 2017; De Schryver et al 2014; FAO 2013). AHPND symptoms include empty gut and atrophied, pale hepatopancreas (Kumar et al 2020; Li et al 2017; Lai et al 2015). Shrimp ponds infected by AHPND, experience high levels of mortality, reaching up to 100 percent death rates in several cases (FAO 2013). AHPND is caused by the bacteria known as *Vibrio parahaemolyticus*.

Vibrio parahaemolyticus was identified as causative agent of AHPND (To et al 2020). It is in the same family that causes cholera (*Vibrio vulnificus*) and it resides in brackish saltwater. This bacterium is halophilic, it requires salt and is also dependent in the temperature of a specific habitat (Center for Disease Control and Prevention 2013). It is a self-limiting bacterium that can lead to acute gastroenteritis in humans, but in relation to AHPND, it turned out to be fatal in shrimp farms.

Premna odorata is a medicinal plant traditionally used in the Province of Albay and used to treat tuberculosis in the Philippines. It is also known as Alagaw, a native tree of the

Philippines, often used in treatments related to vaginal irritation (Lirio et al 2017; Quisumbing 1978). It is part of the seven components of the herbal preparation called "Pito-pito". Alagaw is a small hairy tree, ranging from 3 to 8 meters high and with leaves of 10-20 cm long. Flowers are greenish-white or nearly white, and fruits are dark purple and rounded. *P. odorata* is often found in secondary forests with low altitudes from Batan Islands, and Northern Luzon to Mindanao. It can also be found from India to Myanmar, Nepal, Taiwan, China, Indochina, Japan, Thailand, Malaysia, and Australia. The plant exhibits immunomodulatory potential, showing high response to immune activity (Gokani et al 2007). This study aims to assess the immunomodulatory effects of leaf extracts to AHPND-infected *L. vannamei*. Results of this research will be essential in the improvement and development of the shrimp aquafeed industry.

Material and Method

Sampling. Sampling of *Litopenaeus vannamei*, collection of *Premna odorata* and leaf extract preparation experiments were accomplished from September 2015 to March 2016. *Litopenaeus vannamei* juveniles were taken from Batangas, Philippines. Shrimps were acclimated for three days in the wet laboratory of Thomas Aquinas Research Complex (TARC), University of Santo Tomas and were placed in glass tanks with 25°C water temperature and salinity of 15ppt. Samples were fed during day and night with commercial feeds.

Preparation of leaf extract. The Leaves of *Premna odorata* were collected from Odiongan, Romblon, Philippines. It was identified and verified at Jose Vera Santos Memorial Herbarium, Institute of Biology, University of the Philippines, Diliman and UST Herbarium. The leaves were air-dried until moisture content becomes constant. They were grinded into powder and were soaked to 95% ethanol. It was filtered using Whatman® filter paper (Sigma Aldrich, Germany). The leaf ethanolic crude extract was concentrated with Eyela Rotavap at 40°C. The crude extract was properly labeled after extraction and stored at -20°C.

Incorporation of extract to feeds. The crude ethanolic extract was prepared using 1%, 3% and 5% concentration. 2% starch was added as binder to commercial feeds. It was molded into pellets and was dried in the oven.

Bacterial culture preparation. *Vibrio parahaemolyticus* culture that was used in preparation for the challenge test was obtained at the Research Center for Natural and Applied Sciences (RCNAS), TARC, UST. The said culture was used for the lethal dose 50 (LD_{50}) experiment. It was prepared with the Tryptic Soy Broth (TSB) according to the number of the experimental and controlled set-ups. Bacterial culture was carried out for 18 hours to get the desired concentration of 10^2 CFU/mL. The bacteria in the culture broth were determined using McFarland standards in order to have the right concentration in this experiment.

Infection assay (immersion). Infection tanks (6L) were cleaned, and 900 mL of filtered brine water and aeration were added. A total of 208 shrimps were placed to the infection tanks before the inoculation of bacteria. Experimental tanks were prepared by adding 9L clean filtered brine water. Immersion was conducted by adding the inoculum (volume of cultured broth) in a separate flask together with the sterile TSB+ up to 100mL and the culture was mixed thoroughly before transfer. Culture broth and TSB+ broth was added slowly to the infection tanks and tanks were covered after infection. Immersion of the shrimps lasted for an hour with aeration. After one (1) hour, shrimps were transferred to the experimental tanks and were observed daily.

Monitoring of water quality. Regular checking of the salinity of each tank was done using of a refractometer (Brix, NJ, USA). Each tank was maintained at 15ppt of salinity. Filtering devices were used, and water was changed every three days to maintain its cleanliness.

Prophylactic experiment. Prophylactic measure is designed to prevent diseases. In the

prophylactic set-up, the response of *L. vannamei* observed before and after bacterial challenge. A total of 160 shrimps were used for the prophylactic set-up. Ten sampling days were allotted for the said set-up. Five (5) tanks were prepared for the prophylactic experimental set-up containing 32 shrimps. Each of the five tanks were designated with its corresponding treatment: *Premna odorata* (PMO) 1%, 3%, 5%, a control (infected; fed with commercial feeds) and a naive (uninfected; fed with commercial feeds). Shrimps were fed with their corresponding feeds at 2% body weight for 7 days, and then infected with *Vibrio parahaemolyticus* on the 7th day and fed for the next 5 sampling days. There were ten (10) sampling points, before infection (0, 1st, 3rd, 5th, 7th day) and after infection (7th, 8th, 10th, 12th, and 14th day), in which shrimps were sacrificed for haemolymph extraction. Sampling was done in triplicates.

Therapeutic experiment. The therapeutic set-up is designed to investigate the infected shrimps' response to the leaf extract enriched feeds. The said set-up involved a shorter amount of sampling days. There were 5 sampling days for the said set-up. Five (5) tanks were prepared for the therapeutic measure, same as the prophylactic set-up. Five (5) tanks from the said set-up contained 18 shrimps each for the immune parameters. A total of 90 shrimps were used for therapeutic measure. Shrimps were infected with *Vibrio parahaemolyticus* through immersion and were fed at 2% body weight for 7 days with their corresponding feeds: PMO 1%, PMO 3%, PMO 5%, negative control (infected; fed with commercial feeds) and a naive (uninfected; fed with commercial feeds). Three shrimps had their haemolymph extracted from each THC/PO tank during the 0, 1st, 3rd, 5th, and 7th day.

Measurement of immune parameters

Total haemocyte count (THC). A volume of 0.1 mL haemolymph was extracted from each shrimp sample using a syringe with 0.9 ml anticoagulant sodium citrate and was placed in a microcentrifuge tube. The haemocyte solution was stained with trypan blue and was mixed using a vortex. Neubauer ruling haemocytometer was used to count haemocytes and was observed under a light microscope.

Phenoloxidase activity assessment. Phenoloxidase activity (PO) was measured by detecting dopachrome that was produced from L-dihydroxyphenylalanine (L-DOPA). Haemolymph was centrifuged at 800 rpm for 3 mins at 4°C. Pellets were rinsed and resuspended in 1 ml cacodylate-citrate buffer. The supernatant was discarded after each suspension. The experimental PO activity was measured by transferring the suspension in a sterilized microcentrifuge tube. The cell suspension was incubated at room temperature and zymosan was added to act as an elicitor. L-DOPA followed with cacodylate buffer was added in both conditions. A spectrophotometer was used to measure the optical density at a wavelength of 490 nm.

Superoxide dismutase assay (SOD). Beauchamp and Fridovich (1971) method was used with slight modification to measure superoxide dismutase activity. The shrimp muscle was homogenized with phosphate buffer centrifuged at 6,000 rpm for 5 minutes at 4°C. The supernatant was incubated at 65°C for 5 minutes then placed on a microplate. EDTA, methionine, NBT, and riboflavin were added, and the crude extract was left to stand in florescent light. A spectrophotometer was used to measure SOD activity at a wavelength of 560nm.

DPPH (1,1-diphenyl-2-picrylhydrazyl) assay. DPPH procedure is adapted from Jacinto et al (2011) and Hou et al (2004). A total of 1.2mg of DPPH was dissolved in 10 mL absolute ethanol to make the three hundred (300) µM DPPH free radical solution. Ninety-five microliters (95 ul) of DPPH solution was dispensed to 96-well plates, five microliters of 4% *P. odorata* crude extract was then added to make a final volume of 100uL per well. Gallic acid was used as the positive control and DMSO as the negative control. It was wrapped gently with aluminum foil and incubated for 45 minutes at 37°C. The absorbance was read at 517 nm and computed using the

following formula:

$$\text{Percent free radical scavenging activity} = \frac{AbsDMSO - Abs\ extract}{AbsDMSO - Abs\ GallicAcid}\ x\ 100$$

Growth measurements. The body weight (g) of shrimps was measured weekly for three weeks and the specific growth rate (SGR) was calculated based on Sweilum (2006) formula:

SGR = (final Weight – initial Weight) / days x 100

Feed conversion ratio (FCR). The number of feeds given, and weight gain of the shrimps was measured for each week to compute for the feed conversion ratio based on Sweilum (2006) formula:

FCR = weight of feeds in / weight gain

Data analysis. Analysis of variance at 5% confidence level was used to compare the difference between treatments utilized. The data was expressed in mean standard deviation.

Results

Growth analysis. Three random samples per tank were weighed and their averages were computed for the monitoring of their specific growth rate (SGR). The SGR indicates the increase in general weight of the shrimps in a span of 3 weeks. Moreover, it identifies which of the samples can yield the most favorable growth of the organism from all treatments. Growth response of shrimps was monitored and those incorporated with 1%, 3%, and 5% extracts of *Premna odorata* (PMO) yielded no significant differences. Figure 1 showed that the feeds incorporated with PMO did not affect the growth response of the shrimps.

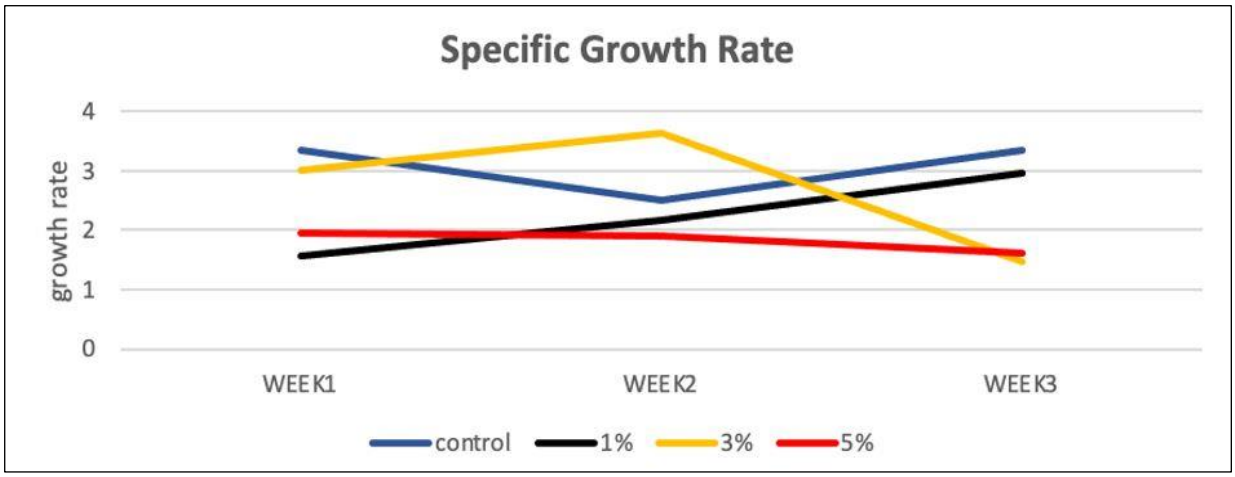

Figure 1. Specific growth rate (SGR) of *L. vannamei* fed with feeds incorporated with PMO extracts (1%, 3%, 5%) and commercial feeds (control) for three weeks (21 days).

Feed conversion ratio (FCR). FCR shows the ratio of feeds given to the weight gain of the shrimps for three weeks (Figure 2). This shows that 3% PMO has the lowest ratio for three weeks. A decreasing ratio for 1% PMO was observed and the 5% PMO increased greatly during the third week.

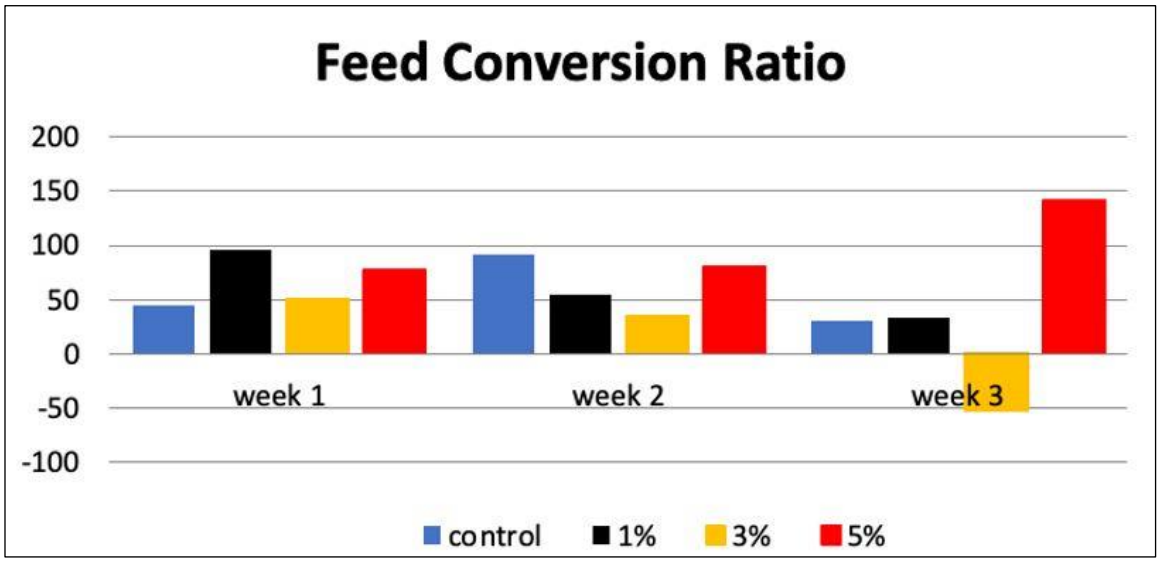

Figure 2. Feed conversion ratio (FCR) of *L. vannamei* fed with 1%, 3% and 5% PMO extracts, and control (commercial feeds) for three weeks (21 days).

Total haemocyte count (THC). Figure 3 showed that there is an increasing trend from day 0 up to day 14 for shrimps fed with 5% PMO. Feeds incorporated with extracts have shown higher THC compared to negative and naive set-ups. Data acquired for both therapeutic and prophylactic set-ups, showed a continuous increase in the THC. There was further increase in the THC after infection, as the shrimps were able to combat against the presence of AHPND. Highest THC was recorded with the feeds incorporated with 5% PMO with a value of $1.7x10^7$ cells/mL. On the other hand, in the therapeutic set-up the highest value recorded was seen with the feeds treated with 5% *PMO* extract yielding a value of $9.3x10^6$ cells/mL suggesting that the shrimp immune system was activated for defense against AHPND.

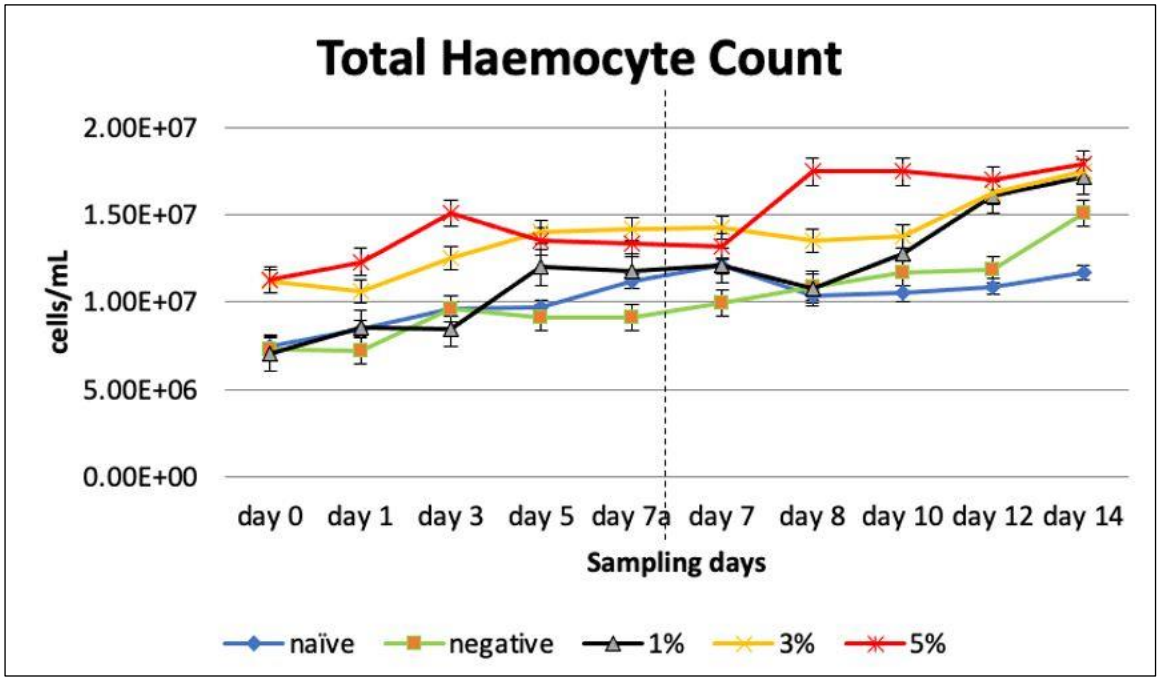

Figure 3. Total haemocyte count (THC) of *L. vannamei* in prophylactic set-up fed with feeds incorporated with PMO extracts (1%, 3%, 5%), no treatment (naive) and commercial feeds (control) for two weeks (14 days).

Based on the phenoloxidase graph (Figure 4), the prophylactic set-up, naive had the highest PO recorded at day 3 with a value of between 0.200 - 0.250. Feeds incorporated with 5% *P. odorata* extract had the highest PO recorded at day 7 post infection with a value between 0.100- 0.150. The graph also showed that feeds incorporated with 5% extracts had visible response during the post-infection compared to the other setups where there are minimal variations in the values starting from day 8 up to day 14 post infection. PO assay showed that in the prophylactic set-up, after the infection, there was an increase in day 7B with the feeds incorporated with different concentrations of *P. odorata.* This indicates that the shrimps were mounting defenses against the presence of AHPND. After day 7 there was a continuous decrease due to the ability of the shrimps to cope with the presence of AHPND thus they were able to maintain their internal balance. For the therapeutic set-up, there was an increase in the values in day 0, that might be due to the adaptive mechanism of shrimps in response to AHPND infection. According to Ascencion et al (2020), the innate immune system of *L. vannamei* contains cellular and other components that work synergistically or individually which protects the said organism during infection. The values remained close to almost stagnant in D1-D5 (Day 1 to Day 5) due to the ability of the shrimps to cope through homeostasis. An increase in day 7 occurred on the other hand, as the shrimp's way to adjust to the changes in the environment.

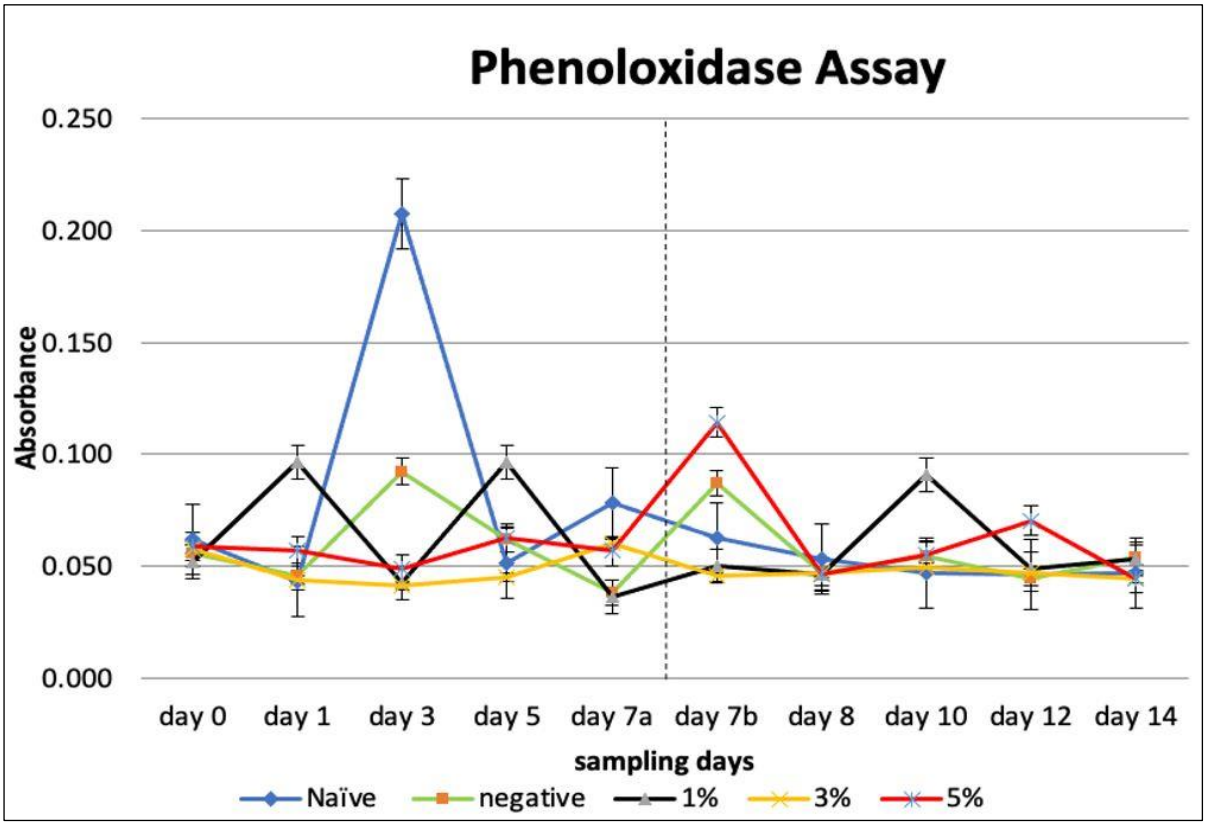

Figure 4. Phenoloxidase Activity (PO) of *L. vannamei* in prophylactic set-up fed with feeds incorporated with PMO extracts (1%, 3%, 5%), no treatment (naive) and commercial feeds (control) for two weeks (14 days).

SOD data showed highest peak at D3 on feeds incorporated with 5% *P. odorata* extract with values between 1.000 – 1.200 (Figure 5). A decrease in SOD values on feeds incorporated with 3% and 5% starting from D5 up to D8 with values ranging between 0.400-0.600, and there was an increase in all the set-ups at D10 with minimal variations on SOD values until D14.

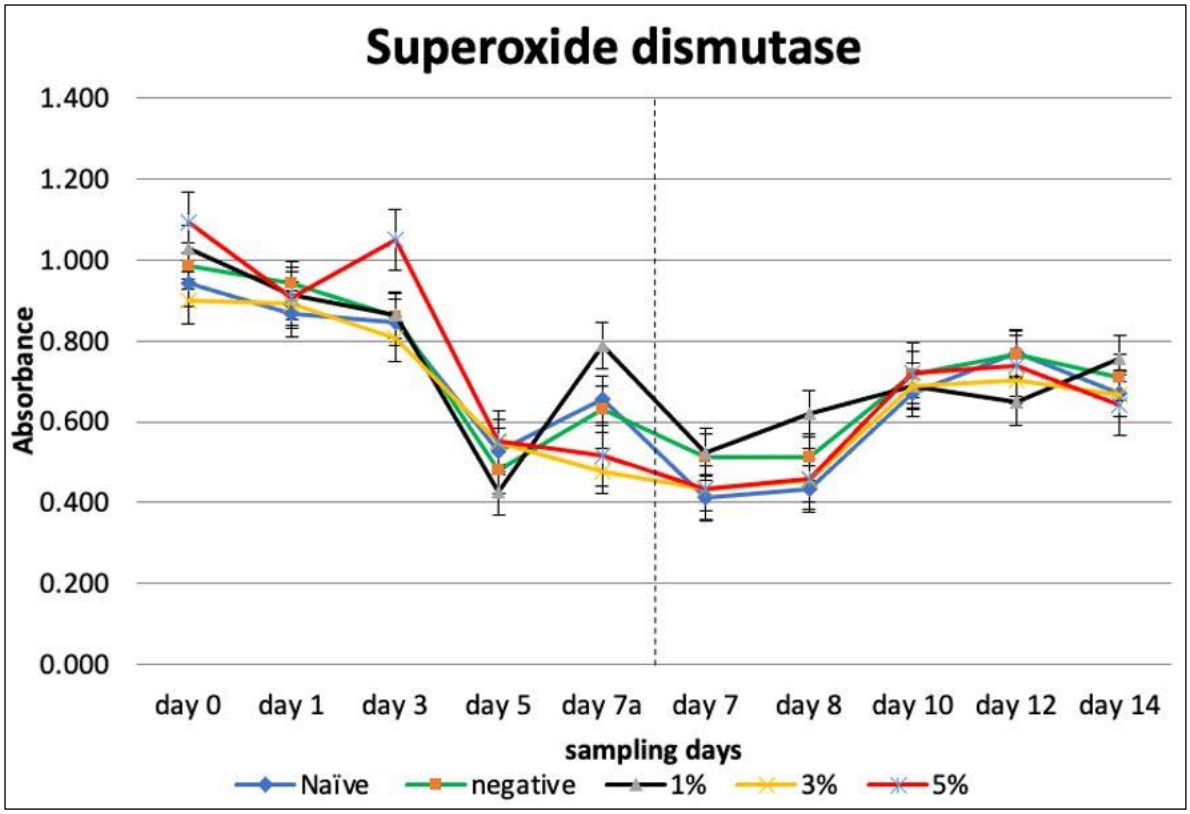

Figure 5. Superoxide dismutate (SOD) of *L. vannamei* in prophylactic set-up fed with feeds incorporated with PMO extracts (1%, 3%, 5%), no treatment (naive) and commercial feeds (control) for two weeks (14 days).

Therapeutic set-up. The therapeutic set-up is designed to test the infected shrimps' response towards the extracts. The said set-up involved a shorter amount of sampling days. There were 5 sampling days for the said set-up.

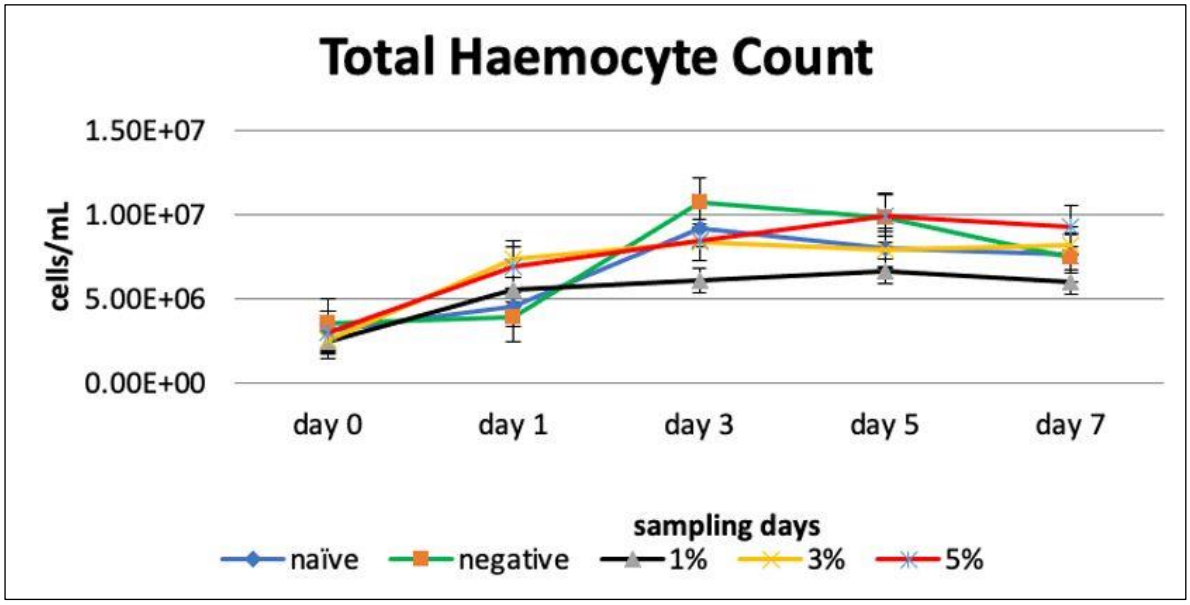

Figure 6. Total haemocyte count (THC) of *L. vannamei* in therapeutic set-up fed with feeds incorporated with PMO extracts (1%, 3%, 5%), no treatment (naive) and commercial feeds (control) for one week (7 days).

The naive and negative set-ups showed the highest THC increase on day 3 with $9.23x10^6$ and $1.07x10^7$ cells/ml, respectively and gradually decreasing up to day 7 (Figure 6). For the 1% PMO, the highest increase was observed on D5 with $6.63x10^6$ cells/ml and a minimal decrease on day 7. For the 3% PMO, the highest increase was seen on day 3 with $8.40x10^6$

cells/mL while 5% PMO showed the greatest increase on day 5 with $9.93x10^6$ cells/ml. These results indicated that increase in number of THC in the shrimps fed with *P. odorata* extract is an ideal treatment against APHND.

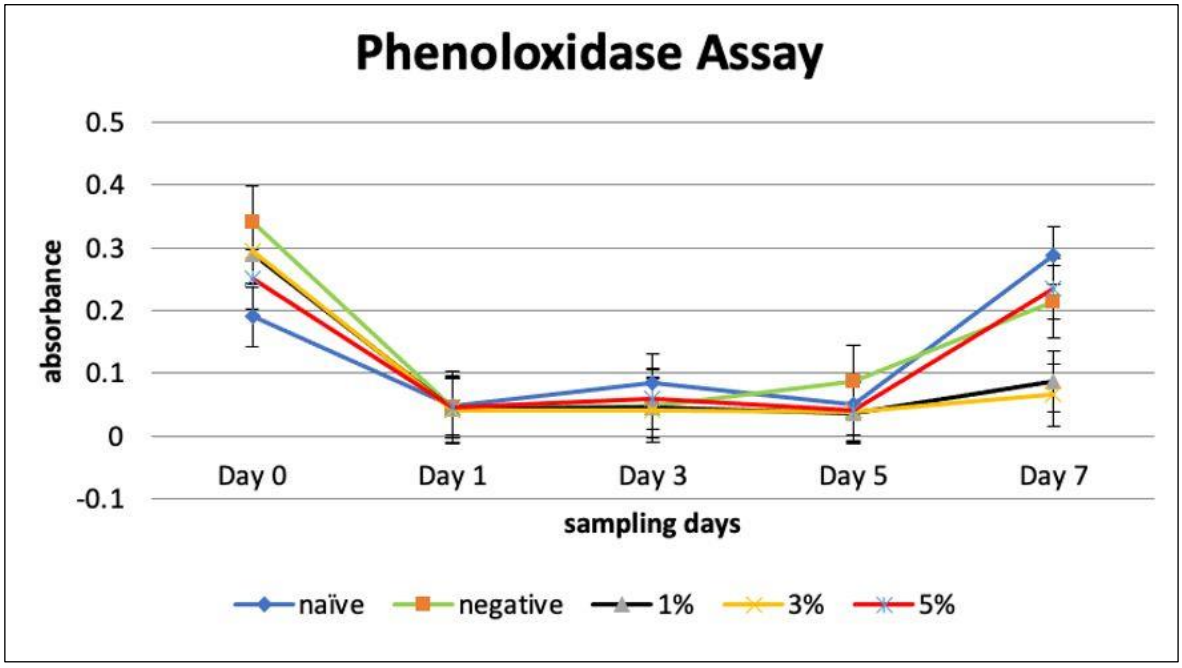

Figure 7. Phenoloxidase assay (PO) of *L. vannamei* in therapeutic set-up fed with feeds incorporated with PMO extracts (1%, 3%, 5%), no treatment (naive) and commercial feeds (control) for one week (7 days).

Figure 7 shows that after the infection, there was a noticeable PO decrease on the absorbance values from D0 to D1 for all the set-ups. On the 5th sampling day, there is a decrease in the values of the set-ups, except for the negative set-up which clearly displayed an increase from 0.048 to 0.087. At Day 7, all the set-ups surged

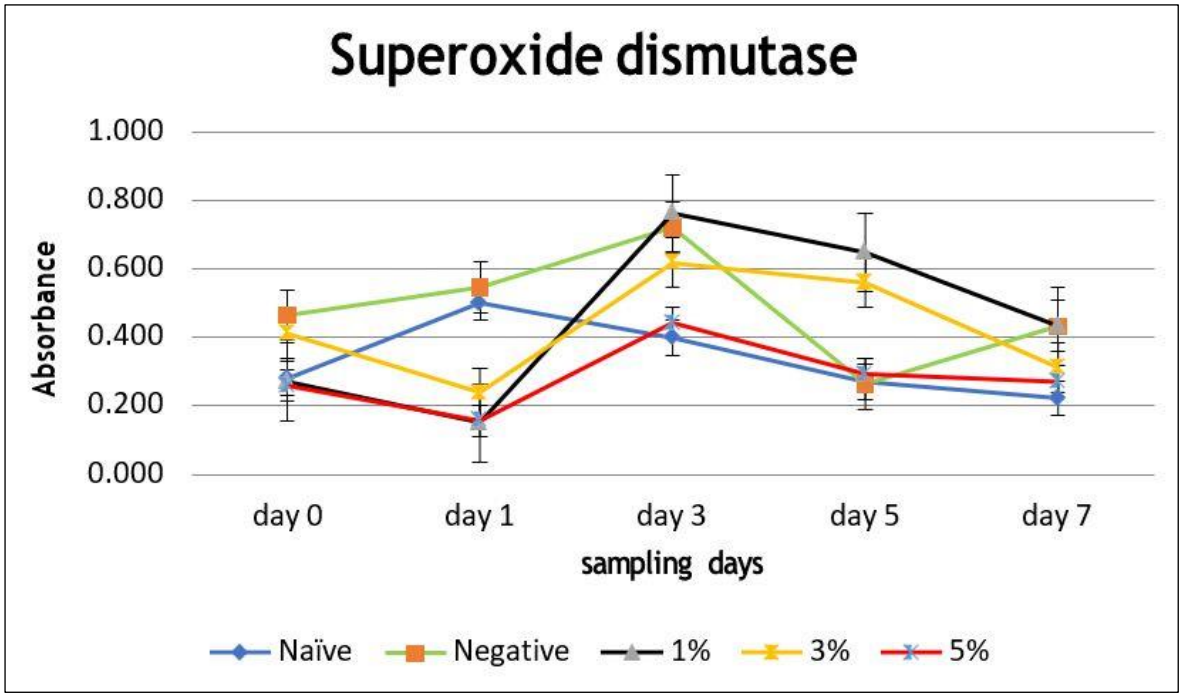

Figure 8. Superoxide dismutase (SOD) of *L. vannamei* in therapeutic set-up fed with feeds incorporated with PMO extracts (1%, 3%, 5%), no treatment (naive) and commercial feeds (control) for one week (7 days).

Based on Figure 8, the naive set-up showed an increase from day 0 to 1 from 0.280 to

0.502, with its absorbance value continuously decreasing up to day 7. For the negative set-up, the highest increase was on day 3 with an absorbance value of 0.720. A sudden decrease followed on day 5 with an absorbance value of 0.265 and a slight increase was observed on day 7. For the 1%, 3% and 5% PMO, the highest increase was seen on day 3 with a value of 0.762, 0.618, and 0.442, respectively then gradually decreasing up to day 7, this could be due to the increase related to stress. This increase in the relative protein content 48 hour after exposure to immunostimulants may be related to the increase in the proteins related to stress (Campa-Cordova et al 2002).

Phytochemical components. In relation to the antioxidant properties, phytochemicals may carry out biological or biochemical activity that has protective or preventive properties in relation to diseases. Qualitative phytochemical test indicates the presence of antimicrobial properties, the test showed the phytochemical components of *P. odorata* crude ethanolic extract has the following constituents: terpenoids, flavonoids, saponins and phenolic constituents.

Diphenyl-picrylhydrazyl (DPPH) Assay. This test is used to measure the free radical scavenging activity of *P. odorata.* The assay makes use of colorimetry in such a way that change in color from purple to yellow indicates the presence of antioxidant properties. Gallic acid is used as the positive control with 100% free radical scavenging activity. *P. odorata* has shown a great potential to scavenge free radicals with a percentage of 79% (Figure 9).

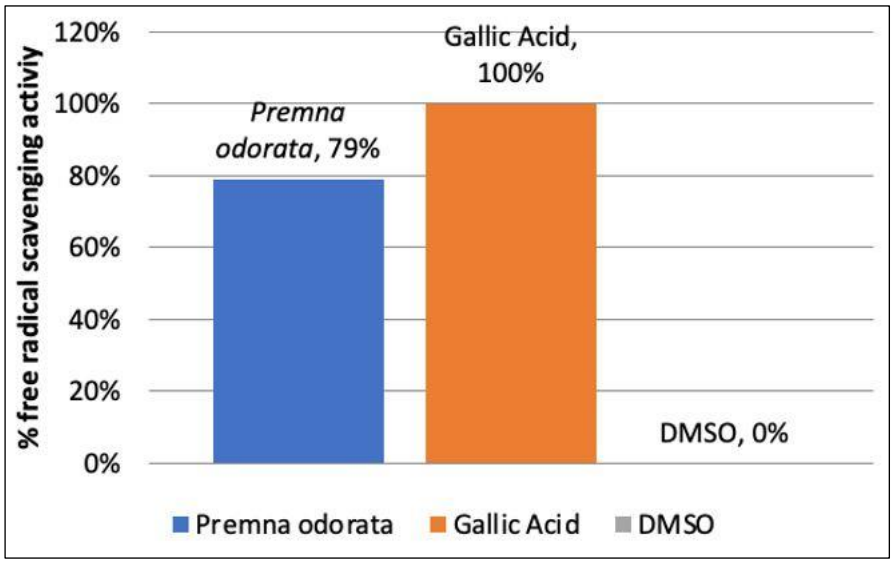

Figure 9. Diphenyl-picrylhydrazyl (DPPH) assay of *P. odorata* crude extracts. *P. odorata* showed 79% free radical scavenging activity.

Discussion. Feed conversion ratio (FCR) is the efficiency of an animal to convert feeds in order to increase body mass. Feed conversion ratio is the ratio of feeds in grams to the weight gain in grams. It is defined as the efficiency of an animal in converting feeds to increase body mass. The lower FCR value on fish meal indicates that the shrimps had converted the feeds more efficiently into flesh (Jabeen et al 2004). High FCR value shows conversion of feeds is not that efficient for growth. FCR results showed that 3% PMO has the lowest FCR compared to other concentrations which means the shrimps were able to eat the feeds and thus supporting the FCR studies of Jabeen et al (2004). The study also shows that FCR is primarily related to the sizes of the shrimps showing similar results based on the study of Wyban et al (1995).

Total haemocyte count (THC). Total haemocyte count (THC) depends on the condition of the body against infection. Haemocytes decrease dramatically during an infection (Smith & Soderhal 1983; Smith et al 1984). THC depends on the condition of the body as it combats against infection. Blood circulation of invertebrates such as shrimps is open during haemocyte distribution in both tissues and vascular system. Therefore, build up or motion of cells from tissues into haemolymphs causes a rise in THC values (Tsing et al 1989). Cell

lysis or expansion of cells from haemolymph to tissue may cause a decline in THC values (Pipe & Coles 1995). Another factor that may cause a decline in THC values of shrimps is the change in physiological form as enhanced vulnerability to bacterial infection (Ford et al 1993). THC results showed that the increasing number of haemocytes of infected shrimps fed with extracts have responded to the bacteria. This could be attributed to the phytochemicals found in the *P. odorata* extract which have the potential interactions as a natural remedy to improve the inflamed liver function (Elmaidomy et al 2020).

Phenoloxidase assay (PO). Phenoloxidase assay is the test that exhibits the reactions or immune response and wound healing abilities in the melanization pathway. Phenoloxidase is the last enzyme in the melanization cascade associated with antimicrobial activity in invertebrates. As it is cytotoxic, most invertebrates store phenoloxidase in the inactive form prophenoloxidase. Quantifying PO is through colorimetry and by converting the substrate L-dopa into dopachrome and measured spectrophotometrically at 490nm (Ardia 2008). In melanogenesis, both enzymatic and non-enzymatic plays an important role. The process is responsible for repairing tissues, and encapsulation of multicellular pathogens as well as defending against other pathogens i.e., bacterial, viral agents and fungi. Invertebrate PO requires a complex system for the inhibition and activation which involves different cell types such as plasmocytes and haemocytes, proPO inhibitory systems, signaling, PO zymogens and even PO itself (Gonzalez-Santoyo & Córdoba-Aguilar 2012). According to Suphantharika et al (2003), an increase in phenoloxidase activity in response to an immunostimulant coincides with an increase in THC. PO data post-infection proved that the shrimps were able to respond to *V. parahaemolyticus infection* by activating phenoloxidase that helps in defense reaction. This corroborates the results from previous immunomodulatory test conducted by Gonzalez-Santoyo and Córdoba-Aguilar (2012). The results of THC and PO is a normal effect of infection from bacteria as supported by Hsieh et al (2008).

Superoxide dismutase (SOD). In eukaryotic and prokaryotic cells, SOD is the enzyme that acts as the first line of defense. It serves as the protection against poisons produced by superoxide radicals. Under the family of metalloenzymes, it protects the body against the poisonous effects caused by superoxide radicals produced in cell compartments. This enzyme generates the disproportionation of superoxide radicals into H_2O_2 and O_2 (Corpas et al 2006). In some studies, extracts from plant are acting in resemblance to SOD by direct formazan production inhibition (Thring et al 2009). SOD results showed that during the prophylactic and therapeutic set-ups, the decrease of absorbance values after the infection was due to the shrimps' maintenance of homeostasis while the increase on the following days was due to the combat against superoxide radicals. The observed increase in SOD activity in the presence of an immunostimulant supports the findings of Campa-Cordova e al (2002). The shrimps were able to combat infection because of the disproportionation of superoxide radicals (Corpas et al 2006). The high antioxidant potential of the crude extract of *P. odorata* species could be attributed in the presence of its phytochemical compounds.

Conclusions. Our research report for the first time that feeds incorporated with *P. odorata* can be an effective immunomodulator for shrimp against AHPND. It showed high levels of total haemocyte count, phenoloxidase activity and superoxide dismutase activity compared to commercial feeds. Due to the non-significant effect in growth, the feeds incorporated with *P. odorata* can be used as additive to the commercial feeds. In the prophylactic set-up, on the other hand, there is a significant difference which means that the feeds incorporated with *P. odorata* exhibit immunomodulatory properties and have aided in the combat of AHPND. Our findings will be valuable in mitigating the spread of Acute Hepatopancreatic Necrosis Disease.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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