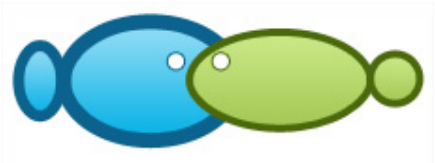

The immunomodulatory effect of *Citrus microcarpa* peel in *Macrobrachium rosenbergii* challenged with *Vibrio alginolyticus*

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Abstract. The world aquaculture market regard *Macrobrachium rosenbergii* as a highly valued commodity having a significantly large size which contributes to its marketability. Like any invertebrate, *M.rosenbergii* lacks an adaptive immune system making them vulnerable to bacterial and viral disease outbreaks which often lead to mass mortality and eventual production loss. A bacterial pathogen responsible for vibriosis, *Vibrio alginolyticus* is one of the major causes of losses in shrimp aquaculture production. In this study, we report the efficiency of crude extracts from *Citrus microcarpa* (locally known as calamansi) peel, an abundant waste product, as an immunomodulation agent for *M.rosenbergii.* The resulting crude extract was incorporated into commercials feeds and given as treatment in different percentages to prophylactic and therapeutic setups. Immune parameters such as total hemocyte count (THC) and phenoloxidase (PO) activity were assessed. For both set ups, shrimps fed with 1% calamansi-peel feed had the greatest increase in THC and PO activity in comparison with the 3% and 5% concentrations and the control groups. Challenge tests showed that shrimps fed with the same feed concentration exhibited significant survival percentages. These findings showed the calamansi-peel extracts' capability to induce bacterial resistance against vibriosis caused by *V.alginolyticus*. Our results provided evidence that the calamansi peel extract's immunomodulation makes it a potential feed additive for the Philippine shrimp aquaculture industry.
Key Words: *Macrobrachium rosenbergii*, *Vibrio alginolyticus*, vibriosis, *Citrus microcarpa*, immunomodulation, phenoloxidase.

Introduction. Losses in the worldwide production of *Macrobrachium rosenbergii* (De Man, 1879) can be owed to a variety of factors such as poor farm management and the presence of pathogens (Pridgeon & Klesius 2012). Known to cause vibriosis, *Vibrio* sp. is a pathogen that is able to induce mortality in marine (Alday-Sanz et al 2002) and freshwater shrimps (Alambra et al 2012) which often lead to disease outbreaks (Pridgeon & Klesius 2012; Yeh et al 2010).The presence of visible black spots on shrimp's cuticle, constriction of hepatopancreas tubules and slow clotting are some of the manifestations and symptoms of infection (Alday-Sanz et al 2002).

M.rosenbergii like other crustaceans possess only an innate immune system (Hoffman et al 1999), unable to adapt to their previously encountered pathogen. Hemocytes serve as their primary defense where each type possesses distinct cytoplasmic granules that serve a specific purpose. For instance, hyaline cells play a role in phagocytosis and melanin production. On the other hand, semi-granular and granular cells function in encapsulating and releasing of cytotoxic products by initiating the prophenoloxidase (proPO) activating system (Söderhäll & Cerenius 1998; Jiravanichpaisal et al 2006). Phenoloxidase, the terminal enzyme of the proPO system, is an indicator of fungal and bacterial presence being activated by the cell wall polysaccharide component β-1,3-glucan (Hou & Chen 2005).

In search for effective means to counter bacterial infection, most have turned to the use of drugs such as antibiotics to prevent bacterial infection. However, the danger in the use of such substances is the development of drug-resistant bacteria (Pridgeon & Klesius 2012; Harikrishnan et al 2011). An alternative approach is the use of immunomodulants from plant extracts which function in altering the immune response by either activating it or suppressing its function. Apart from the fact that they possess a broad spectrum of activity against pathogens, plant-based immunomodulants are considered economical as well as eco-friendly making them an ideal solution (Harikrishnan et al 2011).

Citrus microcarpa, a locally abundant commodity, has been known for its activity against a wide range of bacteria including *Vibrio* sp., *Escherichia coli, Edwardsiella* sp. among others (Musa et al 2008; Wei et al 2008; Lee & Najiah 2009). A previous work evaluated the efficacy of *C.microcarpa* extract as an immunostimulant for African catfish culture against Edwardsiellosis. Fish fed with enhanced feeds had significantly higher antibody response and lower cumulative mortality compared to those fed with normal feeds (Wei et al 2014). However, the effect of *C.microcarpa* peel, a locally abundant waste product, as an immunostimulant for fresh water prawn is not yet reported.

Here, we report the use of *C.microcarpa* peel as an immostimulant for *M.rosenbergii* for the first time. Immune parameters such as total hemocyte count (THC) and phenoloxidase (PO) activity were evaluated. Challenge test was also performed to assess the immune enhancing capability of the extract as a feed additive against *Vibrio alginolyticus* infection.

Material and Method. Sampling of *M.rosenbergii* and succeeding experiments were accomplished from July 2013 until March 2014.

Sample collection. Four hundred (400) juvenile shrimps (2-4 grams) were purchased from the Southeast Asian Fisheries Development Center (SEAFDEC) in Binangonan, Rizal and National Freshwater Fisheries Technology Center-Bureau of Fisheries and Aquatic Resources (NFFTC-BFAR) Munoz, Nueva Etaretarecija. Shrimps were acclimated and fed with commercial feeds for three (3) days in five (5) thirty five (35) liter plastic tanks maintained at room temperature (25-29°C).

Preparation of Citrus microcarpa peel shrimp feed. *Citrus microcarpa* peels were collected from Marventure Corporation in Tonsuya, Malabon and brought to the University of Santo Tomas Herbarium for proper identification. The peels were cut into small pieces, sun-dried for 48 hours and pulverized. Different concentrations of the calamansi peel shrimp feed were prepared according to the method of Lotaka & Piyatiratitivorakul (2012) with minor modifications. Three concentrations: 1%, 3%, and 5% calamansi peel powder were produced and incorporated in the commercial feed with 2% starch. The mixture was molded into strips to produce pellets. The enhanced feeds were oven-dried at 60^{o}C for a day and were stored at 4^{o}C until use.

Culture of V.alginolyticus. *Vibrio alginolyticus* obtained from the Philippine National Collection of Microorganisms (PNCM) in University of the Philippines- Los Baños (UPLB) in Laguna was cultured in Nutrient Broth with 2% NaCl and was incubated for 24 hours at 25^{O}C and stored at 4°C prior to use. The procedure from the work of Alambra et al (2012) was then followed applying minor modifications. Bacteria from the nutrient broth was inoculated in nutrient agar (NA) medium (with 6% NaCl) and stored at room temperature overnight. A single colony was transferred from the NA plate to two (2) mL nutrient broth and was subjected to overnight incubation to generate the bacterial stock. Subsequently, one (1) mL of stock culture was then diluted to one (1) L nutrient broth (NB) and was incubated overnight on a horizontal shaker. At the end of incubation period, one (1) mL of the culture suspension was serially diluted 10-fold with dechlorinated water. Utilizing the pour plate method of colony counting, a bacterial concentration of 3 x 10^4 CFU/ mL was established for the stock culture. Three dosages: 10^1, 10^2, 10^3 – colony forming units (CFU/mL) were tested and mortality was observed

for 5 days. The dosage with 10^2 CFU/mL concentration elicited 50% mortality and was selected for the succeeding challenge tests and measurement of immune parameters.

Quantification of immune parameters. Immune parameters such as the total hemocyte count (THC) and phenoloxidase activity (PO) were observed and quantified. For both the therapeutic and the prophylactic set ups, a total of five (5) tanks were allotted mainly for the naïve, control and three experimental set-ups (1%, 3% and 5% concentrations) with 20 shrimps per tank. Sampling was performed in triplicates at various time points for (a) therapeutic set ups and (b) prophylactic set ups divided into (i) pre-infection and (ii) post-infection periods. A 3-mL sterile syringe (22 gauge) with 0.9 mL anticoagulant solution was used to extract approximately 0.1 mL of the hemolymph from the ventral sinus of each shrimp in every duration. The mixture in each syringe was divided into two portions: 500µL for THC and 500µL for PO activity determination.

For the measurement of THC, Giemsa stain was applied on the samples and dispensed together into the chambers of the Neubauer© haemocytometer. Results were then viewed and counted under a compound light microscope (Olympus). Following the method of Hernandez-Lopez et al. (1996) with slight modifications, PO activity was spectrophotometrically quantified through the formation of dopachrome generated from L-dihydroxyphenylalanine (L-DOPA). The hemolymph extracts were pooled and centrifuged at 800rpm for 3 min at 4°C. The supernatant was discarded and pellet was resuspended in 1mL cacodylate-citrate buffer (0.01 M trisodium cacodylate, 0.45 M sodium chloride, 0.10 M trisodium citrate, pH 7.0) and was centrifuged. The supernatant was discarded and the pellet was resuspended in 200µL cacodylate buffer. The tube was incubated in room temperature (25^oC) for 10 minutes. Subsequently, fifty (50) µL of zymosan was added to the tubes serving as an elicitor. After 5 minutes, 50 µL of L-DOPA was added, followed by 800µL of cacodylate buffer. The cell suspension was placed in a microcentrifuge tube then transferred to a 96-well microplate for the determination of PO activity. The optical density was measured at a wavelength of 490nm using a BIOTEK© ELISA (USA) Microplate Reader.

Bacterial Challenge. In the challenge test, a total of eighty (80) shrimps were immersed for three (3) hours in tanks infected with *V.alginolyticus* at a bacterial concentration of 10^2 CFU/mL. Following immersion, the shrimps were transferred and sorted into eight (8) plastic tanks with four (4) liters of water each (10 shrimps per tank). For both therapeutic and prophylactic set ups, one (1) tank was allotted for the positive control set up while the other (3) three were for the three concentrations (1%, 3% and 5%) of calamansi peel extract feed. The mortality data was then observed and recorded for three hundred thirty six (336) hours.

Statistical Analysis. The results were statistically evaluated using the GraphPad Prism v.6 software. One-way ANOVA (analysis of variance) was used for the challenge test data while measurements from the THC and PO activity utilized the Tukey's HSD Test to determine the significant differences. A confidence interval of 95% was set for each statistical tool.

Results and Discussion. Shrimps fed with 1% feed concentration exhibited the most significant increase in total hemocyte count (THC) ($p<0.05$) and phenoloxidase (PO) activity ($p<0.05$) in comparison with the succeeding concentrations and control groups. For the therapeutic set up, the administration of the feed (1% concentration) brought about a steady increase in the 1^{st}, 3^{rd} and 5^{th} hours which was followed by a drastic increase during the 24^{th} (29% increase from the 5^{th} hour) and the 72^{nd} hours (28% increase from the 24^{th} hour) (Figure 1).

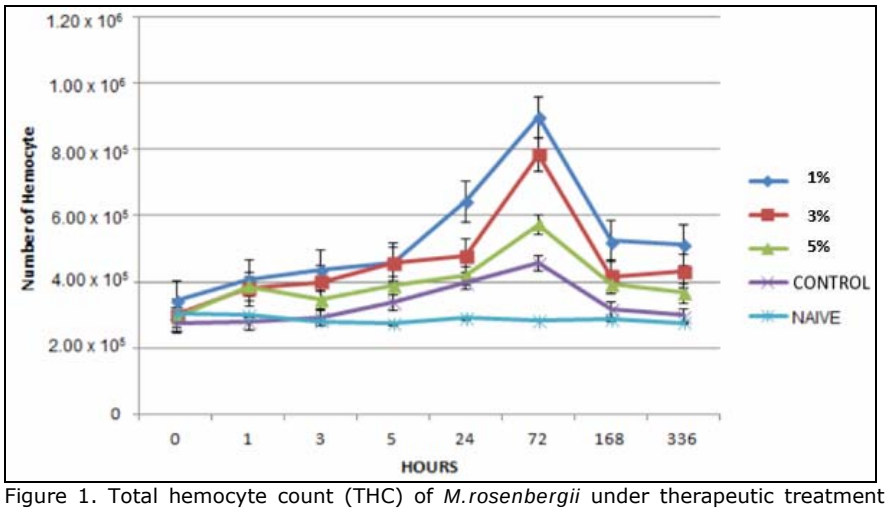

Figure 1. Total hemocyte count (THC) of *M.rosenbergii* under therapeutic treatment with 1%, 3% and 5% *C.microcarpa* peel extract in feed, no treatment with infection (Control) and Naive for 336 hours (14 days) ($p<0.05$).

The similar immediate increase within the 24th and 72nd hour can be observed for the prophylactic set ups pre-infection and post-infection (Figure 2 and 3 respectively). A trend of decreasing THC levels were evident shortly after its increase in figures 1, 2 and 3. This can be attributed to *V.alginolyticus* bacterial infection which can lead to increased stress levels resulting into increased immune response (Yeh et al 2010). Consistent with the trend of decrease after increased THC levels, the study of Cheng et al (2005) states that shrimps exposed to a pathogen exhibit an increase in THC for the first few days of exposure followed by a decline in THC levels and finally recovery towards the initial value.

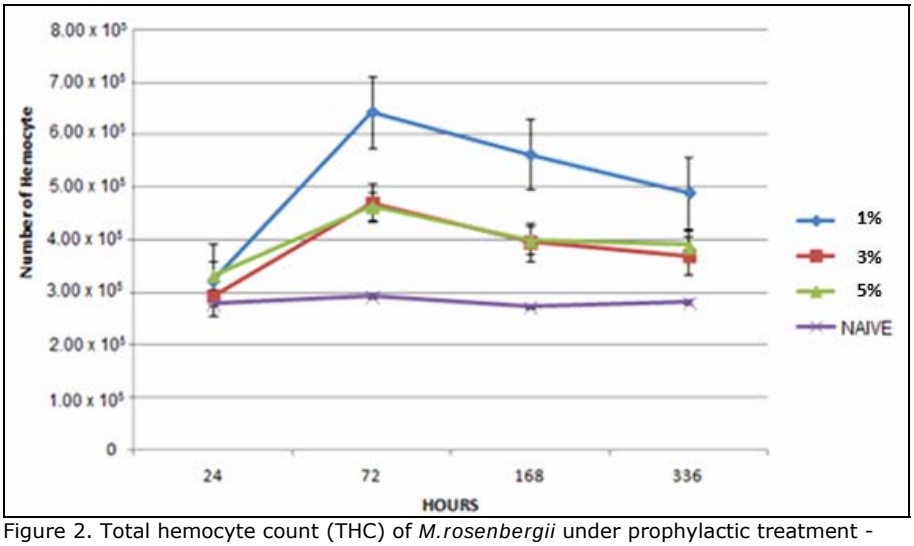

Figure 2. Total hemocyte count (THC) of *M.rosenbergii* under prophylactic treatment - pre-infection with 1%, 3% and 5% *C.microcarpa* peel extract in feed and Naive for 336 hours (14 days) ($p<0.05$).

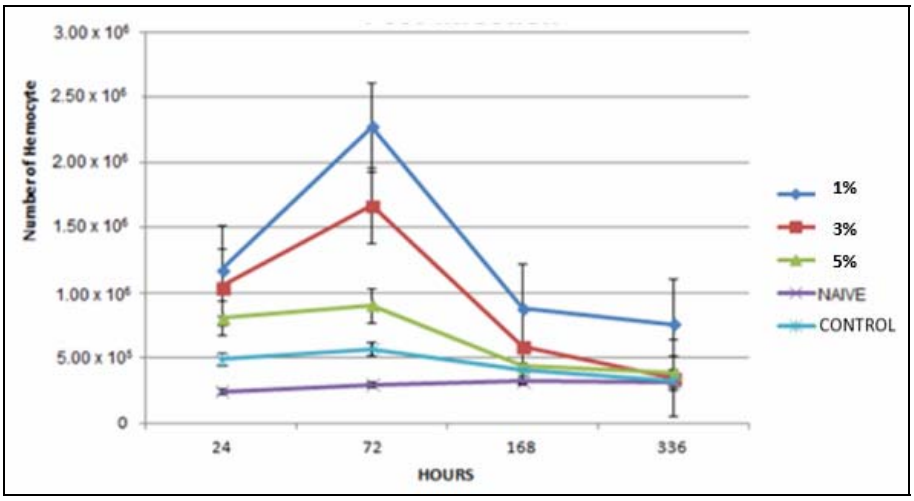

Figure 3. Total hemocyte count (THC) of *M.rosenbergii* under prophylactic treatment - post-infection with 1%, 3% and 5% *C.microcarpa* peel extract in feed, no treatment with infection (Control) and Naive for 336 hours (14 days) ($p<0.05$).

Focusing on the PO activity, the shrimps fed with enhanced feeds in the prophylactic set up had the highest activity on the 168th hour of the pre-infection period in comparison with the positive control and naïve group (Figure 4). A similar trend can be observed in the prophylactic set up post-infection but with a slightly slower increase in comparison with the pre-infection stage (Figure 5).

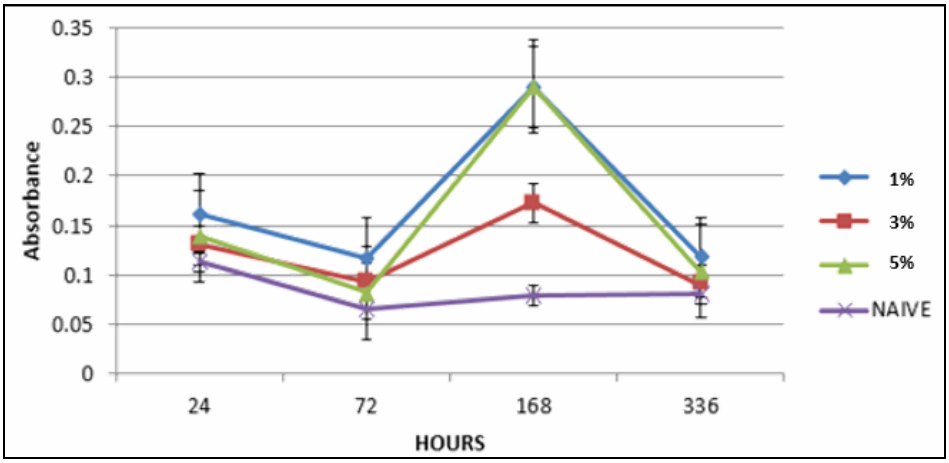

Figure 4. Phenoloxidase (PO) activity of *M.rosenbergii* under prophylactic treatment - pre-infection with 1%, 3% and 5% *C.microcarpa* peel extract in feed and Naive for 336 hours (14 days) ($p<0.05$).

Sung et al (1998) explains that the inherent increase in PO activity was initiated by the prophenoloxidase cascade leading to the activation of the melanization pathway. β-glucans located from the cell walls of fungi and gram negative bacteria such as *V.alginolyticus* are considered microbially-derived PAMPS which upon binding activate the prophenoloxidase cascade (Amparyup et al 2013). This explains the increase seen in the control tanks where shrimps where infected but provided with only commercial feeds (Figure 5 and 6).

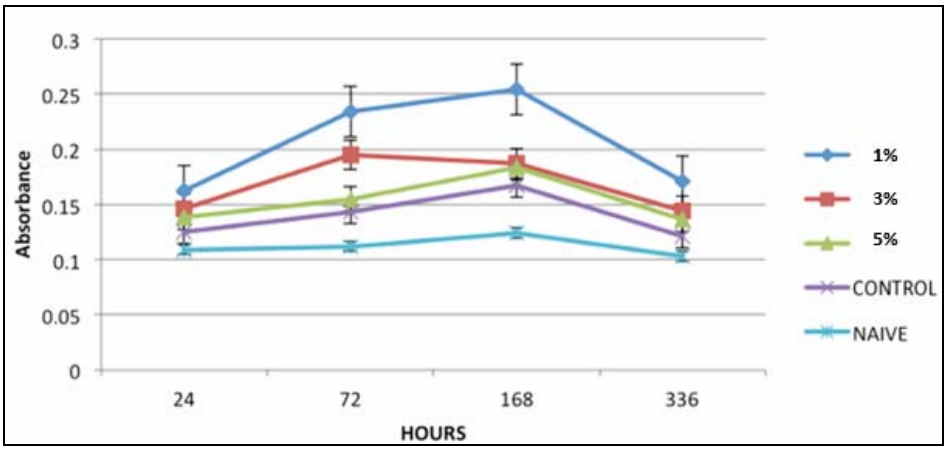

Figure 5. Phenoloxidase (PO) activity of *M.rosenbergii* under prophylactic treatment - post-infection with 1%, 3% and 5% *C.microcarpa* peel extract in feed, no treatment with infection (Control) and Naive for 336 hours (14 days) ($p<0.05$).

Consistent with these results are the noticeable increase following the first few hours (1st, 3rd and 5th hours) of infection in the therapeutic set up (Figure 6). Taking this into consideration, it can also be noticed that the 1% concentration of *C.microcarpa* peel extract additive exhibited the highest PO values for immune response (Figure 5 and 6). Since *C.microcarpa* peel together with other citrus fruits have been known to harbor rich amounts of Vitamin C (Okwu 2008; Hoyle & Santos 2010; Lim 2012) they are highly associated with increased immunity in shrimps serving as elicitors of immune response. Earlier studies have established that Vitamin C serves as a vital feed additive since deficiencies in penaeid shrimp can result to inadequate growth, poor feed conversion, lessened stress resistance, low survival rate, incomplete molting, inefficient wound healing and melanised lesions under the exoskeleton (He & Lawrence 1993; Chen & Chang 1994; Shiau & Hsu 1994).Previous studies have also shown that Vitamin C supplementation is associated with increased survival rates, THC, PO, superoxide anion (O_2^-) production ratio and feeding efficiency in marine shrimps (Lee & Shiau 2003; Maggioni et al 2004; Yang et al 2004). Likewise, there have been reports indicating the same findings for Vitamin C in freshwater prawns *Macrobrachium nipponsense* (Yang et al 2004) and *Macrobrachium malcolmsonii* (Sahoo et al 2005; Asaikkutti et al 2016).

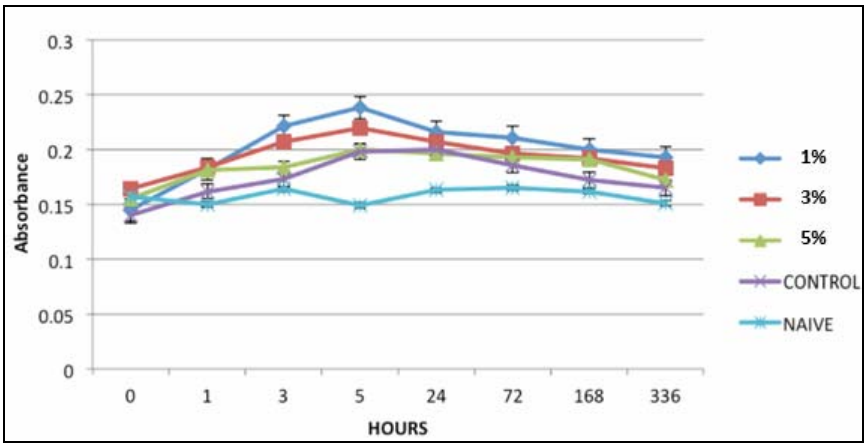

Figure 6. Phenoloxidase (PO) activity of *M.rosenbergii* under therapeutic treatment with 1%, 3% and 5% *C.microcarpa* peel extract in feed, no treatment with infection (Control) and Naive for 336 hours (14 days) ($p<0.05$).

Apart from results of increased immune response, feeding trials with 1% concentration in all set ups had considerably higher survival rates compared with the infected controls (Figure 7 and 8). At the 336th hour (14th day) post-infection (p.i.), the survival rates of the *V.alginolyticus* infected controls were 35% for the therapeutic (Figure 7) and 31%

for the prophylactic (Figure 8) set ups. Shrimps with 1% concentration enhanced feeds have shown 71% (therapeutic and prophylactic) survival 336th hour p.i. for both set ups while those with 3% (Therapeutic – 59%, Prophylactic – 59%) and 5% (Therapeutic – 59%, Prophylactic – 41%) concentration showed significantly lower survival rates. The apparent higher efficiency of the 1% concentration over the 3% and 5% can be explained by the feed additive's (C.microparpa peel crude extract) palatability. Other studies performing feeding trials (Avenido & Serrano 2012; Tantikitti 2014) have emphasized that increased concentration of the feed additive often exceeds the shrimp's basal nutritional requirements making it unpalatable for consumption. In agreement with this trend, the results of Asaikkutti et al (2016) have revealed that increased supplementation of Vitamin C exceeding optimal concentration results in decreased THC levels and survivability for freshwater shrimps. Similarly, this is also true for other organisms such as yellow catfish (Liang et al 2017), soft-shelled turtles (Wang & Huang 2015), grass carp (Li et al 2014) and kuruma shrimp (Nguyen et al 2012). Attractants or stimulants with low molecular weight are most of the time purposely included in feeds involving plant proteins to increase palatability at higher concentrations (Tantikitti 2014). Since attractants were not utilized in the study to improve palatability in feed concentrations, it follows that the 3% and 5% concentrations have significantly less palatability in comparison with the 1% concentration.

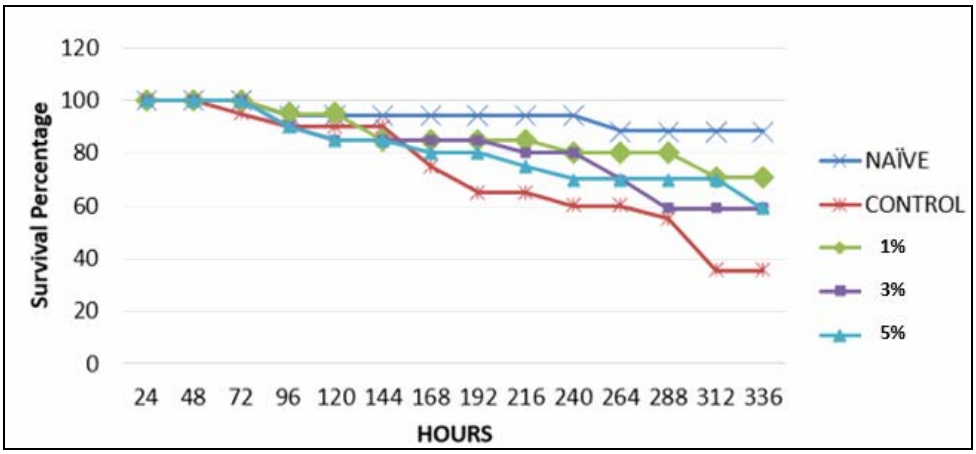

Figure 7. Survival rate of *M.rosenbergii* under therapeutic treatment - with 1%, 3% and 5% *C.microcarpa* peel extract in feed, no treatment with infection (Control) and Naive for 336 hours (14 days).

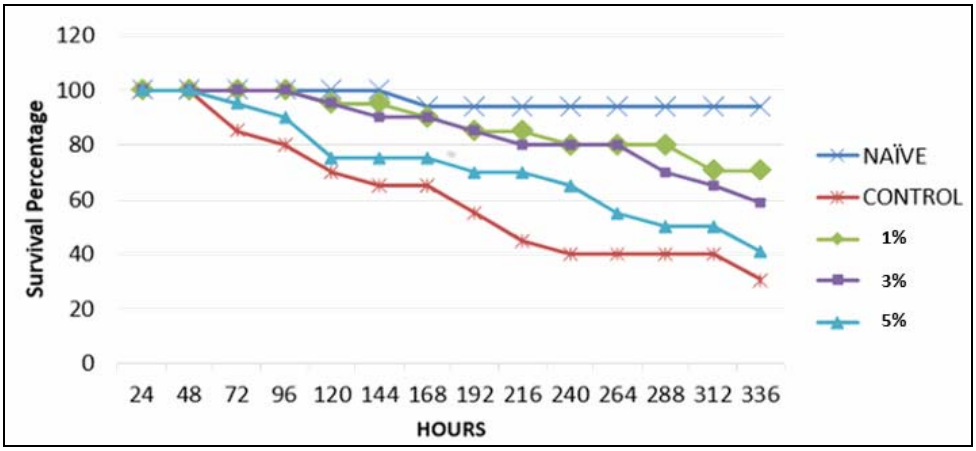

Figure 8. Survival rate of *M.rosenbergii* under prophylactic treatment - post-infection with 1%, 3% and 5% *C.microcarpa* peel extract in feed, no treatment with infection (Control) and Naive for 336 hours (14 days).

In comparison with other immunostimulants, a reputable aquaculture website in 2015 (www.thefishsite.com) recommended the use of Vitamin C together with Vitamin E, phospholipids, trace minerals, carotenoids and essential fatty acids over the stimulants derived from the cell envelopes of microorganisms such as lipoproteins, lipopolysaccharides and polysaccharides. The work of López et al (2003) reported that feed additives with Vitamin C deliver enhanced immune response compared to β 1,3-glucan-based (polysaccharide) immunostimulants since the nutritional status of shrimps are significantly improved. Additionally, β 1,3-glucan-based diet supplementation in the work of Zhao et al (2012) showed that high concentrations (500mg/kg and 1000mg/kg) of the polysaccharide resulted in decreased immune response in comparison with lower concentrations (250mg/kg). Further, the study of Solidum et al (2016) explained that β glucan-based supplementation when administered at high concentrations resulted in overstimulation thereby causing immunosuppression and increased mortality in shrimps. This is the primary reason why immunostimulants from microbial cell envelopes are being discouraged in shrimp farm use (www.thefishsite.com).

Aside from the nutritional and immunological benefits of Vitamin C in citrus fruits such as *C.microcarpa*, anti-microbial activity also plays a role in increased disease resistance and survival rates. Previous studies have revealed that *C.microcarpa* can serve as immunostimulants for African catfishes challenged with Edwardsiellosis, providing increased resistance against disease and serving as an antimicrobial agent (Wei et al 2014). Congruent with this study, an isolated 2-Hydroxypropane-1,2,3-Tricarboxylic Acid from *C.microcarpa* has shown an anti-microbial property against fish pathogens (Lee and Naijah 2009). In addition to this, *C.microcarpa* have also been shown to exhibit antimicrobial activities on a wide range of bacteria such as *V. alginolyticus, V. parahaemolyticus, V. vulnificus, Aeromonas hydrophila, Citrobacter freundii, Edwardsiella tarda, Escherichia coli, Staphylococcus aureus, Streptococcus agalatiae* and *Streptococcus aginosus* (Musa et al 2008). Similarly, wax, hexane (Johann et al 2007), methanol and acetone (Khushwaha et al 2012) extracts from the peels of citrus fruits have been shown to inhibit bacterial and fungal growth. With this, the utilization of immunostimulants from plant extracts particularly citrus fruits has proven to be an efficient alternative to antibiotics (Harikrishnan et al 2011) not only due to improved immune response (Asaikkutti et al 2016) and nutritional status (López et al 2003) but also due to decreased risk of overstimulation and immunosuppression.

Conclusions. Taken all together, our findings suggest that ingestion of crude extract of *C.microcarpa* peel can activate shrimp immune response and at the same time improve survival rates. The study demonstrated that a minimal feed concentration of 1% can effectively enhance the immune system of *M.rosenbergii* by elevating the levels of THC and PO activity. Survival rates were significantly high even with the presence of bacterial infection highlighting its capability to induce bacterial resistance against *V.alginolyticus*. Finally, our results established that citrus fruit waste products can be used in applications of immunostimulants leading to a cost-effective and eco-friendly alternative feed additive for the shrimp aquaculture industry.

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Conflict of Interests. All authors declare that they are not currently affiliated or sponsored by any organization with a direct economic interest in subject of the article.

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