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Research Article

Journal of Aquaculture and Technical Development

Immunostimulatory Effect of *Hygrophila spinosa* Enriched Diet in Freshwater Prawn, *Macrobrachium malcolmsonii* against *Vibrio alginolyticus*

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Abstract

The present study was investigating the immunomodulatory effect of *Hygrophila spinosa* enriched diet on immune response and disease resistance in freshwater prawn, *Macrobrachium malcolmsonii* against *Vibrio alginolyticus*. The total heamocyte count (THC) and prophenoloxidase (pro PO) activity of *M. malcolmsonii* fed with *H. spinosa* enriched diet at 1.0 g showed significant level between weeks 1 and 4, but 0.01 g and 0.1 g diets shown low level. The respiratory burst (RB) activity and superoxide dismutase (SOD) activity significantly enhanced when prawn fed at 0.1 g and 1.0 g herbal extract enriched diets; however, it did not significantly enhanced in prawn fed at 0.01 g herbal diets during the experimental period. The phagocytic (PC) activity was significantly low when prawn fed at 0.01 g and 1.0 g diets whereas 1.0 g diet was obtained significantly enhanced. The percentage (%) of clearance efficiency (CE) was significantly enhanced in prawn fed at 0.1 g or 1.0 g diets against pathogen. The mortality was low in prawn fed at 0.1 g and 1.0 g herbal enriched diets than fed with 0.01 g diet against pathogen. This study concluded that *H. spinosa* extract enriched diet can positively modulate the immune system and decreased mortality in *M. malcolmsonii* against *V. alginolyticus* infection.

Keywords: *Hygrophila spinosa*, Immune response, *Macrobrachium malcolmsonii*, Supplementation diet, *Vibrio alginolyticus*

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Received Date: November 25, 2019

Accepted Date: December 05, 2019

Published Date: December 12, 2019

Citation: Devi G, Balasundaram C, Ramasamy H (2019) Immunostimulatory effect of *Hygrophila spinosa* enriched diet in freshwater prawn, *Macrobrachium malcolmsonii* against *Vibrio alginolyticus*. J Aqua Tech Deve 2: 002.

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Introduction

Freshwater prawn farming is popular in South East Asian countries recently because of their high price and market demand in both domestic and export markets. In India, the largest species of prawns are of interest for aquaculture such as *Macrobrachium rosenbergii*, *M. malcolmsonii*, and *M. gangeticum*, respectively in monoculture and in polyculture with compatible carps [1,2]. Among, Indian River prawn, *M. malcolmsonii*, is the second largest fast-growing freshwater prawn after *M. rosenbergii* and one of the most economically important species in the aquaculture industry. *M. rosenbergii* is widely distributed throughout India, especially in the river draining into the Bay of Bengal. *M. malcolmsonii* culture was suffered due to viral disease such as Infectious hypodermal and hematopoietic necrosis virus (IHHNV), *Macrobrachium* muscle virus (MMV), Appendage deformity syndrome (ADS), Slow growth syndrome (SGS), Branchiostegal blister disease (BBD), and Idiopathic muscle necrosis (IMB); infected prawn is weakened swimming ability, reddish discoloration of cuticles, muscular atrophy, growth retardation and deformities [3]. Several bacterial disease outbreaks have been occurred such as *Vibrio* spp., *Aeromonas* spp., *Pseudomonas* spp., and *Lactococcus garviae* which caused high mortalities in *Macrobrachium* hatcheries [4-11].

In decapods crustaceans, hyaline cells are generally involved in phagocytosis, which is very important process of eliminating micro-organisms or foreign particles of the host [12,13]. A series of reactive oxygen species (ROS) are produced during phagocytosis. Starting this process, the membrane-bound enzyme complex, NADPH oxidase, assembles after binding of a foreign particle to the cell, and reduces molecular oxygen to superoxide anion (O_2^-), subsequently leading to the production of hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radicals (OH·) [14]. Superoxide anion is the first product released during this process known as respiratory burst (RB), and plays an important role in anti-bacterial activity [15]. A number of natural and herbal based immuno stimulants have been reported to enhance the innate and adaptive immune system in prawns and shrimps [5,10,11,16,17].

Hygrophila spinosa T. (Acanthaceae), commonly known as Gokulakanta /Talmakhana, is a well-known medicinal plant widely distributed throughout India, Srilanka, Burma, Malaysia, and Nepal. The leaf, root, and seed of the plant are being traditionally used for the treatment of rheumatism, inflammation, jaundice, hepatic obstruction, urinary infection, oedema, gout, diabetes, bacterial infection, etc. [18]. It was contains apigenin 7-o-glucuronide, alkaloids, flavonoids, and steroids in the chloroform extract, as well as alkaloids, flavonoids, tannins, and steroids in the alcoholic extract of the roots, leaves, and flowers of *H. spinosa* [19-21]. The anti-inflammatory and antipyretic potentials of alkaloids, steroids, and flavonoids have been report-

ed [22-26]. There has been no information concerning the immune response of *H. spinosa* on *M. malcolmsonii* against diseases. Accordingly, the purpose of the present study was examining the effect of *H. spinosa* enriched diet on immune response and disease resistance in *M. malcolmsonii* against *V. alginolyticus*.

Material and Method

Plant extract and herbal diet preparation

H. spinosa plant was collected from locally and the identification was done by Plant Science Department. The roots were collected from the plants, washed thoroughly with tap water to rid them of dirt. After washing, the roots were dried under shade to make them suitable for grinding and the dried plant roots were grounded in a mechanical grinder. After it was sieved then stored in an air tight sterile container for further use. One hundred grams of coarsely powdered was successively extracted with 85% ethanol and then filtered. The successive extraction was performed by a cold maceration process for seven days with daily agitation twice following Cooper and Gunn [27] and Singh et al. [28]. The solvent was evaporated using a rotary vacuum evaporator (Buchi, Flawil, Switzerland). The residues obtained after evaporation were stored at -20°C until used for the experiment.

Supplementation diet

The formulated diet and the ingredients are shown in (Table 1). The ingredients of the experimental diet were well mixed and extruded by a pellet extruder (EX 920, Matador, Denmark). Four experimental diets prepared of the pellet with 0 g (control), 0.01 g, 0.1 g, and 1.0 g of *H. spinosa* extracts were sprayed to the basal diet slowly, mixing evenly in a drum mixer, after which it was air dried under sterile conditions for 12 hrs. The control basal diet was added the same volume of solvent without the extracts. The pellets were dried in an oven at 30°C for 18 hrs, packed, and stored in a freezer at -20°C until used. The proximate composition of the diets were quantified following AOAC method comprised 48.9% crude protein, 8.1% crude lipid, 7.1% crude ash, and 13.6% crude carbohydrate.

Ingredients	%
Fish meal	25
Rice bran	20
Soya flour	13
Wheat flour	16
Ground nut cake	15
Tapioca powder	9
Aminovit	1.5
Vitamin mix	0.5
Proximate analysis Carbohydrate	12
Protein	47.2
Fat	7.9
Ash	18.9

Table 1: Feed composition for prawn.

Vibrio alginolyticus

Diseased shrimps were collected from local prawn farms and the muscle was dissected out and culture on tryptic soy agar (TSA, Difco) plates incubated at 28°C for 18 hrs, subsequently they were incubated on thiosulfate-citrate-bile sucrose (TCBS, Difco). One dominant colony was selected and re-streaked onto TSA to obtain pure cultures. The biochemical characteristics were carried out with commercial API 20E Kits (ATB System, bioMerieux) and the biochemical reactions were compared with the reference strain *V. alginolyticus* ATCC (American Type Culture Collection). The genomic DNA was purified by DNA purification Kit (No. A1120, Promega) and the polymerase chain reaction (PCR) tests were carried out using specific PCR primers for identification of *V. alginolyticus* 16S rDNA according Ruimy et al. [17].

Experimental animal

M. rosenbergii were obtained from a commercial farm, reared in 500 mg^{-1} round holding tanks for 2 weeks, and fed formulated diet (Table 1) until they were used for the experiment. The water quality such as temperature 26-32°C, water transparency 30-60 cm, pH 7.0-8.5, dissolved oxygen > 5 mg l^{-1}, free CO_2 < 8 mg l^{-1}, hardness 100-50 mg l^{-1}, total alkalinity 80-150 mg l^{-1}, NH_4^+-N 0.02-0.20 mg l^{-1}, calcium 30-80 mg l^{-1}, phosphorus 0.01-0.90 mg l^{-1}, and nitrogen 0.05-90.5 mg l^{-1} were maintained during the experiment.

Experimental design

After 2 weeks of acclimatization, prawn were divided into four groups of 25 prawns in 250 l tanks and fed with 0 g, 0.01 g, 0.1 g, and 1.0 g of *H. spinosa* extract supplementation diets at the rate of 10% of body weight twice a day. There were three replicate tanks per treatment were maintained. After 30 days of feeding, all groups except control were injected intraperitoneally (i.p.) the ventral sinus of the cephalothorax with 50 µl PBS containing *V. alginolyticus* at 3.7 x 10^7 cfu ml^{-1} whereas control group injected with same volume of PBS. On weeks 1, 2, and 4 post-infection, six prawns randomly collected from each tank to hemolymph blood samples for hematological and immunological assays.

Sample collection

Haemolymph (500 µl) was withdrawn from the ventral sinus of each prawn into a 1 ml sterile syringe (25 gauge) containing 0.9 ml anticoagulant solution (trisodium citrate 30 mM, sodium chloride 0.34 mM, EDTA 10 mM, pH 7.55, osmolality adjusted with 0.115 Mglucose to 780 mOsm kg^{-1}). A drop of the anticoagulant-haemolymph mixture was placed on a haemocytometer to measure THC (Leica DMIL, Leica Microsystems,Wetzlar GmbH, Germany) and the remaining of the haemolymph mixture was used for immunological assays.

Prophenoloxidase (proPO) activity

The proPO activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L^{-DOPA}). The details of measurements were described previously [29,30]. The optical density (OD) of the prawn PO activity was expressed as dopachrome formation per 50 µl haemolymph.

Respiratory Burst (RB) activity

The respiratory burst (RB) of haemocytes was quantified using the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anion (O_2^-) according Liu and Chen [29]. The OD at 630 nm was measured using a microplate reader (Model VERSAmax, Molecular Devices, Sunnyvale, CA, USA) and the RB was expressed as NBT-reduction per 10 µl haemolymph.

Superoxide Dismutase (SOD)

Superoxide dismutase (SOD) activity was measured by its ability to inhibit superoxide radical dependent reactions using the Ransod kit

(Randox, Crumlin, UK). The details of measurements were described previously [31]. A reference standard for SOD was supplied with the Ransod kit. One unit of SOD was defined as the amount required inhibiting the rate of xanthene reduction by 50% and the specific activity was expressed as SOD unit's ml^{-1} [32].

Phagocytic (PC) activity

Briefly, 200 µl of haemolymph was mixed with 200 µl of sterile anticoagulant for PC activity. The methods for the measurements of PC activity were described previously [29]. Two hundred haemocytes were counted and the PC activity, defined as percentage phagocytosis (PR) was expressed as:

PR = [(phagocytic haemocytes)/(total haemocytes)] x100

Clearance Efficiency (CE)

The CE was measured following the method of Adams [33] and the CE to *V. alginolyticus*, defined as percentage inhibition (PI), was calculated as:

PI=100- [(cfu in test group)/(cfu in control group)] x100

Cumulative mortality

There are twenty prawn were used in each group. All groups were used three replicate groups. The preparation of bacterial culture, challenge study, and disease were same in previous section.

Statistical analysis

Data was conducted to compare the significant differences among treatment using the SAS computer software (SAS Institute Inc., Cary, NC, USA). For statistically significant differences, it was required that $P < 0.05$.

Results

Hematology

The THC was significantly low when *M. malcolmsonii* fed with herbal enriched diet at 0.01 g and 0.1 g against *V. alginolyticus*. However, the THC did not significant change when prawn fed at 1.0 g herbal enriched diet against pathogen (Figure 1).

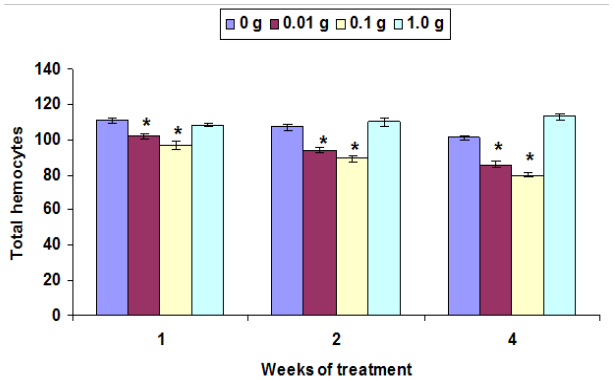

Figure 1: Changes in total haemocytes counts (THC) of *M. malcolmsonii* (mean ± S.E; n = 6) fed with herbal enriched diet against *V. alginolyticus*. Data at the same exposure time with asterisks are significantly different (P < 0.05) among treatments.

Prophenoloxidase (proPO) activity

The proPO activity significantly low when prawn feeding at 0.01 g and 0.1 g herbal enriched diets during the experimental period when compared to control against pathogen. However, prawn feeding with 1.0 g herbal enriched diet did not significantly change against pathogen from weeks 1 to 4 (Figure 2).

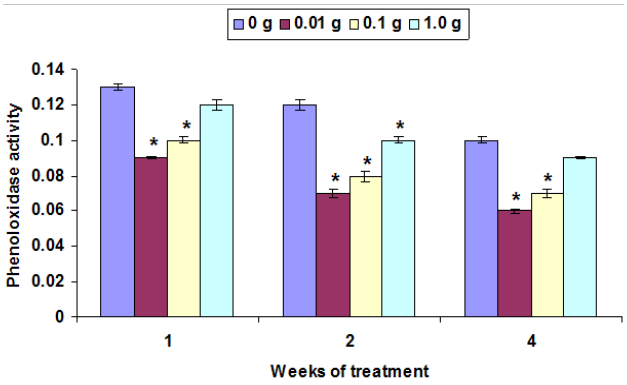

Figure 2: Prophenploxidase (proPO) activity *M. malcolmsonii* (mean ± S.E; n = 6) fed with herbal enriched diet against *V. alginolyticus*. Data at the same exposure time with asterisks are significantly different (P < 0.05) among treatments.

Respiratory Burst (RB) activity

The RB activity did not significantly enhanced at any time when prawn feeding with 0.01 g herbal enriched diet against pathogen. However, the RB activity significantly enhanced in prawn feeding with 0.1 g and 1.0 g herbal enriched diets against pathogen as compared to control (Figure 3).

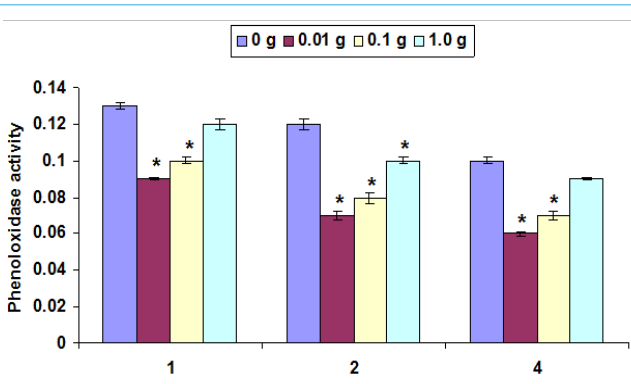

Figure 3: Prophenploxidase (proPO) activity *M. malcolmsonii* (mean ± S.E; n = 6) fed with herbal enriched diet against *V. alginolyticus*. Data at the same exposure time with asterisks are significantly different (P < 0.05) among treatments.

Superoxide Dismutase (SOD) activity

The SOD activity significantly enhanced when prawn fed with 0.1 g diet but did not found significant at 0.01 g or 1.0 g diets against pathogen when compared to control on first week. Prawn feeding with 0.1 g and 1.0 g herbal enriched diets significantly enhanced the SOD activity on weeks 2 and 4 when compared to control against pathogen. However, the activity was not significant when fed with 0.01 g diet on weeks 2 and 4 (Figure 4).

Phagocytic (PC) activity

The PC activity significantly low in prawn fed at 0.01 g and 0.1 g herbal enriched diets from weeks 1 to 4 when compared to control against pathogen. However, prawn fed at 1.0 g diet significantly increased the enhanced the PC activity during the experiment (Figure 5).

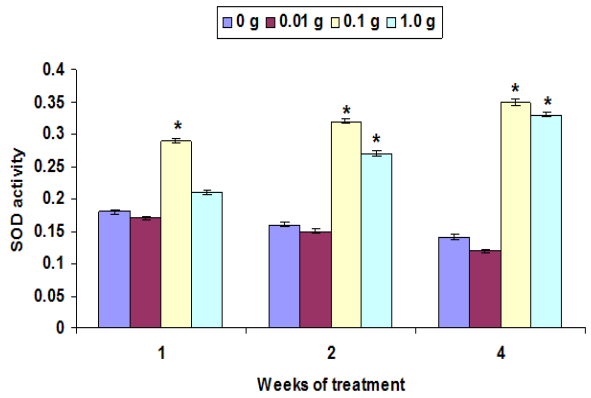

Figure 4: Superoxide dismutase (SOD) activity of *M. malcolmsonii* (mean ± S.E; n = 6) fed with herbal enriched diet against *V. alginolyticus*. Data at the same exposure time with asterisks are significantly different (P < 0.05) among treatments.

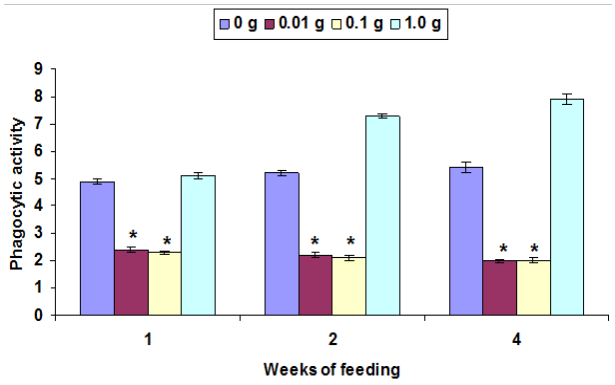

Figure 5: Phagocytic (PC) activity of *M. malcolmsonii* (mean ± S.E; n = 6) fed with herbal enriched diet against *V. alginolyticus*. Data at the same exposure time with asterisks are significantly different (P < 0.05) among treatments.

Clearance Efficiency (CE)

The CE did not significant change when prawn fed at 0.01 g herbal diet on weeks 1 to 4 when compared to control against pathogen. However, the CE significantly increased when prawn fed at 0.1 g and 1.0 g herbal diets when compared to control. All diet supplementary doses of herbal extract enriched diet significantly increased the CE on second week against pathogen (Figure 6).

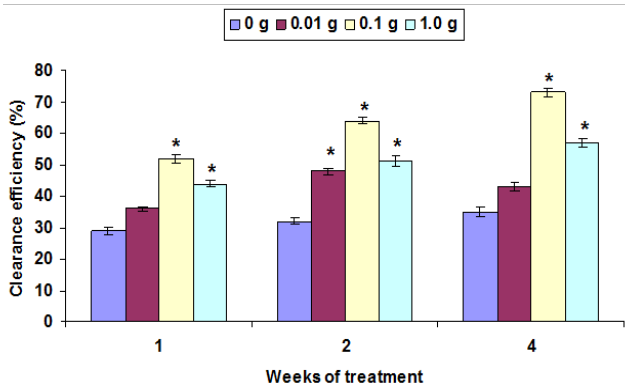

Figure 6: Clearance efficiency (%) of *M. malcolmsonii* (mean ± S.E; n = 6) fed with herbal enriched diet against *V. alginolyticus*. Data at the same exposure time with asterisks are significantly different (P < 0.05) among treatments.

Disease resistance

The cumulative mortality was found 40% and 35% in groups feeding with 0.1 g and 1.0 g herbal extract enriched diet against pathogen. The mortality was found 55% in prawn feeding with 0.01 g diet against pathogen. Prawn fed with basal diet without herbal extract was found 90% mortality against pathogen (Figure 7).

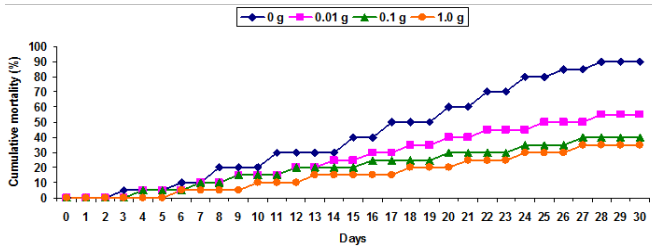

Figure 7: The percentage (%) of cumulative mortality (CM) of *M. malcolmsonii* fed with herbal enriched diet against *V. alginolyticus* for 30 days.

Discussion

In the present study, higher protection in terms of mortality was found 40% and 35% in *M. malcolmsonii* feeding with 0.1 g and 1.0 g *H. spinosa* extract enriched diets against *V. alginolyticus*. The mortality was found higher at 55% when prawn fed at 0.01 g diet. In a previous study indicated that in *L. vannamei* increased its susceptibility to *V. alginolyticus* infection by decrease in salinity, as well as the presence of ammonia, nitrite, and copper sulphate in the rearing water and when treated with noradrenaline against the same pathogen [29,31,34-36]. In this study prawn fed with basal diet and challenged with *V. alginolyticus* higher mortality. Pacific oyster *C. gigas* that had been challenged with pathogen *V. splendidus* when subjected to mechanical stress increased the mortality [37]. *C. gigas* injection with noradrenaline, a key component of the neuroendocrine stress response system, also caused higher mortality [38]. Therefore, the present result was suggested that *V. alginolyticus* involved in the physiological changes in *M. malcolmsonii*.

In the present study, the THC significantly decreased when *M. malcolmsonii* fed with herbal enriched diet at 0.01g and 0.1g against *V. alginolyticus*. The circulating THC in *L. vannamei* displayed higher THC and proPO activities [34,35,39,40]. In the present study, the THC did not significant change when fed at 1.0g herbal enriched diet against pathogen. The circulating THC was affected by extrinsic factors like temperature and salinity variations, as well as nitrite and Cu_2C in *L. vannamei* and *L. stylirostris* [34,35,40,41]. In this study, proPO activity significantly low when prawn feeding with 0.01 g and 0.1 g herbal enriched diets during the experimental period against *V. alginolyticus*. Both THC and proPO activity in *M. rosenbergii* were significantly higher at pH 7.5-7.7 and 30-31°C [42]. In this study, prawn feeding with 1.0 g herbal enriched diet did not significantly change against pathogen from weeks 1 to 4. Exposure of common shrimp, *Crangon crangon* to polychlorinated biphenyl 15 (PCB 15) resulted in significantly decreased THC and PO activity [43]. The PO activity was significantly decreased in both *L. vannamei* and *M. rosenbergii* when exposure to ammonia-N at a concentration of 0.55 mg l^{-1} [29,44] and nitrite-N at 9.87 mg l^{-1} and Cu_2C 10 mg l^{-1} [34,35]. In the present study, prawn feeding with 1.0% herbal enriched diet significantly enhanced against pathogen, as indicating that herbal diet modulate the immune response in *M. rosenbergii*.

In this study, the RB activity significantly enhanced in prawn feeding with 0.1 g and 1.0 g herbal enriched diets against pathogen. The releases of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) were considered to play a more important role in shrimp microbicidal activity than hypochlorites (OCl^-) and myeloperoxidase (MPO) [45]. In *L. vannamei* injection with fungicide propiconazole induced an increase of O_2^- at day 6, but caused a dose-dependent decrease in O_2^- at day 13 [41]. It was proposed that the decreased production of O_2^- in hypoxic *P. stylirostris* was due to the decrease of THC, and that the activity of NADPH oxidase responsible for the production of O_2^- was not affected under hypoxia [40]. Similarly, *L. vannamei* decreased its release of O_2^- when exposure of to 11.10 mg l^{-1} ammonia-N for 48 hrs, to 9.87 mg l^{-1} nitrite for 96 hrs, or to 20 mg l^{-1} Cu_2C [29,34]. The SOD significantly enhanced when prawn fed with 0.1 g and 1.0 g herbal enriched diets on weeks 2 and 4 when compared to control against pathogen. However, the activity was not significant when fed with 0.01 g diet on weeks 2 and 4. This fact suggests that the activity of NADPH oxidase responsible for the release of O_2^- decreased with decrease in the activity of SOD responsible for scavenging O_2^-. The fact that the SOD activity recovered later than that of the RB suggests that the prawn received herbal enriched diet causes immunomodulation to scavenge O_2^- to other reactive oxygen intermediates (ROIs) including (H_2O_2).

The PC activity significantly low in prawn fed at 0.01 g and 0.1 g herbal enriched diets from weeks 1 to 4 whereas fed at 1.0 g diet significantly increased during the experiment in this study. The CE significantly increased when prawn fed at 0.1 g and 1.0 g herbal diets and all diet supplementary doses of herbal extract diet on second week against pathogen. Phagocytosis is an important cellular defence mechanism, whereas CE is an important humoral defence mechanism in molluscs and crustaceans [46,47]. A significant reduction of PC activity and CE against *V. alginolyticus* was observed in *L. vannamei* following exposure to Cu2C [35] and ammonia-N [29]. A significant reduction in phagocytosis of *Bacillus* cereus was also observed in the *C. maenas* when exposure to Cd2C [48]. The PC activity and CE decreased in *P. monodon* against for *V. harveyi* following exposure to O_2^- [49]. However, noradrenaline had a dose-dependent inhibitory effect on phagocytosis in *C. gigas* [38]. In conclusion, the present study documented that *M. malcolmsonii* feeding with *H. spinosa* extract enriched diet experienced an increase in susceptibility to *V. alginolyticus*. In addition *H. spinosa* plays an important role in immune modulation by decreasing THC, proPO activity, and PC activity, and CE in *M. malcolmsonii* against *V. alginolyticus* infection.

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