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Can *Zingiber officinale* immunomodulation in *Macrobrachium rosenbergii* (de Man) against *Vibrio alginolyticus*?

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Abstract

The present study was investigated the protective efficacy of *Zingiber officinale* extract enriched diets at 0 g, 0.01 g, 0.1 g, and 1.0 g per kilogram in giant freshwater prawn, *Macrobrachium rosenbergii* (de Man) against *Vibrio alginolyticus*. The total hemocyes (THC) significantly increased in prawn fed at 0.1 g and 1.0 g diets on weeks 1 and 2 and all the doses of the diet on week 4 as compared with control against *V. alginolyticus*. The prophenoloxidase (proPO) activity significantly enhanced all doses of the diet during the experiment as compared to control. The respiratory burst (RB) activity significantly enhanced when prawns were received at 0.1 g and 1.0 g diets on first week and all the doses of the diet on fourth week. The superoxide dimutase (SOD) activity did not significant change between weeks 1 and 2, but it was enhanced on fourth week at 0.1 g and 1.0 g diets. The phogocytic activity was significantly enhanced in all the doses of the diet during the experiment. The clearance efficiency was significantly increased in 0.1 g and 1.0 g diets between weeks 1 and 2 and all the doses of the diet on week 4 as compared with control. The survival rate was 70% and 83% with 0.1 g and 1.0 g diets compared with 0.01 g diet in *M. rosenbergii* against *V. alginolyticus*. Therefore, this present results suggested that feed supplementation with *Z. officinale* positively modulate the immune system and protect *M. rosenbergii* from *V. alginolyticus* infection.

Keywords: *Macrobrachium rosenbergii*; Non-specific immunity; Supplementation diet; *Vibrio alginolyticus*; *Zingiber officinale*

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Introduction

The global aquaculture production was at 167 tonnes million tonnes (MT), of which 74 MT contributed by intensive aquaculture in 2015 [1]; but global demand of the aquaculture products is to be expected to increase above 635 MT in 2020. Among the aquaculture species, the giant freshwater prawn, *Macrobrachium*

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rosenbergii is one of most popular and economically important farmed inland crustacean species in the world belong to the family Palaemonidae. This species is surviving a wide range of salinity levels during its life cycle ranging from 0 to 18‰ [2]. The major aquaculture production of the giant freshwater prawn is mainly in Bangladesh, China, India, Myanmar, Thailand, Vietnam, etc. Among, the output of farmed, *M. rosenbergii* alone contribute 51% about 42 tonnes worth US$ 2.13 billion in 2008 [3]. In India, total freshwater prawn production was 10,152 metric tonnes in 2016 (MPEDA, 2018). However, the culture of *M. rosenbergii* has been seriously affected by many bacterial and viral diseases, results significant economic losses [5-11]. Therefore, the enhancement of the immune status of the prawn is essential to reduce stress and economic loss to be associated disease outbreaks.

In order to prevent and control of the diseases, prophylactic chemo-therapeutants and antibiotics were used in intensive aquaculture. However, the abuse of broad-spectrum of these therapeutants has resulted in an increased number of antibiotic-resistant bacteria, which impact on the food safety for human. Therefore, aquaculture farmers need to reduce the application of chemo-therapeutants and more focusing the use of vaccines, probiotics, natural or plant based immunostimulants [12-38]; they are enhancing the growth, protection or reduced mortality from diseases, immune system, regulation of growth and immune related genes.

Traditional Chinese medicines (TCMs) have been used as immunostimulants to treat human and animal diseases for thousands of years [39]. Traditional Korean medicines (TKMs) also reported to treat many animal and human diseases [17]. The TCMs and TKMs are easily available and cheap to prepare, and are effective with fewer side effects during treatment of diseases [17,39]. They contain many active components and other constituents, including organic acids, alkaloids, polysaccharides, anthraquinones, volatile oils, flavonoids, glycosides, tannic acid substance, trace elements, other immune active factors, which can strengthen the metabolism of aquatic animals, improve the composition of protein and enzymes, and enhance the growth of animals. The immunostimulating activity of herbal components has been most widely studied in several fish and shellfish [12-19,21,24,26-34,37,38].

Zingiber officinale Roscoe (family, Zingiberaceae), known commonly as ginger, is a worldwide cookery for spice and flavoring agent in thousands of years. These plants were identified many phytochemical bioactive compounds, such as gingerols, shogaols [40], diarylheptanoids [41], phenylbutenoids [42], flavanoids [43], diterpenoids [44], and sesquiterpenoids [45,46] which are contain many antibacterial, antioxidant, and cytotoxic components. However, the application of the herb on immunological function in prawn is not well understood. Therefore, this study to be conducted the efficacy *Z. officinale* through oral administration with different concentrations in *M. rosenbergii* and find-out immunological parameters against *V. alginolyticus* infection.

Materials and Methods

Herbal extract and diet preparation

Zingiber officinale 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, they were dried under shade to make them suitable for grinding. The dried plant roots were grounded in a mechanical grinder and sieved than stored in an air tight container for further use. One hundred grams of coarsely powdered was successively extracted with 85% ethanol. The successive extraction was performed by a cold maceration process for seven days with daily agitation twice following Singh et al. (2007). The solvent was evaporated using a rotary vacuum evaporator (Buchi, Flawil, Switzerland). The residues were obtained after evaporation and stored at -20 °C until used for

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the experiment. 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) used as control diet. Four experimental diets prepared of the pellet with 0 g (control), 0.01 g, 0.1 g, and 1.0 g of *Z. officinale* 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 h. 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 h, packed, and stored in a freezer at -20 °C until used. The proximate composition of the diets was quantified following AOAC method comprised 52.3% crude protein, 8.3% crude lipid, 7.4% crude ash, and 14.9% crude carbohydrate.

Table 1: Composition of the feed for *M. rosenbergii*.

Ingredients	Composition (%)
Groundnut oil cake	45
Soybean meal	18
Fish meal	17
Rice bran	17
Mineral and vitamin mix[a]	2.8
Carboxy methyl cellulose	0.2

[a] Each 250 g vitamin and mineral mixture provides vitamin A (5,000,000 IU), vitamin D_3 (100,000 IU), vitamin B_2 (0.2 g), vitamin E (75 units), vitamin K (0.1 g), calcium pantothenate (0.25 g), nicotinamide (1.0 g), vitamin B_{12} (0.5 mg), choline chloride (15 g), calcium (70 g), manganese (2.75 g), iodine (0.1 g), iron (0.70 g), zinc (1.5 g), copper (0.2 g) and cobalt (0.05 g).

Culture of Vibrio alginolyticus

V. alginolyticus was isolated from diseased prawn according to Liu et al. [47]. It was cultured on tryptic soy agar (TSA supplemented with 2% NaCl, Difco) for 24 h at 25 °C before being transferred into 10 ml of tryptic soy broth (TSB supplemented with 2% NaCl, Difco), where it remained for 24 h at 25°C as stock culture for tests. The broth cultures were centrifuged at 7155 g for 15 min at 4 °C. The supernatant fluids were discarded and the bacterial pellets re-suspended in saline solution for further use.

Experimental animal

M. rosenbergii (20-25 g) were obtained from a commercial farm and acclimated in the laboratory for 2 weeks before conducting the experiment. The prawns were immediately examined health status upon arrival. During the acclimation period the prawns were provided control or basal diet (Table 1). The unfed feed and faecal materials were removal daily. Similarly, 50% of water was renewed daily basis. The water temperature 28±2 ºC, pH 7.2-8.0, total hardness 75-100 mg l^{-1}, dissolved oxygen at 6-7 mg l^{-1}, and ammonia at <0.1 mg l^{-1} where was observed during the experimental period.

Experimental design

After acclimation period, the prawns were distributed into four groups of 25 prawns maintained in 100 l tanks and fed with 0 g, 0.01 g, 0.1 g, and 1.0 g of *Z. officinale* extract enriched diets at the rate of 10% of their body weight twice in a day. All experimental groups were run in three replicate tanks. After 30 days of feeding with 0.01g, 0.1 g, and 1.0 g of *Z. officinale* extract enriched diet groups were

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injected intraperitoneally (i.p.) into the ventral sinus of the cephalothorax with 50 µl PBS containing *V. alginolyticus* at 1.5 x 10^7 cfu ml^{-1}. However, the control group fed with 0 g or without of *Z. officinale* extract enriched diet group was injected i.p. with the same volume (50 µl) of PBS alone. After 1, 2, and 4 post-infection with *V. alginolyticus*, there are six prawns were randomly collected in each experimental tank and collect hemolymph for hematological and immunological studies.

Sample collection

On 1, 2, and 4 week post-injection with *V. alginolyticus*, 100 µl haemolymph 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 M, EDTA 10 mM, pH 7.55, osmolality adjusted with glucose to 780 mOsm kg^{-1}). A drop of the anticoagulant-haemolymph mixture was placed on a haemocytometer to measure total hemocytes (THC) using an inverted phase-contrast microscope (Leica DMIL, Leica Microsystems, Wetzlar GmbH, Germany). The remainder of the haemolymph mixture was used for subsequent immunological assays.

Phenoloxidase (PO) activity of haemocytes

The PO activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) following the procedures of Hernandez-Lopez et al. [48]. The diluted haemolymph was centrifuged at 700 g at 4 °C for 20 min. The supernatant fluid was discarded and the pellet was rinsed, re-suspended gently in 1 ml cacodylateecitrate buffer (sodium cacodylate 0.01 M, sodium chloride 0.45 M, trisodium citrate 0.10 M, pH 7.0) than centrifuged again. The pellet was re-suspended with 200 µl cacodylate buffer (sodium cacodylate 0.01 M, sodium chloride 0.45 M, calcium chloride 0.01 M, magnesium chloride 0.26 M, pH 7.0). Aliquot (100 µl) was incubated for 10 min at 25-26 °C with 50 ml of trypsin (1 mg ml^{-1}), which served as an elicitor. Fifty microlitres (50 µl) of L-DOPA were added, followed by 800 µl of cacodylate buffer 5 min later. The optical density (OD) at 490 nm was measured using a Hitachi U-2000 spectrophotometer (Tokyo, Japan). The control solution was used for the background PO activity in all test conditions, and consisted of 100 µl of cell suspension, 50 µl cacodylate buffer (to replace the trypsin) and 50 µl of L-DOPA. The OD of background PO activity ranged from 0.02 to 0.05. The OD of the prawn PO activity was expressed as dopachrome formation per 50 µl haemolymph.

Respiratory burst (RB) activity of haemocytes

RB activity of THC was quantified using the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anion (O_2^-), as described previously [49]. The OD at 630 nm was measured in triplicate using a microplate reader (Model VERSAmax, Molecular Devices, Sunnyvale, CA, USA) and the RB was expressed as NBT-reduction per 10 ml haemolymph.

Superoxide dismutase (SOD) activity of haemocytes

The SOD activity was measured by its ability to inhibit superoxide radical dependent reaction using the Ransod Kit (Randox, Crumlin, UK). Briefly, the reaction mixture (1.7 ml) contained xanthine (0.05 mM) and 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT, 0.025 mM) dissolved in CAPS 50mM (pH 10.2) and EDTA (0.94 mM). In the presence of xanthine oxidase (80U l^{-1}, 250 µl), superoxide and uric acid were produced from xanthine. The superoxide radical reacted with INT to produce a red formazan dye. The OD was measured at 505 nm, 37 °C, and the rate of reaction was estimated from the absorbance readings 30s and 3 min after adding xanthine oxidase and compared a reference standard SOD supplied by Ransod Kit. One unit of SOD was defined as the amount required inhibiting the rate of xanthine reduction by 50% and the specific activity was expressed as SOD units ml^{-1} [50].

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Phagocytic activity of haemocytes

The method for the measurements of phagocytic activity was described by Liu and Chen [49]. Two hundred haemocytes were counted in each samples and the phagocytic activity, defined as phagocytic rate (PR) and expressed as follows: PR = [(phagocytic haemocytes)/(total haemocytes)] x100.

Bacterial clearance efficiency

The number of colonies in the control and treated group as clearance efficiency, defined as percentage inhibition (PI) of *V. alginolyticus* which is calculated as follows: PI = 100 – [(cfu in test group)/(cfu in control group)] x 100.

Statistical analysis

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

Results

THC level

The THC did not significantly increase in *M. rosenbergii* that received *Z. officinale* enriched diet at 0.01 g and challenge with *V. alginolyticus* on first and second week. The THC significantly increased in prawn was received at 0.1 g and 1.0 g *Z. officinale* enriched diets from weeks 1 to 4 when compared to control against *V. alginolyticus* (Figure 1).

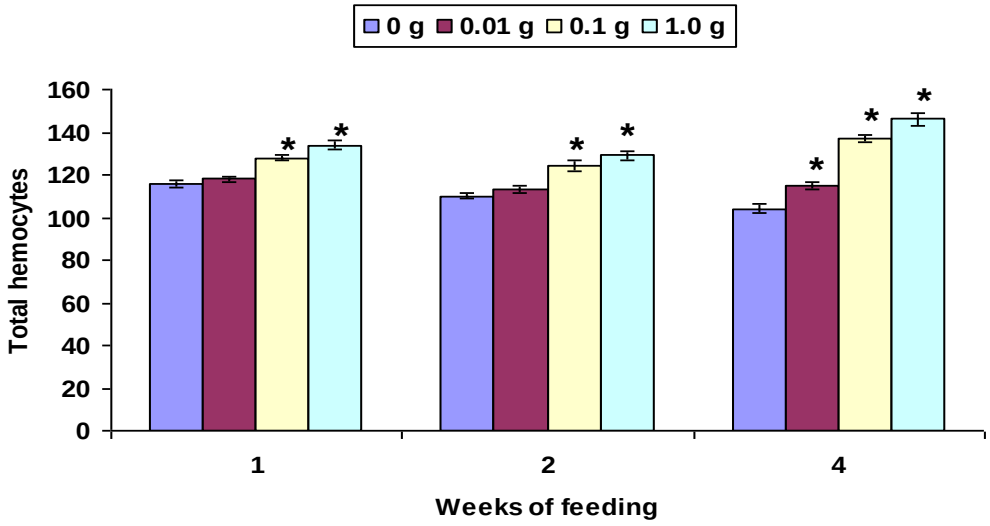

Figure 1: The total hemocytes (THC) of *M. rosenbergii* (mean ± SE) that received *Z. officinale* enriched diet and challenge with *V. alginolyticus* at chosen week. Data are significantly different ($P < 0.05$) among treatments at the same exposure time indicated with asterisks.

PO activity

In prawn fed with 0.1 g and 1.0 g diets on first week and all the doses of the diet on second week and fourth week significantly enhanced the PO activity as compared to control against pathogen. However, the PO activity did not significantly enhance in prawn fed at 0.01 g diet on first to the control against pathogen (Figure 2).

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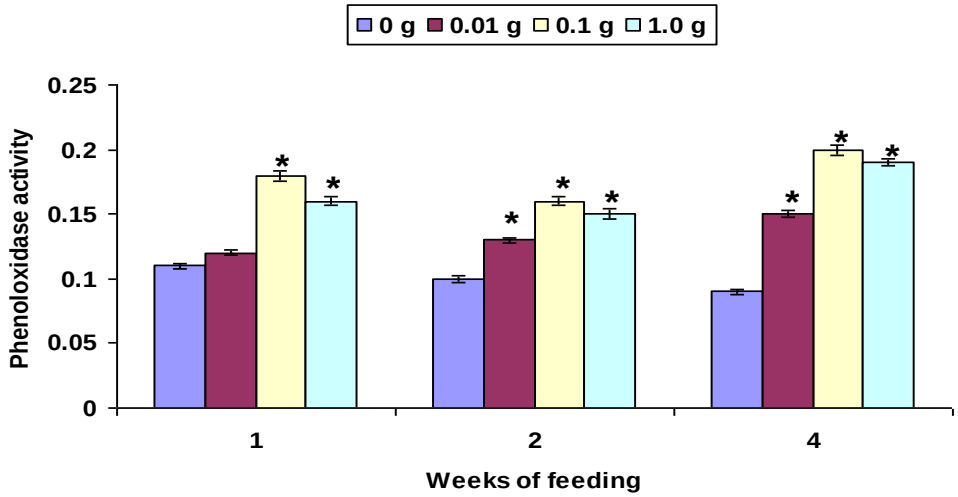

Figure 2: The phenoloxidase (PO) activity of *M. rosenbergii* (mean ± SE) that received *Z. officinale* enriched diet and challenge with *V. alginolyticus* at chosen week. Data are significantly different ($P < 0.05$) among treatments at the same exposure time indicated with asterisks.

RB activity

All the doses of the diet significantly enhanced the RB activity on second week. However, the RB activity significantly enhanced in prawn fed all the diet on first and fourth week as compared to control against pathogen except in 0.01 g diet on first week (Figure 3).

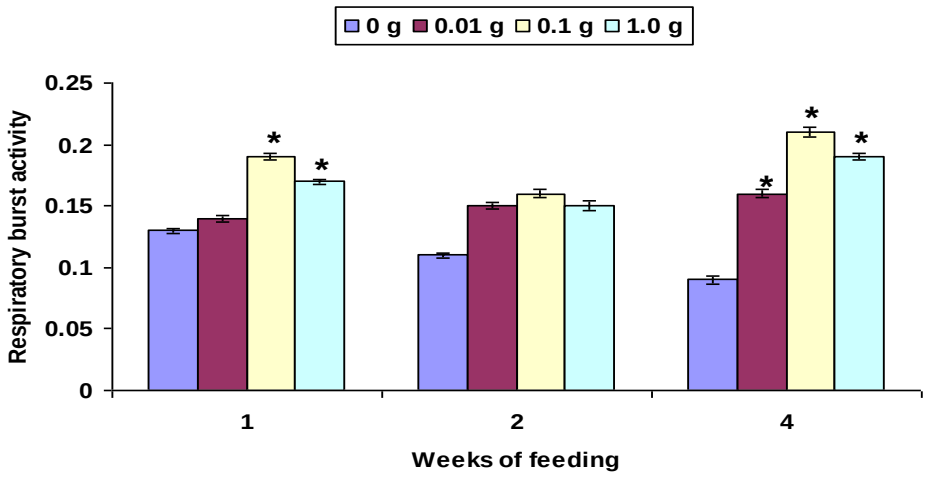

Figure 3: The respiratory burst (RB) activity of *M. rosenbergii* (mean ± SE) that received *Z. officinale* enriched diet and challenge with *V. alginolyticus* at chosen week. Data are significantly different ($P < 0.05$) among treatments at the same exposure time indicated with asterisks.

SOD activity

The SOD activity did not significantly enhance almost all the diet on first and second week. However, the SOD significantly enhanced in prawn fed at 0.1 g and 1.0 g diets on forth week against *V. alginolyticus* (Figure 4).

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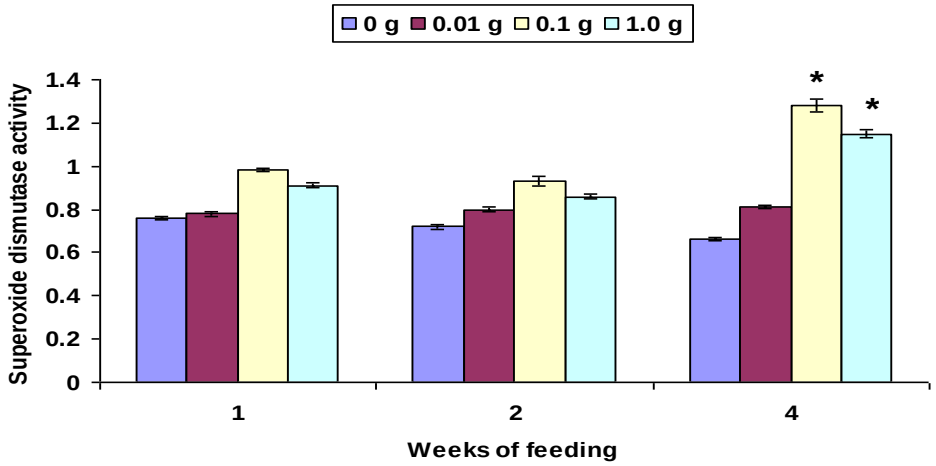

Figure 4: The superoxide dimutase (SOD) activity of *M. rosenbergii* (mean ± SE) that received *Z. officinale* enriched diet and challenge with *V. alginolyticus* at chosen week. Data are significantly different ($P < 0.05$) among treatments at the same exposure time indicated with asterisks.

Phogocytic activity

The phogocytic activity significantly enhanced all the supplementary diets from weeks 1 to 4 when compared to control against pathogen. However, this activity did not significant changed when prawn fed at 0.01 g diet on first week (Figure 5).

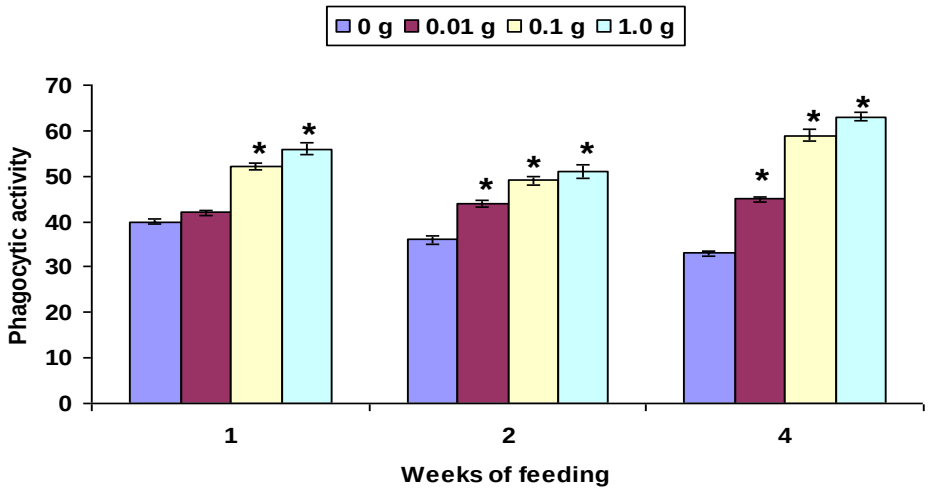

Figure 5: The phogocytic activity of *M. rosenbergii* (mean ± SE) that received *Z. officinale* enriched diet and challenge with *V. alginolyticus* at chosen week. Data are significantly different ($P < 0.05$) among treatments at the same exposure time indicated with asterisks.

Clearance efficiency

The clearance efficiency did not significantly enhanced in prawn fed at 0.01 g diet on weeks 1 and 2. However, the clearance efficiency significantly enhanced when fed 0.1 g and 1.0 g diet on second week and all the diet on fourth week in prawn against pathogen (Figure 6).

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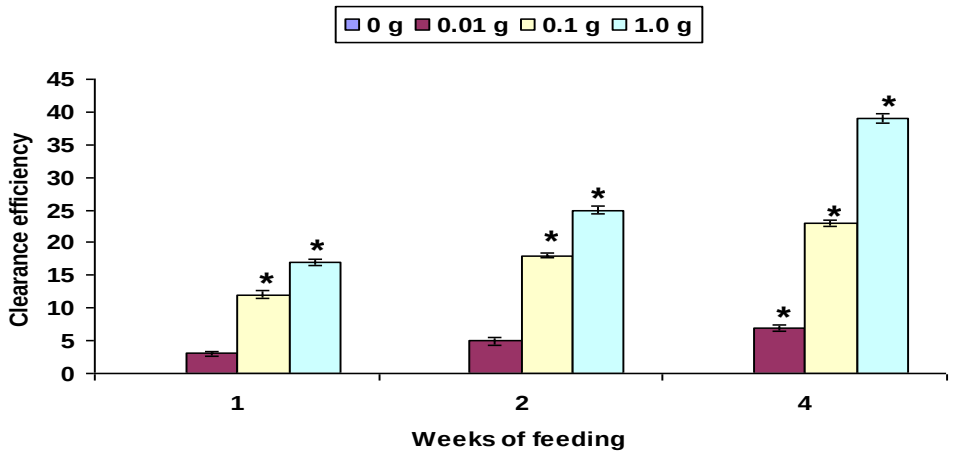

Figure 6: The clearance efficiency of *M. rosenbergii* (mean ± SE) that received *Z. officinale* enriched diet and challenge with *V. alginolyticus* at chosen week. Data are significantly different ($P < 0.05$) among treatments at the same exposure time indicated with asterisks.

Survival rate (RA)

The SR was 89.2%, 85.4%, and 83.2% in prawn fed at 1.0 g diet on weeks 1, 2, and 4. However, the SR was also high with 78.2%, 72.2%, and 70.6% at weeks 1, 2, and 4 in prawn fed at 0.1 g diet; but it was 71.4%, 64.6%, and 38.2% when prawn fed with 0.01 g diet (Figure 7).

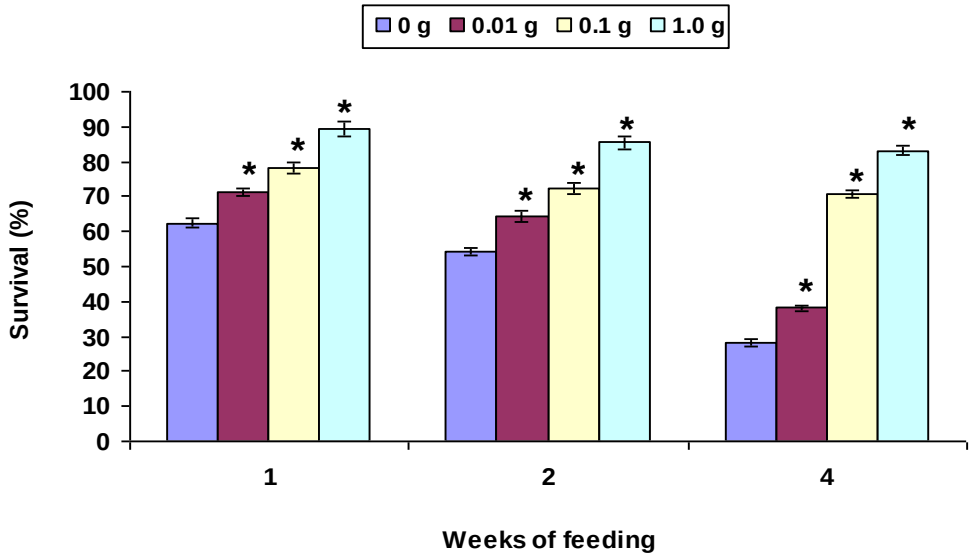

Figure 7: The survival rate of *M. rosenbergii* (mean ± SE) that received *Z. officinale* enriched diet and challenge with *V. alginolyticus* at chosen week. Data are significantly different ($P < 0.05$) among treatments at the same exposure time indicated with asterisks.

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Discussion

In decapod crustaceans, circulating haemocytes are generally classified into hyaline, semi-granular, and large granular cells [51] which are involved in phagocytosis and production of melanin via the prophenoloxidase (proPO) system [52]. The phenoloxidase (PO) is the terminal enzyme in the proPO system and is activated by several microbial polysaccharides and β-1,3-glucan [53]. A number of reactive oxygen species are produced during phagocytosis which process the membrane bound enzyme complex, NADPH oxidase, assembles after binding the cell to a foreign particle, and reduces molecular oxygen to superoxide anion (O_2^-), subsequently leads to the production of hydrogen peroxide (H_2O_2), singlet oxygen ($1O_2$), hydroxyl radical ($OH\cdot$) and other reactive compounds [54]. Among the O_2^- is the first product released from RB that plays an important role in microbicidal activity [55]. Few studies were reported that polysaccharides and glucan extracted from microbes to increase the survival and non-specific immune response in fish and shrimps. Similarly, administration of yeast glucan by oral or immersion to increased resistance in *P. monodon* against *V. vulnifus* [56], *M. japonicus* against *Vibrio* [57], and *P. monodon* against *V. damsela* [58]. Brown algae *M. pyrifera* and *L. nigrescens* were extracted sodium alginate extract to increase the resistance of *L. vannamie* against *V. alginolyticus* [59,60]. Similarly, the survival rate in the present study recorded low mortality in term of protection *M. rosenbergii* from *V. alginolyticus* when fed at 1.0 g diet than with 0.01 g and 0.1 g diets. Therefore, the herbal extract of *Z. officinale* showed positive effects on preventing *V. alginolyticus* infection in *M. rosenbergii*.

The PO activity significantly enhanced in prawn fed with 0.1 g and 1.0 g diets on first week and all the diet on 2nd and 4th week. The RB activity significantly enhanced all the diet on 1st and 4th week in this study. These result are in agreement in *P. monodon*, *M. marsupenaeus*, *L. vannamei*, *M. Malcolmsonii*, *M. rosenbergii*, and *M. nobilii* which had been received yeast glucan, fungus glucan, zymosan, lipopolysaccharide (LPS), and herbals by immersion or dietary intake increased their PO activity and RB activity [10,30,56,60-62] which indicate that prawn increasing immune ability. Sodium alginate extracted from *M. pyrifera* and *L. nigrescens* has been reported to increase the PO activity, RB activity in *L. vannamei* [59,60]. In the present study, *Z. officinale* extract indicate that increased the THC, PO activity, and RB activity in *M. rosenbergii* against *V. alginolyticus* confirmed to involved immunomodulation. The SOD activity significantly enhanced in prawn fed at 0.1 g and 1.0 g diet on 4th week whereas the phogocytic activity enhanced with all the supplementary diets from weeks 1 to 4 against *V. alginolyticus*. An increase of superoxide anion together with an increase the activity of SOD suggests that not producing too much higher concentrations of superoxide anion in prawn when treated with *Z. officinale* herbal extract. The fungus glucan or herbal has been reported to increase the phagocytic activity of *M. japonicas* [57] and *P. monodon* [30,64]. In addition, oral administration LPS extracted have been increased the phagocytic activity in *M. japonicus* [61].

The clearance efficiency in this study significantly enhanced when prawn fed at 0.1 g and 1.0 g diets on 2nd week and all the diets on 4th week against pathogen. Both the phagocytic activity and clearance efficiency in *M. rosenbergii* increased to *V. alginolyticus* indicate that *Z. officinale* protect in prawn from *V. alginolyticus* infection, and it were correlated well with the resistance to *V. alginolyticus* and *A. hydrophila* when the shrimp or prawn received sodium alginate or herbal by injection or dietary administration [30,59,60]. The exact mechanisms of these changes in *M. rosenbergii* against *V. alginolyticus* are currently unknown. Further extensive research is needed to evaluate the immune stimulatory effect and disease resistance of the *Z. officinale* extract in other prawn or shrimps against different pathogen before to inclusion of feed additive for sustainable aquaculture.

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