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Effect of *Shilajit* enriched diet on immunity, antioxidants, and disease resistance in *Macrobrachium rosenbergii* (de Man) against *Aeromonas hydrophila*

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ABSTRACT

The effect of diet supplemented with Shilajit, a multi-component natural mineral substance on the antioxidant activity, immune response, and disease resistance in freshwater prawn, *Macrobrachium rosenbergii* (de Man) against *Aeromonas hydrophila* is reported. The total hemocyte count (THC) and phagocytic activity significantly increased with 2 g kg^{-1} supplemented diet on first week and with other enriched diets on weeks 2 and 4. The respiratory burst (RB) activity and glutathione peroxidase (GPx) activity were significantly increased with 2 g kg^{-1} supplemented diet on weeks 1 and 2 whereas 2 and 4 g kg^{-1} diets on week 4. The phenoloxidase (PO) activity increased significantly with 2 g kg^{-1} diet only on second week and with other enriched diets only on fourth week. The superoxide dismutase (SOD) activity increased significantly with any enriched diet during the experimental period except with 6 g kg^{-1} diets on first week. However, the glutathione reductase (GR) activity was enhanced significantly only with 2 g kg^{-1} enriched diets on weeks 2 and 4. The cumulative mortality of the prawn fed with 2 and 4 g kg^{-1} enriched diets was 10% and 15% whereas with 6 g kg^{-1} diet the mortality was 20%. The results suggest that diet enriched with Shilajit at 2 g kg^{-1} or 4 g kg^{-1} positively enhances the antioxidant activity, immunity, and disease resistance in *M. rosenbergii* against *A. hydrophila*.

1. Introduction

Freshwater prawn culture has been recognized as an eco-friendly alternative for sustainable prawn production. In many countries the freshwater prawn, *Macrobrachium rosenbergii* (de Man) is an economically important venture because of its high commercial value [1]. It is a highly priced elite food product in both domestic and export market; *M. rosenbergii* is euryhaline and survives in a wide range of salinities between 0 and 18‰ [2]. In many countries its farming has been expanding significantly during the last decade. However the juveniles and adults of *M. rosenbergii* suffer high mortalities especially in hatcheries [3–5] due to several disease outbreaks such as appendage deformity syndrome (ADS) [6] and bacterial pathogens such as *Vibrio* spp., *Aeromonas* spp., and *Pseudomonas* spp., and *Lactococcus garviae* [7,8]. Among, these, *Aeromonas* spp. is considered to be the major threat in the commercial cultivation of *M. rosenbergii* in Taiwan [9] and Brazil [10] including India [11,12].

In the prevention and control of prawn diseases large quantities

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of antibiotics and chemicals are applied; vaccines are another eco-friendly measure. However the traditional measures build up drug-resistance in pathogens; vaccines are pathogen specific and hence novel strategies to control bacterial diseases in aquaculture are needed. The effect of plant products on innate and adaptive immune response in fish and shellfish diseases was reviewed [13]. It is encouraging to note that in *Penaeus monodon* dietary administration of polysaccharide gel obtained from the fruit-rind of *Durio zibethinus* could significantly increase the immune response and disease resistance against *Vibrio harveyi* and white spot syndrome virus (WSSV) [14]. In *M. rosenbergii* administration of *Withania somnifera* and *Eichhornia crassipes* supplemented diets positively enhance the immunity and survival rate against *A. hydrophila* and *L. garvieae* [15,16].

Shilajit is a blackish-brown exudates of variable consistence obtained from the steep rocks of specific mountain regions of the world at altitudes between 0.6 and 5 km [17]. In India it is found in Himalayas region at altitudes between 1 and 5 km on the cave walls embedded in rocks or as rock exudates from Arunachal Pradesh in the East to Kashmir in the West [17,18]. Though shilajit has similar physical properties and qualitative chemical composition, there is a regional variation in the ratio of individual components. Shilajit/humus consists of humic (80–85% of total organic mass) and non-humic (15–20%) substances [18], organic matter (60–80%), mineral matter (20–40%), and ~5% trace elements. Shilajit contain more than 85 minerals in ionic form and humic substances (mainly fulvic and humic acid) [19]. It contains 14–20% humidity; 18–20% minerals; 13–17% proteins (with marked- α-amylase activity); 4–4.5% lipids; 3.3–6.5% steroids; 18–20% nitrogen-free compounds; 1.5–2% carbohydrates; and 0.05–0.08% alkaloids, and a number of amino acids [20]. Shilajit comprise 65 organic compounds namely, albumins, coumarins, free fatty acids, organic acids including adipic, succinic, citric, oxalic and tartaric, waxes, resins, polyphenols, essential oils and vitamins like B_1, B_{12}, etc. [21,22]. Shilajit also contain a number of active constituents such as dibenzo-α-pyrones and related metabolites, such as tirucallane triterpenes, small peptides consisting of non-protein amino acids, some phenolic lipids, small tannoids, and FA [21].

In many countries Shilajit has been used for centuries as a traditional medicine [17] in treatment of genitourinary diseases, diabetes, digestive disorders, nervous diseases, tuberculosis, chronic bronchitis, asthma, jaundice, anemia, eczema, bone fractures, osteoporosis [23–25] kidney stones, edema, spondylitis, hemorrhoids, injured muscles, bone fractures, and diseases such as osteoporosis and other diseases; it is also used as a rejuvenator and an internal antiseptic [17,26,27]. Shilajit has potential use such as anti-inflammatory, anti-fungal, anti-ulcerogenic, anxiolytic activity, anti-allergic, analgesic, anti-diabetic, memory enhancer, and an antioxidant [18,23,28–31]. For therapeutic applications it is administered in the form of an aqueous extract to activate phagocytosis and cytokine release by murine peritoneal macrophages [32], stimulate osteoblastic differentiation of mesenchymal stem cells [33], induce the proliferation of lymphocytes in the cortical thymus layer and increase migration of these cells into thymus-dependent zones of the lymph nodes and spleen [34]. In the aqueous extract of Shilajit humus comprise fulvic acid (FA) as the primary organic substance endowed with for many biological and medicinal properties [25,29] effective in the treatment of disorders including gastritis, diarrhea, stomach ulcers, dysentery, colitis and diabetes mellitus [17,25] and stimulate neutrophil and lymphocyte immune function [35,36]. FA has broad spectrum antimicrobial property on a variety of bacteria, including *P. gingivalis*, *F. nucleatum*, *S. mitis*, *A. actinomycetemcomitans*, *E. faecalis*, *S. mutans* and also create a cytotoxic environment for cancer cells [37]. The complement system plays an essential role in innate immunity, contributing to inflammatory responses and the destruction and removal of pathogens. Likewise, the removal of complement by fixation has also been proposed to be a potential therapeutic strategy for treating inflammatory diseases [38]. It is also used in the form of an aqueous extract for therapeutic applications as an immuno stimulant and anabolic food additive [36]. To our knowledge this is the first study on the protective efficacy of dietary supplementation with Shilajit on antioxidant activities, innate immune function, and disease resistance in *M. rosenbergii* against *A. hydrophila*.

2. Material and methods

2.1. Diet

The basal diet (control) comprised mackerel meal, dehulled soybean meal, and corn gluten meal as the protein source; wheat flour, α-potato starch, and wheat gluten as carbohydrate and fish oil as lipid source in addition with vitamin and mineral premix (Table 1). The dietary Shilajit (Aravind Annai Herbals, Chennai, India) was incorporated with the basal diet at doses of 2, 4, and 6 g kg^{-1} by evenly mixing with the basal diet thoroughly. The enriched feeds were dried in a vacuum freeze drier for 15 h, ground, and extruded by passing through 5 mm mesh sieve. The prepared diets were stored at −20 °C until used for the experiment. The proximate composition of the experimental diets was quantified following AOAC method.

2.2. Pathogen

A. hydrophila (MTCC 646) was obtained from Institute of Microbial Technology, Chandigarh, India. The pathogenic nature of *A. hydrophila* was confirmed by inoculating into *M. rosenbergii* and reisolation according to Yogananth et al. [39]. *A. hydrophila* was grown with agitation at 37 °C in a 250 ml conical flask containing tryptic soy broth (TSB; Merck) to log phase. The culture was harvested by centrifugation at 3500 × g for 20 min at 4 °C. Bacterial pellets were washed twice with sterile 0.15 M phosphate buffered saline (PBS) at pH 7.2. The bacterial pellets were resuspended and

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

Ingredients	Composition (%)			
	Basal	2 g	4 g	6 g
Mackerel meal	55	55	55	55
Dehulled soybean meal	12	12	12	12
Corn gluten meal	5	5	5	5
Wheat flour	12	12	12	12
α-potato starch	2	2	2	2
Wheat gluten	6	4	2	0
Fish oil	5	5	5	5
Vitamin premix[a]	2	2	2	2
Mineral premix[b]	1	1	1	1
Shilajit	0	2	4	6
Proximate constituents				
Crude protein	46.86	46.42	46.67	47.12
Crude carbohydrate	16.72	16.51	16.18	16.35
Crude fat	14.67	14.84	14.43	14.55
Crude ash	10.84	10.96	11.24	11.51
Crude fiber	3.72	3.78	3.83	3.92

[a] Vitamin mixture providing the following concentration per kilogram diet; vitamin A 5000 IU; vitamin D 400 IU; vitamin E 20 mg; thiamin mononitrate (B1) 4 mg; riboflavin (B2) 6 mg; nicotinamide 50 mg; pyridoxine hydrochloride 3 mg; calcium pentohenate 10 mg; cyanocobalamine (B12) 2 mg; ascorbic acid (vit C) 100 mg; biotin 0.1 mg.

[b] Trace mineral mixture use providing the following concentration (ppm) copper 10; iron 100; manganese 50; zinc 50; cobalt 0.05; and iodine 0.1.

divided into aliquots and stored in TSB supplemented with 15% (v/v) glycerol at −70 °C until used. The identity of the bacterium was confirmed by morphological, pictorial, and biochemical characteristics including the following reactions: motile, Gram-negative, cytochrome oxidase positive, glucose positive, arginine dihydrolase positive, ornithine decarboxylase negative, ONPG positive, esculin positive, sucrose positive, L-arabinose utilization, and fermentation of salicin [40] and the followed by PCR for confirmation of genus and species, following Ghatak et al. [41].

2.3. Experimental animal and design

Healthy freshwater prawn, *M. rosenbergii* (20–25 g) were obtained from a commercial farm and acclimatized in the laboratory for 2 weeks before experimentation and fed with the control (basal) diet. The prawns were examined for their health status immediately upon arrival with standard microbiological method. Ten percent of water was renewed daily to remove the unfed and fecal materials. The water temperature 28 ± 2 °C, pH 7.2–8.0, total hardness 75–100 mg l^{-1}, dissolved oxygen at 6–7 mg l^{-1}, and ammonia concentration <0.1 mg l^{-1} were measured during the experimental period. Prawns were divided into five groups of 25 each in triplicate kept in 250 l tanks and fed with (i) unchallenged control fed without Shilajit enriched diet, (ii) *A. hydrophila* challenged control fed without Shilajit enriched diet and Shilajit supplementation diets at (iii) 2 g kg^{-1}, (iv) 4 g kg^{-1}, and (v) 6 g kg^{-1} doses at the rate of 10% of their body weight twice a day. After 30 days of feeding, all groups except the unchallenged control were injected between the second and third abdominal segments with 50 μl PBS containing *A. hydrophila* at 1.3 × 10^7 cfu ml^{-1}. The unchallenged control group received 50 μl PBS alone. On weeks 1, 2, and 4 of post-infection, six prawns were randomly collected from each tank, anaesthetized with MS-222 (NaHCO3 and tricaine methanesulphonate; Sigma Chemicals) 1:4000 in dechlorinated water for 2 min to collect hemolymph samples for hematological, antioxidant activities, and immunological assays. A group of 20 prawns were used in each experimental group including control group separately as mentioned above to observe cumulative mortality for a period of 30 days.

2.4. Hematological analysis

The hematological parameters such as total hemocyte counts (THC; cells ml^{-1}) were assayed using a Burker hemocytometer. The hemocytes on the Burker hemocytometer were observed under phase contrast microscope and counted manually in all 25 squares (= 0.1 mm^3).

2.5. Respiratory burst activity

Respiratory bursts of hemocytes were quantified using the reduction of nitro blue tetrazolium (NBT) to formazan as a measure of superoxide anion (O_2^-) production according to Tseng et al. [42]. Respiratory bursts are expressed as NBT-reduction in 10 μl of hemolymph.

2.6. Phenoloxidase (PO) activity

PO was spectrophotometrically measured by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) following a previous study [42]. The optical density of the shrimp's PO activity was expressed as dopachrome formation in 50 μl of hemolymph.

2.7. Phagocytic activity

Two hundred microliters of hemolymph was collected from the ventral sinus, mixed with 200 μl of sterile anticoagulant, and then used to measure the phagocytic activity following Tseng et al. [42]. The phagocytic activity was defined as the phagocytic rate (PR) as follows: PR = [(phagocytic hemocytes)/(total hemocytes)] x 100%.

2.8. Antioxidant activities

Total superoxide dismutase (SOD) activity was determined by spectrophotometrically at 420 nm according to Marklund and Marklund [44]. One unit of SOD activity is defined as the amount of the enzyme needed to effect 50% dismutation of superoxide radical per minute. Glutathione peroxidase (GPx) activity was assayed, based on the rate of NADPH oxidation at 340 nm, by the coupled reaction with glutathione reductase (GR). The specific activity was determined using the extinction coefficient of 6.22 mM cm^{-1} [45]. GR activity was determined spectrophotometrically, measuring NADPH oxidation at 340 nm [46]. One unit of GPx or GR activity is defined as the amount of the enzyme that consumes about 1 μmol of substrate or generates 1 μmol of product per min. Activity was expressed as international milliunits (mU) per milligram of protein.

2.9. Statistical analysis

One-way analysis of variance (ANOVA) was used to analyze the data. When ANOVA identified differences among groups, Tukey's multiple comparisons test was used to examine the significant differences among treatments using the SAS computer software (SAS Institute, Cary, NC, USA). Before the analysis, the percentage data were normalized by an arcsine-transformation. Statistically significant differences were accepted at $p < 0.05$.

3. Results

3.1. Hematology

The THC did not significantly increase in the infected prawn when fed with Shilajit-enriched diets on first week except with 2 g kg^{-1} diet as compared to control. However, THC significantly increased when fed with each enriched diets on weeks 2 and 4 (Fig. 1).

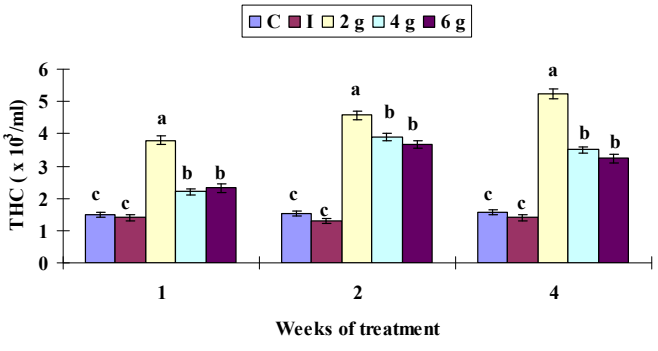

Fig. 1. Total hemocyte counts (THC) of *M. rosenbergii* fed with Shilajit-enriched diets against *A. hydrophila*. Data (mean ± SE, n = 6) with different letters significantly differ ($p < 0.05$) among treatments. C: unchallenged control, fed without Shilajit-enriched diet, I: challenged control, fed without Shilajit-enriched diet.

3.2. Respiratory bursts

The respiratory bursts activity did not significantly enhance in any Shilajit-enriched diet except with 2 g kg^{-1} diet on weeks 1 and 2 when compared to control; while it was significantly enhanced with 2 and 4 g kg^{-1} diets on fourth week (Fig. 2).

3.3. PO activity

No significant differences in PO activity were observed among the three diet on first week. Shilajit-enriched diet with 2 g kg^{-1} significantly enhanced than that of other diets as compared to control on second week. On the other hand, it significantly increased with any enriched diet on fourth week (Fig. 3).

3.4. Phagocytic activity

Diet enriched with 2 g kg^{-1} Shilajit significantly enhanced the phagocytic activity as compared to other diets on first week. However, all enriched diets significantly elevated the phagocytic activity on weeks 2 and 4 (Fig. 4).

3.5. SOD activity

The SOD activity was significantly enhanced when infected prawn were fed with any enriched diet during the experimental period as compared to control. However, the SOD activity was not significantly enhanced with 6 g kg^{-1} Shilajit-enriched diet on first week (Fig. 5).

3.6. GPx activity

The GPx activity did not significantly change in the control as well as 4 and 6 g kg^{-1} enriched diet fed groups on weeks 1 and 2. However, it significantly increased in the infected prawn fed with 2 g kg^{-1} Shilajit-enriched diet. The GPx activity was enhanced significantly on fourth week except in 6 g kg^{-1} diet fed group (Fig. 6).

3.7. GR activity

The GR activity did not significantly change among the experimental group on first week. However, the GR activity was significantly enhanced with 2 g kg^{-1} Shilajit-enriched diet on weeks 2 and 4 as compared to control against *A. hydrophila* (Fig. 7).

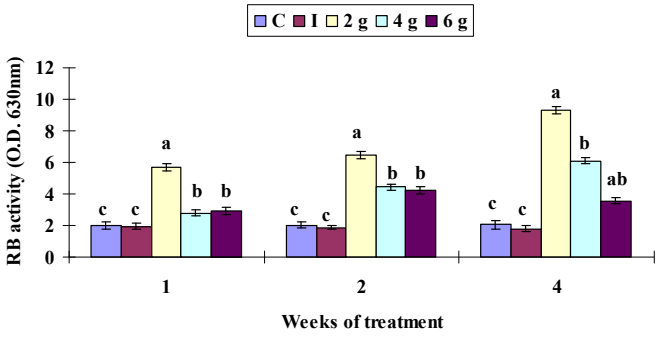

Fig. 2. Respiratory burst (RB) activity of *M. rosenbergii* fed with Shilajit-enriched diets against *A. hydrophila*. The statistical information and label as same in Fig. 1.

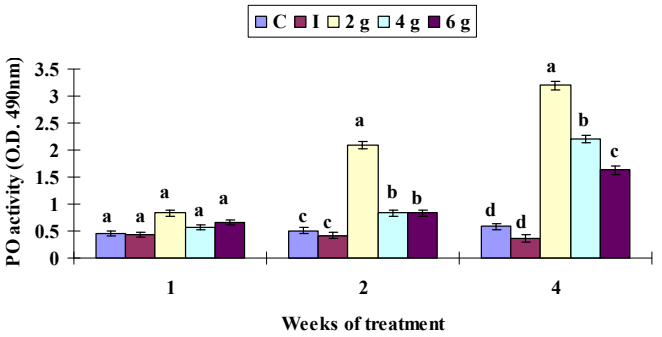

Fig. 3. Phenoloxidase (PO) activity of *M. rosenbergii* fed with Shilajit-enriched diets against *A. hydrophila*. The statistical information and label as same in Fig. 1.

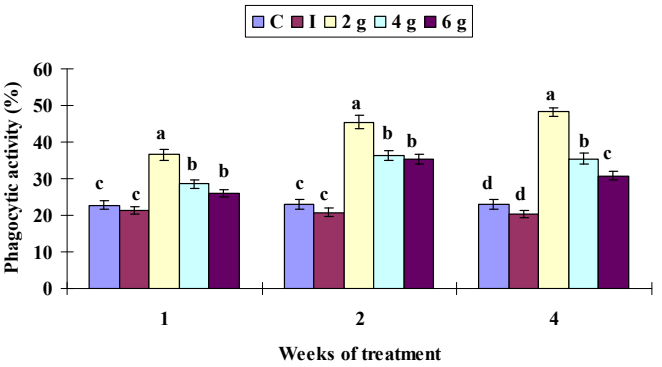

Fig. 4. Phagocytic activity of *M. rosenbergii* fed with Shilajit-enriched diets against *A. hydrophila*. The statistical information and label as same in Fig. 1.

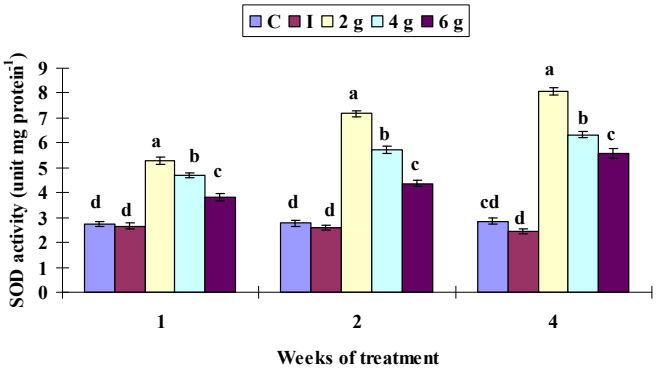

Fig. 5. Superoxide dismutase (SOD) activity of *M. rosenbergii* fed with Shilajit-enriched diets against *A. hydrophila*. The statistical information and label as same in Fig. 1.

3.8. Disease resistance

The low cumulative mortality in the infected prawn fed with 2 and 4 g kg^{-1} Shilajit-enriched diets was 10% and 15% during 30 days. However, the infected prawn fed with 6 g kg^{-1} Shilajit-enriched diet suffered 20% mortality for 30 days. The infected prawn fed with basal diet without Shilajit suffered 90% mortality. There was no mortality in the non-infected control group fed with basal diet (Fig. 8).

4. Discussion

Globally especially in South East Asia, the freshwater giant

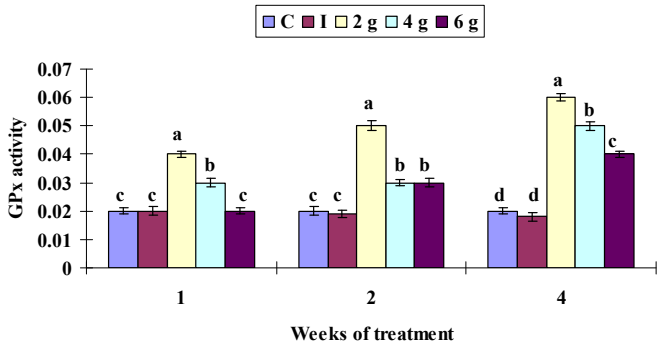

Fig. 6. Glutathione peroxidase (GPx) activity of *M. rosenbergii* fed with Shilajit-enriched diets against *A. hydrophila*. The statistical information and label as same in Fig. 1.

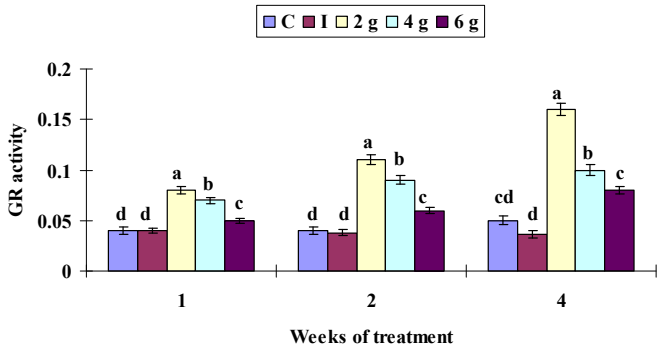

Fig. 7. Glutathione reductase (GR) activity of *M. rosenbergii* fed with Shilajit-enriched diets against *A. hydrophila*. The statistical information and label as same in Fig. 1.

prawn, *M. rosenbergii* is considered as a potential candidate species for sustainable aquaculture. However the production of prawns in countries including India has been vulnerable by many factors including diseases leading to significant economic loss in nursery and grow-out ponds. Among the bacterial pathogens, motile *Aeromonas* cause high mortality in prawn culture. In aquaculture, application of traditional medicine is disadvantageous; to overcome the drawbacks use of traditional antibiotics and chemotherapy application of natural mineral supplements or herbals with multifunctional active principles can be ideal alternatives. A number of studies have demonstrated that rare earth minerals possess antibacterial properties [47,48]. Dietary supplementation with rare earth elements causes bacterial flocculation by changing the structure and altering the surface charge of bacterial membranes [49]. The Azomite (a natural mineral) enriched diet shows better growth performance, survival rate, and enhance the immunomodulatory response in the freshwater fish, *Oreochromis mossambicus* against *A. hydrophila* [50] Jaleel et al. [51] have demonstrated that the Azomite supplemented diets enhances the immune response and growth performance of fingerlings of freshwater ornamental fish Koi carp, *Cyprinus carpio*.

In line with these findings Shilajit a natural mineral has been reported to be beneficial in the treatment of peptic ulcer [24,29]; further it is also used as a memory enhancer, a neuro-protective, anti-inflammatory, and anti-oxidant [17,23,24,52,53]. Shilajit increase SOD, catalase (CAT), and GPx activities in corpora striatum and frontal cortex [53]; it significantly decreased carrageenan-induced edema in rats [18]. These effects may be related to the anti-inflammatory and neuroprotective effects of Shilajit [24]. Few studies have reported to increase of proinflammatory cytokines such as TNF-α, IL-0β, and IL-6 [54]. Anti-edema effects of Shilajit could be related to its antioxidant and anti-inflammatory effects [17]. However, there is no report on the effects of Shilajit on immune response, antioxidant pathway, and disease resistance in giant prawn, *M. rosenbergii* against diseases.

Crustacean haemocyte play an important role in the hosts' immune response including recognition, phagocytosis, melanization, cytotoxicity, and cell-cell communication; their count varies greatly in response to infection, environmental stress, and endocrine activity during moulting cycle [54]. THC is one of the most important parameters used to assess immune response and can reveal the resistance to infectious pathogen. The THCs of crustaceans have a main role in clotting, exoskeleton hardening, and elimination of pathogens [3]. In the present study the THC was significantly increased in infected prawn when fed with 2 g kg^{-1} on first week while all Shilajit-enriched diets on weeks 2 and 4. These results are agreement with *M. japonicus* fed with BG for 14 days had 1.2 to 1.5 times increased THCs than those fed without β-glucans (BG) against penaeid rod shaped DNA virus (PDRV) [55]. Therefore, these results suggested that haemocytes may play an important role in cellular defence [56]; however, the THC in *Aeromonas* infected *M. rosenbergii* fed without Shilajit-enriched diet declined to the control value. Similarly in *P. monodon* the THC was considerably decreased to 60% of its pre-infection level when infected with white spot syndrome virus (WSSV) after 24 h as reported by Chang et al. [57]. Hence, the THC decline-than-normal numbers of circulating THC in crustacean species correlate well with a susceptibility to pathogens which may play an important role in cellular defence [56].

In the present study the respiratory bursts activity was

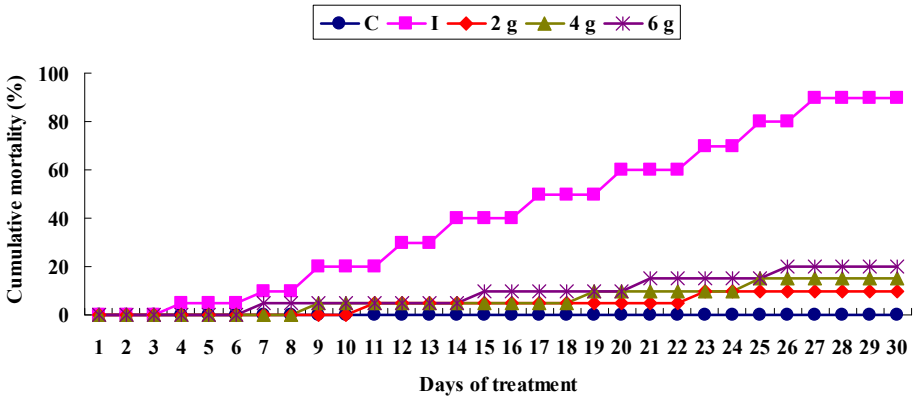

Fig. 8. Cumulative mortality (n = 20) of *M. rosenbergii* fed with Shilajit-enriched diets against *A. hydrophila* for 30 days. The label as same in Fig. 1.

significantly enhanced with 2 g kg^{-1} Shilajit-enriched diets on weeks 1 and 2 while it was significantly enhanced with 2 and 4 g kg^{-1} diets on fourth week. In fish and shellfish the increased respiratory burst activity can be correlated with increased bacterial pathogen killing activity of phagocytes or hemocytes which is an important indicator of cellular immunity [58,59] However, the respiratory bursts activity declined the infected prawn fed without Shilajit-enriched diet as indicating the prawn's susceptibility to *Aeromonas* pathogen. The reactive oxygen intermediates (ROIs) formed by phagocytic cells during the process of phagocytosis measurements of the phagocytic activity and respiratory bursts are widely used to evaluate the defense ability of the host against pathogens. The excessive accumulation of ROIs is extremely toxic to host cells which are minimized by various enzymatic antioxidants [60]. Respiratory burst is a post-phagocytic event, which release ROS including superoxide anion; a number of reactions lead to the production of hydrogen peroxide, singlet oxygen, hydroxyl radical, and numerous other reactive products [61]. The anti-superoxide anion ability indicates the activity of scavenging the superoxide anion in the body; it can also reflect the release of superoxide anion by respiratory burst [62].

In this study, the infected prawn fed with all Shilajit-enriched diet had an increased PO activity on forth week which contributing to disease resistance against *A. hydrophila*; thus, the higher PO activities in prawn fed with Shilajit-enriched diets may reflect the higher immunity contributing to microbicidal actions against *A. hydrophila*. In crustaceans, the circulating haemocytes are not only involved in the production of melanin via the prophenoloxidase (proPO) system but also in coagulation, exoskeleton hardening, elimination of pathogens [3]; it also influence molting in crustaceans, development of organs, reproductive status, nutritional condition, and disease prevention [63]. Indeed the proPO system is acknowledged as the most important immune system in crustaceans [64]. However, any Shilajit-enriched diet did not significantly increase the PO activity in infected *M. rosenbergii* on week 1 and 2 which may have contributed to the disease susceptibility against *A. hydrophila*.

The increase in secretion of oxygen radical in response to infectious pathogen has been demonstrated in crustacean [65]. The ROIs are formed by phagocytic cells during the process of phagocytosis; hence the measurements of phagocytic activity and respiratory bursts are widely used to evaluate the defense ability of the host against pathogens. Phagocytosis, the most common cellular defense reaction in combination with humoral components, constitutes the first line of defense against parasites or other intruders that evade the physico-chemical barrier of the cuticle. Phagocytes produce lysosomal enzymes which efficiently degrade and remove foreign material. All enriched diets significantly elevated the phagocytic activity in this study on weeks 2 and 4. ROIs are released during RBs of phagocytosis, which represents a defensive function against invasive pathogens. The present results are agreement when fed with dietary banana supplement in infected *M. rosenbergii* [66]. Under normal physiological condition, the harmful effects of ROIs are effectively neutralized by the antioxidant defense system of an organism, which includes enzymes like SOD, CAT and various peroxidase, and small antioxidant molecules like ascorbate, sugars and polyunsaturated fatty acids [66]. However, in the infected prawn fed with all enriched diets the phagocytic activity did not significantly enhance on first week except with 2 g kg^{-1} diet in the present study.

The SOD activity was significantly enhanced in infected prawn fed with any Shilajit-enriched diet during the experimental period against *A. hydrophila*. The superoxide released by processes such as oxidative phosphorylation is first converted to hydrogen peroxide and then reduced to water. This detoxification pathway is the result of multiple enzymes with SOD catalyzing the first step and then CAT, GPx, and various peroxidases removing hydrogen peroxide [67]. GPx is identified as a very potent antioxidant that protects the body from damage due to oxidation by free radicals [68]. Therefore, to achieve sustainable aquaculture, it is important to seek effective methods of alleviating or eliminating environmental stress. For example, Selenium is an essential trace element for organisms and it has a biochemical role in synthesizing selenoproteins such as GPx, which is an antioxidant enzyme. The activity of SOD has been measured in the shrimps *Palaemontes argentinus* and *Litopenaeus vannamei* [43,69]. When low levels H_2O_2 prevail organic peroxides are the preferred substrate for GPx whereas with high H_2O_2 concentrations, they are metabolized by CAT [70]. In our study, GPx and GR activities increased slightly on first week and then significantly after 2nd week. SOD could constitute a good molecular-bioindicator for oxidative stress and acute pollution [71]. Since the antioxidant enzymic activities of prawn fed Shilajit-supplemented diets were significantly higher than those of prawn fed the basal diet, this could enhance the antioxidant enzymic activity.

On the other hand, GPx activity significantly increased in any Shilajit-enriched diet fed group only on fourth week. However, the GR activity significantly increased with 2 g kg^{-1} Shilajit-enriched diet on weeks 2 and 4. Induction of antioxidant enzymes is an important line of defense against oxidative stress in fish and shellfish [72]. The present results are agreement with recent studies in *P. monodon* on the changes in some antioxidant enzymes activity such as SOD, CAT, and GPx, and oxidative damages (lipid peroxydation) in various tissues when infected with WSSV [73,74]. In addition, several studies show that changes in SOD activity [66] and in SOD, GPx and CAT expression follows bacterial or virus challenges/infection [75,76]. It has also been suggested that the antioxidant defenses operate at a very much lower rate in infected shrimps despite the higher requirement for dismutation of harmful free radical formation during infection by pathogens [74]. Based on this claim, it has been proposed that dietary supplementation of products with antimicrobial and antioxidant properties may be a promising disease prevention option for increasing resistance of shrimps to pathogens [74]. Mathew et al. [74] reported a concomitant increase in lipid peroxidation and a drop in antioxidant enzyme activities of SOD, CAT, GPx and Glutathion-S-transferase in the digestive gland, muscle and hemolymph of *P. monodon* infected with WSSV. They conclude that the antioxidant defence system was affected by the viral infection and that the tissue antioxidant status operated at a lower rate in *P. monodon* despite the higher requirement for dismutation of harmful radical formation during WSSV infection.

According to traditional Indian knowledge, Shilajit acts as a tonic, laxative, expectorant, diuretic, anti-bilious, immuno-modulator, lithotriptic, and anti-hypertensive medicine when given orally; when applied externally it acts as an antiseptic, analgesic, deobstruent and germicide. Intra peritoneal administration of Shilajit at doses of 20 and 50 mg kg^{-1} per day for 21 days induced a dose dependent increase of antioxidants such as SOD, CAT, and GPx activities in the frontal cortex and striatum of rats when compared with the control because of the presence of dibenzo-α-pyrones and fulvic acid [25]. Recent studies report that Shilajit possesses immunomodulatory capabilities such as increasing white blood cell activity eliciting different degrees of murine peritoneal macrophages and activating splenocytes of animals at early and later stages of tumour growth [17]. The cumulative mortality of the infected prawn fed with 2 and 4 g kg^{-1} Shilajit-enriched diets is 10% and 15% whereas the group fed with 6 g kg^{-1} diet suffered 20% mortality. In the present study, SOD and GPx activities increased together with an increase in GR and phagocytic activity in infected

prawn fed with Shilajit-enriched diets. The results strongly suggest that 2 g kg^{-1} Shilajit-enriched diet positively enhances the immune response and antioxidant activities and disease resistance in *M. rosenbergii* against *A. hydrophila.* Shilajit contains several potential active substance(s) and the exact mechanism on immune response and antioxidant activities and disease resistance of the Shilajit remain unknown. Further detailed immunology and molecular studies are required in other prawn against pathogens.

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