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Immunomodulatory effect of *Withania somnifera* supplementation diet in the giant freshwater prawn *Macrobrachium rosenbergii* (de Man) against *Aeromonas hydrophila*

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ABSTRACT

The effect of *Withania somnifera* extract supplementation diets on innate immune response in giant freshwater prawn *Macrobrachium rosenbergii* (de Man) against *Aeromonas hydrophila* was investigated. The bacterial clearance efficiency significantly increased in prawn fed with 0.1% and 1.0% doses of *W. somnifera* supplementation diet against pathogen from weeks 1–4 as compared to the control. The innate immune parameters such as, phenoloxidase activity, superoxide anion level, superoxide dismutase activity, nitrate, and nitrite concentrations were significantly enhanced in prawn fed with 0.1% and 1.0% doses of *W. somnifera* supplementation diet from weeks 1–4 against pathogen. The total hemocyte counts (THC) significantly increased in prawn fed with 0.1% and 1.0% doses diet from weeks 1–4 against pathogen as compared to the control. These results strongly suggested that administration of *W. somnifera* through supplementation diet positively enhances the innate immune system and enhanced survival rate in *M. rosenbergii* against *A. hydrophila* infection.

1. Introduction

The giant freshwater prawn *Macrobrachium rosenbergii* (de Man) is an important freshwater farmed inland crustacean species in many countries because of its high commercial value [1] and survival in a wide range of salinity between 0 and 18‰ [2]. *M. rosenbergii* farming has dramatically expanding many countries in the last decade. The world production of the prawn species had increased to over 2,00,000 tonnes in 2002 [3] and the value of the prawn was US$ 102.22 million in Malaysia during 2004 which the production of giant freshwater *M. rosenbergii* alone contributed US$ 2.82 million or 0.28% of the total value [4]. The intensification of prawn aquaculture linked with deteriorated pond environment and resultant stress as well as poor quarantine in many prawn hatcheries and farms in West Indies, China, Taiwan, India, and Thailand have witnessed the epizootics of *M. rosenbergii* nodavirus (*Mr*NV) [5–8]. In India, juveniles and adults of *M. rosenbergii* have been suffered a major setback due to occurrence of appendage deformity syndrome (ADS) [8] and several disease outbreaks have occurred due to bacterial pathogens [9,10] such as *Vibrio* spp., *Aeromonas* spp., and *Pseudomonas* spp., and *Lactococcus garviae* infection [11] which caused high mortalities in hatcheries [12–14]. Among, *Aeromonas* spp. is considered to be the major threat to the commercial cultivation of *M. rosenbergii* aquaculture in Taiwan [12] and Brazil [15] including India [16–19].

To prevent and control prawn diseases were used large quantities of antibiotics and vaccines. Application of the antibiotics develops drug-resistance bacteria while vaccines are specific for pathogen, thus novel strategies to control *Aeromonas* spp. are needed for prawn culture. Several studies have been reported that the application of herbal or other immunostimulants in fish and shrimp farming for enhancing immune response and reduction of disease impacts [16,17,20–23]. Heat inactivated *V. anguillarum* was used as immunostimulant and reduce the mortality in post-larvae (PL) of tiger shrimp *Penaeus monodon* [24]. The potential immunostimulating properties of natural and commercial plant-derived products were evaluated for immunostimulation purpose in finfish and shellfish aquaculture against viral and bacterial diseases [21–23]. Screening of various Indian medicinal plants and aqueous

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extract of *Cyanodon dactylon* showed strongest antiviral activity against white spot syndrome virus (WSSV) [21,24]. Dietary administration of bovine lactoferrin and selenium enriched-diet influences the immune ability and resistance against *Aeromonas hydrophila* infection in *M. rosenbergii* [25,26].

Indian ginseng, *Withania somnifera* (L. Dunal) belongs to the family Solanaceae which is distributed throughout India that known to possesses anti-inflammatory [27], anti-tumor [28], anti-stress [29], anti-oxidant [30], and immunomodulatory [31] properties. Feed containing *W. somnifera* extract at the rate of 200 mg kg^{-1} significantly reduced the mortalities of juvenile greasy grouper, *Epinephelus tauvina* against *Vibrio harveyi* infection [32] and in *Labeo rohita* against *A. hydrophila* [33]. In this study, we attempted the effect of *W. somnifera* supplementation diet on innate immune parameters including total hemocyte count (THC), phenoloxidase (PO), nitric oxide (NO), superoxide anion (O_2^-), and superoxide dismutase (SOD) production in *M. rosenbergii* against *A. hydrophila* infection.

2. Materials and methods

2.1. Plant extract and herbal diet preparation

W. somnifera was collected from locally and the identification was done by Plant Science Department, Bharathidasan University. The roots were collected from the plants, washed thoroughly with tap water to rid them of dirt. After washing, the roots were dried room temperature under shade to make them suitable for grinding. The dried plant roots were grounded in a mechanical grinder and sieved then stored in an air tight 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 [34] and Singh et al. [35]. 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. 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% (control), 0.01%, 0.1%, and 1.0% doses of *W. somnifera* 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 30–38 °C for 18 h, packed, and stored in a freezer at −20 °C until used. The proximate composition of the diets were quantified following AOAC method comprised 51.3% crude protein, 8.4% crude lipid, 7.5% crude ash, and 14.7% 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).

2.2. Aeromonas hydrophila

The pathogenic bacterium, *A. hydrophila* was isolated from diseased prawns and the bacterium was identification and biochemical characterization according to Kennedy et al. [36]. The bacterium was grown on tryptone soy broth (TSB, Difco) for 24 h at 30 °C. The broth cultures were centrifuged at 5000 g for 15 min at 4 °C and the bacterial pellet washed twice with sterile phosphate buffered saline (PBS; pH 7.4) by centrifuging at the above speed. The pellet was re-suspended in PBS at 10^7 colony forming unit (cfu) ml^{-1} as stock bacterial suspensions for challenge study.

2.3. Experimental animal and experimental design

Healthy giant freshwater prawn, *M. rosenbergii* (20–25 g) were obtained from a commercial farm and acclimated in the laboratory for 2 weeks before experimentation. Prawns were given the control or basal diet during acclimatization period as mentioned in Table 1. The prawns were examined health status immediately upon arrival for standard microbiological method. Ten percent of water was renewed daily to removal of waste feed and faecal 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 four groups of 25 each in triplicate kept in 250 l tanks and fed with 0% (control), 0.01%, 0.1%, and 1.0% doses of *W. somnifera* supplementation diet at the rate of 10% of their body weight twice a day. After 30 days of feeding, all groups were injected between the second and third abdominal segments with 50 μl PBS containing *A. hydrophila* at 1.3×10^7 cfu ml^{-1}. On weeks 1, 2, and 4 post-infection, six prawns were randomly collected from each tank to collect hemolymph for hematological and immunological assays. A group of 20 fish were used in each experiment separately for cumulative mortality.

2.4. Sample collection

The haemolymph of 100 μl was collected from the ventral sinus of each prawn in to 1 ml syringe (26 gauge) containing 900 μl anticoagulant (sodium chloride 0.45 M, glucose 0.1 M, sodium citrate 30 mM, citric acid 26 mM, EDTA 20 mM, pH 4.5) [25]. A drop of haemolymph was placed on a hemocytometer and measure THC and DHC using an inverted phase contrast microscope.

2.5. Bacterial clearance efficiency

A volume of 25 μl haemolymph was taken and diluted (1:14) in TSB and spread on TSA plates in quadruplicate followed by 12 h incubation at 30 °C to obtain bacterial counts. Counts were calculated taking the dilution factor into consideration and recorded as the mean cfu count ± SE for the quadruplicate counts [16].

2.6. Immunological assay

The immunological assay such as phenoloxidase activity (PO) was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA; Hi Media, Mumbai) according to Ashida and Sóderhäll [37]. The superoxide anion assay was quantified as described by the method of Song and Hsieh [38] and the superoxide dismutase (SOD) activity was determined according to Beauchamp and Fridovich [39] using nitroblue tetrazolium (NBT) in the presence of riboflavin. The haemolymph total nitrite and nitrate levels were determination spectrophotometric method according to Sastry et al. [40] with the following modification. Another 100 μl hemolymph samples were