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# The effects of ethanolic extract of *Indigofera longirecemosa* leaves incorporated with basal diet fed on growth and disease challenges on *Vibrio parahaemolyticus* infection of the giant freshwater prawn *Macrobrachium rosenbergii* post larvae

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**Abstract**

The present study was to evaluate the basal diet supplemented with 1%, 2%, 3% and 4% extract of *Indigofera longirecemosa* leaves fed on the growth performance, disease challenge and histopathology analysis of post larvae of *Macrobrachium rosenbergii* for 60 days. The experimental methods designed for the prawns were divided into control, infected prawn, and treated groups fed with control diet and infected prawns treated with different concentration of 1%, 2%, 3% and 4% *I. longirecemosa* leaves extract. All experimental groups were diseases challenged using with intramuscularly injected with 30µl of *Vibrio parahaemolyticus* ($1\times10^7$ cfu/ml) on prawn of post larvae. The disease challenge study was observed by 30day period, the five prawns were randomly selected from each tank for each experimental and control group to haemolymph collection. The cumulative mortality and survival rate were assessed every day. The histological analysis of hepatopancreas examined the end of the experimental periods. The prawns were fed on basal diet supplemented with 1%, 2%, 3% and 4% *I. longirecemosa* leaves showed good growth performance was observed. The specific growth rate of the prawn was observed the highest in the 4 % ethnolic extract incorporated feed treatment compared to all others treatment groups. Prawns fed the 4% diet had highly significantly higher ($p< 0.05$) than the control group. Histopathological examination of the present study observed that hepatopancreas of control prawns exhibited in the well-organized structure of glandular tubular normally seen in the control. *V. parahaemolyticus*. 4% ethanolic extract had significantly more E-cells within their hepatopancreatic tubules compared to other treatments while after 56 days, the hepatopancreatic tubules of prawns in the 4% extract treatment were more closely arranged with significantly more R- and E-cells. In conclusion, this study suggested that the formulation of the experimental diet composition of basal diets supplemented with ethanolic extracts of *I. longirecemosa* were effective as growth promoters and treatment bacterial infection of *M. rosenbergii*.

**Keywords**
*Macrobrachium rosenbergii*; *Vibrio parahaemolyticus;* aquaculture; *Indigofera longirecemosa,leaves*

## Introduction

FAO predicts the global aquaculture production will exceed 100 million tonnes by 2020. With a current world production of 66 million tonnes, in India contributes over 4.2 million tonnes ranking them the world's second largest aquaculture producer. With a continuous rising demand in fish and other aquaculture produce a reduction in operation costs and sustainability is of the greatest importance.

The giant freshwater prawn, *Macrobrachium rosenbergii* (de Man), is an economically important crustacean and is farms in many countries. It is distributed in Indonesia, India, West Java, Central Java, Yogyakarta, East Java and Bali (Nugroho and Emmawati 2004; Nugroho *et al*., 2005). It is a common inhabitant of rivers and estuaries in tropical regions of the world (New, 2005). Fresh water prawn of *M. rosenbergii* is an aquaculture importance owing to its high fecundity, rapid growth, wide range of salinity and temperature tolerance, disease resistance as well as its superior taste and high commercial value ( Johnson, 1982; New, 1995; Roustaian *et al*., 2001). *M. rosenbergii* is highly preferred for culturing due to its fast growth rate, tolerance to wide fluctuations of temperature, salinity and resistance to major diseases. The use of probiotics in the aquatic organisms is increasing with the demand for more environmentally-friendly aquaculture practices (Gatesoupe, 1999). The rising demand for freshwater prawns has caused a significant increase in the number of prawn farms. The major causative agents for diseases in giant freshwater prawns of *M. rosenbergii* were observed by protozoa, fungi, viruses and bacteria (Tonguthai, 1997; Cheng and Chen, 1998; Yoganandhan et al., 2006; Chen *et al*., 2001). The major bacterial disease spreading among farmed shrimp have been known as necrotizing hepatopancreatitis (NHP) and vibriosis which are respectively caused by *Vibrio parahaemolyticus* (Wong *et al*., 2000).

Phytochemicals have been derived from whole plants and plant parts. Several novel drug isolated and chemical structures design for medicinal plants. In aquaculture, medicinal plants are also used as chemotherapeutic and feed additives (Chang, 2000). Medicinal plants have been known as immunostimulant for thousands of years. The medicinal plants have been used as immunostimulant for mice, chickens or human cell lines studies (Tan and Vanitha, 2004; Cao and Lin, 2003; Lin and Zhang, 2004; Lin *et al*., 2006; Shan *et al*., 1999). The application of medicinal plants as natural and innocuous compounds has potential in aquaculture as an alternative to antibiotics and immunoprophylactics.

The medicinal plants of *Indigofera longeracemosa* Boiv.ex Baill. is belongs to the family Fabaceae. The medicinal plants of *I. longeracemosa* is used for an antitode for snake poison. Previously, A novel xanthene, 3-isopropyl-9a-methyl-1,2,4a,9a-tetrahydroxanthene, was isolated from the stem of *I. longeracemosa (*Thangadurai and Viswanathan,2000). Many compounds used for antimicrobial, antiulcerogenic and pharmacognostical activities are reported (Thangadurai and Viswanathan,2000; Perumal and Kala,2009). The chemical composition, cytotoxicity and anticancer activities of the *Indigofera longeracemosa* leaves were reported previously (Suseela and Lalitha, 2015). The present study was to evaluate the basal diet supplemented with 1%, 2%, 3% and 4% extract of *I. longirecemosa* leaves fed on the growth performance, disease challenge and histopathology analysis of *M. rosenbergii* for 60 days.

## Materials and Methods

### Selection of animals and acclimatization

The experiments were conducted at the Gayathri Research Foundation, Palayamkottai, Tamil Nadu. Post larvae of were obtained from a private farm of Kottayam and Allepey in Kerala. They were acclimatized for two weeks for laboratory conditions.

### Diet preparation

The preparation of experimental diets and composition for given in the table -1. The basal diet supplemented with 1%, 2%, 3% and 4% of ethanolic extract of *I. longirecemosa* leaves. The control diet does not contain without the extract. Experimental diets composition was prepared for added with distilled water mixed with extract and basal diet composition. The diets were prepared and air-dried at room temperature for 72h and packed with airtight containers, labelled and stored.

### Growth performance

The experimental study was contacted in the laboratory condition and to assess the growth performance of *M. rosenbergii* for 60 days. Post larvae of *M. rosenbergii* were divided into 5 groups with 10 post larvae in each group. Each prawn was selected approximately weighing about 12 ± 47mg randomly distributed into 50liter tanks (10 prawn /tank) containing aerated recirculated freshwater. Each treatment was three replicates and the prawn were feeding over a period of 60 days. A non-stop aeration to maintain the dissolved oxygen to the optimal level was provided. They were assigned to five treatments (1%,2%,3% and 4%) with three replicate (n=3) per treatment. Group one was considered as control fed with basal diet. Group 2 to 5was fed with basal diet supplemented with different concentration of supplement with ethanolic extract of 1%, 2%, 3% and 4% of *I. longirecemosa* leaves. All prawns were fed with twice daily at 4% of body weight and the daily ration was adjusted. The feed consumption in each aquarium was recorded daily. Dead prawn from each aquarium were collected in the daily weighed. The growth parameters were analyzed for survival rate (SR), weight gain (WG), specific growth rate (SGR) and FCR were determined the following formula

All prawns were counted and weighed as follows:
Survival rate = 100 × (final prawn number) / (initial prawn number)), Weight gain rate (WGR) = 100 × (final body weight - initial body weight) / initial body weight Specific growth rate SGR = (lnWf - lnWi x 100) / t , Feed conversion rate (FCR) = feed intake / (final body weight - initial body weight) were calculated.

Table -1: Feed preparation and composition mixture of control diet and experimental diets

| Ingredients | Composition Control diet (%) | Experimental Diets (%) | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| Groundnut oil cake | 45 | 45 | 45 | 45 | 45 |
| Soybean meal | 18 | 18 | 18 | 18 | 18 |
| Fish meal | 17 | 17 | 17 | 17 | 17 |
| Rice bran | 17 | 16 | 15 | 14 | 13 |
| Mineral and vitamin mix | 2.8 | 2.8 | 2.8 | 2.8 | 2.8 |
| Carboxy methyl cellulose | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |

a Each 250 g vitamin and mineral mixture provides vitamin A (5,000,000 IU), vitamin D3 (100,000 IU), vitamin B2 (0.2 g), vitamin E (75 units), vitamin K (0.1 g), calcium pantothenate (0.25 g), nicotinamide (1.0 g), vitamin B12 (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).

### Disease Challenge assay

Disease challenge study, experimental setup was detailed mentioned in the growth performance method. After feeding,

prawn with different concentration of ethanolic extract of *I. longirecemosa* leaves supplemented with basal diets for 30 days. The challenge test was made by intraperitoneal injection with 0.1/ml suspensions of *V. parahaemolyticus* ($1\times10^7$cfu/mL).

The percentage survival in each treatment was calculated with the following formula

$$\text{Survival rate (\%)} = \frac{\text{Total number of prawn stocked} - \text{Number of prawn died}}{\text{Total number of prawn stocked}} \times 100$$

**Analysis of histopathological study**

For histology analysis, hepatopancreas was removed from the disease and control and treated group were collected. Tissues were fixed at 10% buffered formalin for 24h. These tissues were transferred into new buffered formalin solution. The fixed tissues were processed in automated tissue processor. Then, the tissues were embedded in paraffin block for easy to store and handle. Sectioning using microtome to produce very thin, 4 µm sections that are placed on a microscope slide ready for staining. Finally, the tissues were stained with haematoxylin and eosin and observed under light microscope. The histologic examination to detect any possible pathologic changes resulting from local or systemic disease.

Table-2: Result of weight gain observation of basal diet incorporated with four concentration of *I. longirecemosa* leaves extract fed to post larvae of *M. rosenbergii* for 90 days

| Feed | Days of Experiments | | | | | | | Wait gain(mg) |
|---|---|---|---|---|---|---|---|---|
| | 0 | 15 | 30 | 45 | 60 | 75 | 90 | |
| Control | 12.47±11.26 | 45.47±0.21 | 101.47±0.67 | 204±0.23 | 250±0.26 | 325±0.126 | 432.47±1.06 | 420 |
| 1% | 12.47±11.26 | 45.56±0.54 | 114.47±0.11 | 210±0.26 | 268±0.126 | 356±0.34 | 531.47±0.99 | 519 |
| 2% | 12.47±11.26 | 49.21±0.16 | 153.47±8.29 | 234±4.11 | 279±3.56 | 387±1.29 | 589.47±6.09 | 577 |
| 3% | 12.47±11.26 | 51.41±0.21 | 161.47±6.26 | 256±11.26 | 289±11.26 | 401±11.26 | 609.47±0.71 | 597 |
| 4% | 12.47±11.26 | 57.47±0.87 | 163.47±1.29 | 267±9.21 | 290±10.26 | 435±1.27 | 678.47±7.23 | 666 |

Values are means ± SE of three replication.

Table-3: Results of Specific growth rate (SGR) of *I. longirecemosa* fed on M. rosenbergii

| Feed | Experimental Periods for 60 days | | | |
|---|---|---|---|---|
| | Days 15 | Days 30 | Days 45 | Days 60 |
| Control | 4.8 | 5.2 | 6.89 | 7.44 |
| 1 % Extract + Control Diet | 5.8 | 7.09 | 8.23 | 8.97 |
| 2 % Extract + Control Diet | 6.2 | 7.76 | 8.67 | 10.23 |
| 3 % Extract + Control Diet | 6.45 | 8.11 | 9.12 | 11.76 |
| 4 % Extract + Control Diet | 6.89 | 10.05 | 11.45 | 12.06 |

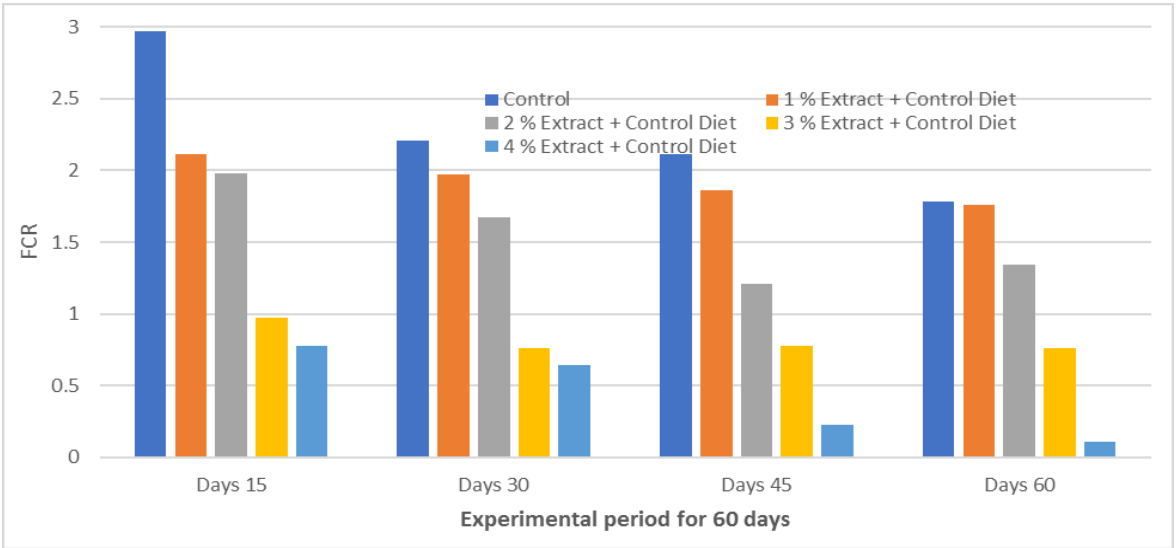


Fig.1: Results of Specific growth rate (FGR) of *I. longirecemosa* fed on *M. rosenbergii*

## Results and Discussion

The results of the present study, growth performance of *M. rosenbergii* fed on *I. longirecemosa* leaves extract incorporated with basal diet for 60 days seen in the table-2. The post larvae of *M. rosenbergii* fed with 4% of additives on *I. longirecemosa* leaves extract showed maximum weight gain was observed (Table-2). The post larvae of *M. rosenbergii* fed with control diet gained lower growth was observed when compared with other all experimental diets seen in the table-2. All experimental diets fed to post larvae were observed by 100 % of survival rate. The control diet was observed 75% of survival rate. According to Daniels *et al*., (1995) reported that highest survival rate of 73.7 to 81.9% by *M. rosenbergii* fed with specially formulated diet in earthen ponds. The specific growth rate was observed good results in supplemented diets on *I. indigofera* leaves fed post larvae of *M. rosenbergii* when compared with the control diet (Table-3). Prawns fed the 4 % diet had significantly higher ($P < 0.05$) than the control. Tidwell *et al*., (1993) reported that the survival and mean individual weight of 78 % and 42g respectively in a monoculture of *M. rosenbergii* using formulated diet containing 32% protein. The FCR rate *M. rosenbergii* post larvae fed with the formulated feeds in the different concentration of ethanolic extract of *I. longirecemosa* given in fig.1. Hossain (2004) reported that FCR varied between 2.18 to 2.43 which were comparatively higher than that of the present study. Das *et al*., (1996) stated that FCR of pellets varied from 2.94 to 6.30. The present study observed that *I. longirecemosa* showed good growth responses on fed with *M. rosenbergii*.

Immunostumilant and the adjuvants used in fish and prawn vaccines are of interest, as they offer an alternative to drugs, chemicals and antibiotics currently used in fish and prawn culture to control the several diseases. In this study, the disease challenged groups showed reduced mortality when compared with the control group. The cumulative mortality of *M. rosenbergii* fed on control diet for 30 days observed by 100 % and experimental diets observed for no mortality. Supplementing diets with 4% of *I. longirecemosa* leaves resulted in better survival rate and reduction in bacterial load when compared than control. Earliest report of five herb composition reduction in viral load with black tiger prawns (Citarasu *et al*., 2006), and helped to decrease *Vibrio* species loaded in post-larvae of black tiger prawn (Velmurugana *et al*., 2010). The hemocyte count are which represent an indicator of the physiological state of the invertebrate. Our study shows that the increasing number of total hemocyte was observed in the all experimental diet compared than the control diet. Hemocyte play a crucial role in the immune system of invertebrates; they are involved in the mediation of both humoral and cellular responses (Rowley and Powell,2007), and have the ability to kill several bacterial species (Genthner *et al*.,1999). Hemocyte counts in crabs have been shown to increase as a result of injury (Tubiash *et al*.,1975). Earlier studies, the number of hemocytes is also a good health indicator of invertebrates. Hemolymph of shrimp, even of those that are healthy, has been shown to contain bacteria (Gomez-Gil *et al*.,1998; Wang *et al*.,2002).

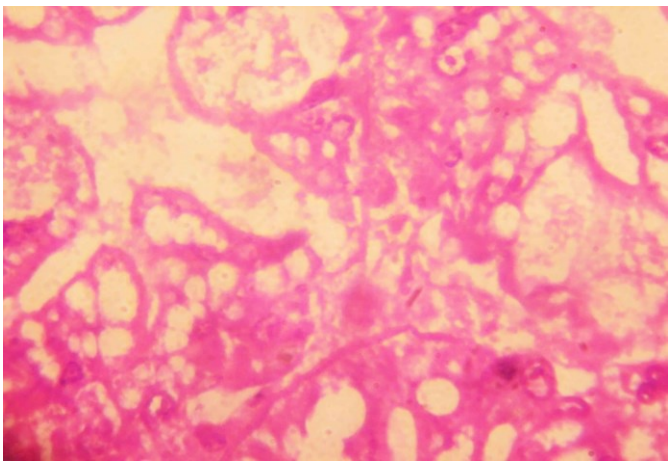

Photo-1: Control fed on *M. rosenbergii* hepatopancreas of histological section (H/E).

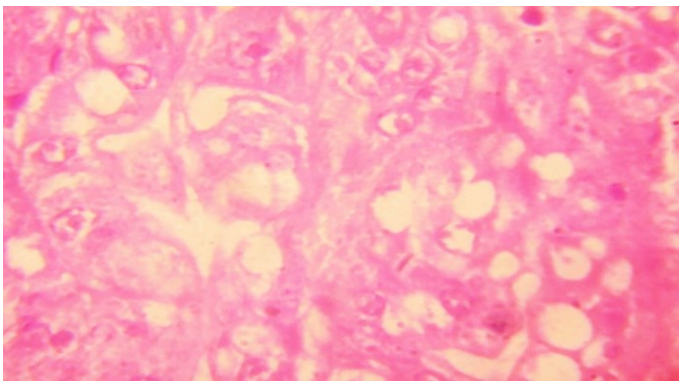

Photo-2: Histological section of *Vibrio parahaemolyticus* infected *M. rosenbergii* hepatopancreas (H/E).

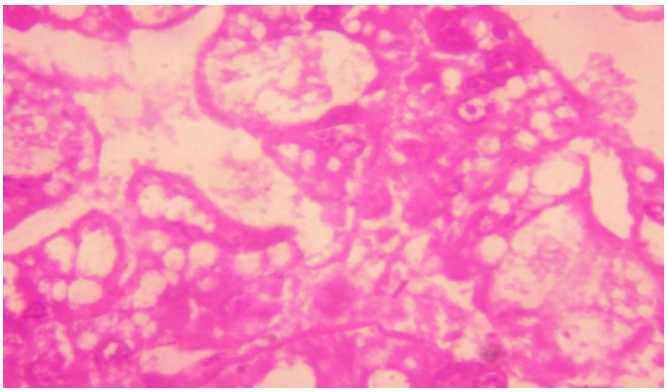

Photo-3: Experimental fed on *M. rosenbergii* hepatopancreas of histological section (H/E).

The hepatopancreas of control prawns exhibited the well-organized glandular tubular structure normally seen in *M. rosenbergii* (Photo-1). The tubules were closed distally, but opened out proximally into ducts which, in turn, united to form longer ducts that were ultimately connected to the digestive tract. The tubule lumen was found to have a 'star' like appearance (Photo-1). A single layer of epithelial cells was found lining the tubules. The cells showed normal differentiation into E (embryonic) cells at the narrow distal end of the tubule, young R (restzellen) cells and F (fibrillenzellen) cells a short distance away from the distal region, and B (blasenzellen) cells in the middle and proximal regions of the tubules (Photo-2). The B-cells exhibited large apical secretory granules, the R-cells were found to contain large amounts of rough endoplasmic reticulum and lipid droplets, while the F-cells were found to be non-vacuolated and deeplystained (Photo-3). The interstitial sinuses between tubules were normal. The control diet fed on *M. rosenbergii* was observed by normal number of R-cells in the tubular epithelium of the hepatopancreas. The *Vibrio* infected group of prawns observed by higher R-cells in the hepatopancreas and abnormal lumen (ALU) and hemocytic infiltration (HI) in the interstitial sinus (IS) were observed. The pathological identification of prawns observed the separation of necrotic cells of hepatopancreas (NCH) from basal laminae (BL); thickened basal laminae (TBL); necrotic tubules (NT) of the hepatopancreas containing tissue debris (TD) in the lumen; occurrence of melanization (MZ) and coagulation (CO) in the thickened basal laminae; walling off of the tubules by hemocytes (HC) around the thickened basal laminae. 4% of the ethanolic extract incorporated with basal diet had observed by E-cells within their hepatopancreatic tubules compared to other treatment groups. Hepatopancreatic tubules were observed in the prawns fed with 4% extract treatment more closely arranged with significantly observed in R and E-cells.These results indicate that ethanol extract of *I. longirecemosa* was capable of activating the immune system of *M. rosenbergii*. The conclusion of the present study, the best specific growth rate and feed conversion ratio observed by 4 % of ethanolic extract *I. longirecemosa* leaves supplemented with basal diet. 4 % of ethanolic extract *I. longirecemosa* leaves formulation, feed is recommended for *M. rosenbergii* post larvae.

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