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Immunostumilant activity of *Drynaria quercifolia* rhizome extract and histopathological analysis of *Vibrio harveyi* infection of *Macrobrachium rosenbergii*

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Abstract

In the present study, we evaluated in the growth parameters of the basal diet incorporated into *Drynaria quercifolia rhizome extract* fed with *Vibrio harveyi* infected prawn *Macrobrachium rosenbergii* post larvae. In the results of control diet incorporated into 4% *D. quercifolia* observed that the best growth performance in weight gains, specific growth rate (SGR) and feed conversion ratio (FCR). In the present study, *Vibrio harveyi* infected prawn *M. rosenbergii* observed by THC and DHC increasing counts in experimental diets treated groups compared than control group. The conclusion of the present study, experimental diets of *D. quercifolia rhizome extracts* is the best feed, growth enhanced and immunostumilant activity for *V. harveyi* infected prawn *M. rosenbergii* and also it is best suitable feed for *M. rosenbergii*.

Keywords: Immunostimulant, *Drynaria quercifolia,* growth performance, immune response, *Vibrio harveyi, Macrobrachium rosenbergii*

Introduction

Growth of aquaculture industry is now significant benefits of productivity, sustainability and profitability. Worldwide, the aquaculture industry has grown at an average rate of 8.9% per year. *Vibrio* spp, which are considered as a significant problem with development of aquaculture sector with severe economic losses worldwide. Approximately, global estimation of disease losses by the World Bank in 1997 reported to the US $3 Billion per year (Subasinghe *et al*.,2001). Due to its high importance in aquaculture, large amount of research had been carried out throughout the world on various aspects of its biology, ecology and aquaculture (Wowor and Ng 2007). *Vibrio harveyi*, and *Vibrio* anguillarum are very dangerous in marine species and these species having been associated with large-scale losses of prwans (Arias *et al*.,1999; Pujalte *et al*.,1999; Frans *et al*.,2011; Diggles *et al*.,2000). *Vibrio harveyi* is a Gram-negative bacterium, ubiquitous in the marine environments and is found free-living in the water column, and in the gut of some marine animals.

The prawn is one of the high market value of aquaculture product. At present, feed is the largest single cost item, as it constitutes 40-60% of operational cost in prawn industry. *Macrobrachium rosenbergii* is a large freshwater prawn native of the Indo-West Pacific from northwest India to Vietnam, Philippines, New Guinea and northern Australia. Giant freshwater prawn *M. rosenbergii* popularly known as 'scampi' has been expanding on India. It has been introduced into many countries for aquaculture. *M. rosenbergii* is a freshwater prawn, but since its larvae require brackish water for survival. Development of feed formula is a major input of hatchery, the availability of cost-effective feeds plays an important role in aquaculture industry (Chunchom *et al*., 2010). The use of artificial feeds with optimized nutritional quality is the need in aquaculture. Success in aquaculture depends on a great extent of sound nutritional practices based on the knowledge of nutrients required by the species cultured. Supplementation of diets of growth inducing substances has the potential to be profitable because of the improved growth rate or reduced culture period (Keshavanath *et al*., 2003).

Natural antioxidants are a wide class of compounds coming mainly from spices, herbs and recently by agriculture byproduct (Moure *et al*., 2001). Several of these antioxidants are utilized for improving animal health (Sutton *et al*., 2006) for organic animal production and for improving the quality of final product (Hamre *et al*., 2004). Many medicinal plants showing immunomodulatory activity have been used instead of drugs because of their low toxicity for the host system, adequate absorption and capability to reach the target organ without much degradation by host enzymes (Arivuchelvan *etal*., 2012). The side effects of synthetic drugs such as presence of antibiotic residues leading to the problem of antibiotic resistance to humans, toxic metabolites remaining in meat and byproducts are a matter of concern in long term usage of synthetic products. Such issues have promoted use of herbal preparations which are considered to be relatively safe and affordable to rural folk. Further, absence of antibiotic or toxic residues in meat and milk products has also encouraged herb based health solutions in veterinary health care sector. Thus, traditional herbal medicines in veterinary practice have great potential as an alternate therapy (Gupta *et al*., 2013).

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Extracts or active principles are isolated from plants, they can influence the immune response in several ways (Hoffmann *et al*.,1997): on cellular level by modulating the proliferation rate of immune cells (e.g. naftoquinones), on humoral level by influencing the antibody production (e.g. polysaccharides) or by modulating cellular functions to increase or decrease cytokine or other mediators production (e.g. phenolic compounds). Most studies have used herbal extracts rather than the purified compounds, therefore there is still suspicion of the efficacy and optimum dosage of herbal plants and their derivatives as immuno stimulators. Hence, more research is required for scientific validation of herbal plants as potent animal immunostimulators (Hashemi and Davoodi, 2012).

Drynaria quercifolia is commonly known as the Oak-leaf fern is a "basket fern" belonging to the Polypodiaceae family. They are a terrestrial, and epiphytic on tree trunks in open forests and rainforests. *D. quercifolia* is native to Western Australia as well as India, Southeast Asia, Malaysia, Indonesia, the Philippines and New Guinea. This species of fern is characterized particularly by having "wooly" rhizomes. Rhizome is bitter and astringent, aqueous extracts how antibacterial activity (Anonymous, 1952; 1986). Meidicnal uses of rhizome is treated for phthisis, hectic fever dyspepsia, cough and typhoid fever (Kirtikar and Basu,1935; Nadkarni, 1982; Dixit and Vohra,1984). The aim of this study is to describe the nutritional study and histological structure of the hepatopancreas of *Macrobrachium rosenbergii* for laboratory method.

Materials and Methods

Selection of animals and acclimatization

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

Prawn and virulence tests

Test animals of *M. rosenbergii* was weighing approximately 0.35g in total body weight were held in tanks (25L). *Vibrio harveyi* was selected for virulence test. The lethal dose 50 % end point (LD_{50}) tests, with batches of 10 animals per dose, were conducted by intramuscular (i.m) injection at the 5th abdominal segment of prawn in 24h bacterial suspension ($1x10^6$ c.f.u. animal) into the test animals. Mortalities were recorded for daily. Lethal dose of 50% mortality of prawn (LD_{50} values) were calculated by standard method.

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 *D. quercifolia* rhizome. 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.

Table- 1: Composition of the basal diet and experimental diets

Composition	Percentage (%)				
	Control	1%	2%	3%	4%
Groundnut cake	50	49	48	47	46
Fish meal	25	25	25	25	25
Rice bran	13	13	13	13	13
Soybean meal	5	5	5	5	5
Vitamin and mineral mixture*	3	3	3	3	3
Vegetable oil	3	3	3	3	3
Carboxymethyl cellulose	1	1	1	1	1

*Each 1000 g vitamin and mineral mixture provides vitamin A-2,000,000 i.u. , Vitamin D_3-100,000 i.u. , vitamin B_2 0.8 g. vitamin E-100mg. vitamin C-0.3mg, vitamin K-0.4g. calcium pantothenate- 1.00 gni cotinamide-0.4 g, vitamin 812-2.4 mg, choline chloride-60 g, calcium-300 g, manganeese- 11g. iodine-0.4 g, iron- 3.00 g, zinc-6.00 g. copper-0.8g and cobalt-0.18 g.

Immunomodulatory activity

The experimental study was contacted in the laboratory condition and to assessed 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 0.350g randomly distributed into 50liter tanks (10 prawn /tank) containing aerated recirculated freshwater. 0.01 ml of 10^6 cfu. of *Vibrio harveyi* injected with 5th abdominal segment of prawns was produced the bacterial diseases for one week. After 24hrs counting of mortality rate and treatment of standardized formulated feeds given in the table-1. Each treatment was three replicates and the prawn were feeding for 60 days experimental periods. 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 replicates (n=3) per treatment. Group one was considered as control fed with basal diet (TC). Group T1 to T4 was fed with basal diet supplemented with different concentration of supplement with ethanolic extract of 1%, 2%, 3% and 4% of *D. quercifolia* rhizome. 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 and weighed. The growth parameters were analyzed for weight gain (WG), survival rate (SR),

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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.

Determination of Haemogram

Haemolymph of *M. rosenbergii* was collected at the end of each week from the ventral-sin us cavity of prawns from each tank using a 26-gauge needle and 1ml syringe containing anticoagulant solution (l :9) (tri-sodium citrate: 0.114 *M,* sodium chloride: 0.10 *M,* p H 7.45). Haematological parameters of total haemocyte count (THC) and differential heamocyte count (DHC) was enumerated by Neubauer method previously described by Campbell, (1995).

The total haemocytes counts (THC) cell per mm^3 of haemolymph were calculated as follows:

$$THC = \frac{\text{Haemocytes in four - 1mm}^3 \text{ x depth factor x dilution factor}}{\text{Number of squares counted}} \times 100$$

Differential haemocyte counts (DHC): Haemolymph smears were stained with May Grunwald-Giemsa and counted. Cells were observed at a magnification of 1000x. Haemocytes were characterized and relative abundance of the cell type were determined by counting 100 cells. The percentage of each type of haemocytes was calculated on the basis of total number of all the haemocytes in a single observation. The DHC was expressed as follows:

$$DHC = \frac{\text{Number of specific type of cells counted}}{\text{Total number of cells counted}} \times 100$$

Histopathology

Healthy and diseased prawn of hepatopancreas were fixed in 10% formalin and processed for paraffin sectioning, then the sections were stained by using hematoxylin and eosin.

Results and Discussion

The results of growth performance of prawn *M. rosenbergii* fed with control diets and the experimental diets was summarized in the table -2. The high growth rate of *M. rosenbergii* was recorded in the 4% of *D. quercifolia* rhizome and the lowest growth rate was observed by control diet (TC). All experimental diets of incorporated with *D. quercifolia* rhizome had a good specific growth rate (SGR) that was higher than that of the control (TC). The survival rate of prawns was the highest rate was observed by all experimental diets. The food conversion rate (FCR) was better results observed in all experimental diets compared than the control (Table-2).

Table-2: Growth parameters on *D. quercifolia rhizome fed on M. rosenbergii*

Parameters	Treatments					
	Control	Disease control	1%	2%	3%	4%
Wait gain (%)	0.55	0.45	0.56	0.67	0.89	0.91
SGR (%/day)	2.34	2.45	2.98	2.99	3.01	3.56
Survival (%)	75	50	100	100	100	100
FCR	1.24	1.96	0.89	0.67	0.54	0.34

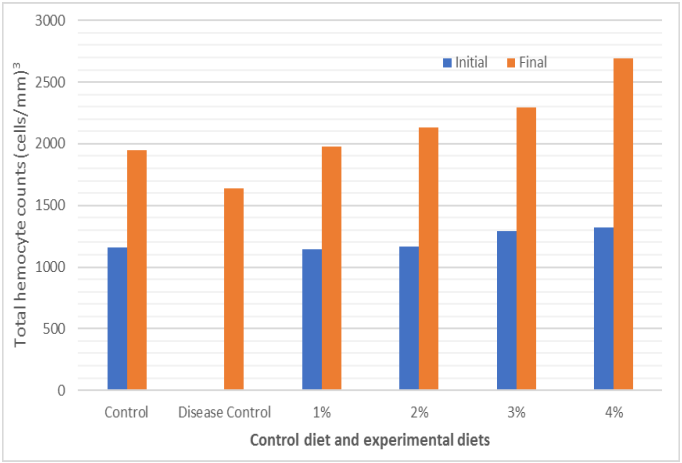

Fig.1: THC observation initial and final day counting *Vibrio harveyi* infection of *M. rosenbergii* fed with control diet and experimental diets

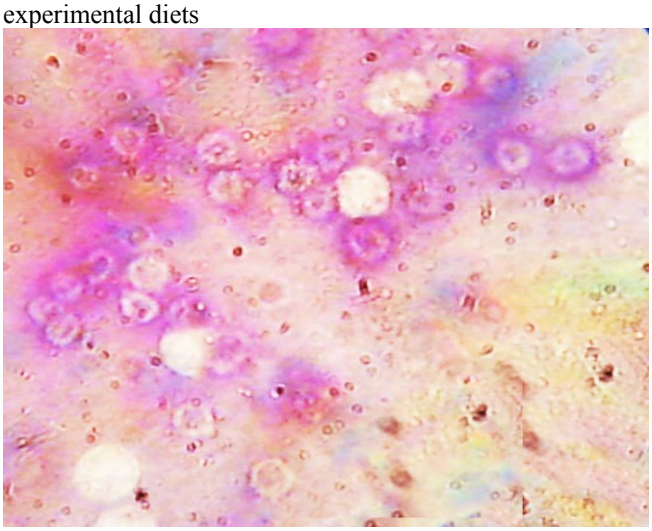

Photo-1: Haemocytes of prawn of *Macrobrachium rosenbergii*

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Table-3: DHC observation initial and final day counting *V. harveyi* infection of *M. rosenbergii* fed with control diet and experimental diets

Type of Cells	Initial Day Control	1 %	2 %	3 %	4 %	Final day Control	1 %	2 %	3 %	4 %
H C	74	81	83	84	81	84	81	82	84	83
SG C	16	17	14	02	13	06	12	7	15	16
GC	10	02	03	14	06	10	7	11	1	1

HC-Hyaline Cells, SGC- Semi Granular Cells, GC- Granular Cells

In the present study, THC and DHC showed the decreased haemocyte counts on diseased prawn of control diets. The THC and DHC gradually increase with an increase in every week of all experimental diets fed with *M. rosenbergii* (Fig.1; Table-3). We observed the present study, *M. rosenbergii* possesses three different types of haemocytes, non-granular hyaline cells, semi granular and large granular heamocytes (Photo-1). These type of haemocytes are cellular immune mechanism functioning, active phagocytic cells ingesting and eliminating invading particles. Earlier studies, oral administration of one of the commercial herbal immunomodulators (ImmuPlus) could enhance non-specific immunity of the adult prawns (Jaya Kumari *et al*.,2004). According to Desouky *et al*., (2012) reported that *Zingiber officinalis* and *Cyanodon dactylon* incorporated basal diets fed on juveniles from all groups THC and DHC was significantly increased compared to the control. It was clear that small granular hemocytes were the most affected cell type by *Z. officinalis* and *C. dactylon* treatment. THC increasing counts observed by experimental treated group and also three types of haemocytes were observed. These cells are fusiform cells, large ovoid cells and small round cells. Earlier, Vazquez *et al*., (1997) reported that 70% fusiform or hyaline haemocytes, 20% granular haemocytes and 10% agranular haemocytes in the intermoult *M. rosenbergii*. In this species, the fusiform hemocyte was the most abundant hemocyte found as opposed to other crustaceans. In the present study observed that injection of bacteria to modified the control group very poor level of THC and differential hemocytes counts. There are numerous works documenting the effects of medicinal herbs as feed additives and growth stimulator (Malar Vidhya and Maria,2013). Crude extracts of *C. longa* fed on *M. rosenbergii* was best effective result observed earlier researcher (Malar Vidhya and Maria,2013; Poongodi,2012). These results indicated that *D. quercifolia* rhizome can be a good choice for using as an additive for prawn diets.

Pathology of Hepatopancreas

The hepatopancreas is considered to be the central organ of metabolism in decapod Crustacea. It is a bilaterally bilobed brown-yellowish organ. Pathological analysis of hepatopancreas of *M. rosenbergii* was studied by histological techniques (Photo-2). The control diet feed with hepatopancreas of healthy prawn of *M. rosenbergii* was observed by large compact ducts and blind ending tubules (Photo-3). Each tubule consists of single layer of epithelial cells enclosed a lumen. The structure is formed by a mass of blind tubules, with scarce intratubular space. Each tubule consists of a cylindrical epithelial layer surrounded by a basal lamina and myoepithelial cells. It is a system of blind tubules consisting of four cell types. The E-cells at the summits of the tubules develop into R-cells (for resorption of nutrients), F-cells (for production of digestive enzymes) and B-cells (function unknown).

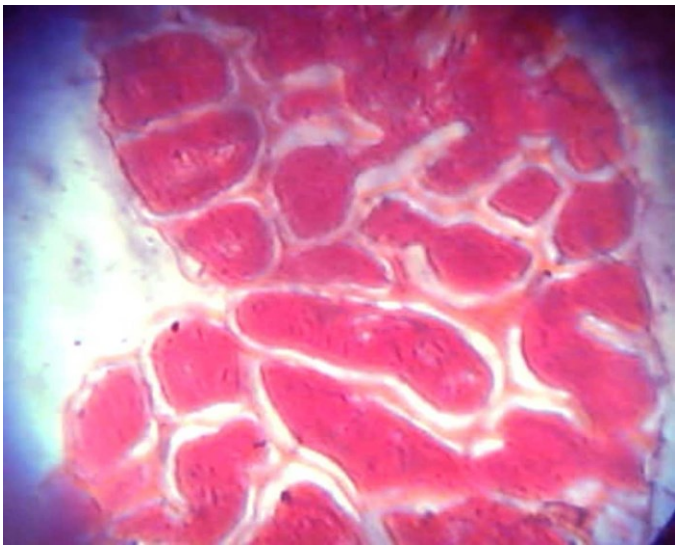

Photo-2: Normal structure of hepatopancreas of *M. rosenbergii*

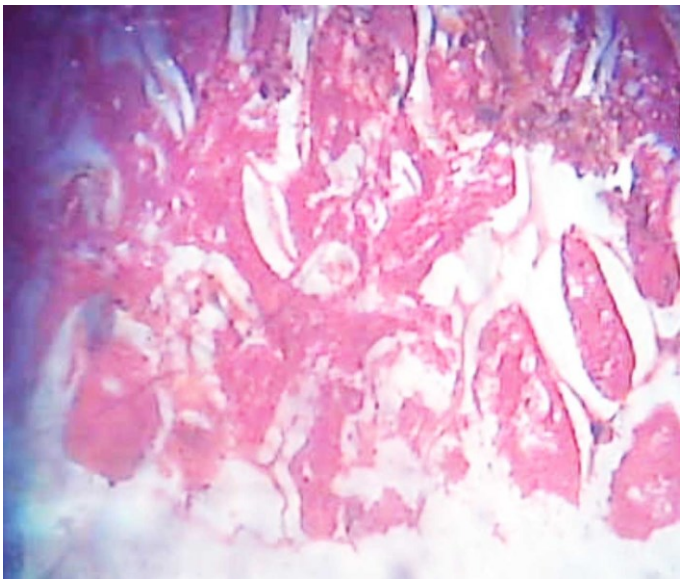

Photo-2: Degenerative structure of Hepatopancreas of *M . rosenbergii*

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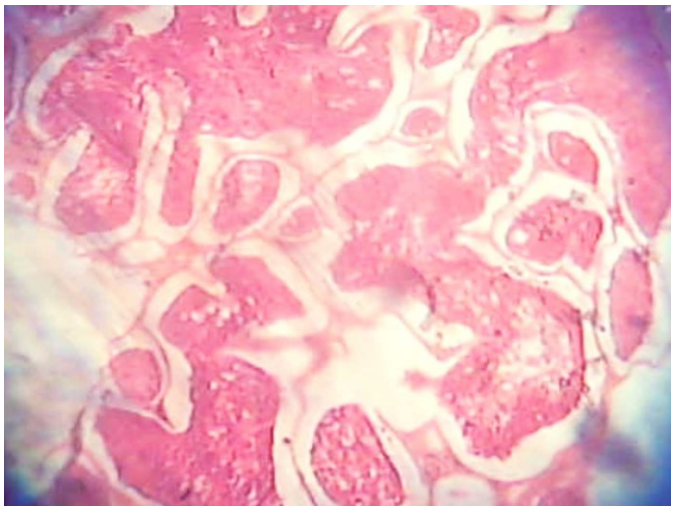

Photo-3: Treated group and normal structure of Hepatopancreas of *M. rosenbergii*

Control diet of disease prawn *M. rosenbergii* observed that hepatopancreatic tubules caused systemic bacterial dissemination, which resulted in marked necrosis (Photo-2). According to Sahoo *et al*., (2007) observed that hepatopancreas had massive and mild hemocyte reaction and crustacean is invaded by a large number of microorganisms are removed by directly phagocytosis, while many others are confined to nodules or clumps of cells. Earlier studies, mortalities due to luminous vibriosis and filamentous bacterial infections and larval mycosis were reported (Karunasagar *et al*., 1994). The present study we observed that intramuscular injection *V. harveyi* induced *M. rosenbergii* fed on control and experimental diets were good results of pathological analysis of hepatopancreas tissues revealed haemocytic nodule formation. According to Vogt (1990), the process of desquamation in the hepatopancreas of *P. monodon* starts with the cells lysis, in particular R-cells, and the neighboring cells protrude in small basolateral extensions, pushing the damage cells into the tubular lumen generating ulcerations. Earlier report, according to Factor and Beekman, (1990) reported that response to the presence of these infected tissues, it has been suggested that some hemocytes may migrate into connective tissue and hepatopancreatic tubules (Fontaine and Lightner, 1974; Smith *et al*., 1984; Sung and Song, 1996). Stains of pathogenic *V. harveyi* in the Phillipines, Thailand, Indonesia and India with similar virulence to *Penaeus monodon* larvae may produce the exotoxins. Markedly effect of different exotoxins in *V. harveyi* strains with different levels of virulence would be consistent with the suggestion of Pizzutto and Hirst, (1995) that the strain specific virulence of *V. harveyi* may be acquired through genetically mobile elements such as plasmids or bacteriophage carrying transposons or insertion sequences (Harris and Owens, 1999). The conditions were typical of the systemic and enteric vibriosis described earlier studies (Egusa *et al*., 1988; Nash *et al*., 1990; Jiravanichpaisal and Miyazakir,1994). The major alterations for both prawn species were observed in the F- and R-cells, and the percentage of the cell type. Bacterial infection of prawn hepatocytes alternated F-cells showed a damaged microvilli border. *Vibrio harveyi* infected animals of *M. rosenbergii* fed with basal diet observed that the endoplasmic reticulum and the mitochondria were more abundant and swelled, and the cristae of the last was damaged. Earlier studies, Jiravanichpaisal *et al*., (2009) reported that moribund crayfish showed that extensive necrotic nuclei and clump-infiltrated hemocytes were found in observed tissues including gill, heart, hepatopancreas and the circulatory system. The present study observed that histopathology is an ideal tool for routine health monitoring and diagnosis, where the changes at the cellular and tissue level due to the pathogen is interpreted to arrive at diagnosis. Our findings result suggested that *D. quercifolia* rhizome extract could increase the immune response of *M. rosenbergii.*

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