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Effect of Herbal Feed Additive on the Growth, Survival and Immune Response of Green Tiger Prawn (*Penaeus semisulcatus*)

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Abstract: Due to the increasing demand for environment friendly shrimp aquaculture, herbs and herbal feed additives have received higher attention. A commercial Herbal Feed Additive (HFA) is a unique blend of selected herbs that act synergistically with each other to help in toning the immune system, protects hepatopancreas and activates several metabolic pathways that help in body weight gain. HFA was evaluated for its effects on the growth, survival, body compositions and the nonspecific immune responses of *Penaeus semisulcatus*. Three test diets were used vital prawn feed with 1 and 2% of the herbal feed additive HFA and the vital prawn feed without the additive (control). At the end of the study, there were no significant ($P>0.05$) differences among the three different diets in terms of shrimp mean weight, total weight, weight gain, Feed Conversion Ratio (FCR) and survival. The phenol oxidase activity was significantly ($P<0.05$) higher in the shrimp fed with 1% HFA than the shrimp fed with 2% HFA and the control. However, there was no significant ($P<0.05$) difference between 2% HFA and the control. The highest phagocytic activity was recorder in shrimp fed with 2% HFA, followed by 1% HFA, then the control. However, there was no significant difference in the PA ($P>0.05$) between the three different feeding regimes. Shrimp fed with 2% HFA contained significantly ($P<0.05$) lower crude protein and higher moisture than those fed with 1% HFA and the control. There was no significant ($P>0.05$) difference in the crude protein and moisture content of the shrimp fed with 1% HFA and the control. There was no significant ($P>0.05$) difference in the fat and the ash content of the shrimp fed with the three feeding regimes.

Key words: Shrimp, herbs, growth, survival, immunity, chemical compositions

INTRODUCTION

Increasing world demand for shrimp has led to a rapid expansion in shrimp farming, diseases always present a risk factor for the sustainability of this activity. Researches in shrimp aquaculture are focused on the development of feed that could meet the animal's nutritional requirements, ensure high growth rate, low mortality rate and enhance their ability to withstand stress in high density culture (Santhanakrishnan and Viswakumar, 1995). Many synthetic substances such as hormones, vitamins, "antibiotics" and other drugs have been used for various activities such as appetizing, growth promotion, immunostimulation and so on in aquaculture (Jayaparkas and Sambhu, 1996). These substances are not preferred in commercial aquacultural operations because of their cost, tendency to form residues and undesirable side effects (Venkatramalingam *et al*., 2007). Consumer's awareness and concerns over food safety have led to the search for alternative substances from natural, biologically active and renewable organic products that could be used as feed additives to replace hazardous synthetic substances.

Due to the increasing demand for environment friendly shrimp aquaculture, herbs and herbal feed additives have received higher attention. Herbs have been widely used in veterinary and human medicine. These natural products are not only safe for consumers but also widely available throughout the world (Direkbusarakom, 2004). For shrimp culture, many studies were conducted to screen various identified herbal extracts as growth promoters or immunostimulant (Chitra, 1995; Citarasu *et al*., 1998; Immanuel *et al*., 2004; Al-Musallam and Al-Ameeri, 2007; Harikrishnan *et al*., 2011; Medina-Beltran *et al*., 2012).

The sustainability of the shrimp industry depends largely on disease control and the health status of shrimp. From this point of view, the immune response is an important parameter to assess shrimp health (Bacherre *et al*., 1995). Invertebrates, including crustaceans, do not have acquired immunity, instead they have an innate immune system that consists of cellular and hormonal reactions. The cellular reactions mediated by hemocytes in clued phagocytosis, nodule formation, encapsulation,cytotoxicity, cell adhesion and hemolymph clotting mechanism (Cerenius *et al*., 2008;

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Sritunyalucksana *et al*., 1999). While humoral reactions involve the prophenoloxidase-activating cascade and immune-related protein such as lysozymes, lectins, lysosomal hydrolytic enzymes and antimicrobial pepides (Chisholm and Smith, 1995; Destoumieux *et al*., 2000; Muta and Iwanaga, 1996; Soderhall *et al*., 1994)

HFA is a unique blend of selected herbs consisting of *Tinospora cardiofolia*, *Terminalia chebula* and *Emblica officianalis* that act synergistically with each other to help in toning the immune system, protects hepatopancreas and activates several metabolic pathways that help in body weight gain.

The commercial herbal feed additive HFA was evaluated in this study for its effect on *P. semisulcatus* growth, survival, body composition and enhancement of the nonspecific immune responses in *P. semisulcatus*.

MATERIALS AND METHODS

Animals: Wild *P. semisulcatus* in the range of 0.5 to 1g were collected from Kuwait bay and used for the study.

Preparation of feed with HFA: Two concentrations (1 and 2%) of the commercial herbal feed additive (VIP Bio Products, India) were evaluated by incorporating the herbal product in the shrimp feed (vital prawn feed, Higashimaru Co, Japan). Briefly, 10 g and 20 g of the herbal product was mixed with 1 kg of the commercial feed, respectively, to get 1 and 2% herbal product incorporated diets. A binder from VIP Bio Products, India was used for mixing the HFA with the feed at the rate of 20 ml/kg feed. The control diet was only the vital prawn feed without the herbal feed additive and binder.

Experimental design and feeding trial: Prior to the start of the feeding trial, experimental shrimp were kept in two circular fiberglass tanks of 2 m^3 capacity and fed with the control diet, the vital prawn feed for one week to acclimate to the experimental conditions. After the acclimation period, the shrimp were transferred to experimental tanks. Three feeding regimes were used in the experiment. Three feed combinations were used vital prawn feed with 1% Herbal additive, vital prawn feed with 2% Herbal additive and vital prawn feed only without herbal feed additive (the control). Each experimental tank was stocked at a rate of 30 shrimp/m^2. For each treatment, three replicates were prepared. Complete randomized design was used for the experiment. The average initial size of the shrimp was 7 g.

The amount of feed provided was 6% (day 1-50) of the total shrimp weight in each tank. According to the weight of the shrimp and the feed consumption throughout the experiment, the amount of feed was reduced to 4% (day 51-100) then, to 3% (day 101-190) of the total shrimp weight. Feeding frequency was 2 times/d. Daily water exchange rate was 100%. Water temperature and the dissolved oxygen were monitored daily. Titanium IC heaters (1000W) were used for each tank to regulate the temperature during the winter (December to February). The duration of the experiment was 190 d. At the end of the experiment, shrimp numbers, individual and total shrimp weight in each tank were recorded.

Phenoloxidase and phagocytic activity: At the end of feeding study, 10 shrimp were used from each treatment to detect the phenol oxidase activity (PO) and Phagocytic Activity (PA). One hundred micro liters of haemolymph was withdrawn from the ventral sinus of the shrimp into 1 ml syringe containing 0.2 ml anticoagulant solution.

Prior to the detection of PO activity, the Haemolymph Lysate Supernatant (HLS) was prepared according to the procedure described by Sung *et al*. (2004). The PO activity was estimated spectrophotometrically using L-3, 4 dihydroxyphenylalanine (L-DOPA; Sigma, USA) as a substrate and trypsin (Sigma, USA) as an elicitor. The haemolymph with the anticoagulant were centrifuged at 120, 000x g for 10 min. The supernatant was discarded. The Haemocyte Lysate Suspension (HLS) was prepared in 0.01 M cacodylate buffer (CAC, PH 7.0) by grinding the cell pellets using a sterile plastic pestle and centrifuged at 120, 000 x g for 10 min. After centrifuging, 25 µl of the supernatant (HLS) was added to the well of 96 well microtitre plate, mixed with 25 µl trypsin and incubated for 15 min at 37°C. For each HLS sample, three replicates were prepared. After incubation, 200 µl of freshly prepared dihydroxyphenylalnine (L-Dopa) solution (1.6 mg in 0.01 M CAC) was added to each well. The optical absorbance at 490 nm was recorded on microplate reader after 2 min. The rate of change of OD of 0.001/min was considered as one unit of PO activity of the sample.

The PA was determined as described by Puangkaew *et al*. (2004) but with certain modification. The haemocyte suspension was prepared in CAC buffer (PH 7.0) and 50 µl of this suspension was seeded into duplicate chamber of an eight well microplate. Phagocytosis assay were carried out using fluorescent polystyrene Latex beads (2 µm; sigma). The bead density was adjusted to 5.3 x 10^3 bead/ml. The cells were incubated at room temperature for 1h. After incubation, the wells were washed with Phosphate Buffer Saline (PBS) twice. The cells were further incubated for 30 min. at room temperature after adding 50 µl of Latex beads (2 µm; sigma) to each chamber. After incubation, the wells were washed with PBS solution. The fluorescence was measured using Bio Tek FLX800 microplate reader at 496nm and emission at 520nm. Fluorescence reading of wells coated with haemolymph suspension from samples were used to obtain relative phagocytic activity of haemolymph.

Proximate analysis of shrimp samples: A random sample of 25 shrimp at the beginning of the experiment were collected, packed in polythene bags, stored at -

70°C and used at the end of the feed experiment for the proximate analysis. At the end of the feeding experiment, 12 shrimp per tank (a total of 36 shrimp for each treatment), were collected, minced, mixed and used for the analysis.

The estimation of water (moisture) content, nitrogen (crude protein), crude lipid and ash for the whole shrimp body was performed according to AOAC (2000).

The chemical compositions of all the samples were measured on dry basis. Moisture content was determined by taking the weight of wet minced shrimp sample; dried it in the oven at 105°C over for 24 h, dry sample was weighed, the difference in the weight representing moisture content of the sample. Ash content was determined by weighing 1.0 g sample and ash at 600°C in muffle furnace for 6 h. The dry sample was weighed, the difference in the weight represent the ash content of the original sample. The crude lipid was determined by Soxhlet method using Tecator Soxtec system HT 1043 extraction unit. The crude protein was determined by Micro-Kjedahl method using FOSS Kjetec 2300.

Data analysis and statistics: Data obtained on the individual final weight, final total weight, weight gain, Feed Conversion Ratio (FCR), survival rate, phenol oxidase activity, phagocytic activity and chemical composition of the shrimp were subjected to a one-way analysis of variance (ANOVA) and Tukey's test at a 5% level of significance using Minitab Statistical Package version 15.0.

RESULTS

Growth performance, survival and feed utilization: The results of growth performance, survival and feed conversion efficiency of shrimp fed with 1, 2% HFA and the control diet are shown in Table 1. At the end of the experiment, there was no significant (P>0.05) difference among the three different feeding regimes in terms of shrimp mean weight, total weight, weight gain, Feed Conversion Ratio, specific growth rate and the survival. The water temperature during the experiment ranged from 22°C to 30°C. The Average weekly dissolved oxygen (DO) concentration was 5.60 mg/l (range 4.98-6.54 mg/l).

Immune assays: The PO value observed was significantly (P<0.05) higher in the shrimp fed with 1% HFA than the shrimp fed with 2% HFA and the control. There was no significant (P<0.05) difference between 2% HFA and the control. The highest PO activity obtained with 1% HFA (107.80±5.62) followed by 2% HFA (71.44±4.80), then the control (58.17±4.39).

The highest PA was recorder in shrimp fed with 2% HFA (261.438±73.5), followed by 1% HFA (248.313±87.3), then the control (233.438±93.7). The relative PA for 2% HFA was 12% and for the 1% HFA was 6.4%.

However, there was no significant difference in the phagocytic activity (P>0.05) between the three different feeding regimes.

Proximate compositions: The proximate compositions of the shrimp fed with the 1%, 2% HFA and the control are shown in Table 2. Shrimp fed with 2% HFA contained significantly (P<0.05) lower crude protein and higher moisture than those fed with 1% HFA and the control. There was no significant (P>0.05) difference in the crude protein and moisture content of the shrimp fed with 1% HFA and the control. There was no significant (P>0.05) difference in the fat and the ash content of the shrimp fed with the three feeding regimes.

DISCUSSION

One of the main strategies in developing a shrimp rearing system is the establishment of a feeding regime that could result in optimal growth, survival and health of the shrimp. The results obtained in the present study

Table 1: Performance of *Penaeus semisulcatus* fed with 1% , 2% herbal feed additive (HFA) and Control (mean±SD)

	Diets		
	1% HFA	2% HFA	Control
Initial Data:			
Mean weight (g shrimp^{-1})	7.26±0.353	7.07±0.146	7.19±0.346
Coefficient of variation (CV)	0.237	217.73±10.60	0.244
Total weight (g m^{-2})	212.20±4.39	0.269	215.80±10.38
Number (shrimp m^{-2})	30	30	30
Final data:			
Mean weight (g shrimp^{-1})	20.84±1.80	0.195	20.26±2.19
Coefficient of variation (CV)	0.242	21.57±0.638	0.294
Total weight (g m^{-2})	526.4±21.0	490.0±20.2	583.1±60.4
Weight gain (g shrimp^{-1})	13.59±1.93	13.18±2.10	14.373±0.345
Weekly weight gain (g shrimp^{-1})	0.506±0.072	0.491±0.078	0.535±0.013
Feed Conversion Ratio (FCR)	4.57±0.181	4.91±0.204	4.15±0.429
Specific Growth Rate (SGR)	0.560±0.058	0.558±0.051	0.584±0.013
Survival rate (%)	84.4±5.10	81±4.00	90±6.65

Table 2: Proximate Composition of *Penaeus semisulcatus* Fed with 1, 2% Herbal Feed Additive (HFA) and Control on dry Matter Basis

	Initial	1% HFA	2% HFA	Control
Composition (%)				
Crude Protein	67.2	[a]71.3	[b]69.7	[a]71.3
Crude lipids	3.8	[a]4.1	[a]4.9	[a]4.8
Ash	15.1	[a]14.3	[a]14.2	[a]14.0
Moisture	1.03	13.9	[a]1.2	10.3
*NFE	[b]1.8	11.2	[a]1.1	9.9

*Means with different superscripts in a row are significantly different (P<0.05).

*Calculated: NFE Nitrogen-free extract = 100 - (crude protein + crude lipids + ash).

clearly indicated that there was no remarkable influence of the HFA on weight gain, feed intake and survival of *Penaeus semisulcatus*.

There was a variation in the individuals final weight in the treated tanks ranging from 11 to 39 g, this variation was higher in the control tanks. The high variation in the shrimp's size had an effect on the statistical analysis of the results and showed no significant differences in the growth when measuring the weight gain of the shrimp. The size variation observed in this study could be attributed to the differences in the growth rate between males and females in *P. semisulcatus*. As no attempts were made in this study to separate the two sexes. Murugan (2005) evaluated HFA for growth promoting of *Penaeus monodon* and he found that HFA significantly increase the growth (33%), promotes regular molting, it delays the onset of the disease (with spot syndrome by 48 h), increases the haemcyte count nearly two folds and it enhances the pigmentation of the animal.

Similar results with herbs were obtained by Yune (2007) in his trial with *Litpenaeus vannamei*. He found no significant difference in the growth of *L. vannamei* between the shrimp fed with diet containing 2% of the herb *Houttuvnia cordata* and the control group. Xiao-hui (2009) obtained no significant difference in the FCR and the survival of *L. vannamei* when fed with diet supplemented with dietary Chinese herbal mixture of *Astragalus*, *Isatis* root, *Honeysuckle* and *Gypsum* in the concentrations of 0.5, 0.9, 1.3, 1.7 and 2.1%, respectively for 56 d. Peraza-Gomez *et al.* (2009) and Medina-Beltran *et al.* (2012) found that feeding *Litpenaeus vannamei* powder of the herbs *Echinacea purpure* and *Uncaria tomentoosa* did not affect the survival and the growth of the shrimp.

Crustaceans have only a nonspecific innate immune response and no long memory which results in the inability of shrimp to protect itself from various pathogens (Sarathi *et al.*, 2007). Regarding humoral defense mechanisms, the measurement of PO activity was proposed as a procedure to evaluate the immune response of shrimp (Rodriguez and Moullac, 2000). Results from several experiments showed that components of the putative proPO activating system could stimulate several cellular defense reactions, including phagocytosis, nodule formation, encapsulation and haemocyte locomotion (Soderhall *et al.*, 1986). Activated haemocytes also could produce extra bactericidal substances, such as H_2O_2 and superoxide anion (O_2) that may increase disease resistance (Song and Hsieh, 1994).

In the present experiment, an increase in the PO activity of the shrimp fed diets incorporated with (1%) HFA indicate the possible potency of HFA to induce nonspecific immune response in the shrimp. Murugan (2005) obtained positive effect of HFA on PO activity with *P. monodon*. Citarasu *et al.* (2006) recorded higher value of proPO when *P. monodon* was fed diet incorporated with methanolic extract of five different herbal medicinal plants like *Cyanodon dactylon*, *Aegle marmelos*, *Tinospora cordifolia*, *Picrorhiza kurooa* and *Eclipata alba* in the concentration 800 mg kg^{-1} feed. Balasubrsmanian *et al.* (2008) found that *in vivo* and *in vitro* administration of 2% of *Cyanodon dactylon* extract enhance the PO activity in the shrimp *P. monodon*. Many other studies have also been reported indicating positive immune responses due to the herbal additive. Xiao-hui (2009) obtained significant higher PO activity in *L. vannamei* serum when fed with diet containing 2.07% Chinese herbal mixture of *Astragalus*, *Isatis* root, *Honeysuckle* and *Gypsum*. PO activity was found to be significantly enhanced when fed with *L. vannamei* diet containing *Cinnamomum kanehira* twing hot-water extract compared to control (Yeh *et al.*, 2009).

Phagocytosis is generally recognized as a central and important way to eliminate microorganisms or foreign particles. In our study, there was no remarkable effect of HFA on the phagocytic activity of the shrimp. Although, the 2% HFA has higher phagocytic activity followed by 1% and the control, there was no significant difference between theses three diets in the phagocytic activity. Similar result was obtained by Vanichkul *et al.* (2010) when they add turmeric extract to the feed of *L. vannamei* for immune enhancement. In their study, phagocytic response of shrimp fed diet incorporated with 12.5 and 25 mg kg^{-1} turmeric extract was high. However, there was no significant differences in the percent phagocytosis between the shrimp fed with turmeric extract and the control.

From the results of the present study, it is clear that 2% HFA has adverse effect on the chemical composition of the shrimp. Although, there was no significant difference among the three diets in the growth of shrimp, protein content was significantly lower in the shrimp fed with the

2% HFA (69.7%) than those fed with 1% HFA (71.3%) and the control (71.3%). The values obtained for the three diets were lower than what Wickins (1976) indicated, where prawn generally is capable of more than 80% protein assimilation. This could be due to the use of artificial diet, the vital prawn feed which contains around 56.8% protein (Al-Ameeri *et al*., 2006) and lower than the protein content of diets based on fresh food (Wouters *et al*., 2001). Similar results with low protein assimilation were obtained by Al-Ameeri *et al*. (2006) where they got 66.6% protein assimilation when they used the Vital prawn feed with *P. semisulcatus*.

Higher amount of crude fat was obtained with 2% HFA over the diets with 1% HFA and the control. In all penaeid shrimp, an increase of total lipids concentration take place mainly due to the ovarian deposition. However, an increasing amount of evidence indicated that a major part of the accumulation of ovarian lipids have originated from the diet (Wouters *et al*., 2001).

In conclusion, it can be stated from our study that there was no remarkable effect of the HFA on the growth and survival of *Penaeus semisulcatus*. On the contrary, there was an adverse effect of 2% HFA on the chemical compositions of the shrimp body. The increased amount of PO activity with HFA indicates the possible potency of HFA to induce nonspecific immune response in the shrimp. There was no remarkable effect of HFA on the phagocytic activity of the shrimp. Though no conclusive benefit was derived by way of growth enhancement due to the feed additive, increased PO activity could be of some help during a production cycle that could suffer due to diseases. However, the potential benefit in a cost to benefit analysis need to be studied further. In addition, controlled challenge test with well known pathogen such as *Vibrio alginolyticus* could give a clear indication on the effectiveness of HFA in enhancing the disease resistance in *P. semisulcatus*.

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