EFFECT OF CHITOSAN SUPPLEMENTED DIET ON SURVIVAL, GROWTH, HEMATOLOGICAL, BIOCHEMICAL AND IMMUNOLOGICAL RESPONSES OF INDIAN MAJOR CARP *LABEO ROHITA*

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Article Received on: 28/03/13 Revised on: 07/04/13 Approved for publication: 11/05/13

DOI: 10.7897/2230-8407.04529

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ABSTRACT

The effect of chitosan incorporated into feed formulations on the growth, hematology, biochemical and immunological response of Indian major carp rohu (*Labeo rohita*) were invest Feed conversion ratio (FCR) and specific growth rate (SGR) of challenged *Labeo rohita* fed chitosan supplemented diet and the control diet at the end of experiment. Data presents mean $\pm$ SD from triplicate determination ($n=3$) for 90 days feeding trial. Different concentrations (0.5%, 0.75%, 1%, 1.25%) and control without chitosan incorporated with total fish feed in the form of dry diets for a period of 90 days. Experimental animals were challenged intra-peritoneally with *Aeromonas hydrophila* on 30th, 60th and 90th day. Hematological parameters determined on the day 30, 60 and 90. The total erythrocyte count, leukocyte count and hemoglobin content were only significant ($P<0.05$) at 1% level and enhanced in chitosan supplemented groups. The biochemical parameters, serum protein, carbohydrate and lipid content were significant ($P<0.05$) compared to control groups. Specific growth rate (SGR) significant at 1% level (55.9%) and lowest in control group (29.7%) and feed conversion ratio (FCR) value was found to be best in 1% chitosan fed group (1.85%) followed by 0.75% group (2.12%) and control group (3.15%). The immunological parameters Lysozyme and agglutination assays were significantly stimulated in chitosan supplemented groups showed highest value in 1% level group on day (53.21 and 18.5) compared to control. The experimental values were statistically significant ($P<0.05$). The present investigation suggested that the chitosan incorporated diets of Indian major carp, rohu fish certainly enhanced the non-specific responses and reduced mortality and also improve the growth through shell fish wastes, that useful for practical aquaculture.

Key Words: Chitosan, *Labeo rohita*, Immune response, Lysozyme activity, Agglutination assay.

INTRODUCTION

Aquaculture is a rapid developing industry. However, unmanaged fish culture practices and adverse environmental conditions affect the fish health leading to production losses¹. Diseases are major bottlenecks in the development and sustainability of aquaculture practices throughout the world². The present trend of intensification in aquaculture is a major concern for the outbreak of disease in fishes are more prone to stress and subsequent infection by pathogen. Fish diseases are great threat to economic viability of any aquaculture practices³. Vaccination may be the most effective method of controlling fish diseases, even though disease caused by bacteria like *Aeromonas hydrophila* has not been controlled by vaccination due to their heterogeneity. Traditional disease control strategies employ antibiotics and chemical disinfectants, but these are no longer recommended practices due to the emergence of bacterial resistance^{4,5}. The use of natural immunostimulants in fish culture for the prevention of disease is a promising new development^{1,6}. Natural Immunostimulants are biocompatible, biodegradable and harmless for the environment and human health⁷. Immunostimulant used in vaccines to amplify the specific immune response or administered as feed additives to modulate non-specific immunity have been demonstrated to play role in protection against diseases in fish⁸. The Immunostimulants enhance the level of duration of specific immune response, both cell-mediated and humoral, following vaccination. Immunostimulants supports to overcome the immunosuppressive effects of stress and those infectious agents that damage or interface with the functioning of cells of immune system. A variety of substance have been shown to have the immunostimulatory effects use of expensive chemotherapeutants and antibiotics for controlling disease have widely been criticized for their negative impacts like accumulation in the tissue as residues, development of the

drug resistance, immunosuppressant and reduced consumer preference for food fish treated with antibiotic⁶. Therefore, instead of antibiotics and chemotherapeutic agents, increasing attention is being paid to the use of immunostimulants for disease control measures in aquaculture. The immunostimulants mainly facilitate the function of phagocyte cells, increase their bactericidal activities and stimulate the natural killer cells, complement system, lysozyme activity and antibody responses in fish and shellfish which confer enhanced protection from infectious diseases^{9a}. Chitosan derived from the chitin is obtained from these discarded exoskeletons by deproteinization, demineralization and deacetylation¹⁰. It arouses interests because of its low side effects, promoting the growth performance, improving immune functions; they contained in chitins of arthropods such as insects and shellfish or the cell wall of fungi¹¹. Although they exhibit various interesting biological activities such as antitumor activity, immunoadjuvant activity, hypolipidemic activity and haemostatic activity¹²⁻¹⁴, the bactericidal activity of chitosan is particularly interesting and has been observed against various bacteria^{15,16}. For these reasons, chitosan is widely used in many different fields, including medicine, food and chemical engineering, pharmaceuticals, nutrition, and agriculture. In aquaculture, it is used as an immunostimulant to protect *salmonids* against bacterial disease¹⁷, enhancing the respiratory burst and phagocytic activities in gilthead sea bream¹⁸⁻²¹ immersion and dietary supplements^{22,23}. However²⁴ reported that depressed growth in *tilapia* after feeding chitin and chitosan, which showed the growth enhancement and health improvement of aquatic animals through symbiotic, is a beneficial and rational strategy and the further studies should be conducted. Indian major carp, *Labeo rohita* is one of the major preferred species in the Indian subcontinent which contributes about 35% of total carp production²⁵. Because of excellent flesh quality,

rapid growth and adaptability to culture conditions, its potential for aquaculture was recognized in recent years²⁶. However, the production continues to be severely affected due to several bacterial and viral diseases^{27,9}. Therefore, the aim of the present study was to determine the Hematology, Biochemical, Serum Lysozyme Activity, Agglutination assay and Immune response in *Labeo rohita* fed with chitosan supplemented diet against *Aeromonas hydrophila*.

MATERIALS AND METHODS

Fish Collection and Maintaining

Labeo rohita, a freshwater carp was used for the present study. Carp fries were collected from Bhavanisagar, Government Fisheries Farm, Bhavani Sagar Dam, Erode, Tamil Nadu, South India, weighing 10±2g (for non-specific humoral assays and disease resistance studies) and 20±5g (for non-specific cellular assays). Fishes were acclimatized for 15 days in 500 L cement tanks. They were kept at the ambient, uncontrolled temperature of 28±2 °C under natural photoperiod and maintained the water quality during this study. Fishes were fed with commercial feed.

Preparation of chitosan

Chitosan were prepared from crab shell waste according to²⁸ with some modifications. The dried *Scylla serrata* mud crab shell waste was collected locally and washed with tap water three times and deproteinised by boiling with 3% aqueous sodium hydroxide for 20mins. After washing off the alkali, the process was repeated three times for the removal of residual protein from the shell and washed with tap water. The deproteinised shell was demineralised with 1.25 N Hcl at room temperature for 1 hrs. Then, the Hcl was drained off and washed thoroughly with tap water followed with distilled water. The chitosan was repaired by deacetylation of chitin by treating with 1:1 (w/w) aqueous sodium hydroxide at 92±3° C for 2 hrs. After deacetylation, the alkali was drained off and washed with tap water and distilled water. The chitosan was dried at room temperature and powdered well by using pestle and mortar.

Diet composition

A pellet feed composed of 40% groundnut oil cake, 33% rice bran, 20% soy bean meal, 5% fish meal and 2% minerals and vitamins mixture (each 250 g minerals + vitamins mixture provides vitamin A 500,000 IU, vitamin D3 100,000 IU, vitamin B2 0.2g, vitamin E 75 U, vitamin K 0.1g, calcium pentathionate 0.25g, nicotinamide 0.1g, vitamin B12 0.6mg, choline chloride 15g, calcium75g, manganese 2.75g, iodine 0.1g, iron 0.75g, zinc 1.5g, copper 0.2g and cobalt 0.045g) was used as control diet (Normal diet). All the ingredients were properly weighed as per their inclusion rates in the five experimental diets. Control=Normal Control feed alone; Diet-1= Normal feed+ 0.5% chitosan; Diet-2 = Normal feed+ 0.75% chitosan; Diet-3= Normal feed+ 1.0% chitosan; Diet-4= Normal feed+ 1.25% chitosan powder and mixed with the normal feed incorporated separately before pelletization in a steam cooked diet²⁸. Fish were fed at the rate of 3% of the body weight of animals in each experimental tank.

Experimental regime

In each experimental tanks, 50 *Labeo rohita* fish (10±0.18 cm length; 13.12±3.2 g weight) were stocked. Experimental fish were fed with formulated diets incorporated with chitosan 0.5%, 0.75%, 1.0%, 1.25% and non-supplemented (controls) diet separately. Length and weight of 10 fish randomly

selected from each pond were measured every 15 days for a period of three months using a measuring scale and an analytical balance.

Bacterial Strain and Challenge Study

Fish fed with chitosan and non-supplemented diets were challenged with *Aeromonas hydrophila* on day 30, 60 and 90. On the day of 30, twenty fish in each group were netted out and were given intramuscular injections of *Aeromonas hydrophila* and then reared in separate experimental tanks. On the 90th day, the remaining fish in the tanks were netted out and injected with *Aeromonas hydrophila*. A challenge study was performed by injecting 100µl of 12 hrs grown culture of *Aeromonas hydrophila* at a concentration of 1.5±0.3×10⁶cfu/ml⁻¹. Total count was determined using Neubauer hemocytometer and total viable count was confirmed by spread plate method. Mortality was recorded daily up to 15 days and relative percentage survival was calculated using the methods²⁹.

$$RPS = 1 - \left\{ \frac{\text{Percent mortality in treated group}}{\text{Percent mortality in control group}} \right\} \times 100$$

Collection of Serum from fish

Fish from each treatment were anaesthetized and blood was collected in 1 ml tuberculin syringe fitted with 24-gauge needle³⁰ and stored in 5 ml test tube without anticoagulant and allowed to clot for 2 hrs, at room temperature in a slanting position. The tubes were centrifuged at 2500 x g for 15 min and the supernatant serum was separated and collected in screw cap sterile eppendorf tubes at 20°C until used for assays.

Estimation of Total Serum Protein

The protein content in serum was estimated by employing Folin-Ciocalteu reagent method³¹. Carbohydrate was estimated³² and Lipid was estimated by³³.

Haematology

Blood hemoglobin was estimated by Haemoglobinometer. Erythrocyte and Leukocytes were counted by the method³⁴ using haemocytometer.

Calculation

$$\text{Count the RBC'S per cu.mm of the blood} = \frac{\text{(No. of Erythrocyte counted} \times 200 \times 4000)}{80}$$

$$\text{Number of WBC (Thousands/cu.mm)} = \frac{\text{(No. Of Leukocyte counted} \times \text{Dilution})}{\text{Area counted depth of fluid}}$$

Serum Lysozyme Activity

Serum Lysozyme activity was measured by adapting the method described³⁵. Serum samples were diluted with phosphate buffer (pH-7.4) to a final concentration of 0.33mg ml⁻¹ protein. In a suitable cuvette, 3ml of *Micrococcus luteus* (IMTECH Chandigarh, India) suspension in phosphate buffer (A450 ¼ 0.5e0.7) was taken, to which 50ml of diluted serum sample was added. The content of the cuvette was read in spectrophotometer at 450nm exactly 60 minutes after the addition of serum sample. The absorbance was compared with standard lysozyme of known activity. The lysozyme activity was expressed as IUml⁻¹ per mg protein of serum.

$$U/ml = (OD1 - OD2/4 \times 0.001 \times 2) \times 1000$$

Agglutination assay

100 µl of PBS buffer was aliquoted into 96 well microtitre plates. The first row was used as control without the extracts. The extracts (100µl) were added into the first well of the second row and a 2-fold serial dilution was made until the last well in row three. Then, 50µl of bacterial pathogen was added to all the wells. They were incubated at room temperature for 1 hrs. The presence of buttons in the centre of the well indicates agglutination and the agglutination titre

value of the extracts were read as the reciprocal of the last dilution showing agglutination.

Statistical Analysis

Statistical analysis of data was performed by One -Way ANOVA with Duncan test at the level of 95% using SPSS 14 (Statistical significance was set at the level of $P < 0.05$ with $\pm$ standard deviation).

Table 1: The Mortality, Survival, Feed Conversion Ratio and Specific Growth Rate of Experimental Fishes

S. No	No. of fish	Mortality (%)	Survival (%)	FCR (%)	SGR (%)
Control	40	15(37.5)	25(62.5)	3.15 $\pm$ 1.16 ^a	29.7 $\pm$ 0.57 ^c
Exp1	40	9(22.5)	31(77.5)	2.12 $\pm$ 1.08 ^{ab}	48.5 $\pm$ 4.71 ^{bc}
Exp2	40	5(12.5)	35(87.5)	2.44 $\pm$ 0.89 ^{ab}	53.3 $\pm$ 1.42 ^{ab}
Exp3	40	3(7.5)	37(92.5)	1.85 $\pm$ 0.76 ^b	55.9 $\pm$ 4.15 ^a
Exp4	40	5(12.5)	35(89.7)	2.02 $\pm$ 0.60 ^{ab}	54.30 $\pm$ 1.86 ^{ab}

*Values with the same superscript in a column do not differ significantly ($P > 0.05$). *Mean $\pm$ SD

Table 2: Biochemical Analysis Different level of Chitosan fed Fishes

Groups	Protein	Carbohydrate	Lipid
Control	30.53 $\pm$ 1.24 ^c	17.76 $\pm$ 0.63 ^c	11.04 $\pm$ 0.90 ^b
Exp1	49.90 $\pm$ 3.81 ^b	21.08 $\pm$ 0.70 ^b	11.51 $\pm$ 0.97 ^b
Exp2	53.68 $\pm$ 1.24 ^a	21.74 $\pm$ 1.30 ^{ab}	12.27 $\pm$ 0.69 ^{ab}
Exp3	56.30 $\pm$ 3.36 ^a	22.72 $\pm$ 0.98 ^a	13.41 $\pm$ 1.49 ^a
Exp4	54.82 $\pm$ 1.50 ^a	22.13 $\pm$ 1.02 ^{ab}	13.23 $\pm$ 1.17 ^a

Table 3: Means and SD for Serum Lysozyme (U ml⁻¹) count for different dosages of Chitosan at Different Days

Days	Control	0.50%	0.75%	1.00%	12.5%
1 st Day	17.60 $\pm$ 0.03	18.36 $\pm$ 0.45	17.81 $\pm$ 0.05	18.21 $\pm$ 0.06	18.27 $\pm$ 0.12
30 th Day	34.25 $\pm$ 0.34	23.42 $\pm$ 0.07	32.45 $\pm$ 0.06	34.56 $\pm$ 0.56	36.75 $\pm$ 0.08
60 th Day	37.65 $\pm$ 0.17	29.01 $\pm$ 0.34	33.22 $\pm$ 0.56	53.21 $\pm$ 0.45	45.67 $\pm$ 0.76
90 th Day	40.96 $\pm$ 0.04	28.67 $\pm$ 0.05	32.67 $\pm$ 0.03	45.32 $\pm$ 0.23	43.21 $\pm$ 0.23

Table 4: Agglutination Titre for different dosages of Chitosan at different Days

Days	Control	0.50%	0.75%	1.00%	1.25%
30 th Day	4.59 $\pm$ 2.01	6.73 $\pm$ 5.24	7.76 $\pm$ 6.03	14.13 $\pm$ 2.40	12.2 $\pm$ 2.08
60 th Day	14.1 $\pm$ 1.86	15.1 $\pm$ 1.60	14.6 $\pm$ 2.95	17.5 $\pm$ 0.77	14.7 $\pm$ 2.12
90 th Day	15.9 $\pm$ 3.12	16.5 $\pm$ 2.25	17.2 $\pm$ 1.85	18.5 $\pm$ 1.27	16.9 $\pm$ 1.73

Figure 1: Weight of *Labeo rohita* fed chitosan different level 0.5%, 0.75%, 1.00%, 1.25% feed and a supplemented control diet for 90 days and weighed every 15 days. $P < 0.05$, indicate a significant difference between fish fed Immunostimulants and those fed the control diet. Data represented as mean $\pm$ S.D

Figure 2: Total erythrocyte counts (RBC) of *Lebeo rohita* fed with different level supplement diets. Data are expressed as mean and S.D ($P < 0.05$). n=30

Figure 3: Total leukocyte counts (WBC) of *Labeo rohita* fed with different level of supplemented diets. Data are expressed as mean and S.D ($P < 0.05$). n=30

Figure 4: Haemoglobuline of *Labeo rohita* fed with different level of supplemented diets ($P < 0.05$). n=30

RESULTS

Mortality and survival percentage of supplemented diet and the control diet, after challenge with *Aeromonas hydrophila* are presented. The mortality percentage was highest 37.5% in the control infected group and was lowest 0.5% chitosan in formulated fed group. The positive effect of 1% chitosan on survival was clearly observed in all the groups except for control group fish. The maximum survival rate observed in 1% Chitosan fed group 37(92.5) %. Feed conversion ratio (FCR) and specific growth rate (SGR) of challenged *Labeo rohita*, fed chitosan supplemented diet and the control diet at the end of experiment. All the statistical values are significantly at $P > 0.05$ %.

Biochemical Analysis

The result of the invitation revealed that the total serum protein content of chitosan among the control and experimental group. The highest protein content was observed in the group fed with 1.0% chitosan feed for 90 days. Control and Exp-3 values 30.53 ± 1.24 and 56.30 ± 3.36 the serum protein was increased significantly in the Exp-3 groups for the 90th day ($P < 0.05$). The concentration of carbohydrate was increased in 1% chitosan feed for 90 days Control and exp-3 values 17.76 ± 0.63 and 22.72 ± 0.98 . Lipid content was observed in the group fed with control and Exp-3 13.41 ± 1.49 and 11.04 ± 0.90 . In this present study, different results were found on the biochemical analysis of *Labeo rohita* and the effects were shown at different levels of chitosan. (Table 2)

Serum Lysozyme activity

Over the experimental periods, the serum Lysozyme activity test group exhibited. The result of the present study revealed the experimental period in the serum lysozyme activity from the serum of *Labeo rohita* was reported highest 53.2 ± 0.45 be decreased in 18.21 ± 0.06 . Significantly in the fish fed for 30, 60 and 90 days with feed supplemented with different concentration of the chitosan ($P < 0.05$) the highest lysozyme activity was observed in the group fed with 1.0% chitosan feed for 90 days. (Table 3)

The agglutinated antibody titres was minimum on 30th day and reached a maximum on the 60th and 90th Days in Exp-3 (1% Chitosan) when compared to the control group (Table 4). Although most peak titres were reached by 90th day (18.5 ± 1.27) and lowest value observed in control group is

15.9 ± 3.12 . The experimental and control statistical values significant at $P < 0.05$. (Table 4)

Growth Estimations of Growth

The initial weight of the fish was 10 ± 2 g in all groups growth of fish fed with chitosan was weighed significantly high at $P < 0.05$ after feeding (Figure 1). The positive effect of chitosan on growth was clearly observed in all groups except control fish. The maximum growth was observed in 1% chitosan incorporated feed group 21.37 ± 0.94 and decreased growth was observed in 0.5% 19.22 ± 0.68 , 0.75% 20.11 ± 0.57 , 1.25% and control fish 18.12 ± 0.66 respectively.

Hematological Studies

Hematological parameters of both control and experimented groups, the hemoglobin content was high in 1.0% 9.3 ± 0.7 . There is no big difference in hemoglobin in other group on the day level of hemoglobin increased both 0.5% and 1.0% chitosan. In 1.0% at the end of the 90 days the hemoglobin level. An increased number of RBC recorded in 0.5% chitosan group then 0.5%, 1.0% group and control at the end of experiment the highest number of RBC were observed in 0.5% 506×10^6 administrated group maximum WBC count was observed in 265×10^4 and minimum was observed in the control. (Figure 2-4)

DISCUSSION

The results of the present study clearly showed that dietary chitosan supplementation enhances the growth of *Labeo rohita* compared to control diet²⁸. It is reported that the addition of 1% chitosan to the growth of carp (*Cyprinus carpio*) resulted in a significant increase. Several species of fish fed with the diet containing 1, 2 and 5% beta-chitosan had the highest weight gain, which indicate that beta-chitosan has growth promoting effect³⁶. Hence, the chitosan may play a crucial role in enhancing the digestion and absorption of nutrients at lower levels. Stimulation of growth by Chitosan has previously been reported in other aquatic animals such as Japanese flounder³⁷, turbot³⁸, tilapia³⁹, shrimp⁴⁰⁻⁴², Crayfish⁴³, grass carp⁴⁴, sea cucumber⁴⁵, rainbow trout⁴⁶, sea bass⁴⁷ and grouper⁴⁸. Immunological approaches to prevent fish diseases have normally involved treatment with antibiotics, chemicals or vaccination against specific pathogens,⁴⁹ while the use of Immunostimulants is a relatively new and developing area¹. Haematological parameters of fish blood are useful tools that aids in diagnosis of disease. It can also be used to study

immunopotentiators. Moreover, leukocytes are one of affecting factors in immunity of fish and leukocyte numbers or the proportion of different cell types has been used as indicators of health of aquatic animals⁵⁰. However, infected fish fed with control diet without chitin or chitosan had decreased RBC, WBC, and Hb which indicated that RBCs are being destroyed by the leukocytosis with subsequent erythroblastosis⁵¹. An increase in MCV may be attributed to the swelling of the erythrocytes that result in a macrocytic anaemia or impaired water balance (osmotic stress) or macrocytic anaemia in fishes exposed to stress⁵² this would increase the affinity for oxygen in the blood⁵³. Lymphocytes, monocytes and neutrophils levels were significantly increased in fish fed with 1.0% chitosan supplementation diet from weeks 1 to 4 but did not significantly change the thrombocytes. Similar results were reported in *mrigal*, brown trout and rainbow trout fed with supplementation diet against pathogens⁵⁴. Therefore, the nutritional quality of feed formulation is of prime importance in aquaculture, especially at the culture level. In our study, the diet composition of crude protein and lipid levels in all chitosan formulated fed groups compare to control. In the proximate value of crude protein and lipid composition were higher in 1% chitosan fed groups than other diet groups. Likewise, direct relationship between the amount of Spirogyra incorporated diet and muscle protein and fat contents in *C. catla* were demonstrated by⁵⁵. Dietary lipids in aqua feeds are an important source of energy and essential fatty acids. Inclusion of 5% Ulva meal at low and high lipid levels significantly improves the growth performance, feed efficiency, nutrient utilization, and body composition of Nile tilapia. Optimum lipid levels results in improved growth rates, feed conversion ratios, nutrient utilization and reduced nitrogen excretion⁵⁶. The Lysozyme activity and Agglutination titres did not increase significantly in any diet on first week whereas it was enhanced significantly when fed with chitosan diet on 30th day, 60th day and 90th against pathogen. The lysozyme is a cationic enzyme that breaks β -1, 4 glycoside bonds between N-acetylmuramic acid and N-acetyl glucosamine in the peptidoglycan of bacterial cell walls. It is known to attack mainly the Gram positive bacteria as well as some Gram negative bacteria⁵⁷. An increased level of protection against fish bacterial infection is correlated to an increment in serum lysozyme levels, phagocytic activity and bactericidal activity of head kidney leucocytes⁵⁸. A number of studies have reported that administration of herbals enhance the respiratory burst of phagocytes in fish against diseases^{59,60}. The increasing trends of serum lysozyme activity in this study might have contributed to the enhancement in the non-specific defence mechanisms⁶¹. Agglutins are group of proteins, which have different specificity for carbohydrate binding. These agglutins are Ca^{2+} dependent and can agglutinate a number of fish bacterial pathogens. Similar findings was recorded in haemagglutinin of *R.salmaninarum* using rabbit erythrocyte was reported. Agglutinating most peak titres were reached by 90 days of *S.torvum* leaves soluble fraction treated *Cyprinus carpio* serum samples compared to control⁶². The results of the present investigation, it can be concluded that chitosan can be incorporated in diet in order to increase immune function and protection against infection of *Aeromonas hydrophila*. A dose of 1% chitosan is optimum to stimulate the immune function of Indian major carp *Labeo rohita* and confer a high degree of protection against the invading bacterial pathogen. Same doses of chitosan were equally effective in stimulating the

growth of *Labeo rohita*. This base line information will of immense importance to the fish farmers of the temperate climatic areas.

ACKNOWLEDGEMENT

I am very thankful to Bharathiar University for providing scientific environment during my research work and also thanks to Aquatic Biotechnology live feed culture research scholars for the support.

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Cite this article as:

Kiruba Aathi, Venkatachalam Ramasubramanian, Venkatachalam Uthayakumar and Subramani Munirasu. Effect of chitosan supplemented diet on survival, growth, hematological, biochemical and immunological responses of Indian major carp *Labeo rohita*. Int. Res. J. Pharm. 2013; 4(5):141-147

Source of support: Nil, Conflict of interest: None Declared