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Dietary Supplementation With Chitosan on Haematology and Innate Immune Response in *Cyprinus Carpio Haematopterus* Against *Aeromonas Hydrophila*

KEYWORDS: *C. philarigus*, Chitosan, SDS-PAGE, FT-IR.

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ABSTRACT *In the present investigation, the exoskeleton from Calapa philarigus was collected and processed through Deproteinization, Deacetyalization and Demineralization methods were used for the preparation of chitosan. The yield of chitosan in C.philarigus was 38.23%. The Fat Binding Capacity of the shells was 619±20% respectively. During the experimental period, Cyprinus carpio haematopterus were fed with chitosan (0.25, 0.50, 0.75, and 1%). The present study shows increase in the RBC Haemolytic activity of chitosan in fish was (0.007±0.000). The Antibacterial activity was higher in (1%) inhibited A.hydrophila (72.61±2.85). The SDS-PAGE of protein was higher in 1%. The biochemical composition protein (54.03±1.38), carbohydrate (18.04±0.99), lipid (9.28±0.33). Thus present study concluded that chitosan supplementation diet was modulated the immune defense, disease resistance against the bacterial diseases.*

INTRODUCTION

Aquaculture is growing rapidly in many regions of the world. Aquaculture products constitute an important food supply wisth increasing economic importance (Sakai,1999). To address the mentioned problems, the use of chitosan as a protective material appears to be a potential alternative. The uses of immunostimulants for disease control measures in aquaculture attract great attention especially in practical aquaculture. The crustacean shells were converted into chitin (Value added biopolymer) and chitosan was used as an immune stimulant (Ramesh *et al.*, 2010). In recent years, applications of chitosan to the field of medicine, food, chemical engineering, pharmaceuticals, nutrition, environmental protection and agriculture have received considerable attention (Chung , Wang, Chen& Li ,2003; Li *et al.*, 2007). Thus, is an interesting compound to study in different applications (Ponce- Jimenez *et al,* 2002, Ponce-Jimenez *et al.*, 2012). In Aquaculture, Chitosan has been found to be useful as an immunostimulants to enhance protection of fish (Anderson and Siwicki,1994; Parama *et al.*, 2005; Cha *et al.*, 2008) and shrimp(Wang and Chen et al. ,2005) against bacterial disease. Hence the aim of the present study an attempt has been made to study the immune response of freshwater *Cyprinus carpio haematopterus* fed with chitosan in different concentration.

Materials and methods:

Collection of Crab raw materials & Extraction of chitosan

The fresh water crabs (*Calappa philargius)* were collected in Coimbatore .It was processed for extraction of chitosan. Isolation of chitosan from crab shell wastes involves three steps demineralization (DM), deproteination (DP), and deaccetylation (DA) (Madhavan and Nair, 1974)

Moisture content

Moisture content of the chitosan was determined by the gravimetric method (Black, 1965).

Water Binding Capacity and Fat Binding Capacity

WBC of chitosan was measured using a modified method of Wang and Kinsella (1976).FBC of chitosan was measured using a modified using a modified method of Wang and Kinsella (1946).

Fourier Transform Infrared Spectroscopy (FTIR)

The process of deacetylation involves the removal of acetyl groups from the molecular chain of chitin, the leaving behind a compound (chitosan) with a high degree chemical reactive amino group (-NH2) .This makes the degree of deacetylation (DD) an important property in chitosan production as it affects the physio- chemical properties, hence determines its appropriate applications (Rout,2001) .

Experimental set up and Diet preparation

Healthy & disease free fishes of (*Cyprinus carpio haematopterus*) fishes were collected from TNFDC, Aliyar Dam, Coimbatore, Tamil Nadu. **Treatment – (T1)** : 0.25 % Chitosan, **Treatment – (T2)** : 0.50 % Chitosan, **Treatment – (T3)** : 0.75 % Chitosan, **Treatment – (T4)** : 1.00 % Chitosan, **Control -**commercial feed. After adding the chitosan with commercial feed it was made into pellets are dried under room temperature.

Growth and mortality

The recorded data on weight was for calculation of the feed conversion ratio (FCR) and specific growth rate (SGR). At the end of experimental periods, the SGR increase per day and (FCR) for all the experimental groups were calculated according to (Ricker 1979).Amend (1981) recorded mortality data used for calculating the relative percentage survival (RPS).

Leukocyte count

Leukocytes were counted by the method of Rusia and Sood, (1992) using haemocytometer.

Bio-Chemical Analysis

The biochemical analysis of the protein was estimated by (Lowry *et al.*, 1951), the carbohydrate was estimated by (Roe, 1955), the lipid was estimated by (Folch *et al.*, 1957)

Anti bacterial assay (Zheng & Zhu 2003)

Antibacterial activity measured following the method of

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Zheng and Zhu (2003) with slight modification.

SDS-Polyacrylamide Gel Electrophoresis (PAGE)
Polyacrylamide gels are prepared by the free radical polymerization of acrylamide and the cross linking agent N-N' methylene bis acrylamide Acrylamide + N N' methylene bis acrylamide Chemical Ammonium persulfate Polymerization + TEMED Polyacrylamide.

Statistical analysis
Statistical analysis was performed using analysis of variance (One-way ANOVA), Student's *t*-test to determine differences between experimental levels. Levels of significance are expressed as ($P < 0.05$).

RESULTS
The present study reported the various physiochemical properties of chitosan extracted from shells.

Moisture, WBC and FBC
The moisture content of the chitosan was found to be 0.48±0.18% WBC, FBC was 619±50% and 525±20% respectively.

FT-IR
The different absorption band within the 4000-400 cm range was recorded in the FTIR spectra of chitosan, prepared from shell. Different stretching vibration bands were observed in the range 3449.06-2519.54 cm^{-1} related to (N-H) in (NH) associated to primary amines.

Growth Rate
The initial weight and length of *Cyprinus carpio haematopterus* was 2.0±0.01, 4±0.01 cm .The chitosan fed fishes gained maximum growth Final weight, 10.02-±0.06 gm, final length 12cm.

SGR, FCR, Survival and Mortality Rate
SGR in the chitosan (1.0%) fed group and the control were 58.05±5.83, 50.01±2.50. FCR values of chitosan (1.0%) and control were 2.34±0.34 and 4.56±0.13. The mortality percentage was 16.66±0.04 % in the control group and survival percentage was 53.33±1.03 in chitosan.

Biochemical Analysis
In the present study, biochemical composition of protein, carbohydrate and lipid. The concentration of final fish muscle protein was increased in all the 5 experimental fishes as compared to the initial and maximum level of protein (25.03±1.56mg/g), (54.03±1.38), control (27.04±2.02mg\g). The concentration of carbohydrates in fish was increased in all the 5 experimental fishes compared to the initial and maximum level of carbohydrate (10.02±0.01mg/g), (18.04±0.99) and control (10.47±0.02mg/g).The content of lipid was initially 7.01±2.4mg/g. The concentration increased slightly which was 9.28±0.33mg/g in chitosan, 7.09±0.11mg/g in control.

Haemolytic Complement Activity
Serum haemolytic activity, measured by the mean number of blood serum units/ml serum was increased by the chitosan supplement (Table.1).In the present study Acetic acid shows higher percentage of (0.0071±0.000) in 1% of chitosan of sheep, (0.0188 ±0.000) in 1% of chitosan fish.

Antibacterial Assay
The antibacterial activity with increasing chitosan concentrations was reported. The highest concentration (1.0%) of chitosan used inhibited *Aeromonas hydrophila* by 83.05±7.4.

SDS-PAGE
The SDS PAGE analysis is the storage of protein concentration was higher in T4.

Table1. Haemolytic Activity of crab Chitosan against Sheep and Fish erythrocyte Suspension (Mean±SD)

Chitosan (mg/kg)	Hemolysis of Sheep Blood (%)			Hemolysis of Fish Blood (%)		
	Ex A	Ex B	Ex C	Ex A	Ex B	Ex C
0.25	0.0135±0.000^a	0.0082±0.000^a	0.0088±0.000^a	0.0138±0.000^c	0.0145±0.000^a	0.0259±0.001^a
0.50	0.0125±0.000^a	0.0071±0.000^b	0.0087±0.000^a	0.0076±0.011^a	0.0121±0.000^a	0.0240±0.007^b
0.75	0.0115±0.000^a	0.0070±0.000^b	0.0081±0.000^a	0.0068±0.016^a	0.0114±0.000^b	0.0219±0.000^c
1.00	0.0100±0.480^a	0.0069±0.000^b	0.0071±0.000^b	0.0040±0.023^b	0.0110±0.003^b	0.0188±0.000^d
Hemolysis Sheep Blood				**F Value**		
			Extraction A (Distilled Water)	**0.883****		
			Extraction B (Ethanol)	**25.283***		
			Extraction C (Acetic Acid)	**0.000 ****		
Hemolysis Fish Blood				**F Value**		
			Extraction A (Distilled Water)	**17.045***		
			Extraction B (Ethanol)	**5.631***		
			Extraction C (Acetic Acid)	**59.892***		

****Significant at 0.01 level; *Significant at 0.05 level, ns- Not significant.**
Mean in a column is significantly (p<0.05)

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Graph 1: Biochemical composition of Cyprinus carpio haematopterus fed with Chitosan different concentration (%)

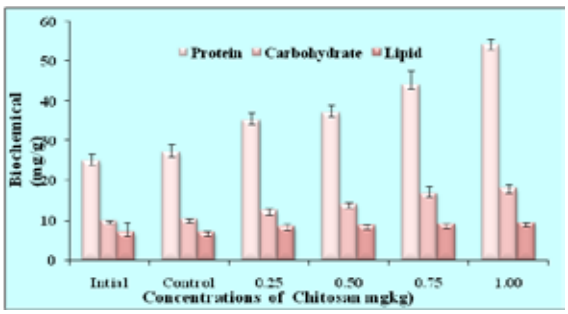
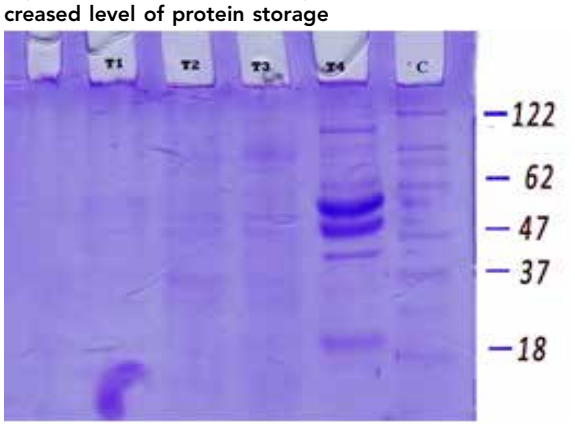


Fig 1: SDS-PAGE was Analyzed T4 was found to increased level of protein storage



Treatment – (T1) : 0.25 % Chitosan, **Treatment – (T2)** : 0.50 % Chitosan,

Treatment – (T3) : 0.75 % Chitosan, **Treatment – (T4)** : 1.00 % Chitosan

Control - (C):commercial feed.

DISCUSSION

In the present study the yield of chitosan from *C. philarigus* was 46.43%. Similar result of 49.7 was reported from shrimp shell by (Pannee Sophonvachiraporn, *et al.*, 2011). The present study , moisture of the chitosan was 0.46±0.03%.The result shows similarities with moisture of chitosan of 2.5±0.11% (Alishahi *et al.*, 2011).According to Cho et al., WBC and FBC of five commercial chitosan products were in the range of 458-805 and 314-535%, but (No *et al.*, 2006) reported lower results of 355-611%. The present study result was 584.33±7.09%. The FBC of *C. philarigus* shells was measured using olive oil. The FBC of chitosan measured 460.33±6.42%. The range of FBC found in the present study (460.30%) was slightly similarly reported by choe *et al.*, 458-805), (314-535) respectively. The chitosan may play a crucial role in enhancing the digestion and absorption of nutrients at lower levels. A significant increase in WBC count was observed in all the treatments for chitosan fed fish. Total protein significantly increased in fish fed with 1% chitosan diets. In the present study significantly increased in haemoglobin contents which were considered to reflect strong innate immunity in fish (Austin, 2011;). The cumulative mortality was lower in chitosan supplemented diets (1%). A similar result was reported in other fishes of chitosan supplementation diets (Dautremepuits *et al.*, 2008; Gopalakannan and Arul, 2006 ;). Based on the above investigation it is evident that chitosan (1%) certainly enhances the immunity of *Cyprinus carpio haematopterus*.

CONCLUSION

Chitosan acts as immunostimulants which appear to improve the immune status and the growth of *Cyprinus carpio haematopterus* in fish farms. It remains for further molecular work to consider the potentiality of chitosan for fish disease control strategies in aquaculture.

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