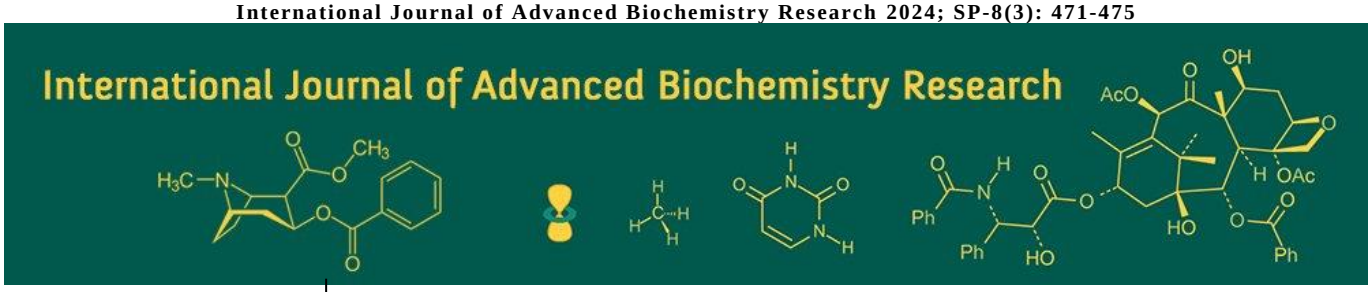

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Iqra Nazir
Department of Aquaculture, GBPUAT, Pantnagar, Uttarakhand, India

Mohd Danish
Department of FRM, GBPUAT, Pantnagar, Uttarakhand, India

Sauliheen Qadri
Division of FRM, SKAUST-K, Srinagar, Jammu and Kashmir, India

Tabinda Shabir Yaswi
Department of Fish Processing Technology, KUFOS, Panangad, Kochi, Kerala, India

Corresponding Author:
Mohd Danish
Department of FRM, GBPUAT, Pantnagar, Uttarakhand, India

Assessment of biochemical parameters (Total serum protein, albumin and globulin) for immunity enhancement in fingerlings of *Cyprinus carpio haematopterus*

Iqra Nazir, Mohd Danish, Sauliheen Qadri and Tabinda Shabir Yaswi

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Abstract
In the current study, the immunostimulatory effects of chitosan, vitamin C, *Curcuma longa, Tinospora cordifolia, Withania somnifera*, and *Allium sativum* on *Cyprinus carpio haematopterus* fingerlings were assessed. A total of 25 fish per treatment group (T_0, T_1, T_2, and T_3) were randomly assigned to receive fingerlings weighing 30.0±2 g each in duplicate. A combination of rice bran, deoiled mustard oil cake, deoiled soybean cake, and vitamin-mineral mixture was used to create four isoproteinous experimental diets. The phytobiotics, Vitamin C and Chitosan were incorporated into diet. Per kilogramme of feed, diet D1 included 2.5 g each of garlic, giloy, turmeric, and ashwagandha whereas the diet D_2 had control feed + (2.5 g of each plant) + 25 mg Vitamin C per kg feed. For every kilogram of feed, diet D3 included 2.5 grams of each plant and 2.5 grams of chitosan. T_0 group fishes were fed with D_0 diet, T_1 with D_1, T_2 with D_2 and T_3 with D_3 @ 5% body weight per day for 122 days. All treatment groups fed the experimental meal showed a significant (p<0.05) increase in the levels of total blood protein, albumin, globulin, A/G ratio, and ALT between the pre-challenged and post-challenged groups. The findings show that the tested phytobiotics, in combination with chitosan and vitamin C, have excellent immunostimulatory effects and have no negative effects on the health of *Cyprinus carpio haematopterus* in a culture system.

Keywords: Total serum protein, albumin, globulin and immunity

Introduction
Asia contributes about 88.91% to the world fish production according to FAO (2016) [5]. Carps contribute to the majority of aquaculture production worldwide, accounting for over 70% of total productivity. These comprise of Indian major carps i.e, Catla (*Calta catla*), Rohu (*Labeo rohita)* and Mrigala (*Cirrhinus mrigala*); Chinese carps viz. Silver carp (*Hypophthalmicthys molitrix*), Grass carp (*Ctenopharyngodon idella*), Common carp (*Cyprinius carpio*) and Big head carp (*Hypophthalmicthys nobilis*). Among these Grass carp (*Ctenopharyngodon idella*) is the highest cultivated carp species contributing about 5.02 mmt, silver carp (*Hypophthalmicthys molitrix*) ranks 3rd contributing about 4.18 mmt and common carp (*Cyprinius carpio*) ranks 4th contributing 3.79 mmt to the world aquaculture production (FAO, 2015) [4]. Aquaculture is a significant industry that is growing annually on a global scale. The Indian fisheries is also contributing to the national food security of the country. India ranks second globally in terms of both total fish production and freshwater fish production. In India, fisheries sector provides employment to about 14.49 million people for livelihood security.

Experimental site and climate
The experiment was conducted in the tarai region of Uttarakhand at the Wet Lab of the College of Fisheries, G. B. Pant University of Agriculture and Technology, Pantnagar, Distt. Udham Singh Nagar. The experiment ran from March 1 until June 30, 2018.

Fish collection and maintenance
At the Fish Seed Hatchery, College of Fisheries, G. B. Pant University of Agriculture and Technology, Pantnagar, three hundred fingerlings of healthy, disease-free *Cyprinus carpio haematopterus* were obtained. Their average body weight was 30.00±2 g. The fingerlings were kept in indoor captive settings in aerated water for two days while they acclimated in cemented tanks.

Treatment combination
T_0 - D_0 Control diet
T_1 - (D_1) with control feed + 2.5 g of garlic + 2.5 g of giloy + 2.5 g of turmeric + 2.5 g of ashwagandha per kg feed
T_2 - (D_2) with control feed + (2.5 g of each of four plants) + 25 mg Vitamin C per kg feed
T_3 - Diet (D_3) with control feed + (2.5 g of each plant four plants) + 2.5 g Chitosan per kg feed
Feeding was done @ 5% body weight daily. The total quantity of the daily diet was given in two installments, once at 9.00 am and then at 5.00 pm.

Experimental design
The entire experiment was carried out under controlled circumstances at the G. B. Pant University of Agriculture and Technology, Pantnagar, in the Wet Lab of the College of Fisheries. In this experiment, twelve equal-sized FRP tanks with a diameter of 1.07 meters and a depth of 0.82 meters were employed in four groups of three duplicates ($4T_3R$= 12). About 600 liters of water were kept in each tank. Every day, the water was aerated. There were twenty-five Amur carp fingerlings in each tank. Fish in the control treatment received a regular diet (D0). The fishes in treatment T_1 were given D_1 diet, T_2 fishes with D_2 diet and T_3 fishes with D_3 diet. The experiment lasted for 122 days. The growth parameters were analyzed every 15th day. Biochemical analysis was done at 0 day, pre challenge, post challenge and withdrawal day.

Biochemical analysis
Total serum protein
Total serum protein was calculated by using following formula

Total serum protein (g/dl) = (Absorbance test / Absorbance of standard) ×6.5

Albumin
The albumin was calculated by using following formula

Albumin (g/dl) = (Absorbance of test / Absorbance of standard) ×4

Globulins
The globulin was calculated by using following formula

Globulins = Total serum protein – Albumin

Albumin-globulin ratio [A/G]
The albumin-globulin ratio was calculated by using following formula

$$\text{Albsumin- globulin ratio} = \frac{\text{Serum albumin (g/dl)}}{\text{Serum globulin (g/dl)}}$$

Serum Alanine aminotransferase (ALT)
The general formula for converting absorbance change into International Units (IU) of activity is: Activity of ALT = ΔAbs./min. x 1768 at 37 °C (IU/L)

Bacterial strain and challenge study
After 102 days of feeding trial, blood samples were collected from treatment and control fish. Suspension of *Aeromonas hydrophila* (designation RTMCX1) was prepared in PBS from freshly grown culture for the challenge experiment from diagnostic bacteriology laboratory, ICAR- DCFR, Bhimtal and maintained at 4 °C in the Department of Aquaculture, G.B Pant University of Agriculture and technology, Pantnagar. Ten fishes from each treatment were challenged intraperitoneally with 10 µl of bacterial suspension (1.5×10^8 CFU/ml) on day 92. Adequate care was taken to avoid injury when the strain came into contact with A. hydrophila. All exposed samples were returned to their respective containers and monitored for their reaction against the injected bacterial strain. Mortality was observed for 10 days. After 102 days of feeding the fish with the D_1, D_2 and D_3 diet, the fishes in the all groups were then fed with control diet for 15 days. After that blood was taken for biochemical analyses.

Statistical analysis
The experimental data were subjected to a two-way analysis of variance (ANOVA) using SPSS (Statistical Package for Social Sciences 2006 version 16.0). The individual mean differences were compared using Duncan's multiple range test. When $p<0.05$ was reached, the values were deemed significant.

Results and Discussion
Total serum protein (TSP)
The observations on total serum proteins in different treatment groups have been depicted in Figure1. The total serum protein (TSP) of amur carp at 0th day varied between 1.88±0.53 to 1.99±0.061 g/dl. The total serum protein of amur carp in pre challenge fishes varied between 2.45±0.006 to 2.76±0.069 g/dl among treatments. In pre challenged group, the maximum value for TSP was recorded as 2.76±0.069 g/dl (T_3). In case of post challenged fishes, the value ranged between 4.94±0.09 to 6.92±0.019. Highest TSP was obtained in T_3 (6.92±0.019 g/dl). Between T_0, T_1, T_2, and T_3 on the 0th day and pre-challenge, as well as between the pre-challenge and post-challenge groups fed experimental diets, the value of TSP differed considerably ($p<0.005$). Abdel-Tawwab *et al.* (2010) [1] discovered that when tilapia were fed green tea and subsequently challenged with *Aeromonas hydrophila*, there was an increase in total serum protein, albumin, and globulin. Harikrishnan *et al.* (2012) [7] also noticed that their was an increase in total serum protein when the fish were fed with 1% or 2% of chitosan diets. The results are in agreement with Siwicki (1989) [13] who found that after feeding carp fish with β-glucan and chitosan their was an increase in the level of total protein content. The values of TSP for the withdrawal period varied between 2.42±0.006 to 2.97±0.169.

Albumin
The observations on albumin in different treatment groups have been depicted in (figure2). The albumin level of Amur carp at 0th day varied between 0.59±0.051 to 0.63±0.532 g/dl. The albumin level of Amur carp in pre challenged

fishes varied between 0.82±0.01 to 0.97±0.03 g/dl among treatment. In pre challenged group, the maximum value for albumin was recorded as 0.97±0.03 g/dl (T_3). The value for fish that had been post- challenged ranged from 1.66±0.01 to 3.29±0.04 g/dl. The T_3 treatment fed with D3 food had the best albumin level (3.29±0.04 g/dl), which was substantially higher than the T_0 control (1.66±0.01 g/dl) ($p<0.05$). The albumin level at the withdrawal period varied between 0.85±0.01 to 0.99±0.04. The value of albumin varied significantly ($p<0.005$) between T_0, T_1, T_2 and T_3 in 0^{th} day and pre challenge as well as between pre challenge and post challenge group fed with experimental diets. The results support the view of Talpur *et al.* (2012) [15] who reported an elevation in albumin level ($p<0.05$) as compared to control when Asian sea bass was fed with garlic supplemented diets and post challenged with *Virio harrveyi*. Fish given Euglena powder showed a substantial change in albumin content at several assays between treatment groups and the control group, according to Das *et al.* (2009) [3]. Same results were observed when *Achyranthes aspera* seed were added in the diet of *Labeo rohita* fingerlings.

Globulin

The profile of globulin in different treatment groups has been presented in (figure3). The globulin level of Amur carp at 0^{th}day varied between 1.29±0.116 to 1.36±0.006 g/dl. The globulin level of Amur carp in pre challenged fishes varied between 1.63±0.02 to 1.79±0.05 g/dl among treatment. In pre challenged group, the maximum value for albumin was recorded as 1.79±0.05 g/dl (T_3) followed by 1.76±0.03 g/dl (T_2) and lowest in 1.63±0.02 g/dl (T_0). In case of post challenged fishes, the value ranged between 3.28±0.05 to 3.63±0.04 g/dl. The best level of globulin was found in treatment T_3 (3.63±0.04 g/dl). Fish blood biochemical examination revealed that T_3/D3, supplemented with 2.5g of each plant and 2.5g of chitosan, had the highest globulin level. Thus, treatment T_3 showed best globulin level which was significantly ($p<0.05$) high as compared to control T_0 as well as other treatments. The globulin level at the withdrawal period varied between 1.66±0.02 to 1.82±0.05. The value of globulin varied significantly ($p<0.005$) between T_0, T_1, T_2 and T_3 in 0^{th} day and pre challenge as well as between pre challenge and post challenge group fed with experimental diet. Similar results were reported by Harikrishnan *et al.*, (2012) [7] who reported elevation in globulin level in *Epinephelus bruneus* fed with 2% chitin or 1% chitosan diets. Subeena and Navaraj (2012) [14] reported enhanced ($p<0.05$) serum protein, albumin, globulin content in *Mystus keletius* fed with herbal diets (*Solanum trilobatum* and *Ocimum sanctum)* as compared to the control group. Sahu *et al*., (2007) [11] reported that *Labeo rohita* challenged with *Aeromonas hydrophila* showed significantly ($p<0.05$) higher globulin level in all the groups in comparison with control when *Curcuma longa* was added in the diet. After feeding *Sparus aurata* with levamisole slight increment in globulin level has been reported by Mulero *et al.* (1998) [10]. The globulin is certainly important for maintaining a good and healthy immune system (Jha *et al.*, 2007) [8].

A/G Ratio

The observations on A/G ratio in different treatment groups have been depicted in (figure 4). The A/G ratio of Amur carp at 0^{th}day varied between 0.45±0.006 to 0.47±0.007.The A/G ratio of Amur carp in pre challenge fishes varied between0.50±0.007 to 0.55±0.006 among treatments. In pre challenged group, the maximum value for A/G ratio was recorded as 0.55±0.006 (T_3) which was significantly higher from T_0, T_1 and T_2. In case of post challenged fishes, the value ranged between 0.50±0.007 to 0.90±0.009. Highest A/G ratio was obtained in T_3 (0.90±0.009). The value of A/G ratio varied significantly ($p<0.005$) between T_0, T_1, T_2 and T_3 in 0^{th} day and pre challenge as well as between pre challenge and post challenge group fed with the experimental diet. The value of A/G ratio during the withdrawal period ranged between 0.51±0.006 to 0.54±0.06. Our results support the findings of Sivaram *et al.* (2004) [12] who reported that the methanolic extracts of herbal plants like *O. sanctum, W. somnifera and Myristica fragrans* significantly improved the albumin–globulin (A/G) ratio in *Epinephelus tauvina* challenged with *Vibrio harveyi*. Chesti *et al.* (2018) [2] reported that feeding of *Allium sativum* upto 1.5% in feed to fingerlings of Amur carp, *Cyprinus carpio haematopterus,* resulted in the significant changes ($p<0.05$) between the pre challenge and post challenge groups in total serum protein, albumin, globulin, A/G ratio, aspartate aminotransferase (AST**)**, serum alanine aminotransferase (ALT**)**.

Alanine Aminotransferase (ALT)

The observations on alanine aminotransferase (ALT) in different treatment groups have been depicted in (figure5). The ALT of Amur carp at 0^{th} day varied between 28.16±0.41 to 30.85±0.52. The ALT content in pre challenged fishes was in the range of 18.96±0.501 (T_3) and 21.75±0.386 (T_0). In case of post challenged fishes, the ALT content was recorded as 15.12±0.388 (T_3) to 19.63±0.314 (T_0). The value of ALT content among 0^{th} day and pre challenged as well as between pre challenged and post challenged fishes varied significantly ($p<0.05$) between the treatments. The experiment conducted by Fazlolahzadeh *et al*. (2011) [6] on Rainbow trout, *Oncorynchu smykiss* fed with garlic rich diet has resulted in variable values of ALT and their was no significant difference in treatments group as compared to control. The values of ALT after withdrawal period varied between 17.86±0.411 to 20.56±0.501**.** Increases in fish total serum protein, albumin, and globulin levels are hypothesized to be linked to a more robust innate immune response. An increase in the total leukocyte count, which is a significant source of serum protein production, including lysozyme, complement factors, and bactericidal peptides, could be the cause of the elevated serum protein content (Misra *et al.*, 2006) [9].

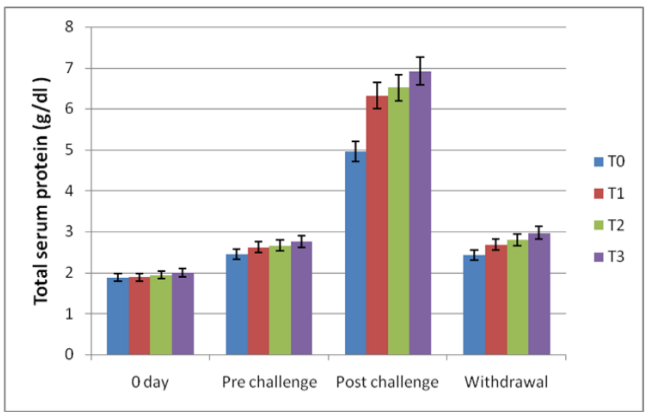

Fig 1: Total serum protein (g/dl) in different treatment groups of *Cyprinus carpio haematopterus* fed with different diet

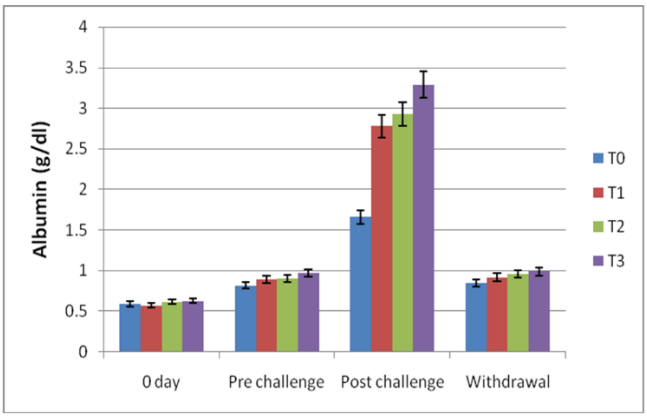

Fig 2: Albumin content (g/dl) in different treatment groups of *Cyprinus carpio haematopterus* raised with different diet

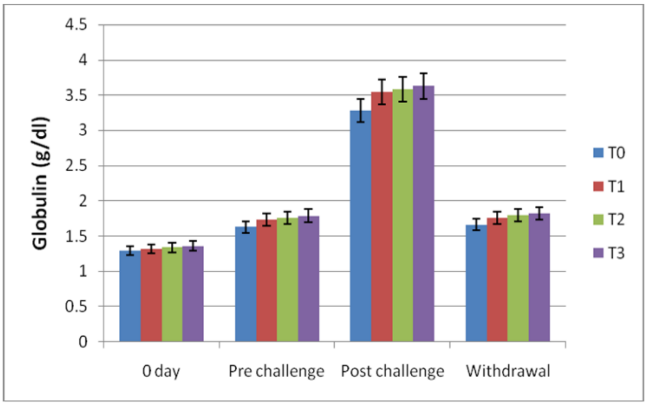

Fig 3: Globulin content (g/dl) in different treatment groups of *Cyprinus carpio haematopterus* fed with different diets

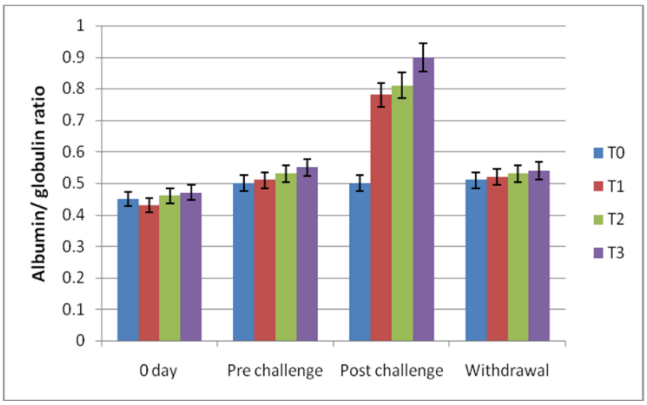

Fig 4: Albumin/Globulin ratio in different treatment groups of *Cyprinus carpio haematopterus* fed with different diets

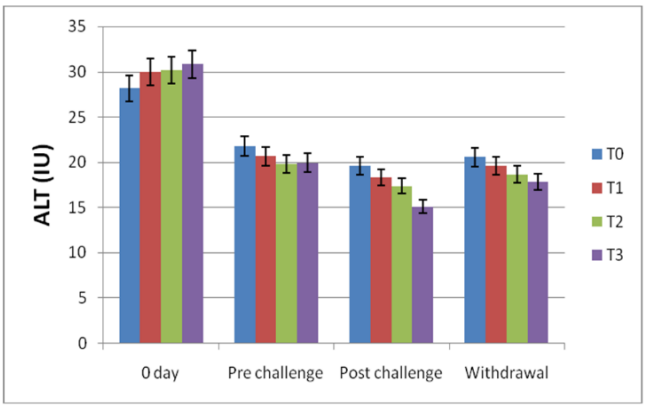

Fig 5: Alanine Aminotransferase (IU/L) in different treatment groups of *Cyprinus carpio haematopterus* raised with different diets

Summary and Conclusion

The fish in this experiment fed diet D3 showed an increase in total serum protein, albumin, and globulin levels which may be an indication that the supplements incorporated in the diet had resulted in the enhancement of the non-specific immune response of fishes and also the increased levels of albumin may facilitate the transport of more humoral compounds as well as phytobiotic extract along with chitosan in the blood.

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