



Immunomodulatory and Growth Promoting Effect of Dietary Fenugreek Seeds in Fingerlings of Common Carp (*Cyprinus carpio* Lin.)

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Abstract

The aim of the study was to determine the immunomodulatory effect of dietary intake of fenugreek (*Trigonella foenumgraecum* L.), seed powder on common carp (*Cyprinus carpio* Lin.). The diets containing 5, 10 and 20 g fenugreek kg⁻¹ of dry feed at the rate of 5% of total body weight was fed to fish for 90 days. Experimental fish stock was challenged intra-peritoneally with *Aeromonas hydrophila*. Total erythrocyte count, haemoglobin count, total serum protein, albumin and globulin content were determined. Results indicate that the values of these parameters were significantly enhanced in fenugreek supplemented groups particularly in 20 mg kg⁻¹ of body weight. Highest lysozyme and nitrobluetetrazolium activity were 65.16 ± 0.585 and 0.989 ± 0.004 respectively in 10 g kg⁻¹ post challenge group. The survival percentage and specific growth rate was higher (98.34%) in 20 mg kg⁻¹ group with respect to control. Furthermore, the antibacterial activity of fenugreek methanolic extract was also observed against two fish pathogens; *Aeromonas hydrophila* and *Pseudomonas liquefaciens*. The findings clearly indicate that the incorporation of fenugreek in diet of common carp fingerlings enhances the non-specific immunity which increases their resistance against infection in fish, suggesting its use in fish diet as a health supplement.

Keywords: *Aeromonas hydrophila*, *Cyprinus carpio*, growth, immunological parameters

Introduction

Aquaculture in Asia has problems with bacterial diseases such as motile aeromonas septicaemia, furunculosis, columnaris, and Edward siellosis. Among these, disease caused by *Aeromonas hydrophila* is most widespread in freshwater fish (Karunasagar et al., 1991). The incidence of drug-resistant bacteria has become a major problem in fish culture. Therefore, the immediate control of all fish diseases is impossible by using only vaccines (Sakai, 1999). The use of antimicrobials for disease control and growth promotion in animals increases the selective pressure exerted on the microbial world and encourages the natural emergence of bacterial resistance. Vaccination may be the most effective prophylaxis for infectious fish diseases but the development of vaccine against intracellular pathogens has not been successful so far. Disease caused by bacteria like *A. hydrophila* has not been controlled by vaccination due to their heterogeneity (Gopalakannan & Venkatesan (2006). However, when applied to hatchery conditions, immunization techniques are not always effective. Immunostimulants may represent an alternative and a supplemental strategy to vaccination in the prevention of diseases in aquatic animals. Some valuable agro-industrial by-products (El-Komy, 2006) such as field crops wastes (Srouf, et al., 2002), aquatic flora (Elmorshedy, 2010), restaurant and market trash (Eissa et al., 1985), spices and medicinal plants (Abd El-Hakim, 2008) are increasingly recognized as substitutes to conventional feed stuff in diets for their immuno stimulatory effects.

Herbs or medicinal plants and spices have been reported to promote various functions like growth, appetite stimulation, anti-stress, immune function, egg hatching rate and also increase disease resistance in fish culture due to presence of different

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active components. Fenugreek is rich in flavonoids, saponins, iron, calcium and zinc (Gupta et al., 1996; Michael & Kumawat, 2003) and benefits the digestive system as a laxative, intestinal lubricant. Geurden et al. (2013) showed that an early short-term exposure of rainbow trout fry to a plant-based diet improves acceptance and utilization of the same diet when given at later life stages. Aqueous leaf extract of *P. niruri* was tested for the potential to be used as an immune stimulant by Muthulakshmi et al. (2016) on other nonspecific and specific parameters and disease-protective property by challenging fish with virulent fish pathogens, result revealed that it can be used either as a routine feed supplement to activate the immune system of farmed fishes or as an adjuvant to enhance the efficacy of vaccines. Feed with leaf extract of *E. hirta* were able to stimulate the specific immune response by increasing the antibody titre value. It was able to stimulate antibody production only up to the 5th day, when fed with higher concentrations of (25 g and 50 g) plant leaf extract. The plant extract showed non-specific immune responses such as lysozyme activity, phagocytic ratio, NBT assay, etc. at higher concentration (50 g) in the *Cyprinus carpio* and in the same concentration (50 g), the leaf extract of *E. hirta* significantly eliminated the pathogen in blood and kidney (Pratheepa & Sukumaran et al., 2014). It was observed that fish have significantly higher survival percentage at higher concentration (50 g) of *E. hirta*, when compared with the control. The objectives of this study are to investigate immunomodulatory activity and growth performance effect of fenugreek (cv. Pant Ragini) at different levels (0.5%, 1 and 2%) when incorporated in common carp diets.

Materials and Methods

Fingerlings of Common carp (*Cyprinus carpio*) measuring total length of 70±2 cm (1.5±0.2 kg weight) procured from fish farm of Fisheries College, Pantnagar and were randomly distributed as a group of 90 fish in the pond. Throughout the study this same sample size was analyzed in triplicate.

Fish feed was prepared by mixing rice bran (17.64%), wheat bran (17.64%), soybean meal (32.35%), fish meal (32.35%), vitamin mineral mixture (1%) and 1% wheat flour. Powder of fenugreek seeds at three different concentrations 0.5%, 1 and 2% was mixed with feed fish diet and pellets are prepared. The various inclusions of feed

were weighed into a bowl, dry mixed and pelleted using a 3 mm pelletizer. Feed consumption or intake (mg) was estimated by subtracting the amount of unconsumed dry feed from the total dry weight of feed offered.

Sampling of fishes was done at intervals of 15 days to assess body weight of fish and the survival rate (%) was calculated by the formula of Baulny et al. (1996). The growth parameters were calculated following standard formulae.

$$\text{Weight gain (\%)} = \frac{\text{Final weight (g)} - \text{Initial weight (g)}}{\text{Initial weight (g)}} \times 100$$

$$\text{Specific Growth Rate (\%)} = \frac{\text{Loge final weight (g)} - \text{Loge initial weight (g)}}{\text{Number of days}} \times 100$$

$$\text{Feed Conversion Ratio} = \frac{\text{Feed given (g dry weight)}}{\text{Body weight gain (g wet weight)}}$$

$$\text{Protein Efficiency Ratio} = \frac{\text{Net weight gain (g)}}{\text{Protein fed (g)}}$$

$$\text{Survival (\%)} = \frac{\text{Total number of harvest fish}}{\text{Total number of stocked fish}} \times 100$$

Blood sampling was carried out for the analysis of the blood parameters, total erythrocyte count (Hendricks et al., 1952), total leucocytes count (Shaw et al., 1930), nitrobluetetrazolium assay and serum lysozyme activity (Chung & Secombes, 1988). Three fish from each treatment were anaesthetized with MS222 (100 ppm, Sigma, USA). Blood was collected from the caudal vein using a syringe, which was previously rinsed with 2.7% EDTA solution. Blood was then transferred immediately to an Eppendorf tube containing a pinch of EDTA powder, shaken gently and kept at 4°C.

For biochemical assays, blood samples were centrifuged (3000 g for 10 min) at room temperature and then serum separated and stored at -20°C until analysis.

Serum protein was estimated by Biuret and BCG dye binding method of Reinhold (1953) using the total protein (Qualigens Diagnostics). Albumin was estimated by the bromocresol green binding method of

Doumas et al. (1971) using albumin kit (Qualigens Diagnostics). After immune modulation trial through feed over 90 day duration, the fingerlings in all the groups were challenged with 100 µl of *Aeromonas hydrophila* at a concentration of $1.5 \pm 0.3 \times 10^6$ CFU ml⁻¹ in PBS as a medium. The bacterial suspension in PBS was inoculated intra-peritoneally in all specimens of all the groups by 1 ml insulin syringe on 30th day and the specimens were re-challenged on 58th day. All the challenged specimens were released back into the respective ponds and were observed for their response. The cause of death was ascertained by re-isolating the infecting organism from dead fish liver. Liver and gill tissue of representative sample fish (moribund/dead) from control and each test group were removed and examined for oxidative stress enzyme activity.

All experimental results are represented as mean $\pm$ SD. The data was statistically analyzed by using statistical package SPSS version 16 in which data were subjected to one way ANOVA and Duncan's multiple range tests was used to determine the significant differences between the means. Comparisons were made at 5% probability level.

Results and Discussion

The highest percentage weight gain 256.66 ± 1.82 was found in 2% fenugreek seed added diet followed by 1% fenugreek seed kg⁻¹ of diet while the lowest 240.50 ± 2.45 percentage weight gain (Table 1) was obtained in the control group. Results obtained in this study, were consistent with the result of studies of Sahu et al. (2007); Maqsood et al. (2009).

Data of specific growth rate (SGR), Feed Conversion Ratio (FCR) and Protein Efficiency Ratio (PER) presented in Table 2 show that the 2% fenugreek dietary supplementation significantly enhanced the SGR, 1.41 ± 0.007 and lowered FCR 3.27 ± 0.022 of *Cyprinus carpio* when compared with the fish fed on control diet. Fenugreek-supplemented diet improved the growth performance which agreed with

findings of Abdel-Zaher et al. (2009) who reported that fenugreek seed as feed additive improved growth parameters in Nile tilapia fingerlings. This observation is also in agreement with the finding of Tonsy et al. (2011).

The highest protein efficiency ratio of 0.531 ± 0.03 was observed in 2% fenugreek diet. Results recorded were found consistent with the results of previous studies of Nikki et al. 1991; Ahmed et al., 2009. The beneficial effect of fenugreek seeds may be due to its contents of active materials according to Dixit et al. (2005) who reported that fenugreek seeds have antioxidant activity. Also, they reported that different fractions of the germinated fenugreek seeds were used to determine their antioxidant potential at different levels. So, our study also signifies the antioxidant activity in fenugreek seeds which may be due partly to the presence of flavonoids and polyphenols.

The data of percentage survival rate is presented in Table 3 in which the highest survival percentage (98.34%) was found when fed with 2% diet while lowest survival percentage (66.67%) was found when fed with 0.5% diet compared to control diet. Gopalakannan and Venkatesan (2006) observed similar results when fish were fed with chitin, chitosan and levamisole for a period of 90 days and then challenged with *Aeromonas hydrophila*. The

Table 2. Effect of Fenugreek supplemented diet on Specific Growth Rate (SGR), Feed Conversion Ratio (FCR) and Protein Efficiency Ratio (PER) of Common Carp

Treatment	SGR	FCR	PER
Control	1.32 ± 0.009	3.54 ± 0.055	0.501 ± 0.004
0.5%	1.36 ± 0.007	3.38 ± 0.025	0.514 ± 0.003
1%	1.37 ± 0.007	3.43 ± 0.012	0.513 ± 0.001
2%	1.41 ± 0.007	3.27 ± 0.022	0.531 ± 0.003

Table 1. Effect of Fenugreek supplemented diet on percentage weight gain of Common Carp.

Treatment	Initial weight (g)	Final weight (g)	Weight Gain (g)	% Weight Gain
Control	12.18	39.80	27.612	226.65 ± 2.32
0.5%	12.14	41.35	29.203	240.50 ± 2.45
1%	12.17	41.91	29.74	244.30 ± 1.90
2%	12.25	43.68	31.43	256.66 ± 1.82

highest growth was recorded in the chitosan fed fish 94.92 ± 9.36 g followed by levamisole 93.25 ± 8.4 g and chitin (63.54 ± 4.7 g). In salinity stress experiment of Roohi et al. (2015), highest survival rate was seen in fish fed 1% fenugreek supplemented diet. Thus these results indicate that fenugreek seeds can be considered as a beneficial dietary supplement for improving the growth performance and blood indices of common carp fingerling.

The increase in total serum protein, albumin and globulin content in experimental groups as compared to control group was observed from the results (Table 4). The highest level of serum protein (3.448 ± 0.017) was found when feed with 2% diet. Thus in our study, total protein of serum was significantly enhanced in all experiments compared

to control ($p < 0.05$) which agrees with findings of Abdel-Zaher et al. (2009). Mohammad et al. (2009) also recorded higher value of total serum protein as compared to control when external food supplement was added in fish diet. The highest level of serum albumin (1.636 ± 0.003 g dL⁻¹) was found when feed with 2% diet while the levels of serum albumin (1.913 ± 0.003 g dL⁻¹) and globulin (2.22 ± 0.09 g dL⁻¹) were found highest at 2% diet during post-challenge. Nayak et al. (2004) reported that the increase in serum total protein indicates that fish are immunologically strong. Rairakhwada et al. (2007) also found that total serum protein content was significantly enhanced in levan fed common carp fingerlings against the infection of *A. hydrophila* while the depressed values were found in control (infected) group. Similar results were obtained by Bruno & Munro (1986) in rainbow trout and Atlantic salmon experimentally infected with *Renibacterium salmonarium*.

The highest hemoglobin content of 9.96 ± 0.06 units and lower hemoglobin content of 8.13 ± 0.08 were recorded with diet supplemented with 2 and 0.5% fenugreek respectively. Post-challenge, the highest hemoglobin content was 9.46 ± 0.46 fenugreek is 1% of diet. Total erythrocyte and leucocyte counts were found highest at 3.21 ± 0.08 g dL⁻¹ and 2.79 ± 0.08 g dL⁻¹ respectively with 2% diet. These results are consistent with the results of Kabir et al. (1978);

Table 3. Effect of Fenugreek supplemented diet on survival of Common Carp by challenge *A. hydrophila*

Treatment	Total no. of fishes	No. of mortalities	Survival (%)
Control	60	35	41.00
0.5%	60	20	66.67
1%	60	12	80.00
2%	60	7	98.34

Table 4. Effect of Fenugreek supplemented diet on Total Serum Protein, Albumin and globulin content of Common Carp by challenge *A. hydrophila*

Treat.	Protein content (g dL ⁻¹)		Serum albumin (g dL ⁻¹)		Globulin level (g dL ⁻¹)	
	Pre-challenge	Post-challenge	Pre-challenge	Post-challenge	Pre-challenge	Post-challenge
Control	1.66 ± 0.14	1.74 ± 0.15	0.78 ± 0.03	0.78 ± 0.01	0.83 ± 0.01	0.94 ± 0.06
0.5%	2.21 ± 0.06	2.62 ± 0.18	1.04 ± 0.03	1.11 ± 0.04	1.07 ± 0.07	1.50 ± 0.09
1%	2.40 ± 0.19	3.23 ± 0.18	1.37 ± 0.06	1.58 ± 0.04	1.59 ± 0.03	1.64 ± 0.06
2%	3.44 ± 0.17	4.13 ± 0.20	1.63 ± 0.03	1.91 ± 0.03	1.81 ± 0.03	2.22 ± 0.09

Table 5. Effect of Fenugreek supplemented diet on Hb, TEC and TLC of *Cyprinus carpio* by challenge *A. hydrophila*

Treatment	Hb content (g)		TEC (million cells/mm ³)		TLC (million cells/mm ³)	
	Pre-challenge	Post-challenge	Pre-challenge	Post-challenge	Pre-challenge	Post-challenge
Control	6.53 ± 0.08	6.23 ± 0.15	1.94 ± 0.15	1.92 ± 0.02	2.61 ± 0.08	2.63 ± 0.11
0.5%	8.13 ± 0.08	7.83 ± 0.29	2.29 ± 0.08	2.13 ± 0.15	2.63 ± 0.08	2.77 ± 0.11
1%	8.83 ± 0.03	8.20 ± 0.46	2.53 ± 0.05	2.40 ± 0.05	2.73 ± 0.12	2.97 ± 0.08
2%	9.93 ± 0.06	9.46 ± 0.43	3.21 ± 0.08	2.66 ± 0.08	2.79 ± 0.08	3.01 ± 0.06

Table 6. Effect of Fenugreek supplemented diet on lysozyme and Nitrobluetetrazolium (NBT) assay.

Treatment	Lysozyme activity (U ml ⁻¹)		NBT activity	
	Pre-challenge (80 days)	Post-challenge (10 days)	Pre-challenge (80 days)	Post-challenge (10 days)
Control	44.330 ± 0.363	44.250 ± 0520	0.5420 ± 0.0003	0.6540 ± 0.001
0.5%	43.750 ± 0.629	54.160 ± 0506	0.5460 ± 0.001	0.640 ± 0.0005
1%	45.580 ± 0546	65.160 ± 0585	0.8913 ± 0.0006	0.9890 ± 0.004
2%	56.330 ± 0.463	83.160 ± 1.12	0.1126 ± 0.004	0.1660 ± 0.004

Rigney et al. (1978) and Bradley et al. (1979) in which TEC, HCT and Hb contents in the control group were lower when compared with the levamisole supplemented groups which may be attributed to activation of the alternative complement pathway by lipopolysaccharide, increased phagocytosis of endotoxins-coated red blood cells and direct lysis by the bacterial toxins or enzymes. In post-challenge, TEC and LEC were found highest 2.66 ± 0.08 and 3.01 ± 0.06 respectively in diet supplemented with 2% fenugreek. Similar findings were reported by Dina et al. (2007) who concluded that TEC and hemoglobin contents were significantly enhanced in the 0.2% levan fed group after the fish were challenged with *A. hydrophila*.

Data represented in Table 6 shows that at 2% fenugreek supplementation the lysozyme activities were 56.330 ± 0.463 and 83.160 ± 1.12 U mg⁻¹ for pre-challenge and post challenge groups respectively. Robertsen et al. (1994) showed that an increased protection against fish bacterial infection correlated to an increment in the serum lysozyme levels, phagocytic activity and bacterial activity of the head kidney leukocyte. Siwicki (1989) described the immunostimulatory activity of levamisole in carp spawners, with treated fish displaying elevated leucocyte and neutrophil numbers and also increase in the lysozyme levels and natural antibody titres.

Studies on neutrophil activity presented in Table 6 clearly showed the enhancing effect of dietary fenugreek supplement on neutrophil respiratory burst activity, which is evident from the increased NBT reduction. Diet supplemented with 1% fenugreek showed maximum increase in the NBT reduction value of 0.8913 ± 0.0006 and 0.9890 ± 0.004 in pre challenge and post challenge groups respectively. Kumari & Sahoo (2006) also demonstrated that respiratory burst (NBT) activity in levamisole fed group was found significantly higher as compared to the CYP-treated control group. Awad & Austin

(2010) also recorded increase in NBT activity as compared to control when external food supplement was added in fish diet.

In conclusion, the findings of this study suggest that fenugreek seeds at 2% dose is beneficial as dietary supplement for improving growth performance, biochemical and blood parameters in common carp fingerlings.

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