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Effect of *Trigonella foenum graecum* seed as feed additive on growth, haematological responses and resistance to *Aeromonas hydrophila* in *Cyprinus carpio* fingerlings

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Abstract
A study was conducted to assess the potential effects of *Trigonella foenum graecum* (fenugreek), as a feed additive on the growth performance, immuno-haematological responses and protection against the bacterial pathogen, *Aeromonas hydrophilla* in *Cyprinus carpio* fingerlings. A control diet and a series of test diets incorporating fenugreek seed at 0.5%, 1% and 1.5% were prepared and fed to the fish daily for eight-weeks. Growth (weight gain, specific growth rate, protein efficiency ratio, feed conversion ratio), haematological (WBC, RBC, haematocrit, haemoglobin content) and biochemical parameters (serum total protein, albumin, globulin, albumin: globulin ratio, blood glucose) were examined among the various treatment groups. The results showed that fenugreek seed as an additive significantly improved survival, weight gain and feed conversion ratio as well as reduced mortalities in the treated groups as compared to the control. Significant increase in erythrocytes, leucocytes, haematocrit, and haemoglobin were observed in treated fish. Significantly higher ($p<0.05$) serum protein and globulin levels were observed in treated fish groups over the control. Moreover, feeding fenugreek based diet resulted in a reduction in serum glucose in treated fish. In conclusion, fenugreek seed as a feed additive led to the improvement of growth and enhanced the immunity of *Cyprinus carpio* against *Aeromonas hydrophilla* infection which is an indication that it could be used as an alternative to antibiotics.

Keywords: *Trigonella foenum graecum; Cyprinus carpio*; *Aeromonas hydrophila*; immune parameters

1. Introduction
Cyprinus carpio commonly known as Common Carp is an important aquaculture candidate species owing to its high demand, fast growth rate, easy availability, wide distribution and commercial importance. The common carp is very versatile, and can live in a variety of habitats including highly degraded areas. It is highly esteemed in the state of Jammu and Kashmir and commands very high commercial value in the market due to its ability to adapt readily to poor conditions, acceptability and high conversion of artificial feed, tolerance to crowded conditions and high quality of its flesh. It has been reported that the intensive farming of the common carp causes stressful environment for the fish, resulting in suppression of their immune response; subsequently leading to disease outbreaks. Common carp is susceptible to many diseases like haemorrhagic septicaemia, or red disease caused by *Aeromonas hydrophilla*. Antibiotics are used to preclude the infections in aquaculture (Subasinghe, 2009) [20], which have undesirable effects on the bacterial population within the aquatic ecosystem (Lalumera *et al.* 2004; Wei *et al.* 2010) [10, 27]. Keeping in mind the adverse effects of antibiotics; their use is not recommended in the aquaculture practices. Therefore, for sustainable development of the aquaculture sector, there is a need to develop alternative therapies for bacterial pathogens that could effectively protect the animals and prevent resistance development of microbes (Defoirdt, *et al.* 2011) [7].
The use of immunostimulants is considered a promising preventive practice that may help to maintain animal welfare and a healthy environment, while increasing production and providing higher profits. Attempts to use the natural materials such as medicinal plants could be widely accepted as feed additives to enhance efficiency of feed utilization and animal productive performance (Mohamed, *et al.* 2003) [14]. Immunostimulant plants are generally recognised as safe; they stimulate the non-specific cellular and humoral defence mechanism in fish and can be ideal alternatives as a source of feed additives for preventing infectious diseases in aquaculture (Maqsood, *et al.* 2011; Talpur and Ikhwanuddin, 2012) [13, 23]. Therefore, attention is being paid to the use of plant based immunostimulants for disease-control measures in aquaculture.

Fenugreek seeds have a long history in Ayurvedic, traditional Chinese, and Arabic Medicine. They have been used for two main pharmacological properties, antidiabetic and hypocholesterolaemic activities (Devi *et al*. 2003; Kumar *et al*. 2005) [8, 21]. They have long been confirmed to have antimicrobial effects (Bhatti *et al*. 1996) [3], antioxidant effects (Mansour and Khalil, 2000) [12] and anti-allergic effects (Thiel, 1997) [26]. Fenugreek seed are also anti-inflammatory (Liu *et al*. 2012) [11] and antipyretic (Ahmadiani *et al*. 2001) [1]. Considering the beneficial effects of fenugreek seed, this study was conducted to evaluate its effect as a feed additive on growth, survival and haematological responses of *Cyprinus carpio* fingerlings challenged with *Aeromonas hydrophilla*.

2. Materials and Methods

2.1 Experimental Fish

Fingerlings of *Cyprinus carpio* of average weight 12.0 ± 2.0 g were purchased from the fish farm of Faculty of Fisheries located at Shuhama campus of Shere Kashmir University of Agricultural Sciences and Technology of Kashmir. In order to ameliorate the handling stress, the fishes were given a mild salt and $KMnO_4$ treatment. The stock was acclimatized under aerated conditions for a period of two weeks. During acclimatization period, the fingerlings were fed with basal diet. The feeding was stopped 24 hours before the commencement of experiment.50% water exchange was done daily and water quality was monitored daily throughout the experimental duration. The temperature was maintained at 16.5 ± 1 °C, dissolved oxygen concentration of 6.0 ± 1 mg l^{-1}, pH 7.5 ± 0.5. Four experimental diets were formulated for the fish; three of them contained fenugreek seed at 0.5%, 1% and 1.5% concentration while as the control group was without any fenugreek supplementation. The fish were fed with treated diets at 4% of body weight in two equal parts twice a day at 10.00 a.m. and 6.00 p.m. Control group was fed with basal diet at the same per cent throughout the experiment.

2.2 Preparation of experimental diet

Ingredient constitution of the basal diet includes fish meal, soybean meal, rice bran, wheat bran, mustard oil cake, wheat flour and vitamins and minerals (pre mixture). Fenugreek seed were purchased from the local market. The whole seed were crushed into powder form using a household electric grinder, and mixed directly with fish feed contents to achieve three modified diets at 0.5%, 1% and 1.5% inclusion rates and control was without fenugreek seed.

2.3 Experimental set up

The fingerlings of *Cyprinus carpio* (12.0 ± 2.0 g) were equally distributed into four experimental groups in triplicate (3 treated and 1 control) based on fenugreek application following a completely randomised design (CRD) using 12 round plastic tanks filled with freshwater and equipped with aeration. Control group was fed with basal diet only while as the treated groups were fed fenugreek seed at 0.5%, 1% and 1.5% inclusion rates. The growth performance including percentage weight gain, specific growth rate (SGR) and feed conversion ratio (FCR) for each group were recorded. After feeding the fish with fenugreek diets for eight weeks, 10 fish from each treatment were randomly selected for the challenge assay. The fish were challenged with *Aeromonas hydrophilla* in intraperitoneal injection with 0.2 ml^{-1} suspensions of 0.9% (w/v) saline.

2.4 Blood collection for haematological and immunological parameters

At the end of the sampling, two fishes were collected from each replicate and anaesthetized with clove oil @ 50 µL l^{-1}. Blood was collected from caudal puncture, using a medical syringe which was previously rinsed with 2.7% EDTA solution. The parameters like total protein, serum glucose, albumin globulin ratio, total erythrocyte count, total leucocyte count, haemoglobin and packed cell volume were recorded.

2.5 Statistical analysis

Results were expressed as the mean ± standard deviation (SD). Data were analysed using one-way ANOVA and differences were considered statistically significant when $p < 0.05$. The comparison of the mean values was done by using Duncan multiple range tests using software program SPSS version 16 for Windows.

3. Results

3.1 Growth performance

Significantly improved weight gain was observed among all the treated groups. Highest weight gain (40.50±0.04) was observed in fish fed with fenugreek seed at 1% (Table 1 & Fig.1a). Similarly SGR, PER and FCR were significantly ($p < 0.05$) improved in the fish groups fed with fenugreek based diet compared to the control (Table 1 & Fig.1b, c, d).

Weight gain (WG %) = 100 x (final weight – initial body weight)/ initial body weight

Specific growth rate (SGR) = 100 x ln (final weight - initial body weight)/duration of experiment

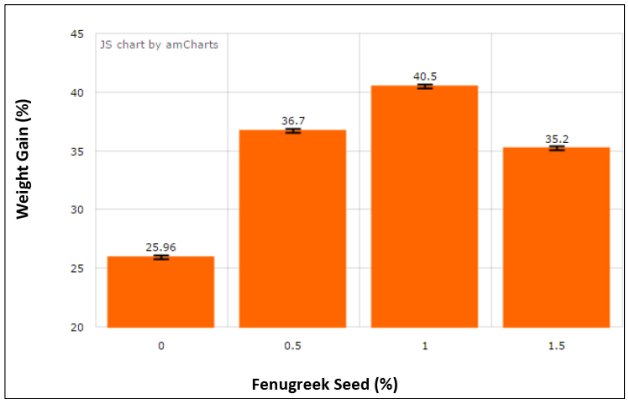
Feed conversion ratio (FCR) =Feed consumed (g, dry weight)/weight gain (g)

Protein efficiency ratio (PER) =weight gain (g)/protein intake (g)

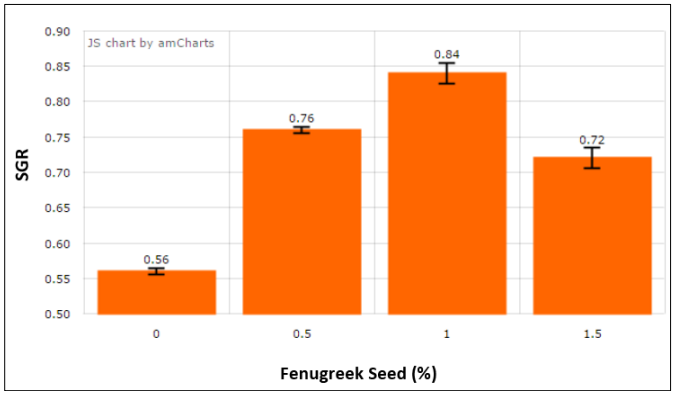
Table 1: Growth parameters of *Cyprinus carpio* fingerlings fed with different levels of fenugreek seed for eight weeks.

Fenugreek seed (%)	Weight Gain %	SGR	FCR	PER
Control	25.96^{a}±0.16	0.56^{a}±0.01	1.60^{d}±.01	1.30^{a}±0.01
0.5	36.70^{b}±0.05	0.76^{b}±0.01	1.40^{c}±0.03	1.71^{b}±0.01
1.0	40.50^{c}±0.04	0.84^{c}±0.03	1.00^{a}±0.01	2.00^{c}±0.03
1.5	35.20^{b}±0.01	0.72^{d}±0.03	1.20^{b}±0.02	1.68^{b}±0.03

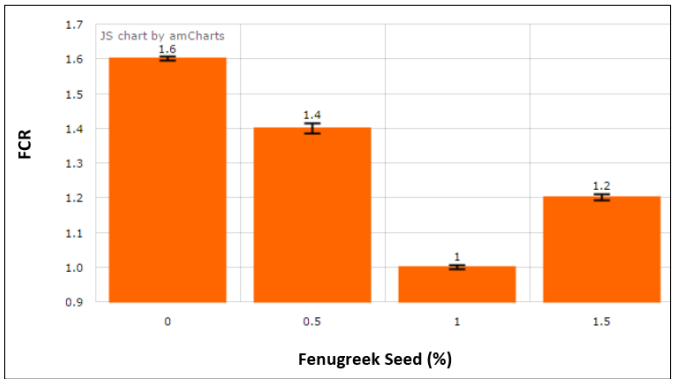
Data were presented as mean±SE (n=3).
SGR-Specific growth rate, FCR-Feed conversion ratio, PER-Protein efficiency ratio.
Values with different superscripts indicate significant difference.



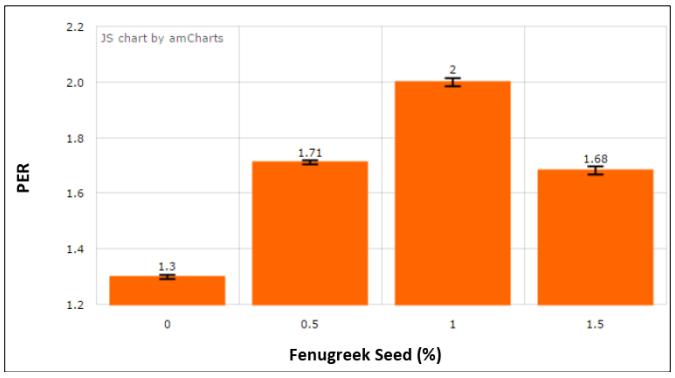
a) Weight Gain (%)



b) Specific Growth Rate (SGR)



c) Feed Conversion Ratio (FCR)

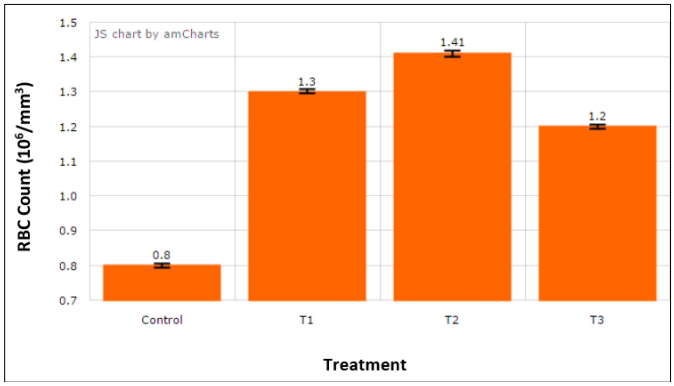


d) Protein Efficiency Ratio (PER)

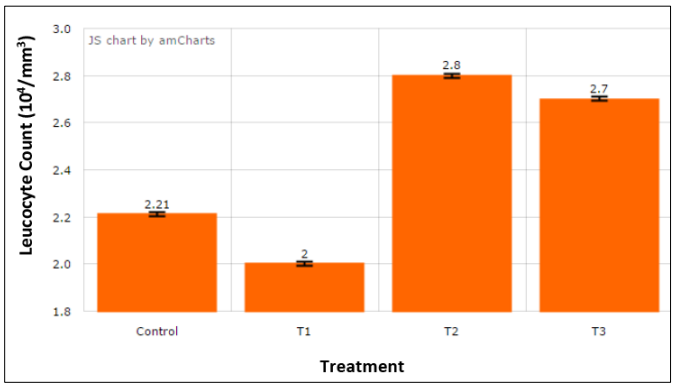
Fig 1: Growth parameters of *Cyprinus carpio* fingerlings fed with fenugreek based diet at different levels for eight weeks (a) Weight Gain(%), (b) Specific Growth Rate (SGR), (c) Feed Conversion Ratio(FCR) and (d) Protein Efficiency Ratio(PER) Data were expressed as mean ± SD; n = 6. Bars having different superscripts are significantly different (P<0.05).

3.2 Haematological analyses

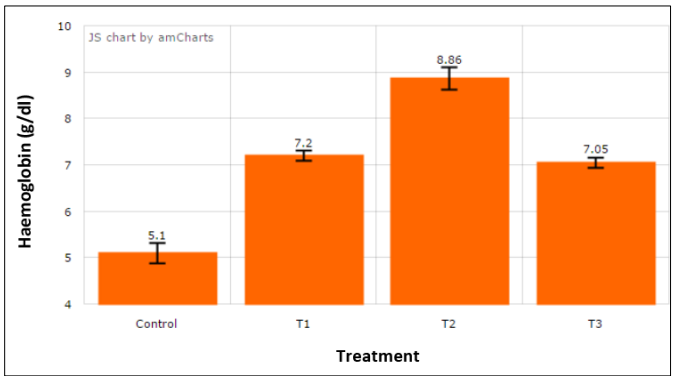
The number of erythrocytes (RBC) and leucocytes (WBC) was significantly higher ($p< 0.05$) in fish that were fed with fenugreek diets as compared to the control. Highest number of RBCs ($1.41\pm0.02\times10^{6}$/ mm^3) was observed in those fish that received fenugreek at 1% inclusion rates. The highest no. of circulatory leucocytes ($2.80^{c}\pm0.01\times10^{4}/mm^3$) were recorded in the same treatment. (Fig.2a, b).The haemoglobin content was highest (8.86±0.46 g/dl) in the fishes fed with fenugreek at 1%, which was significantly higher than the other groups, while as lowest haemoglobin was recorded in the control (Fig. 2c).There was a significant ($p < 0.05$) increase in haematocrit values in those fish fed with the fenugreek based diet as compared to the control (Fig. 2d).



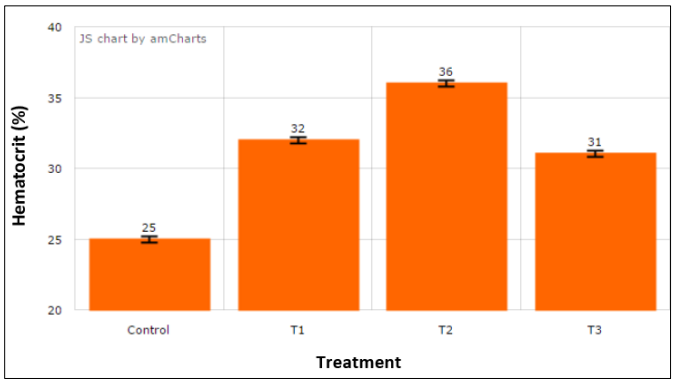
a) RBC count (106/ mm3)



b) Leucocyte count (104/mm3)



c) Haemoglobin (g/dl)



d) Hematocrit (%)

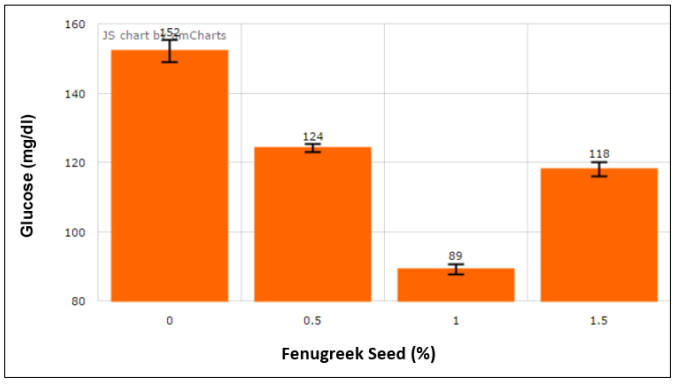
Fig 2: Haematological parameters of *Cyprinus carpio* fingerlings fed with fenugreek based diet at different levels for eight weeks (a) RBC, (b) Leucocyte, (c) Haemoglobin (g/dl) and (d) Haemotocrit (%) Data were expressed as mean ± SD; n = 6. Bars having different superscripts are significantly different (P<0.05)

3.3 Biochemical indices of blood

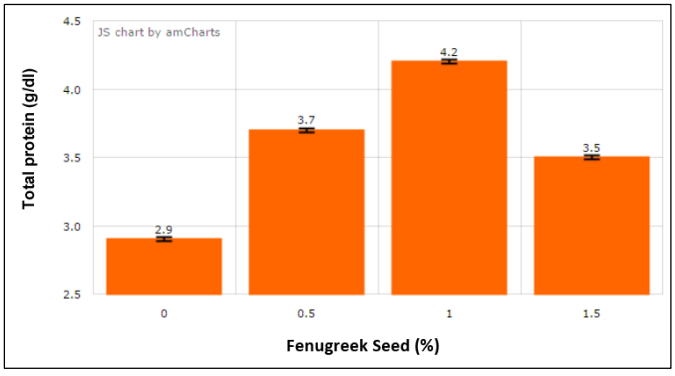
There was a significant difference in the plasma glucose level observed in the various fish groups fed with graded level of fenugreek seed as compared to the control. Lowest serum glucose (89.00±3.21 mg/dl) was recorded in the fish which were fed 1% fenugreek seed while as highest serum glucose (152.00±6.42 mg/dl) was found in the control group (Fig.3a). The highest total protein (4.2±0.01g/dl)content was recorded in those fish groups that were fed with fenugreek based diet at 1% inclusion levels (Fig. 3b). Serum albumin content was significantly higher in all the treatments as compared to the control (Fig. 3c). Also, significantly ($p < 0.05$) higher globulin levels were observed in fish group that were fed with various fenugreek based diets as compared to the control (Fig. 3d).Highest Albumin: globulin ratio was recorded in those fish groups that were fed with fenugreek based diet at 1% inclusion levels.

Table 2: Blood/serum biochemical parameters of *C.carpio* fed with fenugreek based diet at various inclusion levels.

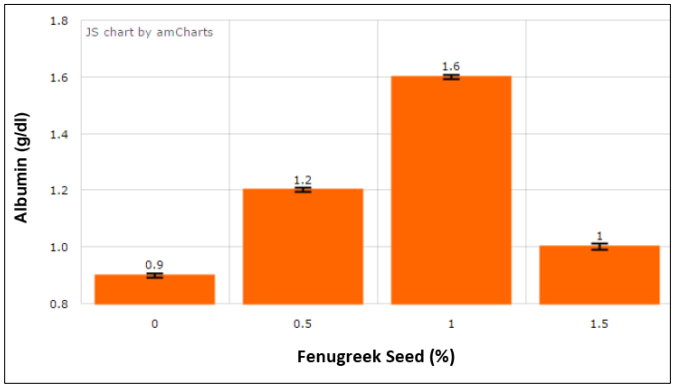
Parameter	Fenugreek seed (%)	Value
Glucose (mg/dl)	Control	152.00^{c}±6.42
	0.5	124.00^{b}±2.30
	1	89.00^{a}±3.21
	1.5	118.00^{b}±4.16
Total protein (g/dl)	Control	2.90^{a}±0.01
	0.5	3.70^{c}±0.02
	1	4.20^{d}±0.01
	1.5	3.50^{b}±0.01
Albumin (g/dl)	Control	0.90^{a}±0.003
	0.5	1.20^{c}±0.002
	1	1.60^{d}±0.003
	1.5	1.00^{a}±0.002
Globulin(g/dl)	Control	2.00^{a}±0.10
	0.5	2.50^{b}±0.11
	1	2.80^{d}±0.10
	1.5	2.50^{b}±0.10



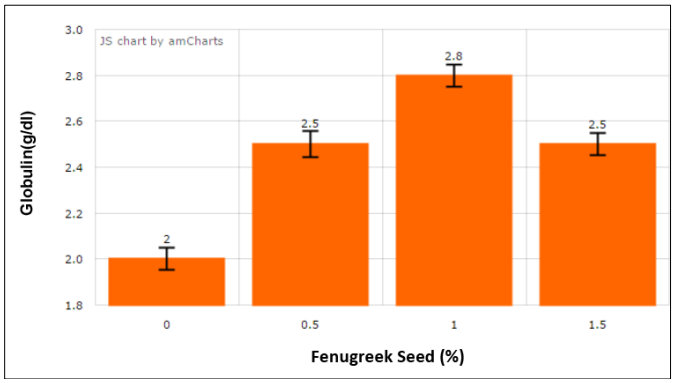
a) Glucose (mg/dl)



b) Total Protein (g/dl)



c) Albumin (g/dl)



d) Globulin (g/dl)

Fig 3: Bio-chemical parameters of *Cyprinus carpio* fingerlings fed with fenugreek based diet at different levels for eight weeks (a) Glucose (mg/dl), (b) Total Protein (g/dl), (c) Albumin(g/dl) and (d) Globulin (g/dl) Data were expressed as mean ± SD; n = 6. Bars having different superscripts are significantly different (P<0.05)

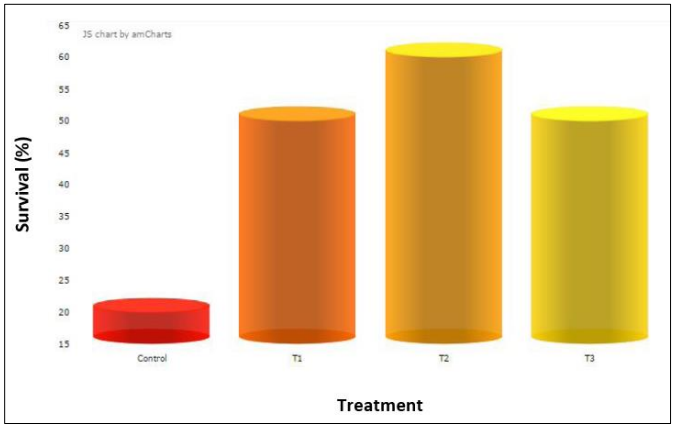


Fig 4: Percentage survival of *Cyprinus carpio* fingerlings of different experimental groups challenged with *Aeromonas hydrophila*

3.4 Resistance against infection

Feeding of the modified diets for eight weeks led to an increase in survival of the common carp fingerlings after challenge with *Aeromonas hydrophilla*. Fish fed with fenugreek seed at various inclusion levels showed significantly ($p<0.05$) higher survival compared to the control. Highest mortality was observed in the control group while as lowest mortality was observed in the group fed with 1% fenugreek seed.

After challenge with *Aeromonas hydrophila*, mortality of fingerlings was recorded over a period of ten days. There was no mortality up to the first twelve hours. Further, till 8th day

there was no mortality. Lowest survival was recorded in the control compared to the treatment groups. The relative percentage survival (RPS) was significantly higher ($P<0.05$) in T_2 group compared to control group.

4. Discussion

In intensive fish farming, fish are mostly affected by stressful conditions which further lead to restrain the immune response and cause infections to occur (Kumari and Sahoo, 2005) [9]. Use of various chemotherapeutants and antibiotics poses the risk of generating resistant pathogens, bio-accumulation and environmental pollution (Raa *et al*. 1992) [22]. Immunostimulants can enhance the non-specific immune response and have broad-spectrum activity, which is helpful in the mitigation of fish diseases, and thus can be used as alternative prophylactic and therapeutic agents in aquaculture practices (Nya and Austin, 2009; Talpur and Ikhwanuddin, 2012) [16, 23]. Medicinal herbs are desirable for stimulating digestion, and have the highest stimulatory influence particularly on bile secretion and pancreatic enzymes activity (Platel, *et al*. 2002) [17]. Moreover, medicinal herbs and their extracts have shown their potential as growth-promoting agents in aquatic animals (Citarasu *et al*. 2002; Sivaram *et al*. 2004) [5, 19].

Present study revealed that feeding of fenugreek seed significantly increased the growth rates and survival of *Cyprinus carpio* fingerlings and also conferred resistance to challenge with *Aeromonas hydrophilla* in terms of better survival. The results of the present study are in agreement with the studies of Nya and Austin (2009) [16], and Talpur and Ikhwanuddin (2012, 2013) [23, 24] who fed garlic diet and ginger diet to rainbow trout and *L. calcarifer* respectively for two weeks and observed significantly higher growth rates in treated fish over the control. Billaud and Adrian (2001) [4] observed that fenugreek seeds contain lysine and L-tryptophanrich proteins, mucilaginous fibre and the rare chemical constituents such as saponins, coumarin, fenugreekine, nicotinic acid, sapogenins, phytic acid, scopoletin and trigonelline, which are thought to account for many of its presumed therapeutic effects.

In the present study, significant difference in the weight gain and specific growth rate among all the treatments was observed. The highest weight gain and specific growth rate was found in the treatment fed with fenugreek seed at 1% while as lowest weight gain and specific growth rate was found in the control group. This indicates that fenugreek seed has a beneficial effect on the growth of the common carp fingerlings. The enhanced growth performance of fish can be correlated to an increase in digestive enzyme activity and low metabolic needs induced by the fenugreek seed. Similar results were observed by Zheng (2009) [29], that supplementation of oregano essential oil increased the growth, antioxidant effect and resistance against *Aeromonas hydrophila* in channel catfish (*Ictalurus punctatus*). The groups fed with fenugreek based di*et al*so exhibited significantly higher protein efficiency ratio as compared to the control. Present results are in agreement with the work of Mohammad *et al*. (2011) [15], who observed that feeding caraway seed meal to *Oreochromis niloticus* fingerlings, improves growth performance, protein efficiency ratio, feed utilization and whole body composition of the fish.

Immunostimulant plants affect the haematological parameters and bactericidal activity of fish (Nya and Austin, 2009; Talpur and Ikhwanuddin, 2012, 2013) [16, 23, 24]. In the present study, the number of RBCs and WBCs were significantly higher in the treated groups as compared to the control. Sahu *et al*. (2007) [18] observed higher RBC count by dietary incorporation of mango kernel in *Labeo rohita* fingerlings as compared to control. Increase in the WBC count depicts that carp fed with fenugreek based diet showed signs of an altered immune response when compared to control group. In post-challenge, WBC count increased and RBC count decreased in all the experimental groups. These findings are in concurrence with those of Talpur and Ikhwanuddin (2012, 2013) [23, 24], who also determined an increase in WBC count after feeding garlic-added feed, ginger diet and neem leaf-diet to *L. calcarifer*.

Increase in haemoglobin contents in the blood indicates that oxygen supply increases thus, improved well-being of fish (Talpur and Ikhwanuddin, 2012) [23]. There was a slight decrease in Hb (g%) in post-challenge as a result of stress because of induced pathogen which might have damaged tissues (Talpur and Ikhwanuddin, 2012) [23]. The results of the present study demonstrate that plasma glucose level reduced significantly in treated groups as compared to the control, which depicted the highest serum glucose. This is in agreement with the reports of Sahu *et al*. (2007) [18] and Citarasu *et al*. (2010) who found that glucose levels were reduced in the aquatic animals fed on herbal immunostimulant diets. During post challenge, the serum glucose increased in all the groups, but the groups which were fed with the fenugreek diet exhibited least value as compared to the control. As there was less serum glucose in the treatments than control, even after challenging with *Aeromonas* isolates, it can be inferred that addition of fenugreek seed in the diet reduces stress in *Cyprinus carpio* fingerlings.

Increase in the serum protein, albumin and globulin levels is thought to be associated with a stronger innate immune response in fish (Wiegertjes *et al*. 1996) [28]. Our results also show an increase in the serum proteins, albumin, globulin and A/G ratio in all experimental diet groups throughout the experimental days.

In the challenge study with pathogenic isolates of *Aeromonas hydrophilla*, the highest relative percentage of survival was observed in the group fed with 1% fenugreek seed (60%), while lowest in the control group. This suggests that supplementation of fenugreek seed in the feed of *Cyprinus carpio* has a positive influence on the survival of *Cyprinus carpio* by resisting *Aeromonas hydrophila.* The decline in fish mortalities among the treated groups as compared to the control could be the result of immune system activation against various pathogens as well as opportunistic bacterial invaders. This is supported by Talpur (2014) [25] who found that peppermint improves the immune response and disease resistance of *Lates calcarifer* against *Vibrio harveyi* infection.

In conclusion, the results of this study indicate that fenugreek seeds can be considered as a beneficial dietary supplement for improving the growth performance and hematological parameters of common carp fingerlings. The use of fenugreek seed as an additive in fish feed needs to be promoted for reducing the losses caused by outbreak of diseases in aquaculture practices.

5. Acknowledgment

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Kashmir University of Agricultural Sciences and Technology (SKUAST-K)

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