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Evaluation of improvement in immunity after application of dietary *Azolla pinnata*, a study of blood serum parameters of *Cyprinus carpio* fingerlings after challenge of *Aeromonas hydrophila*

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Abstract:

The development in aquaculture sector plays major contribution in nations economy. So in fish market the demand for nutrient rich and disease free fishes increasing rapidly. but in another hand major problem arising due to low nutritional level of supplementary food and in other hand major loss occurred due to Fish diseases and the alternative diet used for fish health improvement are mostly costly to can't afford by small scale farmers . The disease outbreak is the sign of lower immune level of fishes due to nutritionally low diet so there is need to develop immunemodulater and best nutritive supplement. so present investigation were focused upon to formulate low coast fish feed with high nutritive value and immune modulator. All the fishes were put in portable plastic pools each portable plastic pools stacked with 15 fishes those were fed with *Azolla pinnata*. Significant results were obtaining in diet containing 350 and 400 g per kilo gram of basal feed. also its shows the greater inhibitory activity against fish pathogens *Aeromonas spp*.

Index Terms: *Cyprinus carpio, , Azolla pinnata ,Aeromonas sp. Fish Feed*

I. Introduction

The Aquaculture sector is expanding it horizons by means of production of food so it been the utmost prospective to convene demand for aquatic food. [1]

Now a days the fish culture in small scale farm level increased .and hence need to produce out nutritional rich and well grown fishes for food purpose. Hence to achieve these goals many farmers using herbal base of feed, for this many protein basis's used with their potential as alternatives to fishmeal in fish culture. Also these diets are without affecting the growth performance of fish [2]

On the other hand, fishes of Cyprinidae family have information about different ranges of Azolla addition levels in fish feed. out of which most of the experiments were shows that feed utilization and boosted growth in *cyprinus carpio*at 10-50 percent of Azolla rich fish feed [3)

Further Azolla is rich with high crude protein ranges 19-30 percent .so that used in fish farm [4] experimental data shows that azolla plant contain protein level up to is 23—30 percent most of are of amino acid and ranges 55 percent [5]

Azolla is a kind of water fern which have tiny branches which floats on water surface, roots remain deep in water. The most extraordinary feature of azolla, it shows symbiosis with nitrogen fixing blue green algae. [6]

So that in present investigation we were used Dry *Azolla pinnata* powder with soybean based diet. In present investigation in the results obtained evidently confirms for utilization of *Azolla maxicana* extract against various fish pathogens. The major aim of investigation to find growth promoting factor and immune modulation in *cyprinus carpio* fingerlings. Here we had chosen *cyprinus carpio* fingerling as test animal so as this formula can be used for wide variety of fresh water fishes having demand in food market.

II. Material and methods

1. **Preparation of Azolla rich diet:** and antibacterial extract preparation

The fish feed was prepared out were shown in table 1 here we were used locally available fish feed ingredients along with autoclaved soybean to reduce amount of anti- nutritional factors , rice bran, wheat and vitamin premix also used with groundnut oil cake. *Azolla pinnata* were sun dryed first and used in powdery form in fish diet. Here we made small pellets so as to became easy to consume fish fingerlings. The macronutrient analysis of *Azolla pinnata were done by* using standard method (AOAC, 2005) [7] the The *Azolla pinnata* powder shows following macronutrients level. Shown in figure 1.further azolla maxicana samples were prepared by using Soxhlet were used for antibacterial study.

2. Serum collection:

For serum collection, another five fishes from each portable plastic pools were anesthetized and collected blood without using anticoagulant it allocated clotting up to for two hour. Compilation of serum with a micropipette and stored (at -20^0C).

2.1: Serum proteins:

Protein kit (Biuret method) used for Serum total protein and albumin determined with the help of the albumin kit -BCG dye-binding method- (Merck). Globulin was calculated by subtracting the albumin values from total protein. Globulin (g%) = total protein(g%) - albumin(g%)

A/G ratio was calculated by dividing albumin values by globulin values.

2.2: Activity of Serum Lysozyme:

Serum lysozyme activity was deliberate by the turbidimetric assay of Parry [4] with the microplate version of Hutchinson/Manning. 0.03% lyophilized *M. lysodeikticus* in 0.05 M, sodium phosphate buffer (pH 6.2) was employed as a substrate. Serum (0.01ml) was added to 0.25 ml of bacterial suspension in a micro plate and the decrease in the absorbance (490 nm) was resolute after 0.5 and 4.5 min incubation (22^0C) using a micro plate reader.

2.3: Serum bactericidal activity and antagonistic study :

Serum bactericidal activity was analyzed for this bacterial culture of *Aeromonas hydrophila* was centrifuged. The OD (optical density) of the bacterial suspension was adjusted. This suspension was then serially diluted 1:10 with PBS five times. Challenge study with *Aeromonas hydrophila* was full-grown on nutrient broth (24 hours at 28°C). After 24 hours the bacterial culture was centrifuged at 3000 rpm for 10 minutes. The pellet was washed three times in PBS (pH – 7.4) and again mixed in PBS and the final concentration was adjusted to 1.6×10^7 CFU ml^{-1}. After 8 weeks experimental period, fishes in all the experimental groups were injected intraperitoneally with 0.2ml of *Aeromonas hydrophila.* survival was calculated using the following formula

$$\textbf{RPS (Relative \% Survival rate)} = \frac{\text{No. of survive fish subsequent to challenge}}{\text{No. of fish injected with pathogen}} \times 100$$

3 : Statistical analysis:

One way ANOVA at a 5% probability level Were used in present investigation

III. RESULTS:

1: Serum total protein against *Aeromonas spp.:*

The serum total protein content of *Cyprinus carpio* fingerlings of different experimental groups is presented in Table 2 The serum total protein content of different experimental groups showed a significant difference ($P\leq 0.05$) between the treatments and the control. The higher total protein content (2.71± 0.05) was found in the fishes which were fed with the ED-3 containing *Azolla pinnata* 350g/kg of diet. and ED-4 containing *Azolla pinnata* 400g/kg of diet.as compare to diet ED-1, ED-2 and control diet which do not contain *Azolla pinnata.*

2: Serum albumin:

The effect of different *Azolla pinnata* treatments on the serum albumin content of *L. rohita* fingerlings is presented in Table 2 and. There was no significant difference (P≥0.05) in the serum albumin content of different treatment groups. But the serum albumin content of all the treatment groups was significantly higher (P≤0.05) than that of the control group fishes (0.82 ± 0.02). The higher value (1.8 ± 0.03) was observed in the B3 group fishes.

3: Serum globulin:

The effect of different *Azolla pinnata* treatments on the serum globulin content of *L. rohita* fingerlings is presented in Table 2 and FIG.3. The serum globulin content was significantly higher (P≤0.05) in all the treated groups compared to control. The higher content (1.58 ± 0.02) was recorded in B3 group fishes. The lowest serum globulin content (0.70^{a}±0.03) was observed in the control group fishes.

4: Serum lysozyme activity:

The effect of different *Azolla pinnata* treatments on the serum lysozyme activity of *Cyprinus carpio* fingerlings is presented in Table 2. Significantly higher (P≤0.05) lysozyme activity was observed in ED1,ED-2. Lowest serum lysozyme activity was observed in the control group, but it was not significantly different (P≥0.05) from the other two treatment groups.

5: Serum bactericidal activity:

The effect of different Azolla pinnata treatments on the serum bactericidal activity of *L. rohita* fingerlings are presented in Table 2 and FIG-5. Significantly lower (P≤0.05) colony count was observed in the B3 group fishes (391± 8.7).

6:A/G Ratio: The serum A/G Ratio of *Cyprinus carpio* fingerlings is presented in Table 2 and The A/G Ratio of B3 (0.64 ± 0.04) and B2 (0.59 ± 0.03) group fishes were significantly lower than B1 (1.06±0.04) and control group (1.09^{b}±0.04) fishes.

2: Percentage survival after challenge study:

The percentage survival of *Cyprinus carpio* fingerlings of different experimental groups after challenging with *A. hydrophila.* Is graphically represented in FIG.3.25. The percentage survival of fishes of the entire Azolla pinnata treated group was significantly higher (P≤0.05) than the control. There was no significant difference (P≥0.05) between the Azolla pinnata treated groups. The higher survival was in the B3 group (80.95 ± 2.4), followed by B1 (78.57 ± 4.1), B2, B3 (71.23 ± 4.1) and control (50 ± 4.1) respectively.

4. Feeding Experimental Setup: Collected fish *cyprinus carpio*were stocked for acclimation in 60 liter portable plastic pools for one week. Fishes were fed two times at 08:00h and at 14:00h. the feeding rate was at 5 % body weight day^{-1} for the whole

rearing period of 30 days, and the amount of feed was adjusted every tenth day following a bulk weighing of each group of fish.

III. Results:

I: Serum total protein against *Aeromonas hydrophilla* : The serum total protein content of *Cyprinus carpio* fingerlings of different experimental groups is presented in Table 2. The serum total protein content of different experimental groups showed a significant difference (P≤0.05) between the treatments and the control. The higher total protein content (2.71± 0.05) was found in the ED-3 group fishes which were fed with the diet 3 containing *Azolla pinnata* 450g/kg of 1kg feed.

2: Serum albumin: The effect of different *Azolla pinnata* treatments on the serum albumin content of *c.carpio* fingerlings is presented in Table 2. There was no significant difference (P≥0.05) in the serum albumin content of different treatment groups. But the serum albumin content of all the treatment groups was significantly higher (P≤0.05) than that of the control group fishes the higher value was observed in the ED-3 fed fishes.

3.Serum globulin: The effect of different Azolla pinnata treatments on the serum globulin content of *L. rohita* fingerlings is presented in Table 2. The serum globulin content was significantly higher (P≤0.05) in all the treated groups compared to control. The higher content was recorded in ED-3 fed fishes. The lowest serum globulin content was observed in the control group fishes.

4: Serum lysozyme activity: The effect of different Azolla pinnata treatments on the serum lysozyme activity of *Cyprinus carpio* fingerlings is presented in Table 2. Significantly higher (P≤0.05) lysozyme activity was observed in ED-1). Lowest serum lysozyme activity was observed in the control group, but it was not significantly different (P≥0.05) from the other two treatment groups.

5: Serum bactericidal activity: The effect of different *Azolla pinnata* treatments on the serum bactericidal activity of *Cyprinus carpio* fingerlings are presented in Table 2 Significantly lower (P≤0.05) colony count was observed in the ED-3 feed.

6: A/G Ratio: The serum A/G Ratio of *Cyprinus carpio* fingerlings is presented in Table 2. The A/G Ratio of ED-3 and ED-2 Fed fishes were significantly lower than and control group fishes.

7: Percentage survival after challenge study: The percentage survival of *Cyprinus carpio* fingerlings of different experimental groups after challenging with *A. hydrophila.* is graphically represented in Fig.1. The percentage survival of fishes of the entire Azolla pinnata treated group was significantly higher (P≤0.05) than the control. There was no significant difference (P≥0.05) between the Azolla pinnata treated groups. The higher survival was in the ED-3.

Discussion:

Filicophyta as the major source of feed during pond culture of macro-phytophagous fish species *Cyprinus carpio and Cirrihinus mrigala were shown better growth,* Feed acceptance trial was conducted for these seven species using *Azolla imbricata* and *Azolla pinnata* species. Had been recorded higher growth rates fed with azolla [7].

Azolla filiculoides in pond and examined its potential as a fish feed. In a feeding experiment with *Tilapia nilotica,*. Diets C and D exerted the same effect on the growth of Tilapia after 3 weeks of feeding with a 17% decrease of growth compared to the control. Based on the results, it was observed that *Azolla* can replace about 20% of tilapia feed, which indicates the beneficial effect of the use of aquatic plant. [8]

Similar kind of study using dry azolla meal as a sole protein source for feeding tilapia *Oreochromis niloticus.* [9] The inclusion levels of *azolla* meal were 0, 15, 20, 30, 40 and 45% on dry weight basis in diet. Comparing growth results, juveniles fed with 15% of *azolla* meal have exhibited best growth followed by azolla free diet. Considering cost of feed, the study recommended to use 45% azolla incorporated diet for tilapia in a fertilized pond. Another study carried out on *Azolla pinnata* shows that the feed macronutrients value along with protein, vitamins, and minerals. Which shows the better alternative for the commercial fish food *Barbonymus gonionotus* [10]

Higher survival the antagonistic effects of *azolla* extract against the fresh water fish pathogens is effective and safe which helpful in the bacterial disease control.

Tables and Figure

Table 1. Ingredient content of experimental diet with *Azolla pinnata*

Ingredients (IN %)	**Experimental Feeds**				
Ingredients	**control**	**ED-1**	**ED -2**	**ED -3**	**ED -4**
Soybean flour	60	60	60	60	60
Wheat	10	10	10	10	10
Rice bran	05	05	05	05	05
Ground nut oil cake	20	20	20	20	20
Azolla pinnata Powder	00	250*	300*	350*	400*
Vitamin Premix	05	05	05	05	05
* gram per 1 kg of basal diet					

Table 2: Various Serum Parameters of *Cyprinus carpio* Fingerlings against *Aeromonas Spp.*

Experimental groups	Blood Serum parameters					
	Serum Total Protein[1]	**Serum Albumin[2]**	**Serum Globulin[3]**	**Serum Lysozyme Activity[4]**	**Serum Bactericidal Activity[5]**	**A/G Ratio**
Control	1.89a ±0.03	0.91a ±0.04	0.67a ±0.04	72.8a ±09.18	456 c ±2.12	1.08 b ±0.02
ED-1	2.7 b ±0.04	1.01b ±0.04	1.00b ±0.01	73.18b ± 10.34	458b ± 11.1	1.09b ± 0.04
ED-2	2.8 c ±0.03	1.03b ±0.03	1.6c ±0.02	94.9a ±9.12	465b ±8.8	0.61a ±0.02
ED-3	2.9 d ±0.02	1.09b ±0.04	1.5c ±0.01	95.65a ±10.35	395a ±9.3	0.66a ±0.04
ED-4	2.10 c ±0.03	1.07 b ±0.03	1.5c ±0.05	96.8a ±6.20	443 b ±8.2	0.69a ±0.04

The mean values bearing different superscript differ significantly ($P \leq 0.05$). [1](gdL^{-1}), [2] (gdL^{-1}), [3](gdL^{-1}), [4](U $min^{-1}mg^{-1}$protein), [5]colony count

Table 3: Percentage of survival after challenge study with Pathogens. *Aeromonas hydrophilla* .

Experimental Groups	*Aeromonas hydrophilla*
Control	42.20 ±4.2
ED-1	80.10± 4.2
ED-2	81.57±3.1
ED-3	87.80±2.4
ED-4	90.10±1.2

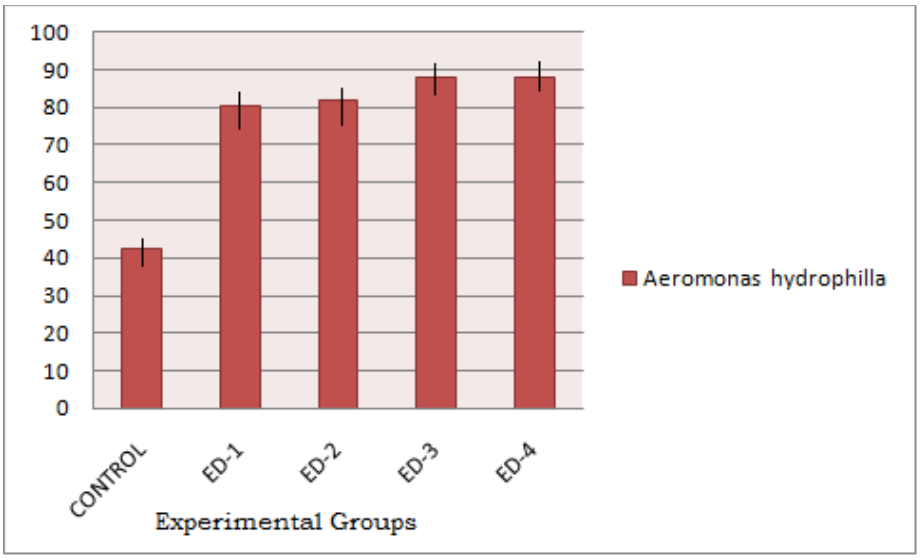

Figure 1 : Percentage of survival after challenge study with Pathogens. *Aeromonas hydrophilla* .

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