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Leaf Powder of *Eupatorium odoratum* Enhances Non-specific Immune Response and Resistance to *Aeromonas hydrophila* Infection in *Cyprinus carpio* (Linn. 1758)

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ABSTRACT

Background: *Aeromonas hydrophila* is considered a major bacterial pathogen in Indian aquaculture. The various experimental trials are being conducted as a control measure against *A. hydrophila* infection in fish. The study evaluated the effect of dietary inclusion of crude dried leaf powder of *Eupatorium odoratum* at 0.5, 1.0 and 1.5% to study the immune response and disease resistance in fingerlings of *Cyprinus carpio* experimentally infected with *A. hydrophila*.

Methods: The fingerlings of *C. carpio* were orally administered crude dried leaf powder of *E. odoratum* at 0.5, 1.0 and 1.5% through feed for 55 days followed by challenge with *A. hydrophila*. The biochemical, haematological and immunological parameters of the fish samples analyzed on 55th and 62nd day of the feeding trial to evaluate the immune response and disease resistance.

Result: The comparative analysis of biochemicals, haematological and immunological parameters confirmed that the fingerlings fed with dry crude leaf powder of *E. odoratum* at 0.5-1.0% showed enhanced leucocytes count, hemoglobin, lysozyme activity, superoxide anion production, bactericidal activity, the total protein and albumin content in pre and post-infected experimental groups. The highest relative survival percentage of fingerlings was recorded in groups fed with 1.0% dietary inclusion of dried crude leaf powder of *E. odoratum*. Thus, the study concludes that dietary inclusion of crude dried leaf powder of *E. odoratum* at 1.0% level imparts improved immune response and resistance against *A. hydrophila* infection.

Key words: *Aeromonas hydrophila*, *Cyprinus carpio*, *Eupatorium odoratum,* Immunomodulation, Plant dry powder.

INTRODUCTION

Outbreak of bacterial, fungal, parasitic and viral diseases has caused a setback to commercial aquaculture, leading to huge economic loss around the world (Mallik *et al*. 2015). In turn, this problem has forced the farmers to adopt a profit motive farm operation system, where synthetic antibiotics, chemicals and drugs are used indiscriminately, causing antimicrobial resistance in aquaculture. It is reported that the several chemical constituents inside the plants *viz.* alkaloids, flavonoids, steroids, phenols, terpenoids, resins, saponins *etc.* are having unique properties to enhance the immune system of finfish and shellfish by enhancing the phagocytic, lysozyme and other complementary activities along with antipyretic, analgesic, anti-inflammatory, anti-stress property, appetite stimulator, growth promoter as well as antimicrobial activity (Citarasu, 2010; Sharma *et al*. 2022). A large number of plants have been used in traditional medicine for the treatment and control of many diseases (Duke, 1987). Even the use of crude powder of plants in aqua-feed results in modulation of the immune response. In many countries, the plant and its products are being used to promote growth, increase natural resistance to infection and in prevention and treatment of various diseases.

The application of multifunctional herbal bioactive compounds in aquaculture systems is a recent activity and few studies have been carried out in fish. Different medicinal plants demonstrated effective non-specific immune responses in fish challenged with pathogenic bacteria (Sahu *et al*. 2007). Moreover, the dose optimization of plant/herbs or their extracts is also suggested to avoid possibility of application of the under or over dosing of the plant/herbal effects. The present study describes the dietary inclusion of crude leaf powder of *Eupatorium odoratum* in fish feed, which induced non-specific immune response and resistance against *Aeromonas hydrophila* infection in common carp, *Cyprinus carpio*.

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MATERIALS AND METHODS

The healthy *C. carpio,* (average length 11±1.5 cm and weight 13±1.0 g) were obtained from Dineshpur, Udham Singh Nagar, Uttarakhand and brought to the wet laboratory of ICAR-Directorate of Coldwater Fisheries Research (ICAR-DCFR), Bhimtal, where the experimental trial was conducted during 2013-2014. The experimental fish were acclimatized for 10 days before they were transferred to the cleaned and disinfected test tanks (FRP, 1000 L capacity) filled with bore well water. During acclimatization, fish were fed with a commercial grow-out pelleted feed @ 4% of their body weight. On every alternate day, 40-50% of water was exchanged.

The experimental diet was prepared with slight modification of the earlier method (Rao *et al*. 2006). The basal and other feed ingredients (fish meal, mustard oilcake (MOC), soybean meal, rice bran, wheat flour, fish oil, veg oil, vitamin and mineral mixture premix and butylated hydroxytoluene) were calculated for one gram and then the feed ration was calculated accordingly for 1.25 kg of experimental feed for three treatments and control. All ingredients (except vitamin and mineral mixture) were mixed thoroughly with the help of hot water to form the dough. The dough was cooked and autoclaved at 121°C for 30 min. Then, the completely cooled dough was mixed with vitamins and mineral mixture and crude dry powder leaf of *E. odoratum* at different concentrations *i.e*. control 0%, treatments 0.5, 1.0 and 1.5%. The pelleted diets were kept for drying overnight in a hot air drier at 60°C.

Two hundred forty numbers of acclimatized fingerlings of *C. carpio* were randomly distributed in twelve rectangular FRP tanks @ 20 no. per tank (0.5 ton capacity each). The fish were subjected to three treatments in three experimental replicates. The completely randomized design (CRD) was followed to set up the present trial. Similarly, the control tank in three replicates, each tank stocked @ 20 no. of fish, was also kept separately to make a reliable comparison with the treatment groups. All the experimental and control tanks were filled with bore well water 24 h before the execution of the stocking process. After stocking, fingerlings were fed with experimental diets @ 4% of their body weight twice a day at 09:00 and 17:00 h for 55 days, prepared from the crude dried powdered leaf of *E. odoratum* at all three concentrations. The water quality parameters were in the optimum range during the experimental period.

On 55th day of the feeding trial, blood samples were collected from experimental fish in the treatment and control groups. Then fish were challenged intraperitoneally with 100 µL of the overnight grown pathogenic strain of *A. hydrophila* (ATCC 35654) suspension at 1.0×10^7 CFU/mL). Mortality and relative survival percentage were recorded over 7 days post-infection.

The blood samples were collected twice on 55th day (pre challenged period) and 62nd day (post challenged period) and 1.5 mL of blood was transferred to the vials containing 2.7% EDTA for estimation of the haemoglobin (Hb), total erythrocyte count (TEC) and total leucocytes count (TLC). To study the biochemical parameters, the serum was separated from blood by keeping the tubes in a slanting position for 4 h and thereafter it was centrifuged at 3941 × g for 10 min at 4°C followed by the collection of straw colored serum and storage at -20°C for further analysis. To estimate the haemoglobin content (g%), the blood was drawn in hemoglobin pipette up to 0.2 marks and transferred to a haemoglobinometer, containing a small amount of N/10 HCl. Again it was diluted with N/10 HCl drop by drop and stirred continuously with a glass rod. The addition of N/10 HCl was continued drop by drop into the hemoglobin tube till the colour matched with the standard glass rod. The reading was taken on haemoglobin tube, showing percentage of haemoglobin (Dacie and Lewis, 1971).

Blood was sucked in RBC pipette up to 0.5 marks followed by RBC diluting fluid up to mark 101. The blood and diluting fluid were mixed properly by shaking pipette and made dilution in the bulbs of the pipette as 1:200. After discarding out the diluted mixture from the lower stem of the pipette, a drop of it was put on the surface of each counting chamber of the hemocytometer, holding the pipettes at a 45° angle. A cover slip was placed on the counting chamber and blood cells were allowed to settle down. Then, the counting chamber was observed under a microscope at 400x and 1000x magnification. Total erythrocyte was counted as per the method reported (Dacie and Lewis, 1971).

Calculation: No. of RBC per cubic mm or $(mm)^3$ =

$$\frac{\text{No. of cells counted} \times \text{dilution} \times 4000}{\text{No. of small sqare counted}}$$

Fresh blood was diluted in WBC diluted fluid and poured into the counting chamber with help of a WBC diluting pipette. WBC was counted from four corners of each counting chamber of the haemocytometer (Dacie and Lewis, 1971).

Calculation: No. of WBC per cubic mm or $(mm)^3$ =

$$\frac{\text{No. of cells counted} \times \text{dilution} \times 10}{\text{No. of 1 sqare counted}}$$

In nitroblue tetrazolium (NBT) assay, 50 µL of blood sample was taken and transferred to 'U' bottom ELISA plate and incubated in a dry bath at 37°C for 1 h to facilitate cell adherence. ELISA plate was washed thrice with 100 µL of PBS to remove non-adherent blood cells. 100 µL of 0.2% NBT was added to the plate as a coloring agent and again incubated for 1 h. Blood cells were fixed with 100 µL of 100% methanol for 2-3 min. The cells were then washed with 100 µL of 70% methanol and allowed to dry. 120 µL of 2 N KOH and 140 µL DMSO were added and mixed properly to dissolve formazene blue precipitation. The absorbance was recorded in ELISA plate reader (Tecan) at 620 nm.

Lysozyme activity was studied by turbidimetric assay, where overnight grown *Micrococcus luteus* (ATCC 49732) in nutrient broth (10^7-10^8 CFU mL^{-1}) centrifuged (7006 × g) to collect the pelleted cell. The pelleted bacterial cell was suspended in 0.05 M sodium phosphate buffer (pH 6.2). Then, 50 µL of serum added to 1 mL of bacterial suspension

and the absorbance was recorded twice at time intervals of 0.5 and 4.5 min in spectrophotometer (Thermo scientific UV 1) at 530 nm. The absorbance decreasing at the rate of 0.001 min^{-1} due to the amount of sample is considered a unit of lysozyme activity.

For bactericidal activity, an equal volume (100 µL) of serum and the bacterial suspension (10^7 CFU mL^{-1}) were mixed and incubated for 1 h at 28°C. The sterile phosphate buffer saline (PBS) was kept as a blank control. The serum-bacteria suspension was diluted at 1:10 ratio with sterile PBS. The diluted suspension (100 µL) was plated onto nutrient agar plates and incubated for 24 h at 28°C. The colonies grown were counted as viable bacteria. The relative percentage survival was calculated as per the formula stated below:

$$\text{Relative percentage survival RPS (\%)} = \frac{1- [(\text{Mortality (\%) in treated group}) \times 100]}{(\text{Mortality (\%) in the control group})}$$

The total serum protein concentration (g/dl) was estimated by the Biuret method (Johnson *et al*. 1999). Albumin concentration (g/dl) in serum (10 µL) was estimated by adding bromocresol green endpoint assay method with the help of Span Diagnostic Kit at 630 nm wavelengths. The albumin content was deducted from total protein to calculate globulin content (g/dl). The albumin-globulin ratio was calculated by using the following formula:

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

One-way ANOVA and Duncan's multiple range test (DMRT) was used to determine the significance difference between the means at 95% confidence level (SPSS version 16.0).

RESULTS AND DISCUSSION

Blood parameters

In pre-challenged fish, the haemoglobin content of the experimental fish was recorded in decreasing order from the control to treatment groups. The maximum value of hemoglobin was recorded in the control (4.5 g%), whereas experimental fish groups fed with *E. odoratum* at 0.5 and 1.5% recorded minimum hemoglobin content of 3.1 g% each. In post-challenged fish, a similar trend was also observed, where the control group demonstrated maximum haemoglobin content as compared to the fish fed with 0.5% *E. odoratum* (Fig 1). Haematological parameters are widely used as an indicative measure of an animal's health status and stress conditions. Haemoglobin content gives an idea about RBC availability and function. In the present study, haemoglobin content showed an increasing trend in the experimental groups fed with *E. odoratum*, which was in accordance to the work done previously (Harikrishnan *et al*. 2003).

Total erythrocyte count (TEC) in the treatment group fed with 1.5% of *E. odoratum* and the control in pre-challenged fish was 0.73×10^6 to 0.84×10^6 cells per 1 mL blood respectively. In the case of the post-challenged fish, the TEC count was also the highest in the control group (Fig 2). Overall total erythrocyte counts in the treatment groups did not vary significantly at different concentrations of *E. odoratum*. The probable reason could be the erythrocyte not demonstrating significant role in the immune response. In the study, the total erythrocyte counts did not get affected ($p>0.05$) by overall disease resistance and immunostimulation of *E. odoratum* incorporated diet, which concurred with a similar finding of the fish fed with *Euglena* incorporated diet over some time (Das *et al*. 2009). In contrary to the above findings, *L. rohita* fed with a diet containing *A. sativum* over certain duration of the experimental trial, has demonstrated increased total erythrocyte counts (Sahu *et al*. 2007).

The gradual increase in the total leucocyte count (TLC) was recorded from the control to treatment groups both in pre-challenged and post-challenged fish, which varied from 8.0×10^3 to 15.0×10^3 cells per mL blood. The comparative analysis of total leucocyte count between pre-challenged and post-challenged fish of the same group resulted in significant ($p<0.05$) increment in TLC in all groups, fed with the experimental diet over 62 days (Fig 3). The leukocyte

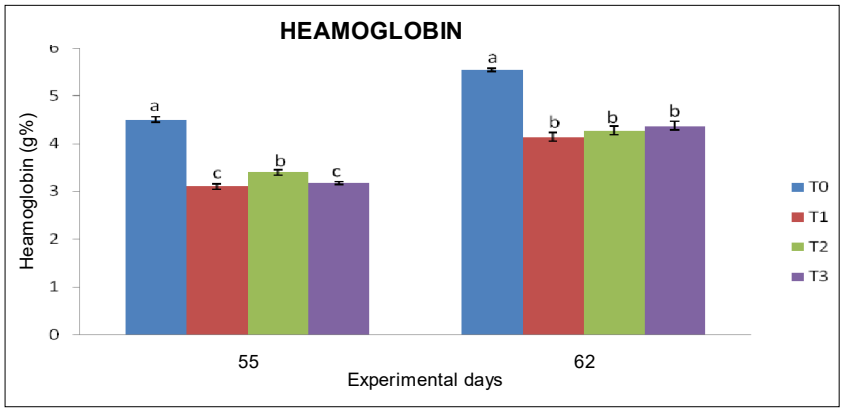

Fig 1: Heamoglobin content (g%) in *C. carpio* fingerlings fed with crude dried leaf powder of *E. odoratum* (T0: Control; T1: 0.5%, T2:1.0% and T3:1.5%) and challenged with *A. hydrophila*. Values (Mean±S.E) bearing same superscript are not statistically significant ($p>0.05$).

plays an important role in non-specific immunity and increased total leukocyte counts (TLC) are indicative of immune stimulation. Along with other immunological parameters, the increased number of white blood cells (WBC) can be considered as an indicator of the health status of fish. In the present study, WBC counts increased significantly in the treatment groups, fed with the diet incorporated with *E. odoratum* powder. This supports the findings of other trial, where *L. rohita* fingerlings fed with after long term administration of dietary glucan through feed (Mishra, 1994).

NBT test (Superoxide anion production)

The value of superoxide anion production was significantly ($p<0.05$) increased from control to treatment groups (0.053 to 0.101) in pre challenged fish. A similar trend was also observed in the case of the post-challenged fish groups (0.076 to 0.120) from control to treatment one (Fig 4). It is reported that the active fish phagocytes are capable of producing superoxide anion (O^{2-}), which limits the growth of pathogenic bacteria. The nature of reactive oxygen is toxic to bacterial pathogens. A variety of natural agents that includes bacteria, bacterial products, β-glucans and garlic are known to stimulate phagocytic activity (Sahu *et al*. 2007). The highest respiratory activity was observed in fish fed with 1.5% of *E. odoratum*. This is in agreement with enhanced NBT activity in *Epinephelus tauvina* fed with diet containing a purified active component of *O. sanctum*, *W. somnifera* and *Myristica fragrans* (Sivaram *et al*. 2004), *L. rohita* fed with 0.5% *Achyranthes aspera* incorporated diet, varying percentages of garlic and *Euglena* powder (Rao *et al*. 2006; Sahu *et al*. 2007; Das *et al*. 2009).

Lysozyme activity

The lysozyme activities recorded in both the pre and post-challenged groups were varied from 125.33 to 267.92 U mL^{-1}. The serum lysozyme activity is considered as one of the important markers of innate immune response in fish. In fish, the serum lysozyme activity is reported to be of leukocyte origin. It helps in enhancing the innate immunity by lysis of bacterial cell wall, stimulating the phagocytosis of bacteria. The increased concentrations of *E. odoratum* supplemented diets (0.5, 1.0 and 1.5%) fed to the treated

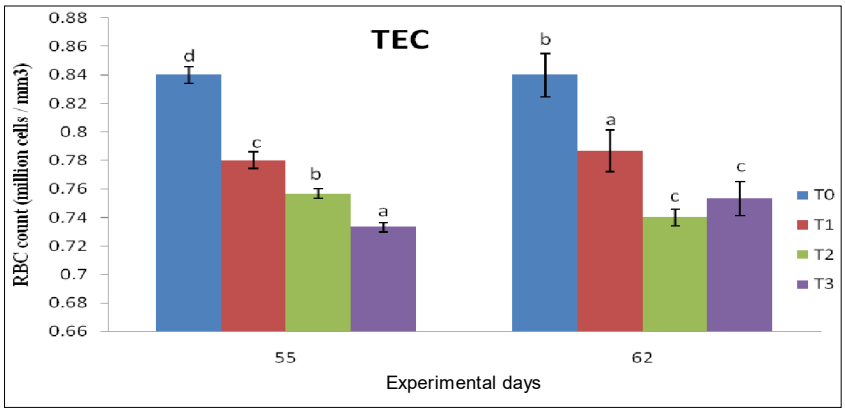

Fig 2: Total erythrocyte count (million cells/mm^3). *C. carpio* fingerlings fed with crude dried powder of *E. odoratum* (T0: Control; T1: 0.5%, T2:1.0% and T3:1.5%) and challenged with *A. hydrophila*. Values (Mean±S.E) bearing same superscript are not statistically significant ($p>0.05$).

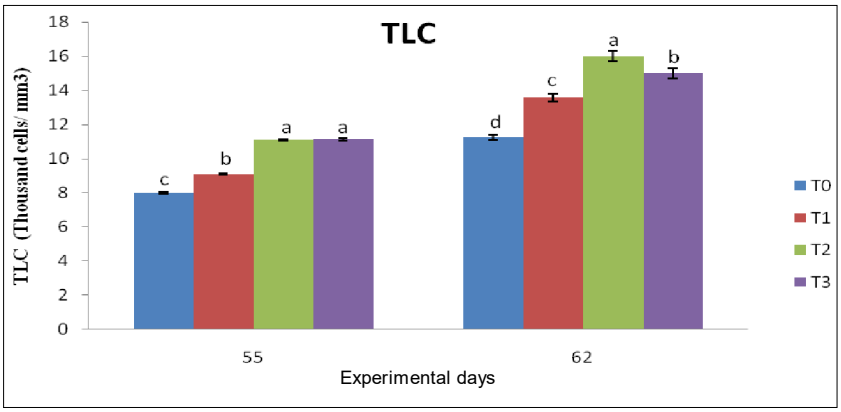

Fig 3: Total leucocyte count (thousand cells/mm^3). *C. carpio* fingerlings fed with crude dried leaf powder of *E. odoratum* (T0: Control; T1: 0.5%, T2:1.0% and T3:1.5%) and challenged with *A. hydrophila*. Values (Mean±S.E) bearing same superscript are not statistically significant ($p>0.05$).

groups, showed increased lysozyme activity ($p<0.05$), thus, establishing the non-specific immune response in *C. carpio* through its dietary inclusion (Guobin *et al*. 2009).

Bactericidal activity

All the experimental groups fed with the diet incorporated with *E. odoratum* showed higher bactericidal activity (33.5 to 53.8%) in both pre and post-challenged fish (Fig 5). The study showed enhanced bactericidal activity with *E. odoratum* feeding trial, supported by the earlier finding (Das *et al*. 2009). Thus, the innate immune response in common carp was positively correlated with the dietary supplementation of *E. odoratum*.

Relative percentage survival (RPS)

The relative percentage survival was highest (61.7%) in the treated group fed with 1.0% of *E. odoratum* (Table 1). The highest relative percentage of survival was recorded in the fish group fed with the experimental diet supplemented with 1% *E. odoratum*. This might be due to the enhanced non-specific immune response of the fish through diet incorporated with *E. odoratum* (Rao *et al*. 2006; Sahu *et al*. 2007). Thus, higher resistance against pathogenic bacteria *A. hydrophila* and increased survival rate of fish in the treated groups implied the improved innate immune responses in common carp by supplementation of dietary *E. odoratum*.

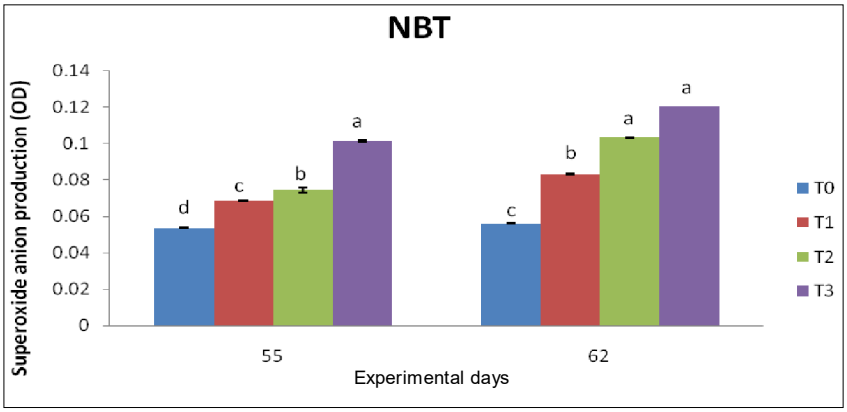

Fig 4: Super oxide anion production. *C. carpio* fingerlings fed with crude dried leaf powder of *E. odoratum* (T0: Control; T1: 0.5%, T2:1.0% and T3:1.5%) and challenged with *A. hydrophila*. Values (Mean±S.E) bearing same superscript are not statistically significant ($p>0.05$).

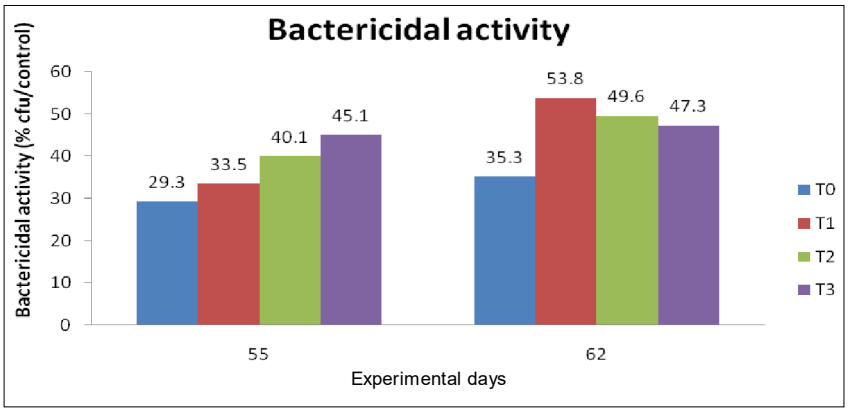

Fig 5: Bactericidal activity *C. carpio* fingerlings fed with crude dried powder of *E. odoratum* (T0: Control; T1: 0.5%, T2:1.0% and T3:1.5%) and challenged with *A. hydrophila*.

Table 1: Relative percentage survival of common carp between 55th and 62nd day of challenge with *A. hydrophila* after fed with diet incorporated with plant dry powder.

Control/ treatments (%)	Total no. of fish	Mortality (number)	Mortality (%)	Survivors (number)	Survival (%)	RPS (%)
0	60	34	56.66	26	43.33	-
0.5	60	25	41.66	35	58.33	26.47
1.0	60	13	21.66	47	78.33	61.77
1.5	60	17	28.33	43	71.66	50.0

Control fish and treatment groups fed with 0, 0.5, 1.0 and 1.5% dried leaf powder of *E. odoratum*.

Total protein, albumin and globulin content and albumin-globulin ratio

In the pre-challenged group, the lowest total protein content (3.45 g/dl) was recorded in fish fed with 1.5% of *E. odoratum*, but increasing in total protein content was observed with increasing concentrations of *E. odoratum* in the feed in the case of the post challenged fish (Fig 6). The albumin content in the pre-challenged groups was recorded in the range of 1.24 to 1.68 g/dl. The post-challenged fish recorded the albumin content in the range of 1.26 to1.9 g/dl in the study

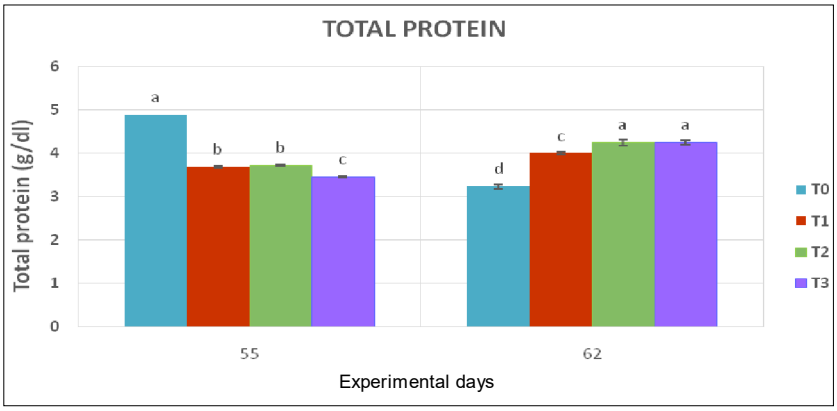

Fig 6: Total protein (g/dl). *C. carpio* fingerlings fed with crude dried leaf powder of *E. odoratum* (T0: Control; T1: 0.5%, T2:1.0% and T3:1.5%) and challenged with *A. hydrophila*. Values (Mean±S.E) bearing same superscript are not statistically significant (p>0.05).

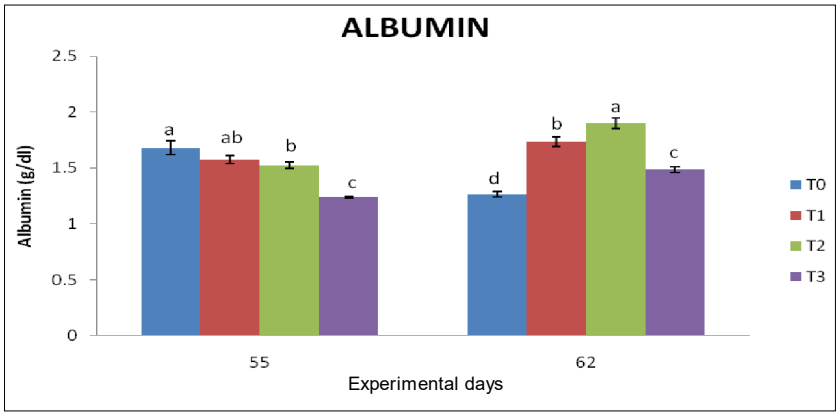

Fig 7: Albumin content (g/dl). *C. carpio* fingerlings fed with crude dried leaf powder of *E. odoratum* (T0: Control; T1: 0.5%, T2:1.0% and T3:1.5%) and challenged with *A. hydrophila*. Values (Mean±S.E) bearing same superscript are not statistically significant (p>0.05).

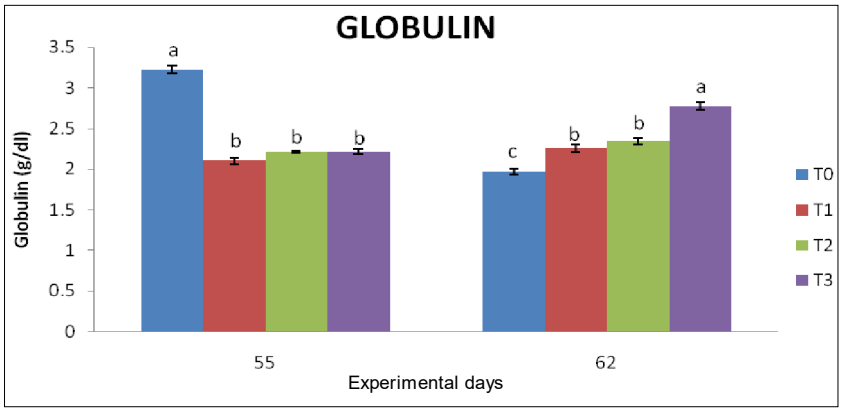

Fig 8: Globulin content (g/dl). *C. carpio* fingerlings fed with crude dried leaf powder of *E. odoratum* (T0: Control; T1: 0.5%, T2:1.0% and T3:1.5%) and challenged with *A. hydrophila*. Values (Mean±S.E) bearing same superscript are not statistically significant (p>0.05).

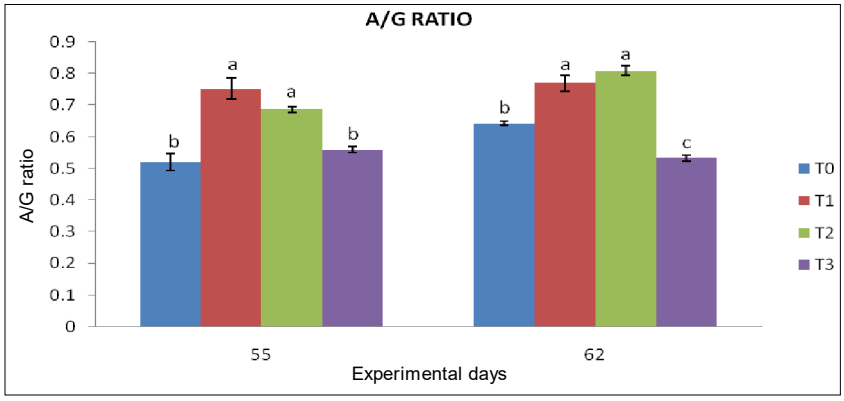

Fig 9: Albumin/Globulin ratio. *C. carpio* fingerlings fed with crude dried leaf powder of *E. odoratum* (T0: Control; T1: 0.5%, T2:1.0% and T3:1.5%) and challenged with *A. hydrophila*. Values (Mean±S.E) bearing same superscript are not statistically significant.

(Fig 7). The globulin content in the pre-challenged fish varied from 2.1 to 3.22 g/dl, whereas in post-challenged fish, it was ranged from 1.963 to 2.776 g/dl (Fig 8). Albumin-globulin (A/G) ratio was varied from 0.52 to 0.81 in both pre and post-challenge fish (Fig 9). Circulating mobile proteins (albumin and globulins) are considered the most important component in blood serum. For maintaining immunological activity, globulin protein is very important in the blood. It is one of the major sources of all immunological active proteins of the blood, which are responsible for the healthy immune system of an animal. Moreover, the enhanced innate immune signature in fish is generally marked with augmented level of albumin and globulin. The fish group fed with 0.5, 1.0 and 1.5% of *E. odoratum* supplemented diets recorded increased levels of albumin and globulin content. Moreover, the presence of high amounts of globulin may infer that 1.5% *E. odoratum* could enhance the immune response of *C. carpio* followed by 1.0% *E. odoratum* supplemented diet (Sahu *et al*. 2007; Das *et al*. 2009).

CONCLUSION

The present study demonstrated that experimental fish, fed with *E. odoratum* enriched diet, enhanced the immune profiles *i.e*. increased serum lysozyme activity, NBT (respiratory burst) activity, phagocytic activity and blood protein profiles, stating that dietary inclusion of *E. odoratum* can act as an plant immunostimulant. Further, it is observed that the experimental groups, fed with the diet supplemented with 1.0% *E. odoratum* showed higher relative percentage survival rate of *C. carpio*, challenged with *A. hydrophila*, thus fortifying the immune stimulatory effect.

Conflict of interest: None.

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