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ORIGINAL ARTICLE

Specific and nonspecific immunostimulation study of *Euphorbia hirta* on *Pseudomonas fluorescens*-infected *Cyprinus carpio*

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Abstract

Context: Infectious diseases are one of the major factors affecting the production of fish worldwide. The pathogens (especially bacteria) affect the immune system of fish and the administration of immunostimulants can increase resistance to infectious diseases by enhancing both specific and nonspecific defense mechanisms.

Objective: In the present study, we have conducted an experiment on the pathogen-infected *Cyprinus carpio* Linn. (Cyprinidae), using *Euphorbia hirta* Linn. (Euphorbiaceae) plant leaves as immunostimulants.

Materials and methods: The aqueous extract of the leaves was prepared and the immunostimulant action was recorded by giving different concentrations of plant extract supplemented diet.

Results and discussion: The results obtained from the studies show that the higher concentration of the extract (50 g/kg diet) provided significant immune response (specific and nonspecific) on the fish. The 50 g/kg leaf extract of *E. hirta* enhanced the phagocytic ratio on 10th and 15th day after the infection. The results of the specific and nonspecific immunostimulation studies are statistically significant.

Conclusion: This work will guide the researchers for the discovery of significant aquaculture nutrients to improve the immunostimulant action on fish.

Keywords: *Pseudomonas fluorescens*, *Cyprinus carpio*, *Euphorbia hirta*, immunostimulant

Introduction

Aquaculture is one of the fastest growing food-producing fields in the world, with an annual average growth rate of 6.9% per year since 1970 and this sector contributed about 36% of the total global fisheries production in the year 2006 (FAO, 2009; Mohanty & Sahoo, 2010). Infectious diseases are a major problem in aquaculture, causing heavy loss to fish farmers. Like man, fish can alter their behavior to counteract or avoid infections and can rely on a fully developed innate (nonspecific) and adaptive (specific) immune system (von Siebenthal et al., 2009). Immunostimulants increase resistance to infectious diseases by enhancing both specific and nonspecific defense mechanisms. Hence, the use of immunostimulants in aqua diet is considered to be safe and effective against various pathogens. The use of immunostimulants in fish culture is a promising new development in the field (Logambal et al., 2000; Dügenci et al., 2003; Rairakhwada et al., 2007).

Euphorbia hirta Linn. (Euphorbiaceae) is a potential candidate and is widely used as a traditional medicinal herb in all the tropical countries. *E. hirta* is also identified as *Euphorbia pilulifera* Linn. (Euphorbiaceae) and has been utilized to treat a variety of diseases including cough, hay asthma, bowel complaints, worm infestation, kidney stones, bronchial affections, as well as low milk

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yield (Johnson et al., 1999; Hore et al., 2006). Literature on these studies showed that *E. hirta* has number of active constituents such as leukocyanidol, quercitol, camphol, quercetin, dihydroellagitannins, dimeric hydrolyzable tannins–euphorbins, and so on (Sudhakar et al., 2006). Extracts of this plant exhibited an antimicrobial activity against *Escherichia coli* Migula (Enterobacteriaceae), *Proteus vulgaris* Hauser (Enterobacteriaceae), *Pseudomonas aeruginosa* (Schröter) Migula (Pseudomonadaceae), and *Staphylococcus aureus* Rosenbach (Staphylococcaceae) (Johnson et al., 1999; Sudhakar et al., 2006). Also, aqueous and aqueous-alcoholic extracts possessed immunostimulant activity and the aqueous extract affected lectin-induced lymphoblast transformation *in vitro* (Szenasi, 1992).

Pseudomonas fluorescens Flügge (Pseudomonadaceae) is an opportunistic bacterial fish pathogen of the freshwater ecosystem, associated with septicemic and ulcerative condition, necrosis of internal organs, external lesions, loss of pigmentation, and so on (Saharia & Prasad, 2001). *Cyprinus carpio* Linn. (Cyprinidae) (Common carp or European carp) is a number one fish in aquaculture and is widespread freshwater fish most closely related to the common goldfish [*Carassius auratus* Linn. (Cyprinidae)]. This fish production is affected by diseases caused by various pathogens in the aqua system and the aquaculture nutritionists have focused their effort to explore new immunostimulants for aquaculture practices to fight against the pathogens (Siwicki, 1989; Harikrishnan et al., 2003; Rairakhwada et al., 2007; Whyte, 2007; Pratheepa et al., 2010). No papers have been found in the available literature on the immunostimulant action of *E. hirta* on *C. carpio* infected with *P. fluorescens*, but the literature shows that the plant posses immunostimulant activity *in vitro* (Szenasi, 1992). Hence, the present investigation was carried out with the objective to stimulate the immune power of *C. carpio* infected with *P. fluorescens* by *E. hirta* as immunostimulant.

Material and methods

Materials

C. carpio Linn. (Cyprinidae) (average weight 45.9 ± 1.5 g) used for the study was purchased from the private fish farm at Kallidaikurichi, Tamil Nadu, India. It was identified by the taxonomist in the Centre for Environmental Sciences and confirmed by experts in Zoological survey of India and a specimen is kept in the museum for ready reference. The experiments were carried out as per the Ethical Guidelines. The oxygen content of the ambient fresh water in the tank (30° ± 1°C) was kept above 50% air saturation and the temperature was controlled between 28°C and 29°C throughout the period of experiments. The experimental fish (including control) were stocked in six troughs with 10 fish each in triplicate in tank (capacity 500 L) at a density of 1 g/L. In order to keep hygienic condition, the tank water was changed on alternative days and the fish were fed with balanced fish diet (Table 1). The water was also analyzed systematically at 7 days interval to maintain optimum levels of dissolved oxygen (6.8–7.2 mg/L), pH (7.7–8.5), and ammonia (0.08–0.12 mg/L) throughout the experiment. The Eppendorf tubes were purchased from Eppendorf (India), Mumbai. The chemicals, media, and other reagents used in the experiments were purchased from Himedia (India), Mumbai and Sigma Fluka, UK.

Preparation of extracts

The plant material of *E. hirta* used in this study was identified by the plant Taxonomists of the Centre for Environmental Sciences and confirmed by the Centre of Advanced studies in Botany University of Madras, Chennai, India. The plant leaves (100 g) were washed to remove the dirt. The leaves were crushed and extracted with water using a Soxhlet extractor. The fraction of the extracts were combined, then centrifuged at 1000 rpm for 5 min, and the supernatant was filtered out to remove the debris. The excess water was removed under reduced pressure, which has given concentrated extract. The experimental diet supplemented with different doses of plant leaf extract viz., 0, 5, 10, 20, 25, and 50 g/kg diet was formulated by adding a prescribed amount of *E. hirta* leaves extract to the pre-steam cooked and cooled diet mixture containing 40.7% protein.

Diet preparation

The experimental diet was prepared by mixing the selected diet ingredients (dry form) with known protein content (tested by Lowry method) accordingly to get 40.7% (Table 1). The mixture of the diet ingredients was wet with water and steam-cooked in batches and cooled. To this basic diet, vitamin mix and cod liver oil were added, then mixed thoroughly for even distribution. The diet was pelletized through hand pelletizer until a size of 1.0 to 4 mm and dried in shade to reduce the moisture content of the diet (<10%).

Pathogen isolation and sensitivity analysis

P. fluorescens was isolated from infected *C. carpio* fish showing the symptoms of hemorrhagic septicemia. After isolation of the strain, it was identified using standard microbial identification test and the identified culture was maintained on tryptose soya agar slopes at 4°C

Table 1. Basal feed ingredients and their protein content.

Feed ingredients	Weight	Protein content (%)
Groundnut oil cake	20 g	09.32
Fish meal	15 g	08.47
Rice bran	130 g	16.38
Tapioca	15 g	04.75
Soya bean meal	20 g	02.00
Vitamin mix (Beplex)	1 tablet	—
Cod liver oil	300 mg	—
Total		40.92

for long-term preservation and was used for infecting healthy fish.

Antibacterial activity of plant leaf extract

The isolated bacterial culture was tested for sensitivity to leaf aqueous extract of *E. hirta* by the disc diffusion method (agar medium). The minimum inhibitory concentration (MIC) of the *E. hirta* against the *P. fluorescens* was determined by the microdilution technique on agar plates (only aqueous extract was used for the study, hence disc diffusion method was used) (Bergan et al., 1997; Wayne, 2008). The extract was prepared using 0 (control), 50, 100, 150, 200, and 250 μg/mL concentrations. A stock solution of 10 μg/mL reference standard of cephalosporin was prepared with sterile distilled water. The pH of the media was adjusted to 7.2–7.4 and the Petri plates were refrigerated overnight (the area of the extract placed was marked). The average size of the inoculum was about 10^5 cells contained in a 2-mm diameter standard loop. When the nutrient agar plates containing the extract and also the control plates having equal volumes of solvent were made ready, the overnight grown broth culture of test organism was spot-inoculated by Checkerboard technique on the marked area of the plates. These were then incubated for 72 h at 37°C. No growth of the organism on the test plate along with growth on the control plate was taken as an indication of antimicrobial activity of the extract. MIC was indicated by the lowest concentration of the extract, which inhibited the bacterial growth (Islam et al., 2008).

Experimental design

The experimental fish, *C. carpio* of uniform size (45.9 ± 1.5 g), were stocked in six troughs with 10 fish each in triplicate (including control). The formulated diet at various concentrations (0, 5, 10, 20, 25, and 50 g/kg) of leaf extract was given separately at 2% of body weight for an epoch of 50 days (the concentrations were fixed for the study from minimum dose). After 50 days of experimental diet dieting, all experimental fish were given only control diet. At the 50th day of immunomodulation, the fish were infected with bacterial pathogen, *P. fluorescens* through intraperitoneal injection at a dose of 1.5×10^4 cells/mL. After 5 days of infection, studies were carried out once in every 5 days up to 20th day to observe the changes in hematological, immunological, and biochemical responses.

Collection of blood and antiserum

The fish were bled serially using tuberculin syringe with a 24-gauge needle from the caudal vein and the blood was collected in EDTA rinsed small serological tubes. The blood (without anticoagulant) collected from fish was kept overnight at 4°C for serum separation. The serum was separated by spinning down at 3000 rpm for 15–20 min in a centrifuge. The supernatant was collected in sterile vials and the serum was kept at 57°C in a water bath for 30 min to inactivate the complement system and then stored at –20°C for further analysis.

Hematology

The red blood cell counts (RBC: 10^6 mm^{-3}) were determined in a 1:200 dilution of blood sample in Hayem's solution and the white blood cell count (WBC: 10^4 mm^{-3}) from a 1:20 dilution of the blood sample in Turke's solution with a Neubaeur hemocytometer. Hemoglobin content (Hb: g/dL) was determined by the cyanomethemoglobin method (Gowenlock, 1996). A 20 μL of the anticoagulated blood was mixed with 4 mL of Drabkin's reagent and kept at room temperature for 4 min and then read at 540 nm.

Specific immune response

Antigen–antibody titration (bacterial agglutination assay)

Circulating antibody titer assay was performed in 96-well microtiter plates using 2-fold dilutions. The titer was recorded as the highest dilution in which visible agglutination (Mat-like observation) was observed. Dot-like formation was considered as negative response (Vallinayagam, 1997).

Nonspecific immune response

Assay of phagocytic activity

The phagocytic activity assay was performed by following the modified method of Sahoo and Mukherjee (2002). Blood (100 μL) was mixed with and equal quantity of bacterial suspension (1:1) in Eppendorf tubes. The density of the bacterial culture was maintained throughout the experiment at 10^4 cells/mL in phosphate-buffered saline (PBS). The mixture was incubated for 20 min at room temperature. After incubation, a thin smear was prepared and fixed with absolute alcohol for 5 min. The smear was later stained with Giemsa stain for 5 min and the phagocytic cells that have engulfed bacteria were counted (under microscope) as positive (Seeley et al., 1990).

The percentage of bacteria ingested phagocytes (phagocytic ratio) was calculated using the following equation:

$$\text{Phagocytic ratio} = \frac{\text{Number of phagocytic cells with engulfed bacteria}}{\text{number of phagocytes}} \times 100$$

NBT assay

One drop of pooled (from six fish) heparinized blood was placed on a coverslip immediately after collection; it was incubated in a humid chamber (60-mm Petri dishes with a wet paper towel) for 30 min at room temperature for the neutrophils to stick on the glass. After incubation, the coverslips containing the cells were transferred upside down to a clean glass slide containing 50 μL of 0.2% filtered nitroblue tetrazolium chloride (NBT) solution and subsequently incubated for 30 min. The dark

blue-stained NBT-positive cells were counted under a microscope (Sahoo & Mukherjee, 2002).

Serum lysozyme activity

Lysozyme activity was analyzed according to Sankaran and Gurnani (1972). A standard suspension of *Micrococcus lysodeikticus* was prepared in 0.066 M phosphate buffer (pH 7.0). The serum (100 μL) was added to 2 mL of bacterial suspension and was incubated at 40°C for 20 min. After incubation, the absorbance was read at 546 nm. The lysozyme content was determined on the basis of the calibration curve and the extinction coefficient was measured. Standard solutions containing 2.5, 5.0, 7.5, 10, and 12.5 μL/mL of hen egg lysozyme in 0.066 M phosphate buffer were used to develop the standard curve.

Statistical analysis

The data collected were statistically analyzed using two-way analysis of variance (ANOVA) to test the effects of experimental diets for all parameters. Student's *t*-test was used to test differences among individual means and the control. The difference was recorded as significant when $P < 0.01$ and $P < 0.05$.

Results

The immunomodulation study of the leaf extract of *E. hirta* on the freshwater fish *C. carpio* (infected with *P. fluorescens*) has provided significant results. The antibacterial sensitivity of the extract against the *P. fluorescens* was performed by microdilution techniques and the MIC was found out from 0 to 25 μg/mL concentration. The extract inhibits the pathogen growth at 200 μg/mL concentration. The standard drug cephalosporin inhibits the pathogen growth at 5 μg/mL. The maximum RBC count in the prechallenged *C. carpio* was observed to be $2.63 \pm 0.034 \times 10^6$ cells/mm^3 with 25 g leaf extract of *E. hirta* per kilogram. After infection with the pathogen, the maximum RBC count of $1.93 \pm 0.012 \times 10^6$ cells/mm^3 was noticed on 5th day with 25 g leaf extract of *E. hirta* per kilogram and 50, 20, 10, and 5 g leaf extracts follow the action.

The control fish showed maximum WBC count of $30.29 \pm 1.089 \times 10^4$ cells/mm^3 and the experimental fish showed higher WBC count of $53.73 \pm 1.909 \times 10^4$ cells/mm^3 with diet having 50 g leaf extract of *E. hirta* per kilogram. After infection with the pathogen, the WBC count has increased with increase in the concentration of plant leaf extract throughout the period of experiment. In all experimental fish, the WBC count was increased up to the 10th day; subsequently, the corresponding values showed a declining trend in the following days. The maximum WBC count of $64.23 \pm 2.202 \times 10^4$ cells/mm^3 was noticed on the 10th day in fish fed with 50 g leaf extract of *E. hirta* per kilogram of diet.

The maximum hemoglobin content of 10.38 ± 0.037 g/dL was noticed in the prechallenged fish fed with diet incorporated with 50 g leaf extract of *E. hirta* per kilogram. Following this, the fish fed with 25, 20, 10, and 5 g leaf extracts of *E. hirta* per kilogram diet have the hemoglobin contents of 10.04 ± 0.132, 9.49 ± 0.022, 9.23 ± 0.039, and 9.06 ± 0.103 g/dL, respectively. After the 5th day of infection, the hemoglobin content was decreased in all fish and the lowest hemoglobin content was observed in fish fed with control diet and the hemoglobin content increased after the 15th day of infection. The higher hemoglobin concentration of 7.53 ± 0.029 g/dL was noticed in fish fed with diet having 50 g leaf extract of *E. hirta* per kilogram diet on the 20th day, which is the highest hemoglobin content in the experimental fish.

The leaf extract of *E. hirta* enhanced antibody production after infection with the bacterial pathogen, *P. fluorescens*, and maximum antibody production was obtained in fish which consumed diet with 50 g leaf extracts of *E. hirta* on the 5th day after infection (10.67 ± 0.577). The results obtained from the study the provided in Table 2. The table clearly indicates that the diet supplemented with 50 and 25 g leaf extracts of *E. hirta* were able to increase the antibody production up to 5 days after infection and thereafter the antibody production has decreased up to the 20th day.

The phagocytic activity study results on the freshwater fish, *C. carpio* fed with *E. hirta*, extract is given in Table 3. In prechallenged fish, the phagocytic ratio increased as the concentrations of the leaf extract increased. After infection with the pathogen, the phagocytic ratio increased and the increasing trend was noticed up to 15 days after infection. In the postchallenged control

Table 2. Effect of different concentrations of leaf extract of *Euphorbia hirta* on antigen–antibody titration ($\log_2$ titer value) in *Cyprinus carpio* infected with the bacterial pathogen, *Pseudomonas fluorescens*.

Concentrations of leaf extract (g/kg feed)	Postchallenge of pathogen (days)				
	5	10	15	20	*t*-value
0	3.33 ± 0.58	3.67 ± 0.58	2.67 ± 0.58	2.00 ± 0.00	—
5	4.67 ± 0.58	4.33 ± 0.58	4.00 ± 0.00	3.33 ± 0.58	2.50*
10	6.33 ± 0.58	5.33 ± 0.58	5.00 ± 0.00	4.00 ± 0.00	3.71*
20	8.67 ± 0.58	7.33 ± 0.58	6.33 ± 0.58	6.00 ± 0.08	5.92**
25	10.00 ± 0.00	8.67 ± 0.58	7.67 ± 0.58	6.67 ± 0.58	6.65**
50	10.67 ± 0.58	9.67 ± 0.58	9.33 ± 0.58	8.33 ± 0.58	10.85**
t-value	—	0.50 NS	0.92NS	1.44NS	—

*$P < 0.05$, **$P < 0.01$, NS—not significant.
Note: Each value is a mean of six individual observations with a standard deviation.

fish, the pathogen enhanced the number of phagocytic ratio for 10 days (51.00 ± 3.010) and thereafter phagocytic ratio was decreased. In all the experimental fish, the phagocytic ratio increased up to the 15th day after infection and subsequently the values showed a slightly decreasing trend.

The number of glass adherent NBT-positive cells obtained from the NBT assay is given in Table 4. It shows that the higher concentrations (50 and 25 g) of leaf extracts were able to increase the number of glass adherent NBT-positive cells substantially. The maximum value of 22.00 ± 3.606 was noticed in the fish fed with diet having 50 g leaf extract per kilogram diet on the prechallenged fish. In control fish, the number of glass adherent NBT-positive cells was maximum up to 10 days after infection, the value being 17.00 ± 0.816 and on subsequent days after infection, the number of glass adherent NBT-positive cells decreased. Among the experimental fish, the maximum value was obtained on the 15th day of infection at 50 g concentration.

In control fish, the serum lysozyme activity was found to be 3.46 ± 0.140 μg/mL before challenging with the pathogen and after infection, the serum lysozyme activity was increased with the maximum of 7.40 ± 0.168 μg/mL on the 10th day. On subsequent days, the serum lysozyme activity was decreased and the value of 4.66 ± 0.281 μg/mL was found on the 20th day after infection in the control fish. The effective enhancement was seen in the fish fed with higher concentration (50 g) of leaf extract of *E. hirta*, the value being 9.79 ± 0.264 μg/mL in the prechallenged fish. After infection with the pathogen, the serum lysozyme activity was increased up to 10 days and the maximum serum lysozyme activity of 16.51 ± 0.106 μg/mL was noticed in the fish fed with diet having 50 g leaf extract of *E. hirta* per kilogram diet.

Discussion

The antibacterial activity of the extract was determined by microdilution techniques using cephalosporin as standard drug. The results obtained from the study shows that the extract has significant activity (200 μg/mL) as compared with the standard drug (5 μg/mL) against *P. fluorescens*. Hence, this plant extract was used to study the immunostimulant activity of the *P. fluorescens*-infected fish.

The hematological studies results such as total RBC count, hemoglobin content, and WBC count reveal that the fish fed with the leaf extracts of *E. hirta* enhanced the hematological responses in *C. carpio*. It is also observed that the RBC count was higher in fish fed with diet having 25 g *E. hirta* leaf extract and 50 g leaf extract yielded maximum WBC count and hemoglobin content. These results are in agreement with the previous studies. A study of Cooper et al. (1963) reported that mitochondria play a significant role in iron metabolism in developing

Table 3. Effect of different concentrations of leaf extract of *Euphorbia hirta* on phagocytic ratio (%) in *Cyprinus carpio* infected with the bacterial pathogen, *Pseudomonas fluorescens*.

Concentrations of leaf extract (g/kg feed)	Prechallenge of pathogen	Postchallenge of pathogen (days)				
		5	10	15	20	*t*-value
0	33.33 ± 1.53	45.00 ± 0.82	51.00 ± 3.01	36.33 ± 1.53	26.67 ± 1.25	—
5	46.67 ± 1.70	47.67 ± 0.47	51.67 ± 1.53	53.33 ± 1.25	50.00 ± 0.82	2.55*
10	51.67 ± 1.25	52.67 ± 4.42	56.33 ± 1.25	61.67 ± 4.65	53.00 ± 2.16	3.55**
20	55.00 ± 0.82	59.00 ± 0.82	65.00 ± 2.05	66.67 ± 1.70	60.67 ± 0.94	4.76**
25	57.00 ± 3.58	60.33 ± 1.25	65.33 ± 0.82	69.67 ± 0.47	64.00 ± 0.82	5.15**
50	63.00 ± 0.82	69.00 ± 0.81	70.00 ± 5.04	69.67 ± 0.90	66.00 ± 1.41	6.45**
t-value	—	0.81NS	1.65NS	1.25NS	0.31NS	—

*$P < 0.05$, **$P < 0.01$, NS—not significant.
Note: Each value is a mean of six individual observations with a standard deviation.

Table 4. Effect of different concentrations of leaf extract of *Euphorbia hirta* on number of glass adherent NBT-positive cells in *Cyprinus carpio* infected with the bacterial pathogen, *Pseudomonas fluorescens*.

Concentrations of leaf extract (g/kg feed)	Prechallenge of pathogen	Postchallenge of pathogen (days)				
		5	10	15	20	*t*-value
0	11.00 ± 1.00	12.00 ± 2.00	17.00 ± 0.82	13.00 ± 1.53	12.00 ± 2.65	—
5	12.00 ± 2.00	20.00 ± 2.65	24.00 ± 1.00	19.67 ± 0.58	17.67 ± 3.06	2.55*
10	14.33 ± 1.53	22.00 ± 4.00	30.00 ± 1.00	26.00 ± 2.00	23.33 ± 1.53	3.63**
20	16.00 ± 1.00	26.00 ± 4.36	32.00 ± 2.00	29.00 ± 4.58	26.33 ± 3.51	4.45**
25	20.00 ± 1.68	29.67 ± 1.53	35.00 ± 2.00	38.00 ± 1.00	32.00 ± 2.65	5.52**
50	22.00 ± 3.61	33.67 ± 1.25	39.67 ± 0.58	43.67 ± 1.16	41.67 ± 1.53	5.72**
t-value	—	2.22NS	3.66**	2.48*	2.07NS	—

*$P < 0.05$, **$P < 0.01$, NS—not significant.
Note: Each value is a mean of six individual observations with a standard deviation.

erythrocytes. Kumar et al. (2006) in their experiments found that RBC count and hemoglobin content were significantly reduced due to bacterial challenge, but dietary starch (gelatinized and nongelatinized) does not increase the hematological response (RBC count and hemoglobin content), whereas the dietary starch enhanced WBC count. The decreased RBC counts and lower hemoglobin concentration in infected fish indicate that RBCs are being destroyed by the leukocytosis activity in an erythrocytic anemia with subsequent erythroblastosis. The hematological results of the present study reveal that the leaf extracts were able to reduce the pathogen-caused immunosuppression. These hematological responses are stimulated by the synthesis of hemoglobin and the increased RBC count and the WBC count by the *E. hirta* plant leaves.

The specific immune response was analyzed with antibody titer experiment. The antibody titer results are given in Table 2 indicate that the leaf extracts strongly enhanced the primary immune response in the experimental fish than in the control fish. The immunological competence was developed on the plant leaf extract fed fish on 5th day after infection with the pathogen, whereas the control fish developed immune response on 10th day of postbacterial challenge. The higher concentrations (25–50 g) of *E. hirta* were only able to stimulate higher antibody production, but statistically no clear concentration dependency in the enhancement of antibody production was noticed (Table 2). This clearly shows that a single booster dose of leaf extracts is sufficient to elicit a high antibody titer and immunization schedule adopted gives a high degree of protection against the bacterial pathogens tested in common carp. The present findings are in agreement with the earlier studies that the significant increase in hemagglutination antibody titers was observed from *Phyllanthus emblica* Linn. (Phyllanthaceae) in *Cirrhinus mrigala* Hamilton (Cyprinidae) (Mercy, 2006). Swain et al. (2007) recently detected antibody response at first week postimmunization with antigens from *Aeromonas hydrophila* Chester, *E. tarda*, and *P. fluorescens* or a combination of all three that rose significantly ($P < 0.05$) at fourth week postimmunization in all the immunized groups. Chand et al. (2006) found that giant freshwater prawn *Macrobrachium rosenbergii* de Man (Palaemonidae) fed with bovine lactoferrin showed significant increase in agglutination titers. The result from the present study also reveal that the leaf extracts in the diet of carp were able to stimulate specific antibodies against the challenged bacteria and rise humoral immunity as observed in the previous studies of Mohanty and Sahoo (2010).

Phagocytosis is the most common cellular defense mechanism, together with humoral components, it constitutes the first line of defense once a parasite or an intruder has overcome the physiochemical barrier of the cuticle. The fish phagocytic activity can be modulated by a wide range of endogenous and exogenous factors, which enhance or modulate the fish immune system (Secombes, 1994). The present study clearly elicits that the plant leaf extract was able to stimulate the phagocytic activity and enhanced the phagocytic ratio significantly in the studied fish. Phagocytic activity is mediated by cytokines such as macrophage activating factor of peritoneal lymphocytes (Graham & Secombes, 1988). The results of the present study show that chemotaxis process was stimulated by the higher concentrations (50 g) of *E. hirta* on 10th day and 25 g in 15th day after infection. The increase in phagocytic ratio indicates that the leaf extracts stimulate the synthesis of chemotactic factors as observed by Fujiki and Tomoki (1997). A study by Nayak (2010) suggested that phagocytic activity is responsible for early activation of the inflammatory response before antibody production and plays an important role in antibacterial defenses.

In the present study, the phagocytic ratio was maximum upto 10 days after infection with the pathogen. The results also reveal that macrophage migration in the presence of exoantigen enhanced with the incorporation of different concentrations of leaf extract of *E. hirta*.

NBT assay is a quick and inexpensive method, focusing on the ability of phagocytes to reduce the dye by the production of oxygen radicals. In vertebrate phagocytic cells, the oxygen-dependent defense mechanism consists of the generation of reactive oxygen intermediates (ROIs) with powerful microbicidal activity (Allen et al., 1972). In the present study, the NBT-positive cells were found to be significantly increased ($P < 0.01$) with increase in days (upto the 15th day) after infection on fish. This is probably due to the increase in lysozyme activity. Lysozyme production was mainly based on the neutrophils and monocytes present in the blood. This is also supported by the fact that lysozyme activity in fish fed with diet having leaf extract was found to be higher than the control (discussed in the next section), suggesting the production of a greater number of NBT-positive cells in the fish.

The leaf extracts of *E. hirta* administered through the diet enhanced the nonspecific defense mechanism in terms of increased number of activated neutrophils (Table 4). The previous findings supported the view that the external stimulants like plant extracts stimulated the activity of NBT-positive cells in the blood of fish, as evidenced by the results of the present investigation in common carp. This is supported by the fact that serum lysozyme and phagocytic ratio also enhanced in fish fed with diet having higher concentrations of leaf extracts.

Lysozyme is one of the important bactericidal enzymes of innate immunity and is an indispensable tool for fish to fight against infectious agents like production of NBT-positive cells (Lindsay, 1986). The major lysozyme secretory cells of higher vertebrates are the macrophage. Lysozyme activity has been found to be modulated by a range of factors including stress, water temperature, injection of foreign materials, nutrients, and so on (Fletcher & White, 1973). The results obtained from the present investigation show that the increased levels of lysozyme in fish fed with diet having plant leaf extracts could be the result of an increment in the number of

phagocytes (macrophage) that secrete a greater amount of lysozyme. The higher concentrations (50 g) of *E. hirta* significantly enhanced the serum lysozyme activity. The earlier studies of various medicinal plants on serum lysozyme activity show that when *Labeo rohita* was fed with diet having extract of *Achyranthes aspera* Hook F (Amaranthaceae) (Rao et al., 2006) and *n*-PUFA (Misra et al., 2006), the serum lysozyme activity was increased considerably. All the above studies are in agreement with the present findings and shows that the effect of herbal plants on lysozyme activity. This confirms that the herbal formulation plays an important role to enhance the lysozyme activity.

Conclusion

The study concluded that the immunostimulant (*E. hirta* extract) was found to stimulate antibody response, lysozyme, and phagocytosis and other hematological functions in fish at a higher concentration. This work will guide the researchers for the discovery of significant aquaculture nutrients to improve the immunostimulant action on the fish. This study also reveals that the scope of using extracts of *E. hirta* as an immunoprophylactic in the health management in culture of carps.

Declaration of interest

The authors report no declaration of interest. The authors alone are responsible for the content and writing of the paper.

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