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RESEARCH ARTICLE

Immunomodulatory effect of *Aegle marmelos* leaf extract on freshwater fish *Cyprinus carpio* infected by bacterial pathogen *Aeromonas hydrophila*

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Abstract

Context: Aquatic organisms (especially fish) require potent defense mechanisms to protect themselves against pathogen invasion and disease formation. The use of immunostimulants in fish culture can prevent the diseases through augmentation of both specific and non-specific immunity.

Objective: A study was conducted to investigate the efficacy of different dietary doses of *Aegle marmelos* (Linn.) Corr. Serr. (Rutaceae) leaf extract for the immune response and the disease resistance of the freshwater fish, *Cyprinus carpio* Linn. (Cyprinidae) infected by *Aeromonas hydrophila* Chester (Aeromonadaceae).

Materials and Methodology: Hematological, specific immune response, non-specific immune response and enzyme assay studies were performed on fish and were scrutinized after 50 days of feeding trial.

Results: Fish were challenged with *Aeromonas hydrophila* at a dose of 1.5×10^4 cells/mL through intraperitoneal injection, and the hematological changes, the immune response, the enzyme activity and the disease resistance of *Cyprinus carpio* against the pathogen were also studied for 20 days at 5-day intervals.

Discussion: The results obtained from the study demonstrated that the fish fed with leaf extract of *Aegle marmelos* incorporated into feed significantly enhanced the red blood cell count, white blood cell count, hemoglobin, phagocytic activity, nitroblue tetrazolium chloride assay, lysozyme, pathogen clearance and enzyme activity compared with the control group. The survivability was higher in the fish which consumed leaf extract-incorporated feed, and the fish group fed with 5 g diet showed highest percentage survival of the fish.

Conclusion: These results indicate that *Aegle marmelos* stimulates the immunity and makes the freshwater fish *Cyprinus carpio* more resistant to *Aeromonas hydrophila*.

Keywords: *Aegle marmelos; Aeromonas hydrophila; Cyprinus carpio;* immunostimulant; phagocytic activity

Introduction

The intensity of fish farming is affected by many factors including the high sensitivity of fish to stress and the accelerated spread of disease in water. It is important for aquaculturists to focus their efforts to maintain their fish with sound health in order to obtain sustainable economic gains.

Intensive aquaculture practices provide much interest in understanding fish diseases. The fish, which are growing in immunosuppressed conditions, become highly susceptible to disease and the majority of these disease outbreaks are caused by bacterial infections. Bacterial diseases create many practical problems during the culture period of fish and these bacterial diseases can wipe out the entire population of fish in the culture system.

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Hence, aquaculture industries require further development to control disease outbreaks, which constitute one of the major setbacks for successful aquaculture.

The use of immunostimulants in fish culture for the prevention of diseases through augmentation of both specific and non-specific immunity is a promising new development in the field (Anderson, 1992; Rao et al., 2006; Sakai, 1999), since the use of immunostimulants for the prevention of these diseases has not shown any side effects on fish. The use of antibiotics and live vaccines on the fish and on the environment are attractive alternative ways of controlling bacterial infections (Siwicki et al., 1994; Mulero et al., 1998; Logambal & Michael, 2000).

Aeromonads are fish pathogens noted for causing major problems for carp aquaculture. *Aeromonas hydrophila* Chester is more abundant in waters with a high organic load than in relatively unpolluted water (Guojun et al., 2009).

Recent literature on the immunostimulant action of herbal drugs on fish shows that the Chinese herbs *Astragalus radix* Linn. (Fabaceae) and *Ganoderma lucidum* (Curtis) P. Kars (Ganodermataceae) on *Cyprinus carpio* Linn. (Cyprinidae) indicates that the ethanol or methanol triberbal solvent extracts positively influence the immune response and protect the health of goldfish against *A. hydrophila* infection (Harikrishnan et al., 2009). The immunomodulation of *Labeo rohita* Hamilton (Cyprinidae) juveniles was evaluated in terms of immuno-hematological changes after dietary gelatinized and non-gelatinized challenge with *A. hydrophila,* which promoted the growth and protecting immunity in *L. rohita* (Vikas et al., 2007).

A study on the effect of dietary doses of *Euglena viridis* (O.F. Mueller) Ehrenberg (Euglenaceae) on the immune response and disease resistance of *Labeo rohita* fingerlings against infection with the bacterial pathogen *Aeromonas hydrophila* was performed and the study concluded that the *Euglena* stimulates the immunity and makes *L. rohita* more resistant to *A. hydrophila* infection (Das et al., 2009). The plant extract derived from *Cynodon dactylon* (Linn.) Pers. (Poaceae) feed against white spot syndrome virus (WSSV) infection of shrimp *Penaeus monodon* Fabricius (Penaeidae) possessing potential prophylactic action was reported by Balasubramanian et al., 2008. The regulatory roles of rutin extracted from *Toona sinensis* (A. Juss.) M. Roem (Meliaceae) in various functions such as physiological, innate non-specific immune responses to the pathogen *Vibrio alginolyticus* Miyamoto and Sakazaki (Vibrionaceae) in white shrimp *Litopenaeus vannamei* Boone (Penaeidae) were examined, and provided significant effect on immune response (Hsieh et al., 2008). The immunomodulatory effect of microbial levan on *C. carpio* juveniles against *Aeromonas hydrophila* infection shows that the effect is caused by the activation of non-specific phagocytes and the use of microbial polysaccharides, constituting an important parameter in reducing mortality in fish (Rairakhwada et al., 2007).

Cyprinus carpio (Common carp or European carp) is a widespread freshwater fish very closely related to the common goldfish *Carassius auratus* Linn. (Cyprinidae) and is the number one fish of aquaculture. The annual tonnage of common carp produced in China alone exceeds the weight of all other fish such as trout and salmon produced by aquaculture worldwide. The above literature study shows that this fish production is affected by diseases produced by pathogens, and research work is going on in this area to improve or stimulate the immune response of this fish to fight against pathogens (Whyte, 2007).

The present study has been carried out with the aim of improving the immunity of infected fish (*C. carpio)* with herbal drug-containing feed. This feed provided better immunocompetence and disease resistance, in addition to satisfying the dietary nutrient requirements for maximum growth. The experiments were designed to improve the health of fish through the use of *Aegle marmelos* (Linn.) Corr. Serr. (Rutaceae) leaf extracts incorporated into feed as immunostimulant.

Methods

Fish collection and maintenance

Common carp (average weight 45.9 ± 1.5 g, 100 fish in both sex) were purchased from a private fish farm at Kallidaikurichi, Tamil Nadu, India. They were transported to the laboratory in an oxygenated bag and stocked in a tank (capacity 500 L) at a density of 1 g/L. The tank water was changed on alternate days and the fish were fed with a balanced fish diet (Table 1). The excess feed and fecal material were siphoned out once a day. The temperature of the water in the fish tank was kept between 28 and 29°C. Other physiochemical parameters of water were also analyzed systematically at 7-day intervals to maintain optimum levels of dissolved oxygen 6.8–7.2 mg/L,

Table 1. Feed formulation used for the study.

Feed ingredients	Weight (g)	Protein content (%)
Ground nut oil cake	25.3	11.79
Fish meal	25.31	13.85
Rice bran	17.01	2.89
Tapioca	8.01	0.16
Soybean	25.3	12.01
Vitamin tablet	1	-
Cod liver oil capsule	1	-
	102.93	40.7

pH 7.7–8.5 and ammonia 0.08–0.12 mg/L throughout the experiment.

Pathogen isolation and its sensitivity analysis

Aeromonas hydrophila was isolated from diseased fish *C. carpio* showing symptoms of hemorrhagic septicemia. After isolation of the strain, it was identified using a standard microbial identification test and the identified culture was maintained on tryptose soya agar slopes at 4°C for long-term preservation for infecting healthy fish. The isolated bacterial culture was tested for its sensitivity to crude leaf aqueous extract *of Aegle marmelos* by disc diffusion test (agar medium) using 100, 200, 300, 400, and 500 µg/mL concentrations of the crude extract. In consequence of this, the *A. marmelos* incorporated into artificial feed at different concentrations of 0, 5, 10, 20, 25 and 50 g plant leaf extract/kg feed was given as an immunostimulant to *C. carpio*.

Diet formulation

The experimental feed was prepared by mixing the selected feed ingredients with known protein content accordingly to get 40.7% (Table 1). Fresh feed ingredients were procured in dry form and their quality was tested to confirm their protein content by Lowery method. The mixture of the feed ingredients was wetted with water and steam cooked in batches and cooled. To this feed, vitamin mix and cod liver oil were added and mixed thoroughly for even distribution. This feed mixture was then pelletized through hand pelletizer until between 1 and 4 mm and dried in shade to reduce the moisture content of the feed below 10%.

Medicated feed formulation

A. marmelos leaves were collected from Sri Paramakalyani temple Alwarkurichi, Tamil Nadu, India. All the required fresh leaves were taken from a single plant. *A. marmelos* medicated feeds at different doses of 0, 5, 10, 20, 25 and 50 g/kg feed were formulated by adding a prescribed amount of leaf extract to the pre-steam cooked and cooled feed mixture containing 40.7% protein.

Experimental design

The experimental fish *C. carpio* of uniform size (45.9 ± 1.5 g) were stocked in six troughs with ten fish each in triplicate (including control). The formulated feeds at various concentrations (0, 5, 10, 20, 25 and 50 g/kg) were given separately at 2% of body weight for a period of 50 days (the concentrations were fixed for the study from the minimum dose). After 50 days of feeding, all the experimental fish were given only control feed. At day 50 of immunomodulation the fish were infected with bacterial pathogen *Aeromonas hydrophila* through intraperitoneal injection at a dose of 1.5×10^4 cells/mL. After 5 days of infection, studies were carried out once every 5 days up to day 20 to observe the hematological and immunological changes and the biochemical responses. These responses were also studied prior to the challenge of pathogen, which served as control values.

Collection of blood and antiserum

The fish were bled serially using a 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 the 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 and stored in sterile vials. The serum was kept at 57°C in a water bath for 30 min to inactivate the complement system and was 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 (Sigma-Aldrich, UK), and the white blood cell count (WBC) (10^4 mm^{-3}) from a 1:20 dilution of the blood sample in Turk's solution (Sigma-Aldrich, UK), with a Neubaeur hemocytometer (BiosciTech, Mumbai). Hemoglobin content (Hb:gm/dL) was determined by the cyanmethemoglobin method (Gowenlock, 1996); 20 µL of the anticoagulated blood was mixed with 4 mL of Drabkin's reagent (Sigma-Aldrich, UK), and kept at room temperature for 4 min and read at 540 nm with a UV spectrophotometer.

Specific immune response

Antigen–antibody titration (bacterial agglutination assay)

Circulating antibody titers were performed in 96-well microtiter plates using two-fold dilutions. The titer was recorded as the highest dilution in which visible agglutination (mat-like observation) could be seen. Dot-like formation was considered a negative response (Vallinayagam, 1997).

Non-specific immune response

Assay of phagocytic activity

The phagocytic activity assay was performed by the following modified method of Sahoo and Mukherjee (2002).

Blood sample (EDTA mixed) was mixed with an 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 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 engulfing the bacteria were counted (under microscope) as positive (Seeley et al., 1990).

The percentage of bacteria ingested by phagocytes (phagocytic ratio) was calculated by Equation 1.

$$\text{Phagocytic ratio} = \frac{\text{Number of phagocytic cells with engulfed bacteria}}{\text{Number of phagocytes}} \times 100 \quad (1)$$

Nitroblue tetrazolium chloride assay

One drop of pooled heparinized blood (from 6 fish) was placed on a cover slip immediately after collection and was placed in a humid chamber (on 60 mm Petri dishes with a wet paper towel) and incubated for 30 min at room temperature for the neutrophils to stick on the glass. After incubation the cover slips containing the cells were transferred upside down to a clean glass slide containing a 50-µL drop 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 microscope (Sahoo & Mukherjee, 2002).

Serum lysozyme activity

Lysozyme activity was analyzed spectrophotometrically according to Santaram et al. (1997) and Sankaran et al. (1972). A standard suspension of *Micrococcus lysodeikticus* was prepared in 0.066 M phosphate buffer (pH 7). 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 measured. Standard solutions containing 2.5, 5, 7.5, 10 and 12.5 µL/mL of hen egg lysozyme in 0.066 M phosphate buffer were used to develop a standard curve.

Pathogen clearance in blood

Pathogen count in the blood sample was carried out by the following procedure of Vallinayagam (1997). The blood sample was serially diluted and the pathogens were counted by using pour plate technique with specific agar (thiosulfate citrate bile salts sucrose (TCBS) agar, Himedia, Mumbai, India) in the Petri dishes and then incubated at 37°C for 24 h. The values were expressed in cfu/mL.

Enzyme assay

Acid and alkaline phosphatase

Acid and alkaline phosphatase activity was estimated by the following method (Pattabiraman et al., 1998). A serum sample (10 µL) was mixed with 5 mL of substrate solution and the absorbance was read at 420 nm immediately. Then the substrate solution with serum sample was incubated at 37°C for 30 min and the absorbance was read at 420 nm. The optical density (OD) difference was noted and the activity was calculated by using Equation 2.

$$\text{Phosphatase activity } (\mu\text{M PNP/mg protein}) = \frac{\text{OD difference of test}}{\text{OD of standard} \times 1/\text{sample taken}} \times \text{conc. of standard} \times 1/\text{incubation time} \times 1/\text{mg protein} \quad (2)$$

Serum peroxidase

Serum peroxidase was analyzed by the procedure of Murugesan and Rajakumari (2005). To 1.4 mL of amino-antipyrine, 1.5 mL of H_2O_2 was added and to this mixture, 0.1 mL of serum sample was added and the extinction was measured at 510 nm for about 5 min. The solution without serum served as control and the standard solutions containing different concentrations of peroxidase in 0.066 M phosphate buffer were used to construct a standard curve. The results are expressed in IU/mL.

Disease resistance

The effects of plant leaf extract incorporated into feed for disease resistance on groups of fish (n = 20/group) were determined. The fish were artificially challenged with a dose of 1.5×10^4 cells/mL of live virulent pathogen *A. hydrophila*. The mortality was observed for 20 days and the average of a triplicate set was used for expression in terms of percentage of survival.

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 $P < 0.05$. This statistical work was

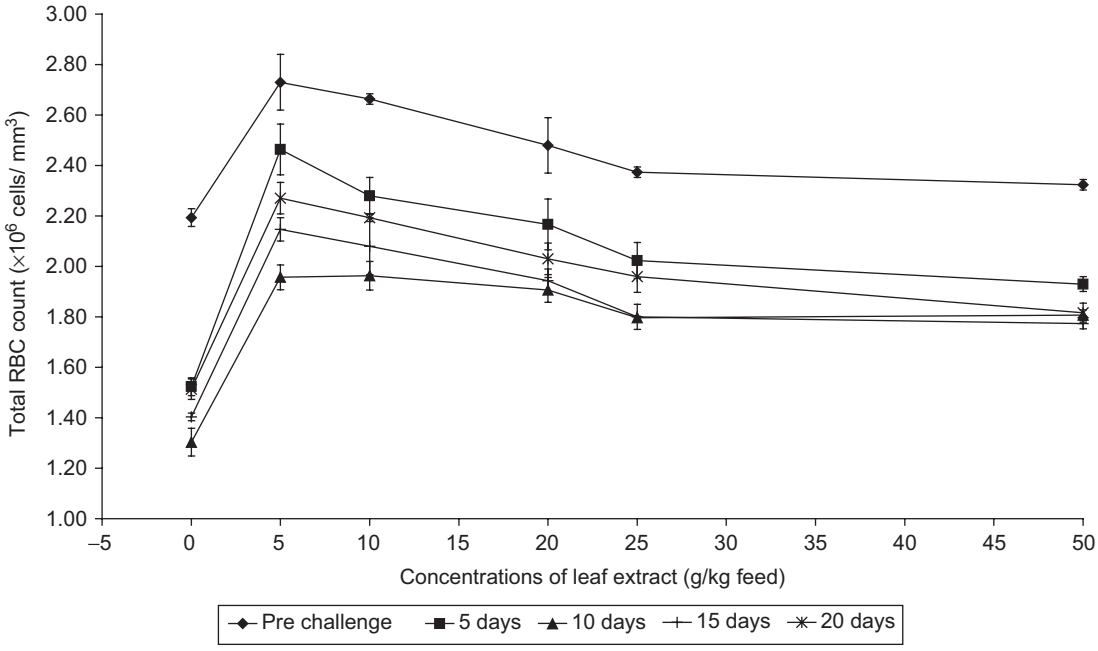

Figure 1. Effect of different concentrations of leaf extract of *Aegle marmelos* on total RBC count (×10^6 cells/mm^3) in *Cyprinus carpio* infected with the bacterial pathogen *Aeromonas hydrophila*.

performed manually with the help of Microsoft Excel program.

Results and discussions

The present study was carried out to evaluate the hematological and enzymatic changes in the freshwater fish *C. carpio* after being artificially infected with the pathogen *A. hydrophila*. The immunomodulatory effect of the leaf extract of *A. marmelos* was found by feeding various concentrations of the extract to the fish. The results obtained from the study are discussed hereunder.

The sensitivity of the *A. marmelos* leaf extract against the pathogen *A. hydrophila* was performed by agar plate diffusion method. The zone of inhibition obtained from the study shows that the extract at various concentrations (100, 200, 300, 400 and 500 µg/mL) has significant activity against the pathogen *(A. hydrophila)*. Earlier literature has also shown that *A. marmelos* extracts possess antimicrobial activity against bacterial species (Madasamy et al., 2003).

Hematological responses

The effectiveness of the 50-day feeding regimes of *A. marmelos* medicated feed at different doses on *C. carpio* and those exposed to *A. hydrophila* for infection were recorded. The fish fed with feeds having leaf extract of *A. marmelos* were able to enhance their total RBC count significantly ($P < 0.05$) and the results obtained show that the maximum RBC count of $2.73 \pm 0.011 \times 10^6$ cells/mm^3 was noticed in the feed having 5 g leaf extract of *A. marmelos*/kg. In control fish, the total RBC count was $2.19 \pm 0.035 \times 10^6$ cells/mm^3. After infection with the pathogen the RBC count was decreased in the control fish up to 10 days after challenge, followed by slight increase in RBC count. The fish fed with feeds incorporating leaf extract of *Aegle marmelos* also showed decreased RBC count up to 10 days after infection; later the RBC count increased marginally. Among all the concentrations of leaf extract incorporated feeds, the fish fed with feed having 5 g ($P < 0.01$) leaf extract of *A. marmelos* showed higher RBC count compared with the other feeds (Figure 1). The feed having 10 and 20 g ($P < 0.05$) leaf extracts of *A. marmelos* showed significant difference in RBC count whereas the fish fed with 25 and 50 g plant leaf extract did not show any significant difference when compared with the control. The results obtained show that the leaf extract of *A. marmelos* has significant influence on the hematology of *C. carpio*.

WBC count

The WBC count was significantly increased in fish fed with feed having leaf extract of *A. marmelos*. After infection with the pathogen, the WBC count increased in fish fed with 10 g leaf extract for 10 days. After 10 days of infection, the WBC count was decreased. In the control, the WBC count increased on day 5 and then decreased

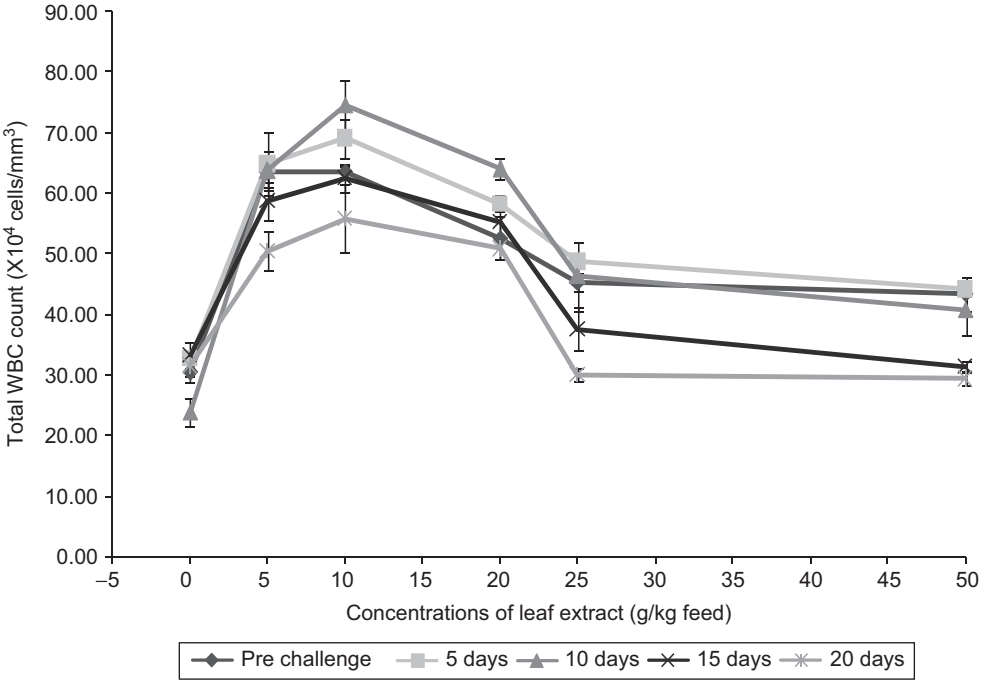

Figure 2. Effect of different concentrations of leaf extract of *Aegle marmelos* on total WBC count (×10⁴ cells/mm³) in *Cyprinus carpio* infected with the bacterial pathogen *Aeromonas hydrophila*.

on day 10, after which the WBC count increased slightly (Figure 2). It is apparent from the figure that the feeds having leaf extract increased the total WBC count up to day 10 after infection with the maximum WBC count of $74.57 \pm 4.004 \times 10^4$ cells/mm^3 for 10 g leaf extract. Statistical analysis decisively indicates that all the concentrations of leaf extracts of *A. marmelos* enhanced the WBC count effectively at 1% level, when compared with the control.

Hemoglobin content

The hemoglobin content of the control fish was found to be 8.93 ± 0.056 g/dL. The hemoglobin content increased in the pre-challenged fish fed with feeds having *A. marmelos* leaf extract, and the maximum value of 11.09 ± 0.094 g/dL was noticed in the feed having 10 g leaf extract, followed by the feeds having 5, 20, 25 and 50 g leaf extracts of *A. marmelos*/kg feed. After infection with the pathogen, the hemoglobin content decreased in all fish, and after 10 days of infection the hemoglobin content increased with a maximum value of 7.53 ± 0.029 g/dL in fish fed with 5 g leaf extract of *A. marmelos* on day 20. Figure 3 reveals that all the values of mean hemoglobin content in challenged fish were significantly less than in pre-challenged fish. Among the challenged fish, the fish fed with feed having 50 g leaf extract/kg showed a lower concentration of hemoglobin throughout the period of experiment. Also it was found that there was no significant difference in the hemoglobin content between the concentrations when compared with the control, except fish fed with feed having 5 g ($P < 0.01$). In general, all the concentrations showed significant variation ($P < 0.05$) in the hemoglobin level from day 5 of post-challenge with pathogen.

The results obtained from the hematological studies show that the fish fed with feeds having the leaf extract of *A. marmelos* significantly enhanced the RBC count, hemoglobin content and WBC count when compared to that of the control fish. Cooper et al. (1963) reported that mitochondria play a significant role in iron metabolism in developing erythrocytes. The results of the present study also suggested that the leaf extract of *A. marmelos* may induce erythropoiesis and lymphopoiesis resulting in the increase of RBC count, hemoglobin content and WBC count. The experiments conducted with feeds incorporating *Acalypha indica* Linn. fed to *Oreochromis mossmbicus* Peters (Anita, 1998); *A. marmelos* fed to *Catla catla* Hamilton (Madasamy, 2003) and *Phyllanthus emblica* Linn. fed to *Cirrhinus mrigala* Hamilton (Diana, 2006) also showed that fish fed with lower concentrations of leaf extracts increased the RBC count, hemoglobin content and WBC count and the same values decreased at higher concentrations as observed in the case of *A. marmelos* in the present study (Figures 1–3). After challenge with *A. hydrophila,* the RBC count and hemoglobin content decreased for 10 days, indicating that the stress may be induced by the pathogens. In almost all infected fish the homostatic processes are extended beyond the normal limits due to stress (Pickering, 1981). Suzumoto et al. (1977) also reported that the changes in blood profile of

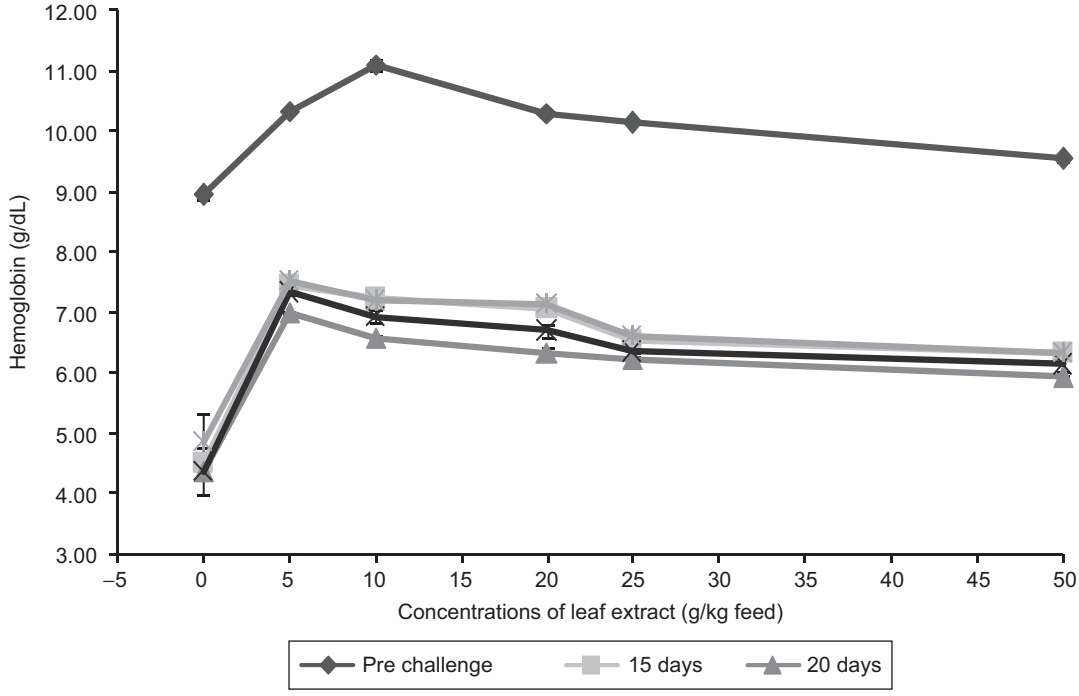

Figure 3. Effect of different concentrations of leaf extract of *Aegle marmelos* on hemoglobin content (g/dL) in *Cyprinus carpio* infected with the bacterial pathogen *Aeromonas hydrophila*.

microbially infested fish, *Oncorhynchus kisutch* Walbaum especially documented a decline in erythrocytes and hemoglobin content. The decreased hemoglobin content may be brought about as a result of the swelling of RBC as well as poor mobilization of hemoglobin from the spleen and other hematopoietic organs in *Ictalurus punctatus* Rafinesque (Scott & Rogers, 1981). These facts support the present finding of significant decrease in erythrocyte and hemoglobin content in fish infected with bacterial pathogens, possibly due to hypochromic microcytic anemia caused by the bacteria. In the extract-treated group, the level of pathogenicity decreased when compared with the control fish. The decreased RBC counts and lower hemoglobin concentration in infected fish indicate that the RBCs are being destroyed by the leukocytosis activity in an erythrocytic anemia with subsequent erythroblastosis (Haney et al., 1992). Kumar et al. (2006) in their experiments found that the RBC count and the hemoglobin content were significantly reduced due to bacterial challenge, but dietary starch (gelatinized and non-gelatinized) had no effect on it, whereas the dietary starch enhanced WBC count. Ellias (1976) suggested that lymphocytes are the executive cells in the specific immune mechanism of fish, and they become stimulated under stress conditions to fight against entry of foreign bodies into the blood stream. In the present investigation, the increase in WBC count may be the result of stimulation caused by the entry of pathogens. The increase in WBC count in fish fed with feeds having leaf extract in the present study may be also due to the induction of active compounds present in the leaf extract, consequent to infection. The hematological results of the present study reveal that the leaf extracts were able to reduce the immunosuppression caused by the pathogen.

Specific immune response

Antigen antibody titration

The antibody response to the pathogen *A. hydrophila* in *A. marmelos* medicated feed at different dose treatments in the experimental fish is presented in Figure 4. The results indicate that the feeds with leaf extract strongly enhanced the primary immune response in comparison with control fish. The fish fed with leaf extract-incorporated feed developed immunological competence on day 5 after infection with the pathogen, whereas the control fish developed immune response on day 10 post-bacterial challenge. This clearly indicates that a single booster dose of leaf extract is sufficient to elicit a high antibody titer and the immunization schedule adopted gives a high degree of protection against the bacterial pathogens tested in common carp. Although all the concentrations of the leaf extract enhanced the antibody response and the lower concentrations (5 and 10 g) of *A. marmelos* were only able to stimulate higher antibody production, statistically no clear concentration dependency in the enhancement of antibody production was noticed. The previous studies indicated that leaf extracts of *Ocimum sanctum* Linn. (Lamiaceae) in *Oreochromis mossambicus* (Logambal & Michael, 2000; Venkatalakshmi & Michael,

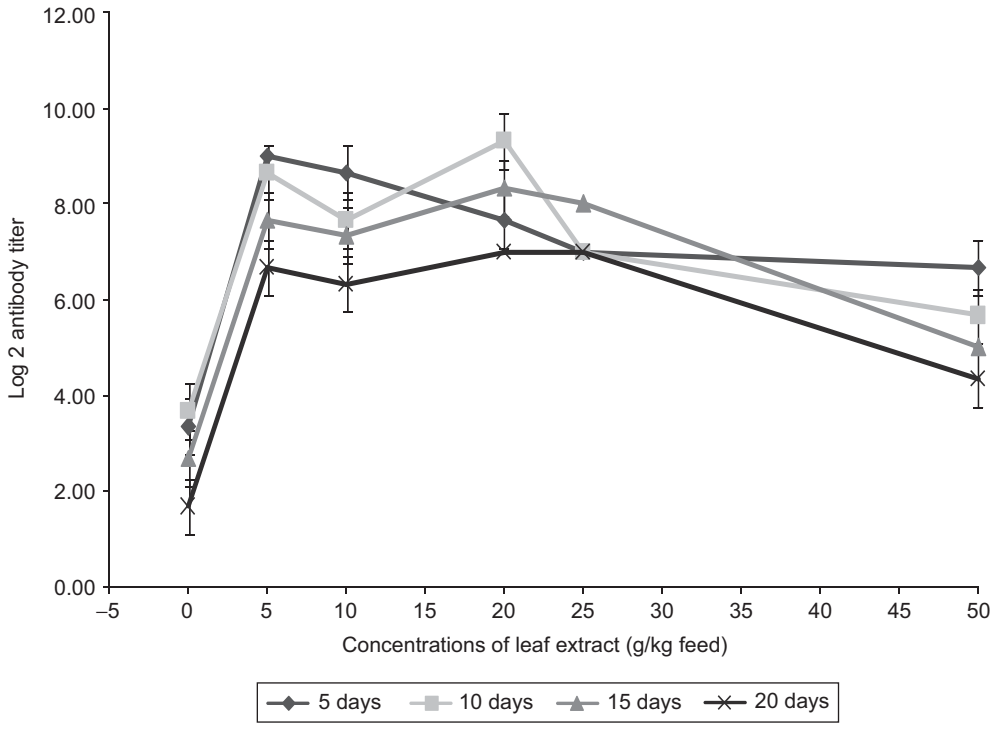

Figure 4. Effect of different concentrations of leaf extract of *Aegle marmelos* on antigen antibody titration in *Cyprinus carpio* infected with the bacterial pathogen *Aeromonas hydrophila*.

2001) enhanced both primary and secondary immune responses to sheep erythrocytes, and they reported that the aqueous extract of *O. sanctum* leaves not only enhanced the magnitude of antibody response, but also significantly shortened the lag period of both primary and secondary immune responses. Davis and Hayasaka (1984) showed that antibody titer rise on days 7 and 14 could be greatly enhanced by an intravenous injection of extract 2 days post-bacterial challenge. Vallinayagam (1997) reported that antibody production predominantly increased when the fish, *O. mossambicus*, were fed with vitamin C at 60 to 70 mg/kg body weight, and the peak response was seen on day 9 after administration. The present findings are in agreement with the reports on significant increase in hemagglutination antibody titers in *O. mossambicus* fed with *Acalypa indica* (Anita, 1998), *A. marmelos* in *Catla catla* (Madaswamy, 2003), and *Phyllanthus emblica* in *Cirrhinus mrigala* (Regina, 2006). The result obtained from the present study also reveals that the leaf extracts in the feed of carp were able to stimulate specific antibodies against the challenged bacteria and raise the humoral immunity.

Non-specific immune response

Nutritional modification has been used to prevent the outbreak or to reduce the severity of infections in fish (Chandra, 1996; Scrimshaw & San Giovanni, 1997). Studies regarding nutritional effects on fish immunity were focused on antibody-mediated immunity against non-specific events, on which fish seem to rely much more heavily than higher vertebrates (Landolt, 1989). In this present investigation, several assays were carried out to test the efficacy of the leaf extract as immunostimulant.

Phagocytic activity

Phagocytosis is the most common cellular defense mechanism, and together with humoral components it constitutes the first line of defense mechanism against a parasite or an intruder. The phagocytic activity of a fish can be modulated by a wide range of endogenous and exogenous factors, which enhance or modulate the fish immune system (Secombes, 1994). The present results clearly show that the leaf extract was able to stimulate the phagocytic activity and enhanced the phagocytic ratio significantly (Figure 5). Phagocytic activity is mediated by cytokines such as macrophage activating factor secreted by peritoneal lymphocytes (Graham & Secombes, 1988). From the results it is also clear that the chemotaxis process was stimulated by the lower concentrations (5 to 10 g) of the leaf extract of *A. marmelos.* The increase in phagocytic ratio indicates that the leaf extract stimulated the synthesis of chemotactic factors as observed in the study of Fujiki and Yano (1997). Phagocytic activity of blood leukocytes were increased in rainbow trout fed with 1% ginger (*Zingiber officinale* Roscoe) (Dugenci et al., 2003). Misra et al. (2006a, 2006b) found that injection of

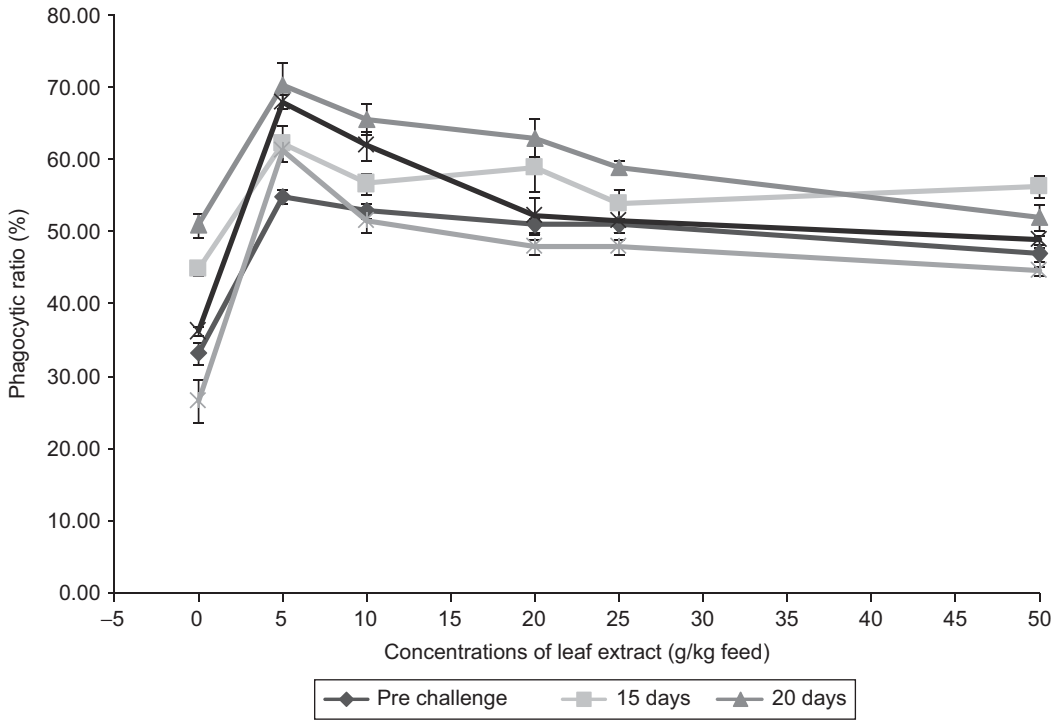

Figure 5. Effect of different concentrations of leaf extract of *Aegle marmelos* on phagocytic ratio (%) in *Cyprinus carpio* infected with the bacterial pathogen *Aeromonas hydrophila*.

different doses of tuftsin enhanced the phagocytic activity in *Labeo rohita* and maximum activity was noticed on day 42 after injection of tuftsin. In the present study, after infection with the pathogen, the phagocytic ratio was increased in all the fish which consumed feed with leaf extract, and the phagocytic ratio was higher up to 10 days after infection. The peak phagocytic ratio of 70.33 ± 3.21% was noticed in fish fed with feed incorporating 5 g leaf extract of *A. marmelos*/kg feed. In the control fish, the phagocytic ratio was found to be 33.33 ± 1.52%, and after infection with the pathogen *A. hydrophila,* the phagocytic ratio increased up to 51 ± 1.73% on day 10 of infection and the ratio further decreased up to day 20 of infection (Figure 5). The fish fed with leaf extract of *A. marmelos* were significantly enhanced ($P < 0.01$) the phagocytic ratio when compared with the control fish and the ratios were maximally significant ($P < 0.05$) on day 10 of infection. The results also reveal that macrophage migration in the presence of exoantigen was enhanced with the incorporation of different levels of leaf extract of *A. marmelos* in fish feed.

NBT assay

NBT assay is a quick, inexpensive method focusing on the ability of phagocytes to reduce the dye by 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; Babior et al., 1973). These phagocytic cells can be elicited upon suitable stimulation by soluble components such as lectins, lipopolysaccharides (LPS), yeast, vitamin C, glucans, zymosan, leaf extracts, etc. The stimulation of the phagocytic cell membrane leads to increased consumption of oxygen, in which the reduction process is catalyzed by a membrane-bound enzyme, NAD(P) H-oxidase giving rise to superoxide (O^{2-}). A number of oxides through various reactions lead to the production of hydrogen peroxide (H_2O_2), singlet oxygen (O_2^1) hydroxyl radical (OH) and numerous other reactive products (Higson & Jones, 1984; Munoz et al., 2000). In the present study the NBT-positive cells were found to be significantly increased ($P < 0.01$) with the increase in days after infection in fish fed with feeds of leaf extracts (Figure 6). In control fish, the number of NBT positive cells was found to be 10 ± 1, and after infection with the pathogen *A. hydrophila* the NBT-positive cells increased and the peak of NBT-positive cells was found on day 10 after infection and the value was 17 ± 0.816. After day 10 of post-infection, the number of NBT-positive cells in the fish was decreased. The same trend of increase in the number of NBT-positive cells was noticed in fish fed with leaf extract of *A. marmelos* feed. Maximum number of 45 ± 0.816 NBT-positive cells was noticed on day 10 in fish fed with feed having 10 g of leaf extract of *A. marmelos*/kg feed. It is clear that a significant increase in the NBT-positive cells was also noticed in fish fed with feed having

5, 10 and 20 g of leaf extract of *A. marmelos* at the 1% level, whereas 25 and 50 g leaf extracts showed variations at the 5% level. Significant enhancement of NBT-positive cells in all concentrations was noticed during the days after infection with *A. hydrophila.* This is probably due to the increase in lysozyme activity. Lysozyme production is mainly based on the neutrophils and monocytes present in the blood. This is also reported by the fact that lysozyme activity in fish fed with feeds having leaf extract concentration was found to be higher than the control, suggesting the production of more NBT-positive cells as found in the present study (Figure 7).

The leaf extract of *A. marmelos* administered through diet enhanced the non-specific defense mechanism in terms of increased number of activated neutrophils. This finding is in agreement with the earlier findings where number of NBT-positive cells in the blood of carp significantly increased at 20 and 30 days post-feeding with

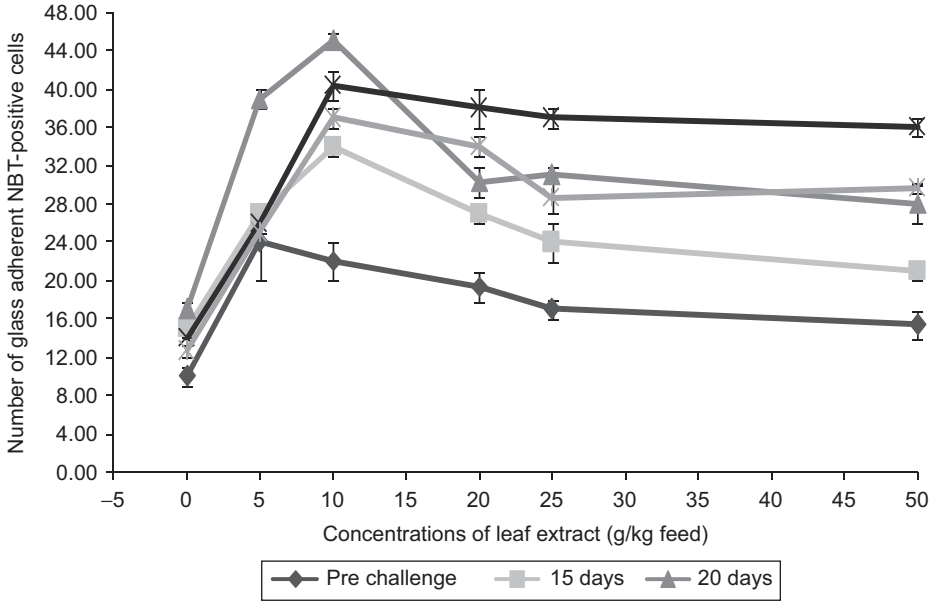

Figure 6. Effect of different concentrations of leaf extract of *Aegle marmelos* on number of glass adherent NBT assay positive cells in *Cyprinus carpio* infected with the bacterial pathogen, *Aeromonas hydrophila*.

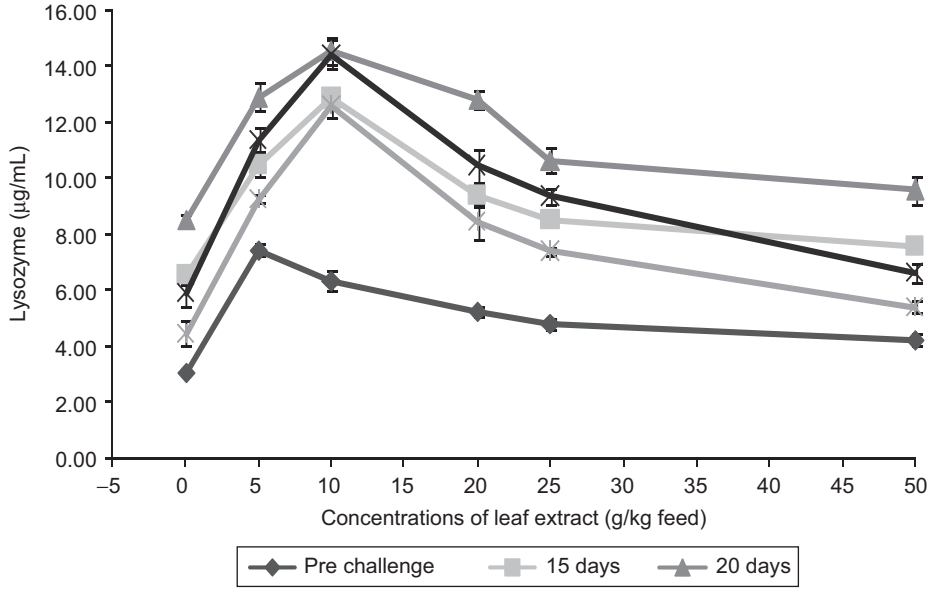

Figure 7. Effect of different concentrations of leaf extract of *Aegle marmelos* on serum lysozyme activity (µg/mL) in *Cyprinus carpio* infected with the bacterial pathogen *Aeromonas hydrophila*.

traditional Chinese medicine (TCM), a formulation from *Astragalus* root and Chinese *Angelica* root at 1% and 1.5% (Jian & Wu, 2004). Water soluble fractions of *Crossandra infundibuliformis* Linn. leaves also enhanced NBT positive cells in *O. mossambicus* (Venkatesan et al., 2001). The fish *Labeo rohita* when fed with gelatinized and non-gelatinized starch with n-3 PUFA were enhanced in the NBT assay (Mirsa et al., 2006b). Kumari et al. (2007) reported that poly herbal formulation (Immunoplus) enhanced the NBT assay in *L. rohita*. The previous findings support the view that external stimulants such as plant extracts stimulate 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 were also enhanced in fish fed with feeds having leaf extracts.

Serum lysozyme activity

The lysozyme activity has been found to be modulated by a range of factors including stress, water temperature, injection of foreign materials, nutrients, etc. (Fletcher & White, 1973; Sakai, 1999). The major lysozyme secretory cells of higher vertebrates are the macrophage cells (Schnydes & Baggioline, 1978). The results of the present investigation indicate that the leaf extract at various concentrations significantly enhanced the serum lysozyme activity in *C. carpio* (Figure 7). The mean values of serum lysozyme activity obtained in *C. carpio* fed with feed incorporated with different concentrations of leaf extract of *A. marmelos* and infected with *A. hydrophila* are represented in Figure 7. From the figure it is clear that in pre-challenged fish, the serum lysozyme activity of 7.44 ± 0.36 µg/mL was observed in fish fed with feed having 5 g leaf extract of *A. marmelos*/kg feed. It is apparent from the figure that the serum lysozyme activity was decreased after 10 days in all the fish fed with feeds having different concentrations of leaf extract, but the reduction was not prominent in the fish fed with feed having 10 g leaf extract of *A. marmelos*/kg feed, when compared to other experimental fish. These increased levels of lysozyme in fish fed with feeds with plant leaf extract could be the result of an increment in the number of phagocytes (macrophage) resulting in the secretion of more lysozyme by these cells. It can also be seen that the lower concentrations (5 to 10 g) of *A. marmelos* significantly enhanced the serum lysozyme activity.

The earlier reported literature also reveals that the medicinal plants are effective in enhancing serum lysozyme activity. Jian and Wu (2004) reported that serum lysozyme activity significantly increased when the fish, was fed with traditional Chinese medicine (TCM) formulation from Astragalus root (*Radix astragali*) and Chinese Angelica root (*Angelica sinensis* (Oliv.) Diels (Umbelliferae). Poly herbal formulation (Immunoplus) (Kumari et al., 2007) and *Mangifera indica* Linn. (Sahu et al., 2007) in the feed of *L. rohita* also enhanced the serum lysozyme activity. All the above studies show the effect of herbal plants on lysozyme activity and are in confirmation with the present study in which the leaf extracts also enhanced serum lysozyme activity.

Pathogen clearance

The average values of pathogen clearance in the blood of *C. carpio* fed with feeds having different concentrations

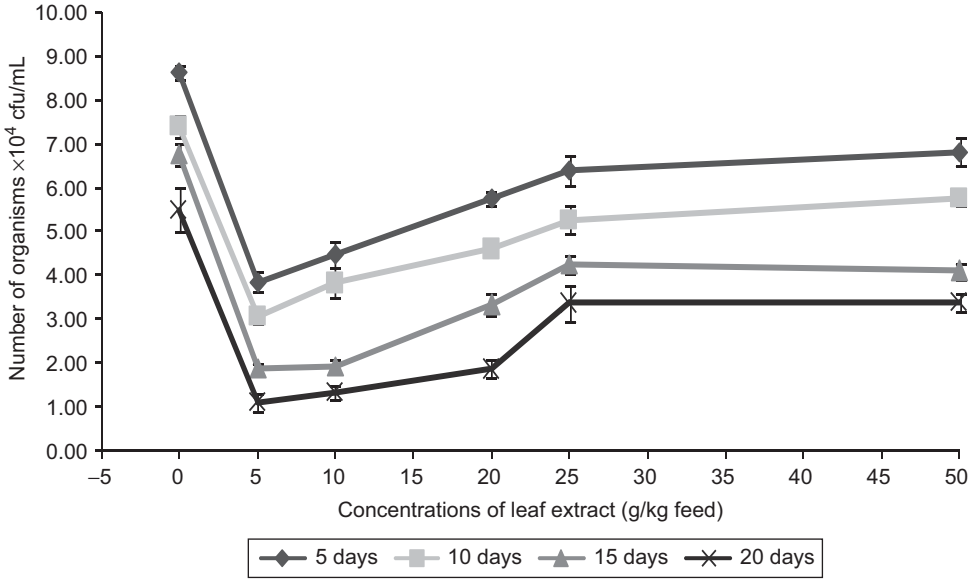

Figure 8. Effect of different concentrations of leaf extract of *Aegle marmelos* on pathogen clearance in the blood of the freshwater fish *Cyprinus carpio* infected with the bacterial pathogen *Aeromonas hydrophila*.

of leaf extract of *A. marmelos* and infected with *A. hydrophila* are graphically shown in Figure 8. From the figure it is evident that control fish showed $8.63 \pm 0.15 \times 10^4$ cfu/mL of pathogen in the blood on day 5 after infection and on subsequent days the pathogen count in the blood was decreased, and on day 20 the count was found to be $5.5 \pm 0.5 \times 10^4$ cfu/mL in control fish. It shows that the fish fed with feeds having *A. marmelos* leaf extract were also able to eliminate the pathogen from the blood significantly when compared with the control fish.

The data on the pathogen clearance in the blood of the freshwater fish *C. carpio* indicate that the feeds with 5 and 10 g leaf extracts of *A. marmelos* were able to eliminate the pathogen significantly at 1% level ($P<0.01$). All other feeds were not found to have a significant impact in respect of pathogen clearance.

The results of the pathogen clearance show that the fish consuming feeds with leaf extract were able to eliminate the pathogen from the blood significantly ($p<0.01$). The leaf extract enabled the fish to eliminate the pathogens on day 10 after bacterial challenge. This is also supported by the results of specific and non-specific immune parameters. The decrease in specific and non-specific immune response after day 10 may be due to the elimination of the pathogen from the fish body. Yoshida et al. (1993) noticed that bacterial counts in the blood and spleen decreased in the fish *Clarias gariepinus* Burchell when treated with glucan. *Acalypha indica* leaf extract in the feed of tilapia was also able to eliminate the pathogen from the kidney and blood (Sukumaran & Anita, 2001). Sukumaran and Vallinayagam (2002) also found that tilapia fed with vitamin C were able to eliminate the pathogen *Pseudomonas fluorescens* Flugge from the blood and kidney. *Labeo rohita* showed better bactericidal activity when they were fed with *Achyranthes aspera* Hook F. seeds (Rao et al., 2006). Sahu et al. (2007) and Misra et al. (2006a) observed increased pathogen clearance in rohu when the fish were fed with *Mangifera indica* and tuftsin incorporated into feeds, respectively. On the basis of these reported results, it may be explained that the total immunostimulation property of the plant extracts may be the reason for better pathogen clearance, as indicated by total specific immune responses.

Enzyme assay

Phosphatase activity

Phosphatase enzyme is a liposome and acts as a non-specific mechanism. Acid phosphatase activity is widely considered to be a valuable parameter of macrophage activation (Sveinbjornsson & Seljelid, 1994).

A. hydrophila-infected fish show an increasing trend of the acid phosphatase activity for 10 days and the maximum acid phosphatase activity of 1.898 ± 0.024 was observed in fish fed with 10 g leaf extract of *A. marmelos* feed. However, the peak value of serum acid phosphatase activity (2.97 µM PNP/mg protein) was observed in fish fed with 20 g leaf extract of *A. marmelos* feed on day 10 after infection. On day 20 after infection, the production was high in fish fed with 10 g extract of *A. marmelos*/kg feed, the value being 1.403 ± 0.085 µM PNP/mg protein (Figure 9). Among the different concentrations tested,

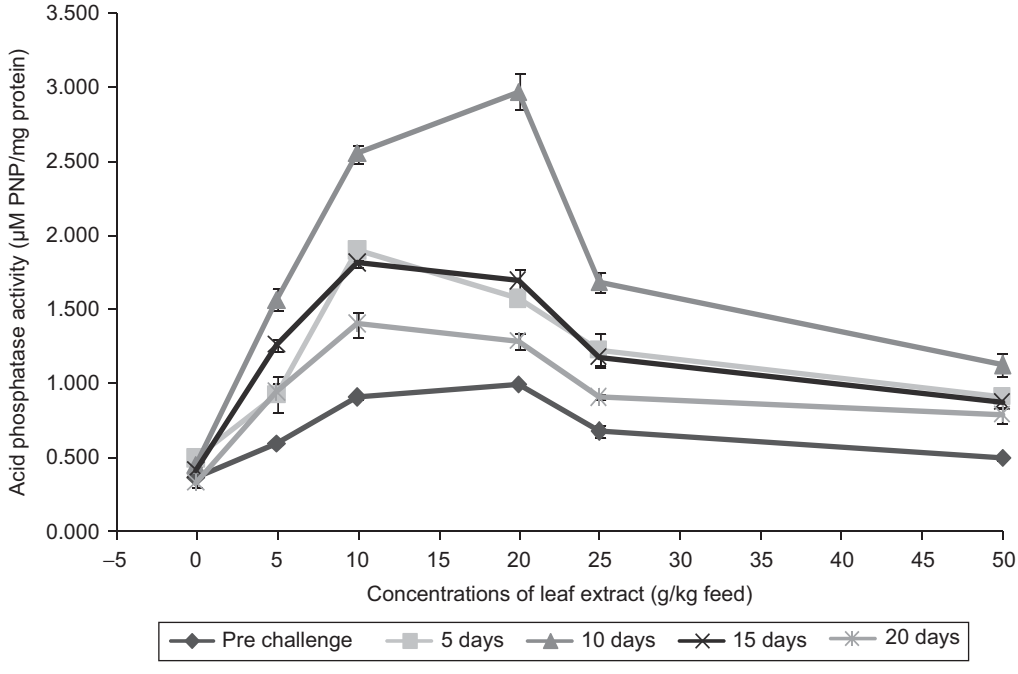

Figure 9. Effect of different concentrations of leaf extract of *Aegle marmelos* on serum acid phosphatase (µg PNP/mg protein) in *Cyprinus carpio* infected with the bacterial pathogen *Aeromonas hydrophila*.

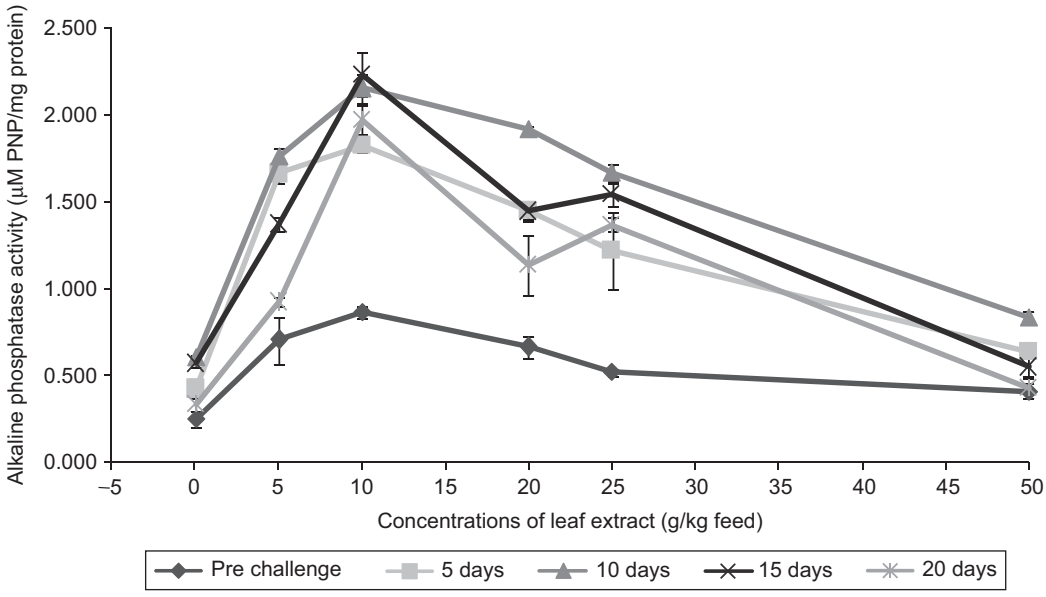

Figure 10. Effect of different concentrations of leaf extract of *Aegle marmelos* on serum alkaline phosphatase (µg PNP/mg protein) in *Cyprinus carpio* infected with the bacterial pathogen *Aeromonas hydrophila*.

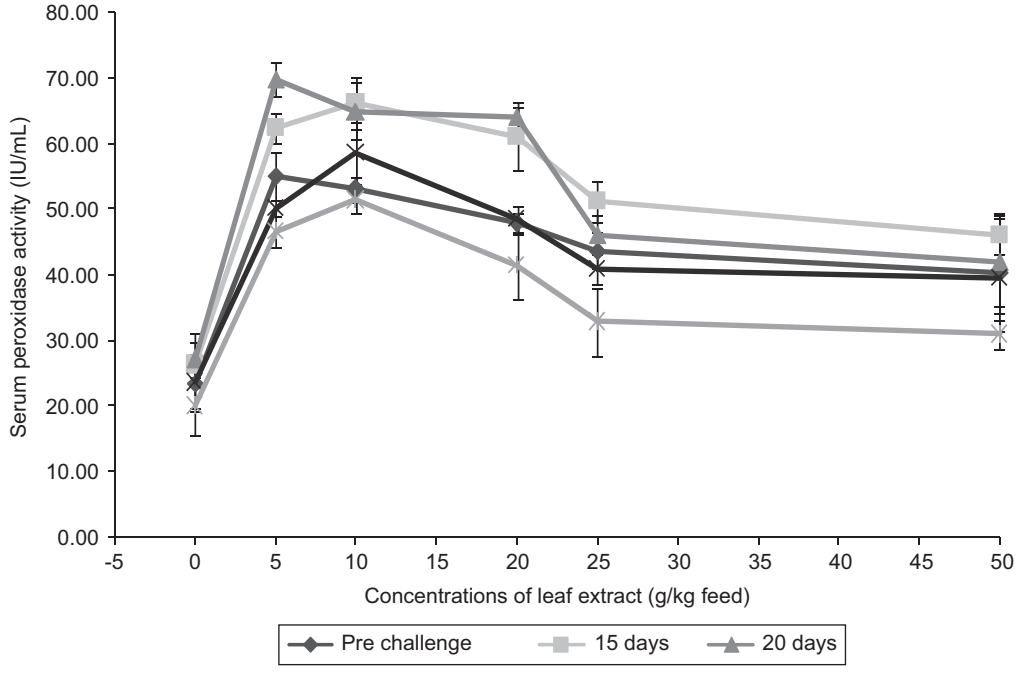

Figure 11. Effect of different concentrations of leaf extract of *Aegle marmelos* on serum peroxidase activity (IU/mL) in *Cyprinus carpio* infected with the bacterial pathogen *Aeromonas hydrophila*.

10 g leaf extract performed better than other concentrations experimented. Feeding the fish with *A. marmelos* significantly enhanced the acid phosphatase activity ($P < 0.01$) when compared with the control, and significant enhancement was seen up to day 15 after infection.

Figure 10 provides information about the alkaline phosphatase activity. It shows that the extracts were able to stimulate the alkaline phosphatase activity and the maximum alkaline phosphatase activity of 0.866 ± 0.034 µM PNP/mg protein was noticed in the fish fed with 10 g extract of *A. marmelos*/kg feed. It is also evident from Figure 10 that among the various concentrations of leaf extract experimented with in common carp, the 10 g extract feed showed the maximum serum alkaline phosphatase activity throughout the experiment. The fish fed with 10 g extract of

A. marmelos were able to enhance the serum alkaline phosphatase up to day 15 after infection, the value being 2.237 ± 0.127 μM PNP/mg protein. All other concentrations were able to enhance the serum alkaline phosphatase activity only up to 10 days after infection.

The results of the acid and alkaline phosphatase activity indicate that the fish *C. carpio* fed with feed having leaf extract of *A. marmelos* showed significant enhancement in the phosphatase activity when compared with control (Figures 9 and 10) suggesting that the enhancement of serum phosphatase activity in fish fed with leaf extract may possibly due to increase of macrophage cells, which in turn produced higher amount of phosphatase enzyme, resulting in enhanced enzyme activity as observed by Dalmo and Seljelid (1995), who reported that lipopolysaccharide (LPS) stimulated the macrophage cells for the higher enhancement of acid phosphatase when compared to the control macrophage cells. Press et al. (1995) noticed the increased amount of acid phosphatase in tissues of Atlantic salmon administered with furunculosis vaccines. Rao et al. (2006) reported that *Achyranthes aspera* in feed enhanced the serum alkaline phosphatase activity in *L. rohita*, as observed in common carp in the present study.

Serum peroxidase

The release of myeloperoxide enzyme is mostly by azurophilic granules of neutrophils during oxidative respiratory burst activity, which is measured through the serum peroxidase activity. Figure 11 reveals that after infection with the pathogen on the control fish, the serum peroxidase activity was increased and the activity was found to be 27.10 ± 3.98 IU/mL up to 10 days after infection, and on subsequent days the serum peroxidase activity decreased. The fish which consumed feed having plant leaf extract showed higher serum peroxidase activity of 54.96 ± 3.67 IU/mL in fish fed with feed incorporating 5 g leaf extract of *A. marmelos*/kg feed, followed by the fish fed with feeds having 10, 20, 25 and 50 g leaf extracts of *A. marmelos*/kg feed. Of all the fish fed with leaf extracts of *A. marmelos*, the maximum serum peroxidase activity of 51.33 ± 1.93 IU/mL was noticed in fish fed with 10 g leaf extract of *A. marmelos*/kg feed. From the results obtained in the present study it is clear that the leaf extract at different concentrations was able to stimulate the serum peroxidase activity as evidenced from the increased enzyme activity in fish fed with feeds incorporating leaf extract of the plant, when compared with control fish. This is also supported by a study conducted by Kumari and Sahoo (2006), which showed that β-glucan enhanced the serum peroxidase activity in *Clarias batrachus* Linn. for about 21 days. In the present study, serum peroxidase activity, which is the indicator of humoral immune response that was found to be high up to 10 days after infection and subsequently the activity decreased, indicating that the leaf extract had stimulation effect on peroxidase activity up to 10 days after infection and subsequently the effect was found to be nullified resulting in the decrease in serum peroxidase activity. Cuesta et al. (2005) found that leukocyte peroxidase decreased in gilthead Sea bream (in both non-parasitized and parasitized) when compared with control. Esteban et al. (2005) noticed that serum peroxidase made no significant difference when sea bream were fed with lactoferrin. Serum peroxidase levels reached a high during weeks 3 and 4 of feeding probiotics to sea bream (Diaz-Rosales et al., 2006), whereas in the present study the enzyme activity was found to be at peak up to 10 days after infection, indicating the moderate effect of plant extract beyond 50 days after feeding with the medicated feed.

Table 2. Survival percentage of the freshwater fish *Cyprinus carpio* infected with the bacterial pathogen *Aeromonas hydrophila*.

Concentrations of leaf extract (g/ kg feed) of *A. Marmelos*	Survival (%)
0	60.0 ± 0.0
5	96.67 ± 2.89 **
10	95.0 ± 5.0 **
20	91.67 ± 2.89 **
25	86.67 ± 2.89 **
50	83.33 ± 2.89 **

*P < 0.05; **P < 0.01; NS, not significant.
Note: Each value is the mean of three individual observations with a standard deviation.

Survival percentage

Table 2 shows the survival percentage of *C. carpio* fed with extract of *A. marmelos* and infected with *A. hydrophila*. From the results obtained, it is clear that the fish fed with feed having 5 g *A. marmelos* showed higher survival rates (96.67 ± 2.98%) when compared with those in other concentrations. It is clear that the fish which consumed 0 g (control) *A. marmelos* showed the survival percentage of 60%. It explained that the extracts of *A. marmelos* enhanced the survival percentage significantly at 1% level.

Conclusion

Results of the present investigation on disease resistance concluded that the incorporation of leaf extract of *A. marmelos* feed protects *C. carpio* fish against the pathogen *A. hydrophila*. The extract when supplementing the feed enhanced protection when challenged with live pathogen *A. hydrophila*. The mechanism by which survival was augmented appears to be positively

correlated with increased phagocytosis, neutrophil activity, lysozyme activity, etc. Among the different concentrations tested the groups fed continuously with feed of lower concentrations (5 and 10 g) of *A. marmelos* resulted in maximum protection (Table 2). Earlier studies have revealed that dietary supplementation enhanced disease resistance as observed in common carp in the present study.

In general, immuno-stimulants were found to stimulate antibody response, lysozyme and phagocytosis and other immunological function in fish (Sakai, 1999). This study also reveals the scope of using extracts of *A. marmelos* as an immuno-prophylactic for health management in the culture of carp. From the study it is concluded that the leaf extract of *A. marmelos* had significant immunostimulant activity against *A. hydrophila* infecting freshwater *C. carpio* fish.

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Declaration of interest

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

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