INTERNATIONAL RESEARCH JOURNAL OF PHARMACY
www.irjponline.com
ISSN 2230 – 8407

Research Article

# *LIMONIA ACIDISSIMA* L. (WOOD APPLE) AS FEED ADDITIVE ENHANCED GROWTH PERFORMANCE, IMMUNE RESPONSE AND DISEASE RESISTANCE OF INDIAN MAJOR CARP, *CATLA CATLA* (HAM.) AGAINST *AEROMONAS HYDROPHILA* INFECTION

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Article Received on: 16/01/15 Revised on: 13/02/15 Approved for publication: 20/02/15

**DOI: 10.7897/2230-8407.06232**

**ABSTRACT**

Disease management has become prominent in aquaculture due to emergence of antibiotic resistant pathogens and overuse of chemotherapeutic agents. One among the developmental strategies to control disease outbreak is by enhancing the fish immunity using immunostimulants. The use of dietary herbal immunostimulants can improve the immune defence of fishes, providing resistance against infections. The present study evaluated the possible effects of *Limonia acidissima* L. fruit (wood apple) supplemented diets on the growth, innate immunity and disease resistance against *Aeromonas hydrophila* in Catla fish. *Catla catla* fingerlings (mean weight 5.0 ± 0.5 g) were separated into four groups and cultivated in 100-L tank. Each group was fed with diets supplemented with 0 g, 1.5 g, 3 g and 6 g per 100 g feed twice daily. Fish were examined for growth and innate immune parameters at 30 days interval up to 120 days. Results revealed that wood apple supplemented diets enhanced the growth and innate immune responses of Catla during the feeding trial. Growth performance, haematological parameters, biochemical parameters and immunological indices significantly ($p < 0.05$) increased in fish fed with experimental diets. The relative survival percentage after *A. hydrophila* challenge increased in fish fed with *Limonia acidissima* diet. Thus, the result suggested that fish fed *Limonia acidissima* Fruit supplemented diet enhanced growth, improved immune system and increased survival rate in *C. catla* fingerlings.

**Keywords:** *Limonia acidissima*, *Catla catla*, Phagocytic activity, Immunostimulant, *Aeromonas hydrophila*.

## INTRODUCTION

Aquaculture is an important source of fish and fish products, which serves as an invaluable source of protein and essential micro nutrients. Globally, the fish food supply has grown remarkably in past five decades, with a growth rate of 3 % per year in the period 1961-2009[1]. Recently it has been estimated that the aquaculture production achieved 62 million tonnes in 2011 and hence, became a rapidly growing animal food production sector in the period 1990-2010[1]. Catla (Family: Cyprinidae) is an economically important Indian major fresh water carp and it is extensively grown in poly-culture systems. The proportion of Catla stocked in India is 30-35 % among the six species composite system with rohu, mrigal, common carp, grass carp, and silver carp and has increased significantly over years[2]. Intensification of aquaculture has developed notably over decades to cope with the increased fish demand, leading to stress factors such as overcrowding, transport, grading, handling and low water quality. These stressors adversely affect the health of cultured fish by causing undesirable physiological changes such as immunosuppression and thus, decreased fish immunity against disease outbreaks[3]. One of the extensively reported limiting factors in the fish poly-culture system is the bacterial infections. Close quartered aquaculture in the absence of health safety measures, promote the spread of pathogens leading to high mortality levels, low meat quality and reduced profit[4-6]. *Aeromonas hydrophila* sustains itself as an important bacterial pathogen and cause persistent disease outbreaks in a variety of fish species especially carps, leading to symptoms like ulceration, exophthalmia and abdominal distension[7,8]. In order to prevent disease outbreaks several antibiotics and chemotherapeutics are commonly used[9]. Consequently, the unchecked use of veterinary drugs presents greater side-effects for the environment and health safety, like development of resistant bacterial strains or the presence of residual antibiotics in the muscles of commercialized fish, leading to human health hazards[10-12]. Accordingly, antibiotics are no longer effective against bacterial pathogens and their use is prohibited in aquaculture system. However, a useful drug and effective vaccine development against the pathogen becomes indispensable[13,14]. One of the promising alternative approaches for controlling fish disease is by strengthening the fish defence mechanism. Innate immunity play a significant role at all stages of fish infection as they highly depend on the non-specific immunity, than mammals[15-17]. Increasing interest in the modulation of fish innate immune system as a preventive measure against disease outbreak has led to the use of plant materials as feed supplement. Herbal extracts are used in fish culture as an alternative for chemotherapeutic agents[18-20]. Plant extracts have been reported to enhance activities like appetite stimulation, growth promotion, immunostimulation, anti-microbial properties in fish aquaculture due to active principles as alkaloids, terpenoids, tannins, saponins, falvonoids, phenolics, steroids or essential oils[21,22]. A large number of medicinal plants such as *Phyllanthus niruri*, *Acalypha indica*, *Azadirachta indica*, *Piper betle*, *Mentha piperita*, *Allium sativum*, *Astragalus membranaceus*, *Lonicera japonica*, *Withania somnifera* have immunostimulant activity and are used to enhance the fish immunity thereby preventing mortalities during disease outbreaks[23-27]. In the present study, we use wood apple (*Limonia acidissima* L.) as a feed additive in fish diets. *L. acidissima* (Family: Rutaceae) fruit is widely known for its medicinal property and used in Indian folk medicine, Ayurveda and Yunani to treat blood impurities, leucorrhoea and urinary problems[28]. It is a deciduous tree indigenous to Indian subcontinent and Sri Lanka[29]. The fruit has high nutritive value and

contains important phyto constituents such as flavonoid, phytosterols, glycosides, saponins, tannins, coumarins, triterpenoids, carbohydrates, vitamins and amino acids[30-33]. Recent studies show that the fruit consist of essential amino acids, wound healing and has rich antioxidant properties[34]. Our earlier studies reported that supplementing *L. acidissima* in fish diets stimulated the growth performance of fresh water carps *Cyprinus carpio* and *Cirrhinus mrigala*[35]. Although our previous study investigated the efficacy of Limonia fruit on growth performance of *C. carpio* and *C. mrigala*, its effect on growth and immunostimulation in *C. catla* is still unknown. Thus, this study is conducted to investigate the effect of dietary supplementation of *L. acidissima* on growth performance, non-specific immune response and diseases resistance against *A. hydrophila* infection in *Catla catla.*

## MATERIALS AND METHODS

### Experimental system and animals

*Catla catla* fingerlings with an average weight of 5.0 ± 0.5 g were procured from Induced carp spawning and seed rearing centre, Tamil Nadu, India Fisheries Development Corporation (TNFDC, Govt. of India), Aliyar, Tamil Nadu, India. The fish were acclimatized for 10 days in laboratory condition in 1000 L fibre reinforced plastic tanks at 30° ± $2^0$C, under continuous aeration. Ammonia and dissolved oxygen contents were maintained using Stringed Bed Suspended Bioreactor (SBSBR)[36], a technology commercialized through M/s Oriental Aquamarine Biotech (P) Ltd, Coimbatore, India. The reactors were activated under laboratory conditions and used in the aquaculture system. The physicochemical parameters of water such as temperature (27- 32$^0$C), pH (7.0 ± 0.8), and ammonia (0.001-0.005 mg/L) were maintained at optimum condition during the experimental period.

### Diet formulation

The *Limonia acidissima* Fruit was collected from Vellingiri hills (Tamil Nadu, India). The fruit was taxonomically identified and authenticated (Voucher No. BSI/SC/5/23/09-10/Tech-319) by Botanical Survey of India, Southern Circle, Tamil Nadu, India. Agricultural University (Govt. of India), Coimbatore, Tamil Nadu, India. The fruits were cleaned and the pulp was scooped. The fruit pulp was shade dried and powdered using electrical blender. Powdered pulp was sieved using 100 µ sieve and stored under dry, airtight condition before incorporating with the basal feed. The basal diet was formulated according to the diet formulation routinely used at TNFDC, Aliyar. Balanced basal diet without *Limonia acidissima* Fruit supplement was used as control feed. The experimental diets were prepared according to our previous studies[35], by supplementing *Limonia acidissima* Fruit powder to the basal diet at varying concentrations (1.5, 3, 6 g/100 g) (Table 1). All the dry ingredients for each of the experimental diets were mixed with deionized water thoroughly by hand. The resulting dough was pelletized using hand pelletizer, air dried in shade at room temperature for 48 h and then stored in dry, airtight containers.

### Proximate analysis of feed

The proximate analysis of the feed was evaluated based on the standard methods of the Association of Official Analytical Chemists[37]. Moisture content was determined gravimetrically in a hot air oven at 100 ± $10^0$C for 24 h. Crude protein content was determined by the Kjeldahl method. Crude lipid was estimated by extraction with petroleum ether (boiling point: 40–60$^0$C) in Soxhlet apparatus. After extraction of the lipid the defatted samples were used for estimation of crude fibre. Ash content was estimated by igniting samples in a muffle furnace (Model No. KSSMF003, Metrolabs, India) at 500 ± $50^0$C for 10 h.

### Experimental design and feeding trials

For growth performance and immune response trials, the experimental facility consisted of 12 tanks (100-L each). *Catla catla* fingerlings were randomly distributed into cultivating system in triplicates (at a density of 25 fish/tank). The feeding trial was performed for four treatment groups: Control group ($T_0$) were fed with basal diet; experimental groups $T_1$ were fed with 1.5 % *Limonia acidissima* Fruit mixed diet, $T_2$ were fed with 3 % *Limonia acidissima* Fruit mixed diet; $T_3$ were fed with 6 % *Limonia acidissima* Fruit mixed diet. The fish were hand fed twice daily (9:00 and 17:00) at 5 % body weight during the feeding trial for 120 days. Ten fish were selected for growth analysis whereas six fish were randomly sampled from groups after each experimental feeding period for haematological and biochemical assays. For bacterial challenge test, six fish were cultivated in non-circulating system, with three-day interval water exchange. All fish were fed their respective diets before challenge study using *A. hydrophila*. Post challenge study was continued up to 10 days. At the end of the post challenge the disease resistance and relative percentage of survival was estimated for all the groups.

### Growth analysis

Growth rate was recorded every 30 days by weighing fish randomly from each tank. Each fish was caught from respective tank and weighed using an analytical balance (Citizen CX 304, India). The fish were carefully returned to its appropriate tank after measurement. The growth performance of fish was evaluated on the basis of Initial body weight (IBW), Final body weight (FBW), Live weight gain (LWG), feed conversion ratio (FCR), protein efficiency ratio (PER), and specific growth rate (SGR) using the following formulae:

$$\text{Specific growth rate (SGR)} = (\text{Ln Final weight} - \text{Ln Initial weight}) / \text{Number of days in trial} \times 100$$

$$\text{Feed conversion ratio (FCR)} = \text{Feed given (dry weight)} / \text{Weight gain (wet weight)}$$

$$\text{Protein efficiency ratio (PER)} = \text{wet wait gain by fish (g)} / \text{Protein intake (g)}$$

$$\text{Live weight gain (LWG)} = \text{Final body weight (g)} - \text{Initial body weight (g)}$$

### Blood and serum sampling for analysis

Each fish was anesthetized with clove oil (Himedia, India) (100 µl / L) of water before collecting blood samples from fish. Blood was drawn from caudal vein of fish by using 1 ml hypodermal syringe and 24 gauge needles which were rinsed with 2.0 % EDTA solution before use. The collected blood was immediately transferred to the test tube coated with thin layer of EDTA and shaken well in order to prevent clotting of blood. Serum was collected without using anticoagulant and was separated from blood by keeping the tubes in slanting position for about 2 h and thereafter it was centrifuged at 1000 × g for 15 min at $4^0$C, followed by collection of straw coloured serum and stored at – $20^0$C for further analysis.

### Biochemical and Haematological analysis

After the experimental feeding trial, a total of 6 fish were randomly selected and serum samples were analyzed for total serum protein (TSP) by following the Lowry's method[38], total serum albumin (TSA) content by Doumas *et al*[39]. Total serum globulin (TSG) (subtracting albumin from total protein) and albumin: globulin ratio (A/G) was also estimated. RBC and WBC count was determined according to the method of Blaxhall and Daisley[40] using a haemocytometer. Haemoglobin content was determined by following the cyanomethemoglobin method of Oser and Hawk[41]. Serum lysozyme activity was determined according to the colorimetric method of Parry *et al*[42].

**Immunological analysis**
**Respiratory burst activity**

The respiratory burst activity of the neutrophils was measured by nitroblue tetrazolium (NBT) assay following the method of Secombes[43]. Briefly, 100 µl of blood was placed into the wells of a flat bottom micro titre plate and incubated at $37^0$C for 1 h to allow adhesion of cells. The supernatant was discarded and the wells were washed three times with 1X PBS. After washing, 100 µl of 0.2 % NBT were added and incubated for 1 h. The cells were then fixed with 100 % methanol for 3 min and washed thrice with 70 % methanol. The plates were air-dried and 120 µl of 2 N potassium hydroxide and 140 µl dimethyl sulphoxide were added to each well. The OD was recorded in an ELISA (Bio Tek, Power Wave 340, India) reader at 620 nm.

**Phagocytic activity**

Phagocytic activity (PA) was determined using *Staphylococcus aureus* MTCC 96 (MTCC, Chandigarh, India) as described by Anderson and Siwicki[44]. A sample (0.1 ml) of blood was placed in a micro titre plate well, 0.1 ml of *S. aureus* 1 x $10^7$ cfu $ml^{-1}$ ($A_{450}$ = 0.5-0.6) cells suspended in phosphate buffered saline (pH 7.2) were added and mixed well. The bacteria-blood solution was incubated for 20 min at room temperature. 5 ml of this solution was taken on to a clean glass slide and a smear was prepared. The smear was air-dried, then fixed with ethanol (95 %) for 5 min and air-dried. The air-dried smear was stained with 7 % Giemsa for 10 min. Two smears were made from each fish. The total of 100 neutrophils and monocytes from each smear were observed under the light microscope (Nikon Eclipse-E300, Japan) and the numbers of phagocytising cells were counted. Phagocytic activity (PA) percentage was calculated as follows.

PA% = (Number of phagocytizing cells x 100)/
Total number of phagocyte cells counted

**Pathogenic bacteria culture**

*A. hydrophila* MTCC 1739 (IMTECH, Chandigarh, India) was cultured in Tryptic soy broth (Himedia, India) at $37^0$C for 24 h. The cultures were centrifuged at 3000 x g for 10 min. The supernatants were discarded and the pellets were suspended in phosphate buffered saline (PBS, pH 7.4). The final bacterial concentration was adjusted to1.8 x $10^6$ cfu $ml^{-1}$ by serial dilution.

***A. hydrophila* challenge**

This experiment was performed according to Jha *et al*.[45] with slight modification. After each treatment period, six fish from each group were selected randomly and injected intra-peritoneally with virulent *A. hydrophila* MTCC 1739 suspension of 0.1 ml (1.8 x $10^6$ cfu $ml^{-1}$). The fish were observed regularly for 10 days and any overt signs of disease including behavioural abnormalities, swelling, and mortality were noted. Any dead or moribund fish were examined bacteriologically to confirm the presence of *A. hydrophila* as per standard procedure. Survival at the end of 10 days post infection was calculated using the following formula

Relative Survival Percentage (RSP %) = [1-(Percent mortality in experimental group/Percent mortality in control group)] × 100

**Statistical analysis**

Data were analyzed using one-way analysis of variance (ANOVA). When overall difference were significant at less than the 5 % level, Tukey's test was used to compare the means between individual treatments. Statistical analysis was performed using SPSS software (version 16 for windows) and the differences were determined to be significant at $p \leq 0.05$.

**RESULTS**
**Growth analysis**

The effects of *Limonia acidissima* Fruit supplemented diets on growth response are provided in Table 2 and Table 3. There was a steady increase in the weight for every thirty days in all fish groups during the whole experimental period. The live weight gain (LWG) of the experimental groups were significantly ($p \leq 0.05$) different as compared to control over the entire feeding period. Significantly higher LWG was observed in fish fed with 3 % *Limonia acidissima* Fruit ($T_2$) supplemented feed than other groups. The results indicate that the specific growth rates ($SGR_s$), feed conversion ratios ($FCR_s$) and protein efficiency rates ($PER_s$) of the experimental groups were significantly ($p \leq 0.05$) different as compared to control group on 30, 60 and 90 days feeding period. However, statistical analyses showed no significant difference in SGR, FCR and PER of experimental and control groups after 90 days feeding period.

**Haematological analysis**

The results of the haematological analysis showed a significantly ($p \leq 0.05$) different RBC level in the experimental groups $T_2$ and $T_3$ , over the entire feeding period as compared to the control ($T_0$) (Figure 1a). The RBC count of $T_1$ was significantly different from control on 30 days experimental period. Significantly higher RBC count was recorded in group $T_2$ whereas lower RBC count was recorded in control. WBC counts were significantly ($p \leq 0.05$) different in experimental groups over entire feeding period as compared to control ($T_0$). Significantly higher WBC count was observed in $T_2$ among other experimental groups while lower WBC count was observed in $T_0$ (Figure 1b). Moreover, the haemoglobin level of groups $T_2$ and $T_3$ varied significantly ($p \leq 0.05$) over the feeding period as compared to control ($T_0$). The haemoglobin content of $T_2$ and $T_3$ groups varied significantly ($p \leq 0.05$) as compared to $T_0$ on all feeding period (Figure 1c). Higher haemoglobin level was observed in $T_2$ as compared to other experimental groups over 30 to 120 days feeding period.

**Biochemical analysis**

In relation to different serological parameters the total serum proteins (TSP), total serum albumin (TSA), total serum globulin (TSG) and albumin: globulin ratios (A/G) were determined. Total serum protein level was significantly different ($p \leq 0.05$) in experimental groups $T_1$,$T_2$, and $T_3$ over the entire feeding period as compared to control group ($T_0$). Significantly higher total protein content was observed in $T_2$ whereas lower serum protein level was observed in control group (Figure 2a). The variations in albumin level was significant ($p \leq 0.05$) in groups $T_1$, $T_2$ and $T_3$ as compared to $T_0$ over entire feeding period (Figure 2b). Among the experimental groups the albumin level was higher in $T_2$ followed by $T_3$ and $T_1$ throughout the feeding period. The globulin content was significantly ($p \leq 0.05$) different in experimental groups $T_2$ and $T_3$ as compared to $T_0$ and $T_1$ over the feeding period (Figure 2c). Higher globulin level was observed in group $T_2$ and $T_3$ over the feeding period whereas the globulin content of group $T_1$ was lower as compared to other groups during entire feeding periods. The albumin: globulin ratio followed the same trend as globulin level and was significantly different in $T_2$ and $T_3$ as compared to control over the feeding period (Figure 2d).

**Immunological indices**
**Serum Lysozyme activity**

A significantly ($p \leq 0.05$) higher lysozyme activity was observed in group $T_2$ and $T_3$ on all feeding period as compared to control from 30 to 90 days feeding period (Figure 3). However, the lysozyme activity decreased in all the groups during 120 days feeding period.

**Phagocytic activity**

Phagocytic activity was significantly ($p \leq 0.05$) different in the experimental groups ($T_1$, $T_2$ and $T_3$) as compared to control ($T_0$) throughout feeding period (Figure 4). Higher phagocytic activity percentage (70.81 %) was observed in groups $T_2$ and (60.74 %) $T_3$ as compared to $T_0$ on 120 day feeding period.

**Respiratory burst activity**

A significantly ($p \leq 0.05$) different respiratory burst activity was observed in fish fed with *Limonia acidissima* Fruit diet over the entire feeding period as compared to the control group ($T_0$) (Figure 5). Higher respiratory burst was observed in experimental groups $T_2$ and $T_3$ on whole feeding periods as compared to $T_1$. Moreover, the respiratory burst activity increased with feeding period (30, 60 days) and was significantly ($p \leq 0.05$) higher at 90 and 120 days treatment period.

**Relative survival percentage**

At the end of the experimental feeding period, the fish from each group were subjected to *A. hydrophila* challenge and observed for 10 days post exposure. There was no mortality of fish up to 48 h on all groups. Fish, fed with different percentage of *Limonia acidissima* Fruit supplemented diet ($T_1$, $T_2$, and $T_3$) showed higher relative survival percentage (RSP) as compared to control ($T_0$) group (Figure 6). The highest relative survival percentage among the experimental group was observed in (90.63 %) $T_2$ during the exposure period. The samples collected from moribund fish of control and experimental groups showed positive for *A. hydrophila* indicating that mortality was caused by pathogen infection.

**DISCUSSION**

In a comprehensive fish culture, fish are often afflicted by stressful environmental conditions leading to deterred immune response[46]. It is well documented that administration of dietary herbal immunostimulants can stimulate the non-specific immune response, elevate disease resistance and improve growth performance of fish and shell fish. Stimulation of the immune response of fish by dietary herbal supplements is of high concern for commercial aquaculture sector[47-49]. This study is the first attempt to investigate the effects of *Limonia acidissima* Fruit as a dietary immunostimulant on the growth performance, innate immunity and disease resistance of Catla. Although studies on *L. acidissima* in fish immunity are still unknown, it is expected that some information about the application of this medicinal fruit in humans will have similar effect in fish. The results of this study showed the potential effect of *L. acidissima* as a growth enhancer and stimulated fish appetence. Our results showed that, fish which received 3 % *Limonia acidissima* Fruit mixed diets exhibited increased weight gain from 30 to 90 days feeding period. However, no significant difference in SGR, FCR and PER was observed between control and experimental groups at 120 days feeding period. This finding elucidated that incorporation of *L. acidissima* in diets meet the optimal value in Catla. Analysis of final body weight and weight gain showed that application of 3 % *Limonia acidissima* Fruit in total diets enhances the optimal growth of the fish. Similar requirement of *L. acidissima* was recorded in *C. carpio* and *C. mrigala* by Teepica *et al*[35], who reported that low concentration of dietary *Limonia acidissima* Fruit supplement could influence a better growth in fresh water carps. It is suggested that 3 % *Limonia acidissima* Fruit supplementation in total diet is the optimal dosage for this carp. This may be due to difference in the individual fish nutrient acquisition, digestibility and feeding quantity. Besides, it has been demonstrated that factors such as species, age, size and stress determine the appropriate usage of plant dietary supplements[50]. Further several herbal supplements are reported to stimulate appetite and enhance weight gain when they are incorporated to cultured fish[51-53]. It is hypothesized that higher dosage of herbal diet supplements has resulted in stimulation of enzyme inhibitors, as most plants contain inhibitors to protect their major components from accidental degradation[54]. Anti-nutrient substances present in plants such as protease inhibitors, amylase, lipase, tannins, saponins, lectins and anti vitamins has been reported to cause disturbance in the gastrointestinal tract[55]. These conditions may have affected the feeding efficiency and digestive process leading to lack of complete nutrient utilization. Although no specific experiment was carried out to determine the anti-nutrients and inhibitors of *L. acidissima* fruit, our results reveal that different percentage of *Limonia acidissima* Fruit play a major role for the growth and feeding efficiency in experimental fish. The proximate composition of experimental and control diets were similar to diet composition of Teepica *et al*.[35] and contained protein, lipid and fibre. Phytochemical analysis of *Limonia* fruit revealed the presence of alkaloids, saponins, flavonoids, phenols, carbohydrates, terpenoids and anthocyanin according to Ilango and Chitra[33]. The bioactive compounds present in *Limonia* fruit supplemented diet may have enhanced fish health by stimulating innate immunity. In the present study, dietary administration of *Limonia acidissima* Fruit enhanced the haematological indices of fish. Observations revealed that catla fed with 3 % *Limonia acidissima* Fruit supplemented diet showed significantly higher RBC levels as compared to other experimental groups over entire feeding period. However, no significant difference in RBC was observed in $T_2$ and $T_3$ as compared to control group ($T_0$) after 90 days of feeding period. The WBC level showed a significant difference in fish fed with *Limonia acidissima* Fruit diet as compared to control. Higher WBC level was observed in 3 % *Limonia acidissima* Fruit diet fed fish as compared to other groups. Moreover, the results indicated a steady increase in the WBC with the feeding period. Herbal immunostimulants can affect the haematological parameters of fishes[56,57]. Significant enhancement in haematological parameters may be due to enhanced erythropoietic centers in (kidney/spleen), decreased erythroclasia and enhanced Fe metabolism. Enhanced RBC and WBC counts following the *Limonia acidissima* Fruit diet feeding indicate the immunostimulant effect and anti-infection properties of wood apple fruit. These results corroborate with the findings of Dina Rairakhwada *et al*.[58] and Talpur and Ikhwanuddin[49], who reported an increase in RBC and WBC count after feeding levan to *Cyprinus carpio* and ginger supplemented feed to *L. calcarifer* fishes respectively. In addition, Misra *et al*.[59] reported an increase in RBC and WBC of rohu following feeding trial with herbal diets for 60 days. In the present study the experimental groups demonstrated a significant increase in haemoglobin content compared to control on the whole feeding period. Haemoglobin content in 3 % *Limonia acidissima* Fruit ($T_2$) diet fed fish increased significantly, followed by $T_3$ and $T_1$ as compared to control, during the whole feeding period. Significant enhancement in Hb content indicated that oxygen supply had increased and thus improved the well-being of fish[57]. A significant increase in total serum protein was observed in all experimental groups compared to control. Increase in serum total protein was reported in *C. catla* fed with ginger[60]. Similar results were reported in *C. carpio* fed with *M. piperita*[61] and *Lates calcarifer* fed with ginger[49]. Stronger non specific immune response is associated with marked increase in the total protein, albumin and globulin levels of fish[25,62,63]. Our study revealed significantly higher albumin and globulin content in *Limonia acidissima* Fruit fed fish as compared to control over the entire feeding period. Furthermore, the results of the present study was in correlation with Abasali and Mohamad[61] who found an increase in serum globulin content in *C. carpio* fed with *M. piperita.* The A/G ratio is an index used to estimate relative changes in the composition of serum or plasma. In our study, the A/G ratio of $T_2$ and $T_3$ was significantly different as compared to $T_0$ over the feeding period. A/G ratio of 3 % *Limonia acidissima* Fruit ($T_2$) diet fed fish was higher at 90 and 120 days feed period. Elevations in serum A/G levels are believed to be related with a stronger innate immune response of fish and are vital fractions for sustaining healthy immune system[45]. As a first line of defense, various factors such as lysozyme, total protein, albumin and globulin present in serum, prevent adherence and colonization of microorganisms[64]. It is extensively accepted that lysozyme is an

essential humoral factor for the innate defence system which inhibits the growth of the pathogens[65]. The lysozyme activity significantly increased in *C. catla* fed with *Limonia acidissima* Fruit supplemented diets as compared to control group. Fish fed with 3 % *Limonia acidissima* Fruit ($T_2$) showed significantly higher lysozyme activity compared to other experimental groups. The enhanced lysozyme activity in this study was in affirmation with previous reports on immunostimulants fed fish[25,49,63]. The significant increase of lysozyme in the present study may be due to up-regulation of lysozyme mRNA which could stimulate the immunity response of fish and may contribute to host resistance against infectious pathogens[66,67]. The results of our study showed higher phagocytic activity in *Limonia acidissima* Fruit diet fed fish groups compared to control group. In this study 3 % *Limonia acidissima* Fruit diet fed fish showed significantly higher phagocytic activity on all feeding period compared to other groups, which evidently indicated that *Limonia acidissima* Fruit diet supplement, enhanced the non specific immunity of fish. Our results are in accordance with the report of Arulvasu *et al.*[60], who observed significantly increased phagocytic activities after feeding *C. catla* with ginger. Phagocytosis is an important defence mechanism in fish against bacterial pathogens[25,62]. In the present study, significantly higher respiratory burst activity was observed in experimental groups as compared to control throughout the feeding periods, which may be correlated with the enhanced immune response in the fish. Significantly higher respiratory burst activity was recorded in $T_2$ as compared to other experimental groups. *L. acidissima* had been well documented to have effective antioxidant properties[33]. The bioactive compounds present in *Limonia acidissima* Fruit acts as antioxidants in a number of ways. The phenolics compounds are attributed by their hydroxyl groups in the termination reaction to rupture the cycle of generation of new radicals[68], while the polyphenol and flavanoids have therapeutic uses due to their stronger antioxidant properties[34]. Respiratory burst involves the synthesis of intracellular superoxide anion that is quantified by nitroblue tetrazolium (NBT) assay[26]. Herbal based immunostimulants have been reported to enhance the respiratory burst activity of fish. Herbs such as *Astragalus membranaceus*[26], *Zingiber officinale*[60], *Mentha piperita*[61], *Achyranthes aspera* seeds[62] were previously reported for enhancing the respiratory burst activity in fishes. Higher respiratory burst activity of the experimental groups in this study indicated the healthy status of macrophage and neutrophil activation. The results of our study revealed that dietary administration of *Limonia acidissima* Fruit was protective against pathogenic invasion as there was an increase in fish survival rate. Fish fed with *Limonia acidissima* Fruit diet showed significant relative survival percentage (RSP) against *A. hydrophila* MTCC1739 infection, compared to control. The dose of bacteria we used for challenge was very high, resulting in complete mortality of fish in control group. Significantly higher relative survival percentage was observed in 3 % *Limonia acidissima* Fruit diet ($T_2$) fed fish than other experimental groups ($T_1$ and $T_3$). The above results indicated that *Limonia acidissima* Fruit supplementation activated the innate immunity and reduced the mortality of fish during pathogen exposure. Similar results in *L. rohita* fed with diets containing *Mangifera indica* kernel showed higher relative survival percentage after challenge with *A. hydrophila*[25]. Further, tilapia (*O. niloticus*) fed with two Chinese medicinal herbs[26] and ethanolic extracts of *Psidium guajava*[69] showed higher survival percentage post exposure with *A. hydrophila*. According to the findings of Rao and Chakrabarti[70], *Catla catla* fed with *Achyranthes aspera* diet for four weeks showed resistance against bacterial pathogens elucidating that immunostimulant at an optimal dose, could enhance the resistance of fish against aquatic pathogens. Kumar *et al.*[20] reported higher survival percentage in azadirachtin fed *C. auratus* exposed to *A. hydrophila*. Similar findings were also reported by Kaleeswaran *et al.*[64] who fed *Cynodon dactylon* mixed diet to *C. catla*.

**Table 1: Composition of experimental diets**

| Materials | Formulation (g/100 g on dry matter basis) | | | |
|---|---|---|---|---|
| | Control ($T_0$) | $T_1$ | $T_2$ | $T_3$ |
| Rice bran | 45 | 45 | 45 | 45 |
| Groundnut oil cake | 20 | 20 | 20 | 20 |
| Maize | 10 | 10 | 10 | 10 |
| Finger millet | 10 | 10 | 10 | 10 |
| Pearl millet | 10 | 10 | 10 | 10 |
| Agrimin forte* | 5 | 5 | 5 | 5 |
| *Limonia acidissima* fruit | 0 | 1.5 | 3.0 | 6.0 |
| Proximate compositions[a] | | | | |
| Crude Protein | 38.83 | 39.23 | 39.14 | 39.21 |
| Crude Lipid | 11.47 | 11.83 | 11.85 | 11.9 |
| Crude fiber | 2.23 | 2.06 | 2.07 | 2.13 |
| Ash | 9.3 | 9.52 | 9.6 | 9.3 |
| Moisture | 6.3 | 6.05 | 6.48 | 6.2 |

Abbreviations: $T_0$ : Control feed; $T_1$: feed mixed with 1.5 % *L. acidissima*; $T_2$: feed mixed with 3 % *L. acidissima*; $T_3$: feed mixed with 6 % *L. acidissima.* * Vitamin and Minerals pre mix, Vit-C free ; Nutritional value (g or mg or IU $kg^{-1}$): Vitamin A - 700000 IU; Vitamin $D_3$ - 140000 IU; Vitamin E - 500 mg; Vitamin $B_{12}$ - 1000 mcg; Folic Acid - 100 mg; Nicotinamide - 1000 mg; Copper - 1200 mg; Cobalt - 150 mg; Iron - 1500 mg; Zinc - 3000 mg; Iodine - 325 mg; Selenium - 10 mg; Magnesium - 6000 mg; Manganese - 1500 mg; Potassium - 100 mg; Calcium - 27 g; Phosphorus - 13 g; Sulphur – 0.72 g; Fluorine - 300 mg [a] Moisture is expressed as percentage of fresh weight; crude protein, crude lipid, and ash are expressed as percentages of dry matter. Each datum is a mean from three separate determinations

**Table 2: Growth parameters of *C. catla* fingerlings fed with different levels of *Limonia acidissima* Fruit diets**

| Groups | IBW (g) | FBW (g) | | | | LWG (g) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 30 days | 60 days | 90 days | 120 days | 30 days | 60 days | 90 days | 120 days |
| $T_0$ | $5.0 \pm 0.13^{a}$ | $19.6 \pm 0.3^{a}$ | $31.3 \pm 0.8^{a}$ | $41.9 \pm 1.5^{a}$ | $49.79 \pm 1.1^{a}$ | $14.6 \pm 0.2^{a}$ | $26.3 \pm 0.7^{a}$ | $36.92 \pm 1.5^{a}$ | $44.7 \pm 1.1^{a}$ |
| $T_1$ | $5.2 \pm 0.15^{a}$ | $23.4 \pm 0.3^{b}$ | $40.2 \pm 0.4^{ab}$ | $43.25 \pm 0.9^{ab}$ | $52.1 \pm 0.4^{ab}$ | $18.2 \pm 0.2^{b}$ | $35.0 \pm 0.4^{b}$ | $38.14 \pm 0.9^{ab}$ | $47.0 \pm 0.4^{ab}$ |
| $T_2$ | $5.2 \pm 0.10^{a}$ | $25.2 \pm 0.4^{c}$ | $43.1 \pm 0.4^{c}$ | $49.8 \pm 1.1^{c}$ | $56.0 \pm 0.4^{c}$ | $19.9 \pm 0.3^{c}$ | $37.6 \pm 0.4^{c}$ | $44.51 \pm 1.1^{c}$ | $50.6 \pm 0.4^{c}$ |
| $T_3$ | $5.2 \pm 0.08^{a}$ | $24.2 \pm 0.2^{bc}$ | $42.2 \pm 0.8^{bc}$ | $47.35 \pm 1.1^{bc}$ | $53.3 \pm 0.6^{bc}$ | $19.0 \pm 0.2^{bc}$ | $36.8 \pm 0.9^{bc}$ | $42.2 \pm 1.0^{bc}$ | $48.1 \pm 0.8^{bc}$ |

Data are expressed as mean ± SE, n = 10. IBW: initial body weight; FBW: final body weight; LWG: live weight gain. Mean values with different superscripts in same column were significantly different ($p \leq 0.05$) from the control

**Table 3: Growth parameters of *C. catla* fingerlings fed with different levels of *Limonia acidissima* Fruit diets**

| Groups | SGR (%/day) | | | | FCR | | | | PER | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 30 days | 60 days | 90 days | 120 days | 30 days | 60 days | 90 days | 120 days | 30 days | 60 days | 90 days | 120 days |
| $T_0$ | 2.0 ± 0.03$^{a}$ | 1.3 ± 0.02$^{a}$ | 1.0 ± 0.02$^{a}$ | 0.83 ± 0.01$^{a}$ | 1.0 ± 0.03$^{b}$ | 1.2 ± 0.04$^{b}$ | 1.25 ± 0.06$^{b}$ | 1.36 ± 0.05$^{a}$ | 2.5 ± 0.08$^{a}$ | 0.4 ± 0.02$^{b}$ | 0.48 ± 0.02$^{b}$ | 0.53 ± 0.02$^{a}$ |
| $T_1$ | 2.2 ± 0.05$^{b}$ | 1.5 ± 0.02$^{b}$ | 1.0 ± 0.01$^{ab}$ | 0.84 ± 0.01$^{a}$ | 0.9 ± 0.03$^{a}$ | 0.9 ± 0.03$^{a}$ | 1.21 ± 0.04$^{ab}$ | 1.31 ± 0.03$^{a}$ | 3.05 ± 0.13$^{b}$ | 0.3 ± 0.01$^{a}$ | 0.47 ± 0.01$^{ab}$ | 0.51 ± 0.01$^{a}$ |
| $T_2$ | 2.3 ± 0.04$^{b}$ | 1.5 ± 0.01$^{b}$ | 1.1 ± 0.01$^{b}$ | 0.85 ± 0.01$^{a}$ | 0.8 ± 0.02$^{a}$ | 0.9 ± 0.02$^{a}$ | 1.07 ± 0.03$^{a}$ | 1.28 ± 0.03$^{a}$ | 3.29 ± 0.11$^{b}$ | 0.3 ± 0.01$^{a}$ | 0.42 ± 0.01$^{a}$ | 0.50 ± 0.01$^{a}$ |
| $T_3$ | 2.2 ± 0.02$^{b}$ | 1.5 ± 0.02$^{b}$ | 1.1 ± 0.01$^{ab}$ | 0.84 ± 0.01$^{a}$ | 0.8 ± 0.01$^{a}$ | 0.9 ± 0.03$^{a}$ | 1.11 ± 0.03$^{ab}$ | 1.32 ± 0.03$^{a}$ | 3.15 ± 0.05$^{b}$ | 0.3 ± 0.01$^{a}$ | 0.43 ± 0.01$^{ab}$ | 0.51 ± 0.01$^{a}$ |

Data are expressed as mean ± SE, n = 10. SGR (%): specific growth rate; FCR: feed conversion ratio; PER: protein efficiency ratio.
Mean values with different superscripts in same column were significantly different ($p \leq 0.05$) from the control

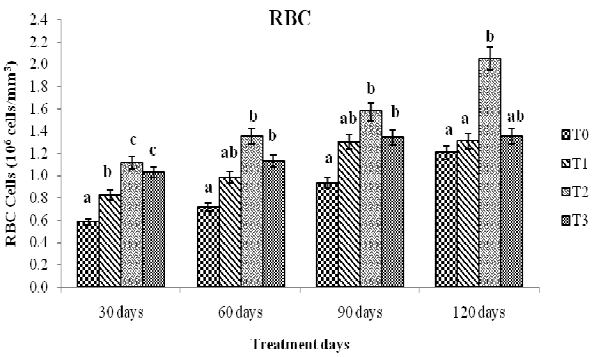


**Figure 1a: Haematological parameters (RBC) of *Catla catla* fed *Limonia acidissima* Fruit diets at different levels for 120 treatment days. Data were expressed as mean ± SE; n = 6. The mean values with different superscripts are significantly ($p \leq 0.05$) different from control among groups**

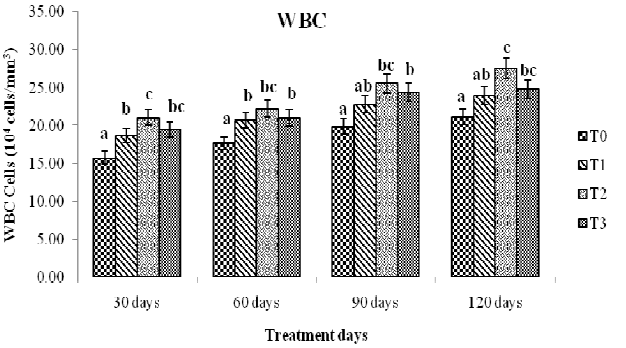


**Figure 1b: Haematological parameters (WBC) of *Catla catla* fed *Limonia acidissima* Fruit diets at different levels for 120 treatment days. Data were expressed as mean ± SE; n = 6. The mean values with different superscripts are significantly ($p \leq 0.05$) different from control among groups**

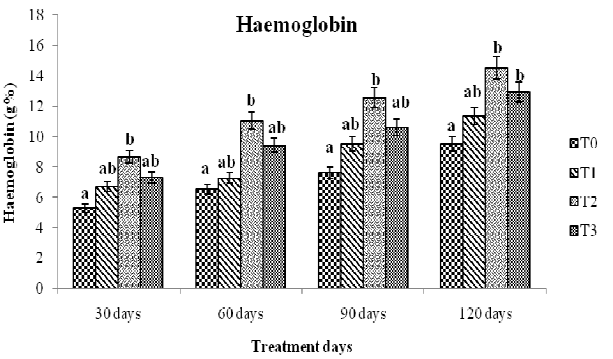


**Figure 1c: Haematological parameters (Hb) of *Catla catla* fed *Limonia acidissima* Fruit diets at different levels for 120 treatment days. Data were expressed as mean ± SE; n = 6. The mean values with different superscripts are significantly ($p \leq 0.05$) different from control among groups**

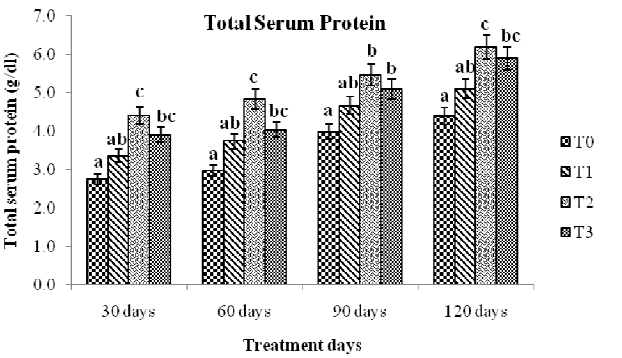


**Figure 2a: Total serum protein (TSP) of *Catla catla* fed *Limonia acidissima* Fruit diets at different levels for 120 treatment days. Data were expressed as mean ± SE; n = 6. The mean values with different super script were significantly ($p \leq 0.05$) different from control among groups**

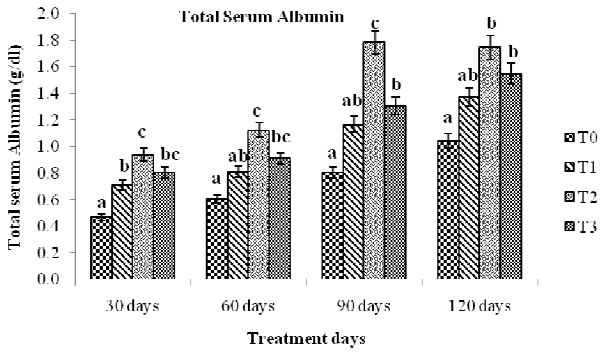


**Figure 2b: Total serum albumin (TSA) of *Catla catla* fed *Limonia acidissima* Fruit diets at different levels for 120 treatment days. Data were expressed as mean ± SE; n = 6. The mean values with different super script were significantly ($p \leq 0.05$) different from control among groups**

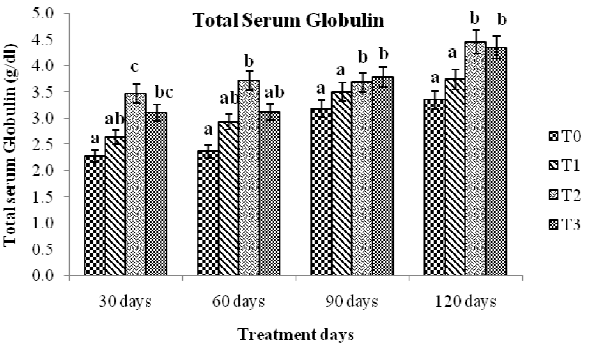


**Figure 2c: Total serum globulin (TSG) of *Catla catla* fed *Limonia acidissima* Fruit diets at different levels for 120 treatment days. Data were expressed as mean ± SE; n = 6. The mean values with different super script were significantly ($p \leq 0.05$) different from control among groups**

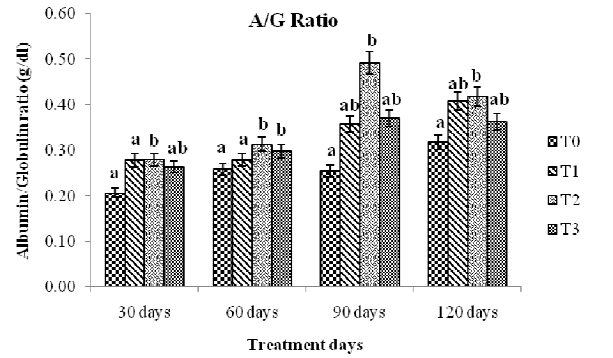


**Figure 2d: Albumin: Globulin ratio (A/G ratio) of *Catla catla* fed *Limonia acidissima* Fruit diets at different levels for 120 treatment days. Data were expressed as mean ± SE; n = 6. The mean values with different super script were significantly ($p \leq 0.05$) different from control among groups**

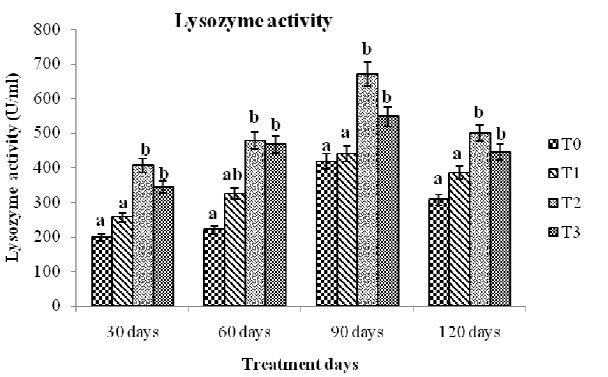


**Figure 3: Effect of *Limonia acidissima* Fruit diet on *Catla catla* lysozyme activity of different groups ($T_0$ to $T_3$) on different treatment days (30 to 120 days). The mean values with different super script were significantly ($p \leq 0.05$) different from control among groups**

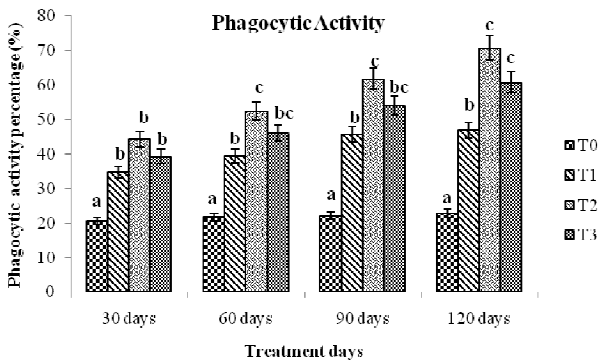


**Figure 4: Phagocytic activity percentage of *Catla catla* fed *Limonia acidissima* Fruit diets at different levels for 120 days experimental feeding trial. Data were expressed as mean ± SE; n = 6. The mean values with different super script were significantly ($p \leq 0.05$) different from control among groups**

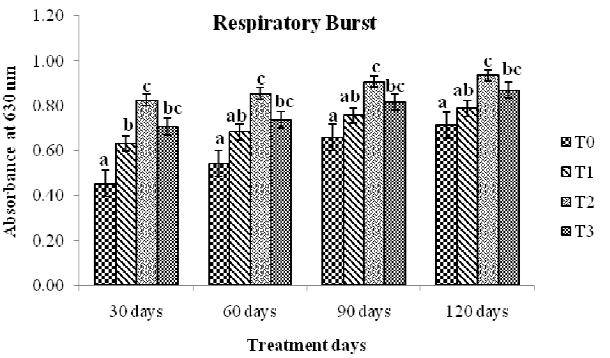


**Figure 5: Respiratory burst: superoxide anion production by blood leucocytes of *Catla catla* fed *Limonia acidissima* Fruit diets at different levels for 120 days of experimental feeding trial. Data were expressed as mean ± SE; n = 6. The mean values with different super script were significantly ($p \leq 0.05$) different from control among groups**

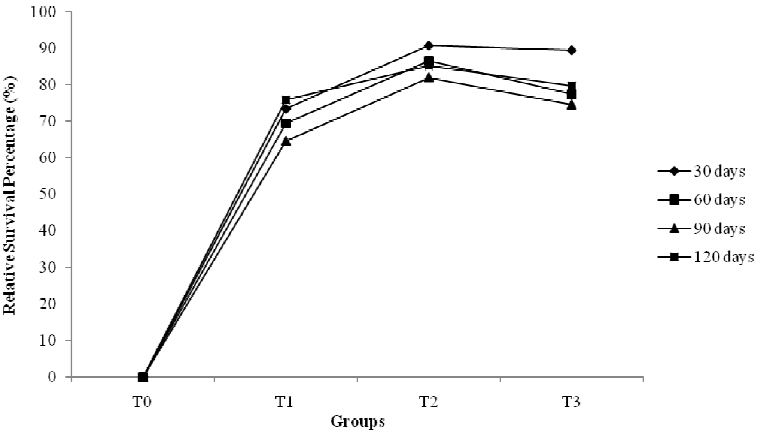


**Figure 6: Relative percentage survival of *Catla catla* fed with *Limonia acidissima* Fruit diets at different levels for varying treatment periods (30 to 120 days) and post exposure with *Aeromonas hydrophila* MTCC 1739. Data were expressed as mean ± SE; n = 6**

## CONCLUSION

Dietary supplementation of immunostimulant can improve animal health and considerably reduce the management costs. Results obtained in this study showed that there is a possibility of using *L. acidissima* as a feed additive to considerably reduce the antibiotic/or disinfectant in intensive aquaculture farms. It is concluded that therapeutic potential of the *L. acidissima* fruit as dietary supplement would enhance the innate immunity of fish as it has reduced the mortality of *C. catla* after challenge with *A. hydrophila*. Present analysis has provided new understanding into immunostimulatory capacity of the *L. acidissima* incorporated in fish diet to avert disease outbreaks and improve the economic growth in the aquaculture industry. However, it remains for further work to validate the *Limonia acidissima* Fruit diet as immunostimulant using molecular analysis with special reference to immune gene expression patterns of *C. catla*.

## ACKNOWLEDGEMENT

This study was financially supported by the Department of Science and Technology, Science and Engineering Research Board (DST-SERB, Govt. of India) (Ref. No. SR/FT/LS-128/2012, Dated 18.12.2012), India. The authors gratefully acknowledge the infrastructure facility provided by Karpagam University, Coimbatore, Tamil Nadu, India. Authors sincerely thank, TNFDC (Govt. of India), Aliyar, Tamil Nadu, India for the extended support in this study.

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**Cite this article as:**
Ponnuraj Srinivasan, Deivamarudachalam Teepica Priya Darsini, Vellingiri Maheshu, J Castro, Jaganathan Dineshbabu, Kanagarajan Manimekalai. *Limonia acidissima* L. (wood apple) as feed additive enhanced growth performance, immune response and disease resistance of Indian major carp, *Catla catla* (Ham.) against *Aeromonas hydrophila* infection. Int. Res. J. Pharm. 2015; 6(2):143-152 http://dx.doi.org/10.7897/2230-8407.06232

Source of support: Department of Science and Technology, Science and Engineering Research Board (DST-SERB, Govt. of India),
Conflict of interest: None Declared