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Effects of lapsi fruits (*Choerospondias axillaris* Roxburgh, 1832) on immunity and survival of juvenile tilapia (*Oreochromis niloticus* Linnaeus, 1758) infected with *Aeromonas hydrophila*

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Abstract
Lapsi fruit (a native from Nepal) is opulent source of essential amino acids, minerals and ascorbic acid and is commonly used for the treatment of cardiovascular diseases in Vietnam, Mongolia and China etc. The phytochemical constituents of lapsi fruit extracts (LFE) are phenol and flavonoid compounds which exhibit potent antioxidant activity to scavenge various free radicals and thus protect from toxic and harmful. A total of 252 fingerlings of *O. niloticus* (average weight 3.84 ± 0.17 g) were randomly distributed in four treatment groups TI (Basal feed + 0% LFE) control, T2 (Basal feed + 0.1% LFE), T3 (Basal feed + 0.2% LFE) and T4 (Basal feed + 0.4% LFE) each in triplicate form. After 60 days of feeding trail highest (p<0.05) weight gain% (273.03%), protein efficiency ratio (1.82) and specific growth rate (2.19) and lowest feed conversion ratio (1.43) in the T3 group fed fish were recorded. Lowest (p<0.05) alanine aminotransferase and aspartate aminotransferase activity was found in the T3 group in both liver and muscle. Incorporation of 0.2% LFE in the feed increased the gill superoxide dismutase activity by 3-fold compared to control group (T1). Highest (p<0.05) respiratory burst activity (0.36), albumin (1.21) and globulin (3.48) contents were observed in the T3 group. When challenged with *A. hydrophila* after 60 days, maximum relative % of survival was noticed in T3 (83.34%) group. Overall, results indicated improvement in the growth, heamato-immunological responses and protected the animals against *Aeromonas hydrophila* infection at 0.2% LFE incorporation in *O. niloticus* while higher dose of LFE incorporation led to stress and immunosuppression.

Keywords: Lapsi, growth, tilapia, immunity, *Aeromonas hydrophila*, survival

Introduction
Aquaculture plays a significant role in eliminating hunger, malnutrition and promoting the socioeconomic status of the poorest of the poor among most of the developing countries. [1] Due to increasing global population and awareness about health benefits, demand for food fish has made tremendous progress over the last two decades. To encounter the demand of fish as the cheapest source of animal protein, aquaculturists are forced to practice 'high input high output' intensive culture. Nevertheless, intensification of aquaculture increases stress level in fish which impairs growth and immune responses against pathogens and eventually leads to outbreak various diseases, resulting into enormous economic losses to the poor farmers. [2, 3] To achieve the sustainable development of aquaculture, control of infectious diseases and maintenance of health of cultured fish is the utmost essential concern. Immune-protection by dietary manipulation has emerged as an important area of research, which is an ideal and sustainable means for enhancing the non-specific immunity of fish. Substances such as chemical agents, herbal extracts, and nutritional factors, stimulate the non-specific defense mechanisms and thus are proved to be efficient in resisting infectious diseases in fish [4] caused by various pathogens.

Aeromonas hydrophila, the main bacterial pathogen of freshwater aquaculture, causes haemorrhagic septicaemia, dropsy, tropical ulceration and ultimately leads to heavy mortality in cultured fishes. [5] Although a large number of costlier chemical formulations such as antibiotics, drugs, pesticides, vaccines, and chemotherapeutics have been attempted to cope up with this situations, but these are not so effective from ecologically safe and environmentally

sustainable aquaculture perspective. On the contrary, use of cheaper herbal extracts as feed additive in the diet of fishes is gaining momentums which are non toxic and reduce the residual load to the aquatic environment. [6] Incorporation of dietary herbal extract not only facilitates in growth augmentation but also leads to boost the non-specific immunity, antioxidization enzyme activity, and disease resistance in fish owing to presence of active phytochemicals. [7, 8] Additionally, dietary herbal extracts have been tested for its efficacy against bacterial challenge in fish. [9, 7, 3] Dietary supplementation of anthraquinone extract, *Zingiber officinale* and *Curcuma longa* stimulated immunity and enhanced resistance against pathogen aggravated stress in fish. [7, 10, 3] Similarly, feed incorporated with herbal extract enhanced the non-specific immune responses in nile tilapia (*Oreochromis mossambicus*) challenged with *Streptococcus iniae* infection. [11]

Lapsi, *Choerospondias axillaris* (Roxb.) belongs to family Anacardiaceae, is a large, dioecious and deciduous fruit tree. The tree is native to Nepal and grows in hills between 850-1900 m above the sea level and also reported from various countries like India, China, Thailand, Japan, Vietnam and Mongolia. [12] Lapsi fruit is opulent source of essential amino acids, minerals and ascorbic acid and commonly used for the treatment of cardiovascular diseases. [13] The main phytochemical constituents of lapsi fruit extract (LFE) is phenol and flavonoid compounds [14] which exhibit potent antioxidant activity to scavenge various free radicals and thus protects from toxic and harmful effects. [15]

Oreochromis niloticus, commonly called as tilapia was introduced in the Nepal over a decade and its culture is significantly progressing due to its ability to withstand a wide range of fluctuating environmental conditions, excellent growth rate at low protein supplemented diets, herbivorous-microphagous feeding habit and resistance to diseases. Physiology of tilapia favors for its cultivation and therefore constitutes substantial share of total aquaculture production in Nepal. Tilapia, one of the candidate species for diversification of aquaculture has been cherished as potential food fish commodity in Nepal.

Investigations of growth and immune responses indicate the physiological wellbeing of the organism in response to feeding of natural herbs. [3] To the best of knowledge, no such studies have been conducted in *O. niloticus* fingerlings fed with LFE supplemented diet and challenged with *A. hydrophila* infection. Hence, the present study aimed to evaluate the efficacy of LFE on growth, haemato-immunological responses and disease resistance against *A. hydrophila* infection by supplementing with basal diet at different doses.

Materials and Methods

Experimental site and experimental animals

The laboratory analysis was carried out at the nutritional and biochemistry division, and the experimental setup was maintained in the wet laboratory of Central Department of Zoology, Tribhuvan University (CDZTU), Kirtipur, Nepal. *O. niloticus* fingerlings (average weight 3.84 ± 0.17 g) were procured from the hatchery of National Aquaculture Centre, Pokhara Valley, Nepal during the month of July, 2015. All procedures were reviewed by the Local Experimental Animal Care Committee, and approved by the institutional ethics committee of CDZTU, Kirtipur, Nepal. Fish were transported, stocked in cement tank (500 L capacity) and left undisturbed during the whole night. The next day, fish were given a salt treatment (5%) to ameliorate the handling stress. The stock was acclimatized under aeration, provided through compressed air for 14 days. During acclimatization, fish were fed with controlled diet having (30% CP and 6% Lipid) at 3% of the body weight. Round the clock aeration was provided. The physico-chemical parameters of the water were within the normal range of tilapia rearing (dissolved oxygen: 5.5-6.0 mgl^{-1}; pH: 7.53–7.92; temperature: 25.0–27.2ºC; alkalinity 46-58 mgl^{-1} and hardness 48-64 mgl^{-1}) throughout the experimental period.

Preparation of crude extract of lapsi fruits

Fruits of lapsi collected from Kathmandu local market were identified by National Herbarium and Plant laboratory, Department of Plant Resources, Ministry of Forests and Soil Conservation, Government of Nepal. After identification, the samples were transported to the Laboratory of Fish Nutrition, Biochemistry and Physiology (FNBP), Central Institute of Fisheries Education (CIFE), Mumbai, India. The crude extracts of the pulp of lapsi fruits were prepared separately by using ethanol (80%) and distilled water. The fruit samples were individually washed with clean tap water and spread over plain sheet of paper for air drying under shade. After drying, the samples were powdered using warring blender. A known quantity (10 g) of lapsi powder was taken in a 250 ml conical flask and added with 100 ml of ethanol (80%). The ethanol lapsi powdered mixtures were kept for 24 hrs on orbital shaker and then, the extract was filtered using muslin cloth to exclude the residues. The extraction was repeated two times, the filtrate pulled together was centrifuged at 10,000 x g for 5 min. and the supernatant was collected. Further, the supernatant was concentrated using water bath at temperature of 70 °C. A greasy final crude material of LFE obtained was transferred to screw-cap bottle, labeled and stored under refrigerated (4°C) condition until use. The total phenolic compound (219.31 mg g^{-1}) and flavonoid compound (88.47 mg g^{-1}) of LFE was determined as per the method described by Ordonez *et al.*, [16].

Experimental design, feed and feeding

Two hundred fifty two fingerlings of *O. niloticus* were randomly distributed in four treatment groups in triplicates following a completely randomized design (CRD). The experimental rearing system consisted of 12 uniform size rectangular glass tank (120 L capacity) containing 21 fish per tank. The total volume of the water in each tank was maintained at 100 L throughout the experimental period.

Four isonitrogenous and isocalorific practical diets were prepared. The fish were divided into four different treatment groups as TI (Basal feed + 0% LFE) control, T2 (Basal feed + 0.1% LFE), T3 (Basal feed + 0.2% LFE) and T4 (Basal feed + 0.4% LFE) (Table 1). The protein and lipid percentage of 31% and 6% respectively were maintained in all the diets. Pelleted feed were prepared using the various ingredients as mentioned in Table 1 and kept in a hot air oven (50-60 °C) overnight for complete drying, packed in polythene bags, and stored at 4 °C throughout the experimental period. Initially, *O. niloticus* fingerlings were fed to approximate satiation level for 60 days twice daily at 3% of the body wt and later adjusted accordingly based on the biomass gain over a period of every 15 days. Daily ration was divided into two parts: about $2/3^{rd}$ of total ration was given at 10:00 h and the rest $1/3^{rd}$ at 17:00 h. Faecal matter and uneaten feed were

siphoned out and two third of the tank water was replenished on alternate days.

Proximate analysis of feed

The proximate composition of the experimental diets was determined following the standard methods of AOAC [17] (Table 1). Nitrogen free extract (NFE) was calculated by difference i.e., NFE = 100 − (CP + EE+ CF+ Ash).

Growth study

Sampling was accomplished to record the fish weight at 15 d interval during the entire experimental period. The growth performances of *O. niloticus* fingerlings were evaluated in terms of weight gain (%), specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER) by using following equation:

Weight gain (%) = (Final weight − Initial weight) / Initial weight x 100;

SGR = 100 x (loge average final weight − loge average initial weight)/Number of culture days;

FCR = Total feed given (dry weight) (g) / Weight gain (wet weight) (g);

PER = Total wet weight gain (g)/Crude protein fed (g);

Tissue homogenate preparation

At the end of the feeding trial, six fish per treatment were sampled and anaesthetized with tricaine methane sulfonate (5 mg l−1) for 2-3 min. The muscle, liver and gill tissues were dissected and weighed carefully after pooling the samples per replicate group. A 5% homogenate was prepared as per method reported by Gupta *et al.,* [2].

Enzyme assays

Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST)

As per the method described by Wotton [18], alanine amino transferase (L-alanine-2-oxaloglutarate aminotransferase; E.C.2.6.1.2) activity was assayed in the liver and muscle tissue homogenates. The substrate comprised of 0.2 M D, L-alanine acid and for aspartate amino transferase (L-aspartate: 2 oxaloglutarate aminotransferase, E.C.2.6.1.1) assay, procedure similar to ALT was adopted except for the substrate comprised of 0.2 M D, L-aspartate.

Alkaline phosphatase (ALP)

The ALP (E.C. 3.1.3.1) activity was determined by the method of Garen & Levinthal [19].

Catalase (CAT) and Superoxide dismutase (SOD)

Catalase (E.C.1.11.1.6) was assayed in the liver and gill tissue as per method of described by Claiborne, (1985) [20]. Superoxide dismutase (E.C. 1.15.1.1) activity was estimated by the method of Misra and Fridovich [21].

Protein estimation

For estimation of protein in the various tissues Lowry *et al.,* [22] method was adopted.

Blood collection

Same six fish from each treatment used for blood collection as for the tissue homogenate preparation. Blood was collected by puncturing the caudal vein using a medical syringe (No.23), which was previously rinsed with 2.7% EDTA solution (as an anticoagulant) and shaken gently in order to prevent haemolysis of blood. The blood samples were used for determination of haemoglobin content, total erythrocyte counts, total leukocyte counts and Nitroblue Tetrazolium (NBT) assay. For serum, another two fish from each replicate and a total of six from each treatment were anesthetized, the blood was collected without anti-coagulant and allowed to clot for 2 h, centrifuged (3000 x g for 5 min) and then kept at -80ºC until use.

Haemato-immunological studies

Total serum protein, albumin, globulin and albumin/globulin ratio

Serum protein was estimated by Biuret and the Bromocresol Green (BCG) dye binding method [23] using total protein and albumin kit (Qualigens Diagnostics, Mumbai). Albumin was estimated by the BCG binding method [24]. The absorbance of standards and tests were measured against the blank in a spectrophotometer at 630 nm. Globulin was calculated by subtracting albumin values from total serum protein. The albumin/globulin ratio (A/G) was calculated by dividing albumin values by globulin values.

Respiratory burst activity (NBT)

Respiratory burst activity of phagocytes was measured using the nitroblue tetrazolium (NBT) assay following the method Stasiack & Baumann [25].

Ascorbic acid analysis

For ascorbic acid analysis same six per treatment were dissected for liver and muscle as for the serum collection. Both liver and muscle tissue was suspended in chilled 5% metaphosphoric acid in the presence of dithiothreitol (DTT) and homogenized for 2 min in an ice bath. The homogenate was centrifuged at 3000 x g for 5 min at 4°C. Ascorbic acid content in the liver and muscle tissues was determined by reverse-phase HPLC (HP 1100, USA) with an ODS column (4.6 × 25 mm, USA) as described by Shiau and Hsu [26].

Challenge study with *A. hydrophila*

After 60 d of feeding, 12 fish from each tank were challenged with virulent *A. hydrophila* strain (ATCC 49140) obtained from Himedia Laboratories, Mumbai, India. *A. hydrophila* was grown on nutrient broth for 24 h at 37^0C in a BOD incubator and harvested by centrifuging the culture broth at 3000 × g for 10 min at 4ºC. The cells were then washed three times in sterile PBS (pH 7.4) and the final concentration was maintained at 1×10^7 cells ml^{-1} by serial dilution. The fish in each experimental group were intraperitoneally injected with 0.5 ml of bacterial suspension. Mortality was observed for all groups for 10 days. Tissues were taken from dead fish for bacteriological culture and to confirm *A. hydrophila* as the cause of death.

The relative percentage survival (RPS) in different treatment groups were calculated by the following formula:

RPS = (Mortality % of control group – Mortality % of treatment group) ×100 /Mortality (%) of control group.

Statistical Analysis

The data were statistically analyzed by statistical package SPSS version 16 (SPSS Inc., Chicago, IL, USA) and mean values of all parameters were subjected to one way analysis of variance to study the treatment effect and Duncan's multiple range tests (DMRT) were used to determine the significant differences if any, between the means. Comparisons were

made at the 5% probability level. Polynomial relationship with dietary LFE was carried out to fit the trend line of liver and muscle aascorbic acid content.

Results

Growth performances

The growth parameters of the experimental animals of different groups at the end of feeding trials are shown in the (Table 2). Supplementation of dietary LFE extract significantly affected the wt gain%, FCR and SGR of different experimental groups. Highest (p<0.05) wt gain% and SGR was observed in the T3 group fed with 0.2% LFE supplementation and were followed by T4 and T2 (Table 2). The reverse trend for the FCR was noticed and lowest value (p<0.05) was detected in T3 followed by T4 and T2 group. Incorporation of LFE significantly affected the PER value of different experimental groups. Highest (p<0.05) PER among LFE fed group was recorded in T3 fed with 0.2% LFE (Table 2) and lowest (p<0.05) was observed in the T2 fed with 0.1% LFE supplementation.

Enzymes assays

Dietary LFE supplementation had significant (p<0.05) impact on AST and ALT activity in both liver and muscle of treated groups. Lower value of AST and ALT activity in the liver was observed compared to muscle. Significantly (p<0.05) lowest AST and ALT activity was found in T3 group in both liver and muscle and the activity significantly (p<0.05) increased in higher LFE fed T4 group (Table 3). A similar trend was observed for ALT activity in the liver. Although ALT activity was lowest in the group fed with 0.2% LFE (T3) but was comparable to 0.4% LFE fed (T4) group.

ALP activity in liver increased with increasing concentration of LFE in the diet and highest (p<0.05) value was observed in the group fed with 0.4% LFE supplementation which was comparable to 0.2% fed group. Supplementation of dietary LFE was found to improve ALP activity in *O. niloticus* fingerling and highest value (p<0.05) was observed in the T3 group which was similar to T4 group (Table 3).

Effects of dietary LFE on the liver and gill catalase activity are presented in Table 4. Highest (p<0.05) liver and gill catalase activity was observed in the T3 group fed with 0.2% LFE supplemented diet and was similar to T4 group fed with 0.4% LFE supplemented diet (Table 4). There was a significant (p<0.05) impact of dietary LFE on SOD activity in both liver and gill among the different experimental groups. In the liver, highest (p<0.05) SOD activity was observed in the T3 and was comparable to T4 group. Significantly 3-fold increase in gill SOD activity was observed in the T3 group fed compared to control group. Lowest (p<0.05) value of gill SOD activity was observed in the control group fed without LFE diet (Table 4).

Total serum protein, albumin, globulin and A/G ratio

A significant difference (p<0.05) in the total serum protein, albumin, globulin and A/G ratios were manifested among the different treatment groups fed with graded level of LFE diet (Table 5). Highest (p<0.05) albumin and globulin contents were manifested in the T3 group compared to all other treatment groups, whereas lower A/G ratio and higher total serum protein content were observed in the T3 group fed at 0.2% LFE diet and was comparable to T4 group.

Respiratory burst activity (NBT)

The production of superoxide radical examined by NBT reduction was significantly (p<0.05) influenced by dietary supplementation of LFE diet (Table 5). Highest (p<0.05) NBT value was observed in the T3 group compared to all other treatment groups. Lowest NBT value among LFE fed group was noticed in T2 group fed with lowest level of LFE incorporation.

Ascorbic acid content

The tissue ascorbic acid content was significantly affected by the LFE supplementation (Fig. 1). Higher content of ascorbic acid was observed in the liver tissue compare to muscle. Ascorbic acid content in the liver was increased with the increasing level of dietary LFE up to 0.2% level of supplementation which showed a second order polynomial relationship in the liver ($Y = -5.558x^2 + 35.99x + 5.673$, $r^2 = 0.837$) (Fig. 1). Similar trend was also observed for muscle ascorbic acid content, which also exhibited a second order polynomial relationship with the dietary LFE ($Y = -0.925x^2$ $7.999x + 14.81$, $r^2 = 0.887$) (Fig. 1).

Relative percentage of survival against *A. hydrophila* challenge

Post feeding trail of 60 days duration, fish were challenged with *A. hydrophila* infection. Initial mortality was observed from the third day post challenge and was confirmed due to the challenge infection with *A. hydrophila*. Mortality was observed up to 10 days. Lower mortalities were observed in fish fed with LFE supplemented diets after challenge with *A. hydrophila*. Among the LFE fed groups, highest relative % of survival was found in T3 (83.34%) followed by T4 (69.45%) while minimum was recorded in T1 group (58.33%). 100% mortality was observed in the control group (Fig.2).

Discussion

Application of natural herbs such as garlic, caraway, fennel, licorice, black seed and fenugreek etc. have been validated for growth promoting properties in aquatic animals. [27, 28] These natural herbal medicine led to significant improvement of feed conversion ratio, protein digestibility and energy retention in tilapia. [29, 30] Present investigation with LFE resulted into significant improvement of the percentage weight gain, SGR, FCR and PER of Nile tilapia fed at 0.2% supplementation level. To corroborate our findings Diab *et al.*, [31] reported highest growth performance in *O. niloticus fed* with 2.5% *Allium sativum* (garlic) supplemented diet. Similarly, significant improvement in weight gain %, SGR and PER of Nile tilapia fed diet incorporated with garlic powder have been reported by researchers. [32, 33] Further, significantly higher SGR in Nile Tilapia fed with *Echinacea purpurea* and *A. sativum* fortified diet has been observed by Aly & Mohamed [34]. Highest (p<0.05) weight gain%, SGR and lowest FCR observed in the T3 group indicates that dietary supplementation of LFE act as a growth promoter. This might be attributed due to improved palatability, digestion and absorption of nutrients in the gut with the dietary incorporation of LFE. Thus, the benign effects of LFE suggest efficient nutrient utilization in diet of *O. niloticus* fingerlings which might be possible due to presence of phenolic and flavonoid content of LFE. [14] However, the surplus dose of LFE in T4 group fed at 0.4% supplementation level might have affected the digestibility of nutrients resulting into negative effects on growth performance as

reported by Kumar *et al.,* [3] in *C. mrigala* fingerlings fed with anthraquinone extract (AE) at 2% and 4%.

Decreased AST and ALT activity in both liver and muscle of T3 group fed with 0.2% LFE as compared to the T4 indicates reduced mobilization of aspartate and alanine via gluconeogenesis for glucose production. Elevated level of transaminase activity in higher LFE fed T4 group might have resulted into stressed situation leading to increased feeding of ketoacids into TCA cycle, there by unsettling oxidative metabolism. [35] Significant reduction of AST and ALT activities in rainbow trout fed a diet containing astaxanthin rich red yeast (*Phaffia rhodozyma*) was observed by Nakano *et al.,* [36]. Our results are supported by the findings of Kumar *et al.,* [3] in *C. mrigala* fingerlings who fed diet incorporated with AE supplementation of 4%. Further, Metwally (2009) indicated a significant decline in the AST and ALT activities in *O. niloticus* fed with *A. sativum* supplemented diet which advocates immune-augmenting and health boosting properties of dietary LFE.

ALP, a zinc-containing metallo-enzyme, plays a vital role for phosphorus metabolism in the body. Drastic increase in ALP activity with dietary doses of 0.01-0.05% *Achyranthes aspera* compared to the control was reported in *L. rohita* by Rao *et al.,* [37]. Increasing trend of ALP activity with concomitant increase in dietary LFE of the current study is supported with the findings of Sharma *et al.,* [38] in *Channa punctatutus* fingerlings fed with high vitamin C and protein dose. Highest activity of ALP in the liver of group fed either 0.4% or 0.2% LFE might be attributed to higher nutrient absorption or probably through accelerating energy metabolism or activating immune system leading to better FCR and growth performance. [39, 2]

The antioxidant enzyme system plays pivotal role to trap the free radicle produced due to abiotic or biotic stress response and hence protects the animal against the oxidative injury. Incorporation of herbs in the fish diet potentiated antioxidant ability and resisted the impact of pathogen aggravated stress [9] and crowding stress [7]. Highest activity of liver and gill catalase in the higher LFE fed T3 and T4 group in the *O. niloticus* led to decline the stress level by improving the health status. In case of SOD activity in the liver and gill, higher LFE fed groups had maximum activity than their non-LFE fed counterparts. Consistent with our findings, Vinodhini & Narayanan [8] indicated that feeding of (500mg/kg diet) *Nelumbo nucifera* and (500mg/kg diet) *Aegel marmelos* in common carp led to increased activities of hepatic catalase and superoxide dismutase. Similarly, catalase and SOD activity in liver tissue homogenate showed significant increase in fish groups fed on diets contained garlic. [32] This indicates that dietary LFE at 0.2% or 0.4% level of incorporation leads to improvement of the health due to its anti-stress ability.

Alteration in the serum biochemical profile specifically reflects the physiological condition of the animal which occurs under influence of certain internal and external factors. [33] Increase in the total serum protein, albumin and globulin levels are strongly correlated with the boost of non-specific defense system in fishes. [2] The A/G ratio is a measurable humoral component of innate immune system. Gamma globulin fraction of globulin is supposed to be associated with virtually all the immunological active protein of the blood. Higher total serum protein, albumin and globulin content observed in the T3 group fed with 0.2% LFE, indicates enhancement of immunological response and reflected with improved SGR, FCR and PER in the T3 group. Our results agree with the findings obtained by Abdal Tawwab *et al.,* [40] in *O. niloticus* fed with *Camellia sinensis* incorporated diet. Similar results were also observed by Kumar *et.al,* [3] who fed diet incorporated with 4% AE in *C. migala* fingerlings. An increase in the total serum protein levels might be attributed due to improved liver functioning which synthesized increased plasma protein or it might be correlated with the increase in the immunoglobulin level and total globulin concentration. [33] Lower A/G ratio observed in the T3 group indicates the presence of high amount of globulin and specifies higher resistance to pathogen infection. [41] These results are supported with increased activity of antioxidant system as well as higher relative percentage of survival of the T3 group.

Incorporation of natural herbs or immunostimulant as dietary additive in fish activates the non-specific immune systems either by enhancing the number of phagocytes/ activating phagocytes or increasing the synthesis of the involved molecules. [11, 3] Increased respiratory burst activity can be associated with increased bacterial pathogen killing activity of phagocytes [42] and thus represents improved immunity status of fish. In our study, fish administered 0.2% LFE showed elevated phagocytic activity which is in agreement with the findings of Nejdet *et al.,* [11] who observed increased phagocytic activity of Nile tilapia fed with herbs like thyme, rosemary and fenugreek. Our findings are further corroborated with the results of Kumar *et al.,* [3] in *C. mrigala* fingerlings fed 0.2% AE in the diet. Upsurge in the phagocytic activity due to LFE incorporation is a strong evidence of enhancement in the innate immune system which is also supported with increased total serum protein, globulin contents and relative percentage of survival of T3 group.

Ascorbic acid is a potent biomolecule, essential for maintenance of several metabolic and physiological functions including the antioxidant system in most fishes. Like other fish species, tilapia too is not capable of vitamin C biosynthesis due to the absence of the enzyme L-gulonolactone oxidase, which is responsible for synthesis of ascorbic acid. [43] Ascorbic acid is extremely labile and easily degraded with the effect of temperature, oxygen, pH and light. However, its derivatives sulfate and phosphates are more resistant to oxidation and retain biological activity in fish. Earlier studies showed that higher ascorbic acid concentration in tissue determines higher tolerance to ambient pollution and better resistance against bacterial infection. [44] Further, Abdel-Tawwab *et al.,* [40] found that dietary incorporation of *C. sinensis* enhanced the weight gain, specific growth rate and RPS of tilapia which is similar to our findings. Increasing trend of ascorbic acid in both hepatic and muscle tissue with the gradual increase of LFE in the diet up to 0.2% incorporation level confers that antioxidant properties of LFE helps to augment growth and to boost immunity in tilapia. However, lower activity in the group fed with higher LFE (0.4%) might have resulted into toxicity due to overdose resulting into immuno-suppression and negative effect on the growth of tilapia. This might be attributed due to detoxification process or preventing peroxidation of cells [45], causing a functional reduction in vitamin C content in both the tissues. Thus, in the present study the optimum level of 0.2% LFE is suitable for the anti-stress and protective role of tilapia fingerlings.

Relative percentage of survival results suggest improved physiological conditions attained by supplementation of LFE.

However, RPS results from fish fed LFE diet at 0.4% indicated immunosuppression and resulted into increased mortality. In this study, highest RPS of 83.34% observed in T3 group supplemented with 0.2% LFE and showed enhanced resistance to *A. hydrophila* infection. Earlier studies demonstrated that tilapia fed a diet supplemented with Rosmarinus *officinalis* [46] and *Cuminum cyminum* [47] led to a significant rise in the survival rate against streptococcal challenge. Our results are supported with the findings of Xie *et al.*, [7] who incorporated 1-2% dietary anthraquinone in the *C. carpio* fingerlings challenged with *A. hydrophilla*. The increased RPS in the T3 group of present study might be due to healthier blood, and higher ascorbic acid content which protected the fish against bacterial infection by augmenting the non-specific defense system. This might also be attributable to improved nutrient utilisation as well as immunostimulating properties of LFE diet.

Conclusions

In conclusion, the results of this study indicates that supplementation of LFE led to improvement in the growth performance, heamato-immunological responses as well as protects the animals against *A. hydrophila* infection, therefore LFE at 0.2% level of incorporation can be used as potent herbal nutraceutical for the culture of *O. nilotiucs* fingerling. The results of the finding also attests that LFE at 0.2% level of incorporation have beneficial effect and further inclusion of higher level results into toxic effects leading to immunosuppression.

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