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Indian Lettuce Extract as Feed Additive Enhances Immunological Parameters in Mono-Sex Nile Tilapia against *Aeromonas hydrophila*

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Authors' contributions

This work was carried out in collaboration between all authors. Author MMMH designed the study, wrote the protocol, and wrote the first draft of the manuscript. Author FY managed the literature searches, analyses of the study performed the spectroscopy analysis and author AK managed the experimental process, author MZR coordinate with the fish farms to get samples and author MEA identified the species of plant. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: The present study evaluated the efficacy of dietary doses of *Lactuca indica* extract on immunological parameters and disease resistance against *Aeromonas hydrophila* infection in mono-sex Nile tilapia, *Oreochromis niloticus*.
Place and Duration: This experiment was performed in the Laboratory of Fisheries and Marine Bioscience (FMB), Jessore University of Science and Technology (JUST), on July to December 2013.
Methodology: *Lactuca indica* extract preparations, harbal diet preparations, *Aeromonas hydrophila* Isolation, specific and non-specific immunological assays (following phagocytic activity and serum agglutination) and challenge test were performed.

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Result: *A. hydrophila* is a more drastic disease producing and common cause of bacterial haemorrhagic septicaemia, it is a gram-negative, non spore-forming, rod-shaped, yellowish colony forming bacteria. Fishes were fed with *L. indica* extract at 0%, 0.5%, 1%, 1.5% and 2.0%; among those 2.0% showed highest significant responses in phagocytic activity, specific growth rate, specific and non-specific immune responses on week 2 and 4 compared to control diet whereas the changes did not manifest on first week. All groups fishes were injected intraperiotonaly (i.p.) with *A. hydrophila* at 3.5×10^{-7} CFU ml^{-1} for analyzing cumulative mortality on 30th day of feeding. *L. indica* enriched diet at 2.0% level resulted in lowest mortality (20%) indicating highest protection (Relative Percent Survival, RPS 75%) from *A. hydrophila* infection than 0.5%, 1.0% and 1.5% doses diet that resulted 72%, 56% and 32% mortalities respectively. 2% dose also showed highest growth compare to other doses.
Conclusion: The results suggest that the dietary supplementation of *L. indica* extract stimulates immunostimulants, reduce mortality and increases disease resistance in *O. niloticus* against *A. hydrophila* infection.

Keywords: Immune response; mono-sex Nile tilapia; Indian lettuce; Aeromonas hydrophila.

1. INTRODUCTION

Mono-sex population of male Nile tilapia one of the most important freshwater fishes in the world produced by treating fry with a synthetic male hormone 17"-methyltestosterone (17"MT) at a treatment regime of 10 $mgkg^{-1}$ food for 30 days [1] culture of mono-sex Nile tilapia might prove effective by introducing a positive approach towards tilapia culture in the world [2]. The first introduction of *Oreochromis niloticus* in Bangladesh was in 1974 from Thailand [3] after 1999 tremendous progress in tilapia farming has been found in Bangladesh [4]. Over last few decades, it has become one of the dominant species of fisheries sector in many Asian countries and it has been currently reached second rank after carp's aquaculture in global production [5]. *A. hydrophila* is a major cause of bacterial infections affecting warm water fishes such as Nile tilapia (*Oreochromis niloticus*) [6]. The disease is most severe when farmed fishes are stressed and the water temperature is high, low DO and high ammonia content. *A. hydrophila* causes disease occurred in three distinct forms, (a) abdominal dropsy, (b) ulcerative, and (c) bacterial hemorrhagic septicemia [7].

Antibiotics and chemotherapeutics used for prophylaxis and treatment in intensive aquaculture have been widely criticized for their negative impacts [8]. Vaccines are the most promising method of preventing diseases. However, a single vaccine is effective against only one type of pathogen [9]. Recently, immunostimulants of herbal origin have been shown to possess the ability to increase disease resistance in fish against a number of diseases by enhancing non-specific and specific defence mechanisms [10].

Lactuca indica belonging to the family *Asteraceae* is an edible medicinal plant widely distributed in Asian countries [10]. *L. indica* extract is used as an anti-inflammatory, anti-bacterial, and anti-diabetic medicine, it is alkaline in nature which helps in blood purification [11] is rich in vitamins minerals, alkaloids, carbohydrates, protein and fiber, prevents constipation, heart diseases, urinary tract infections and iron deficiency [12] is thus beneficial in treatment of anemia and control nervous insomnia, is rich in antioxidant, and has anti-cancerous properties controls cholesterol level [13]. In this backdrop, it was planned to systematically evaluate the dietary administration of *L. indica* extract on immunological parameters and disease resistance against *S. iniae* infection in *O. niloticus*.

2. MATERIALS AND METHODS

2.1 Fish and Management

Mono-sex Nile tilapia*, Oreochromis niloticus* (weight 27.7±1.4 g, N= 375) was obtained from Laal motsho khamar located at Chachra, Jessore and were transported to the laboratory of Fisheries and Marine Bioscience (FMB), Jessore University of Science and Technology (JUST), on July to December 2013. The fish were immediately examined by their movement, eye condition and external lesion to find out their health status and acclimatized in the indoor aquarium (100 L) with recirculating aerated water. Continuous aeration was provided to maintain dissolved oxygen level at 7.5±0.5 mgl^{-1} and one-third of the aquarium water was

exchanged daily and by siphoning the waste materials was removed. During the experimental period water temperature, pH and TDS (total dissolved solid) were 22±0.8°C, 5.94±0.21 and 434±0.29 mgl^{-1} respectively. Fishes were provided with normal feed (without herbal extract) at the rate of 5% of their body weight twice a day at 09:00 and 16:00 h for 3 days but at the first day of their arrival no feed was provided.

2.2 *L. Indica* Extract Preparations

Four (4) kg fresh *L. indica* was collected from Meena Bazar, Dhaka, Bangladesh. The leaves of *L. indica* (International value showed in Table 1) were washed thoroughly with running tap water then sterile distilled water, shade dried and grounded in a mechanical grinder and sieved. The powders were sieved through and 80 µ mesh. The collected powder was kept in sealed plastic container and stored at −20°C until use. The herbal powder (100 g) was mixed with 1000 ml of 95% ethanol in a 2000 ml conical flask and stored at room temperature for the next 7 days and during that time it was agitated daily to ensure complete digestion. The extracts were filtered through Whatman No. 2 filter paper and the filtrated powder was dried under reduced pressure. The residues obtained after evaporation of ethanol was kept in sterilized screw cap glass container and stored at −20°C until use.

Tabel 1. The amount of nutritional value in *L. indica* (in 100 g)

Component	Amount
Carbohydrates	2.2 g
Dietary fiber	1.1 g
Fats	0.2 g
Protein	1.4 g
Water	96 g
Vitamin A	166 µg
Folate (Vitamin B9)	73 µg
Vitamin C	4 mg
Vitamin K	24 µg
Iron	1.2 mg
Energy	10 kcal (60 kJ)

Source: Lifestyle Lounge: Health & Fitness

2.3 Harbal Diet Preparations

The experimental diet was prepared by mixing with locally available mega feed which contains protein: 34%, crude fiber: 6%, crude ash: 18%, moisture: 11%, lipid: 6%, fat: 3% (Source: Spectra fish feed Com. Ltd. Bangladesh). At first mega feed was grinded by a grinder and mixed with *L. indica* extract. All the ingredients were mixed thoroughly by adding water and pelletized by hand and then sun dried. Five different experimental pellet diets were prepared which contained five different percentages of *L. indica* such as 0%, 0.5%, 1.0%, 1.5% and 2.0%. The prepared feed was then sun dried under sterile condition for 3-4 days and stored in a plastic airtight container.

2.4 *Aeromonas hydrophila* Isolation

A. Hydrophila isolated from diseased mono-sex nile tilapia, was used in FMB laboratory for the study. Stocks were grown in brain heart infusion (BHI, Hi-media, Indian) and nutrient broth for 24 hrs at 37ºC and then kept in −20°C until use. The subculture was taken and centrifuged (4000 rpm for 15 min), after centrifugation the supernatant was discarded and the pellet was resuspended in sterile phosphate buffer saline (PBS). The culture was adjusted at 3.5×10^{-7} colony forming units (CFU) ml^{-1} by 10 time's serial dilution and incubated at 37°C for 24 hours. The bacterium was confirmed by some biochemical test (Table 2).

2.5 Experimental Design

The experiment was performed in 100 L rectangular glass aquarium in the department wet laboratory. The fishes were divided into five groups (0%, 0.5%, 1.0%, 1.5% and 2.0%) of 25 fishes each in triplicate. Fishes were provided with adequate aeration and fed at the rate of 3% to 2% of body weight twice a day with the respective diets till the end of experiment (30 days). On the 1^{st}, 2^{nd} and 4^{th} weeks of feeding, fishes were randomly separated from each experimental aquarium to collect blood and mucus for specific and non-specific immunological assays. On 30^{th} day of feeding, all groups' fishes were injected intraperitoneally (i.p.) with 25 µl PBS containing *A. hydrophila* at 3.5×10^{-7} CFU ml^{-1} for analyzing cumulative mortality.

2.6 Growth Performance

The growth performance percentage of weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) were determined according to Chowdhury et al. [14].

$$\text{Percentage of weight (g) gain} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

$$\text{Percentage of Specific Growth Rate (SGR)} = \frac{\text{Final weight (g)} - \text{Initial weight (g)}}{\text{Time (days)}} \times 100$$

$$\text{Feed Conversion Ratio} = \frac{\text{Feed intake per body weight}}{\text{Weight gain}}$$

2.7 Bleeding, Serum Separation (Specific Immune Response Assay) and Colony Count

Blood from the randomly selected fish were drawn directly from the caudal vein with the help of a sterilized 1 ml hypodermal syringe containing EDTA (Ethylene-Diamine-Tetra-Acetic Acid) as an anticoagulant using 24 gauge needles. For serum separation blood was collected without anticoagulant in serological tubes and stored in a refrigerator overnight. The clot was then spun down at 4500 g for 10 min. The collected serum was stored in sterile serum tubes at −20 °C until used for assays. All the procedures were carried out in the sterilized condition. After drawing blood fishes were given 1% KMnO4 dip treatment and released in to the tank. For each group (0%, 0.5%, 1.0%, 1.5% and 2.0%) three culture plates were prepared. Bacterial stock solution was serial diluted for 10 times and 10^{-3}, 10^{-4} and 10^{-5} concentration were selected for further usage. Then 25 µl volume from each (10^{-3}, 10^{-4} and 10^{-5}) diluted solution was mixed with 25 µl separated serum (followed by disc diffusion method) of five different groups of fishes then speeded in different culture plates and finally all plates were placed in an incubator at 37°C for 24 hrs. Then bacterial colonies of all plates were counted.

2.8 Mucus collection and bacteria culture (non-specific immune response assay)

Mucus was collected by scraping the body surface and gill of fishes with a scalpel from five groups (0%, 0.5%, 1.0%, 1.5% and 2.0%) and collected mucus was kept in five eppendorfs separately. Same like as serum and bacteria culture, three culture plates for each group were prepared as followed by disc diffusion method. 25 µl mucus from each group was mixed with same volume of three different diluted bacterial solutions (10^{-3}, 10^{-4} and 10^{-5}) and finally all plates were placed in an incubator at 37°C for 24 hrs. Then non-specific immune response was observed by counting bacterial colonies of all plates.

2.9 Immune Response Assay

The phagocytic activity and serum agglutination titer were quantified by following the modified method of Swan et al. [15].

2.10 Challenge Test

For the challenge test virulent *A. hydrophila* strain were prepared from maintaining the serial dilution. On 30th day of feeding each group fishes were injected intraperitoneally (i.p.) with 0.5 ml of 24 hours cultured *A. hydrophila* which contained 3.5×10^{-7} CFU ml^{-1} challenge strain. The clinical signs and mortality was recorded up to 30 days of post challenge. The cumulative mortality was calculated by following Amend (1981) and Relative Percent Survival (RPS) was calculated as follow

$$\text{RPS} = 1 - \frac{(\%\ \text{Mortality in treated group})}{(\%\ \text{Mortality in control group})} \times 100$$

2.11 Statistical Analysis

Values for each parameter measured were expressed as the arithmetic mean ± standard error (SE). Effects of herbal diets on growth performance, hematological, and immunological parameters were tested using one-way ANOVA and the mean values were compared by using Duncan's multiple range tests at 5% level of significance [16].

3. RESULTS

3.1 Disease Resistance (Challenge test)

The cumulative mortality was lowest 20% when fed with 2.0% supplemented diet compared with control (80%) and other dose diets, which were 32%, 56%, and 72% in case of 1.5%, 1%, 0.5% supplemented diets respectively. In this study 2.0% supplemented diet showed 80% survivality and 75% RPS which was higher than other treatments (Fig. 1).

3.2 Specific Immune Response Assay (Serum, bacteria culture)

Fishes feeding with different doses of *L. indica* (0.5%, 1.0%, 1.5% and 2.0%) did not significantly change immune response on first week. Immune response level significantly increased with 1.5% and 2.0% supplemented diets on week 2 and 4. However, immune response level did not significantly change in control (Fig. 2).

3.3 Non-specific Immune Response (Mucus, bacteria culture)

Fish feeding with 0.5% and 1.0% *L. indica* enriched diet did not significantly enhance the immune response at 30th day in mono-sex Nile tilapia against A. *hydrophila* compared to control diet (0%). Fish fed with 1.5% and 2.0% *L. indica* enriched diet showed significantly enhanced immune response from week 1 to 4 compared to the control (Fig. 3).

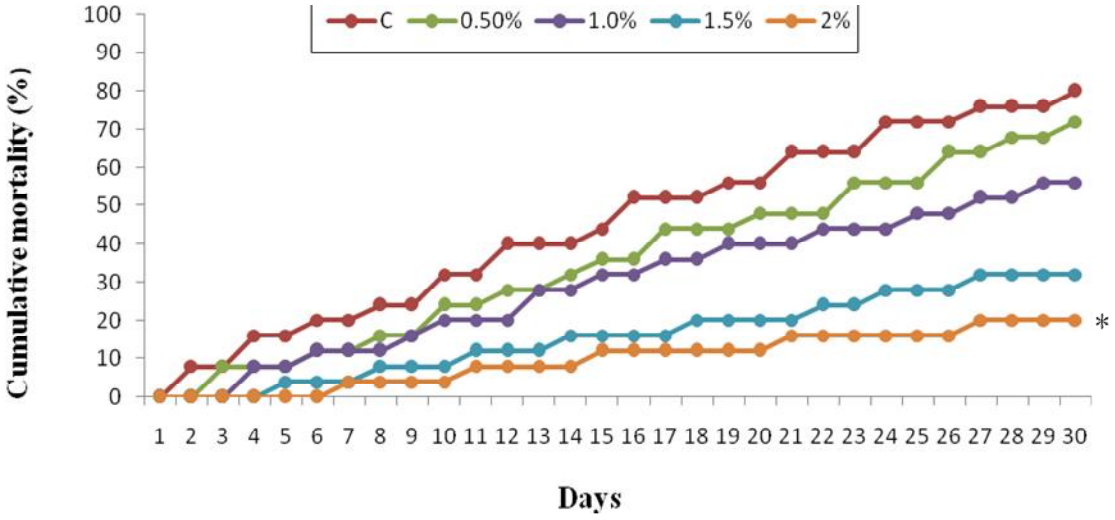

Fig. 1. The cumulative mortality of mono-sex nile tilapia fed with different doses of *L. indica* supplemented diets against *A. hydrophila*
** indicates relatively significant (P<0.05)*

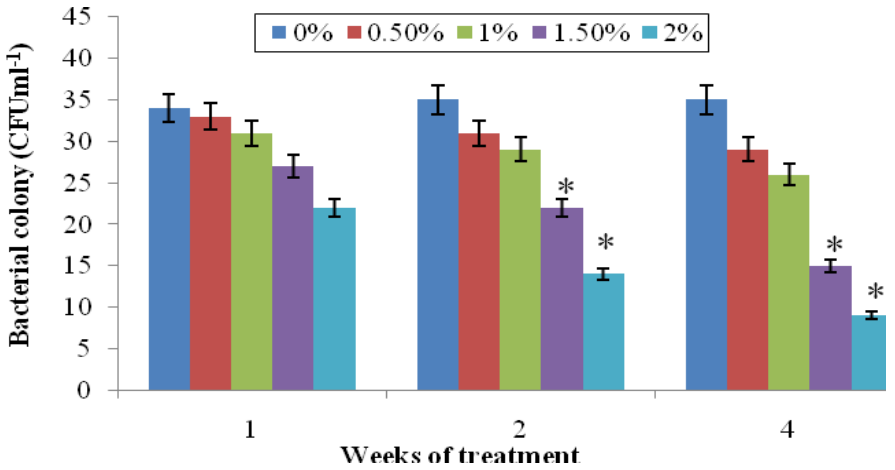

Fig. 2. Bactericidal activity of serum of mono-sex Nile tilapia fed with different doses of *L. indica* supplemented diets against *A. hydrophila*
** indicates relatively significant (P<0.05)*

Table 2. Identifying characteristics of fish pathogenic strain *Aeromonas hydrophila*

Identifying characteristics	*Aeromonas hydrophila*
Colony	Yellowish
Morphology	Small rods
Gram strain	-
Catalase	+
Oxidase	+
Gelatin liquefaction	+
Indole production	+
OF test	F
Arabinose	+
Manitol	+
Sucrose	+
Inositol	+
Esculin hydrolysis	+
Voges-proskauer reaction	+
Ammonium production	-
Glucose	G

Note: + = positive reaction; - = negative reaction; O = oxidation; F = fermentation; G = gas.

3.4 Phagocytic Activity

Phagocytic activity did not significantly enhance with 0.5%, 1.0%, 1.5% and 2.0% enriched diet on first week against *A. hydrophila*. However with 1.5% and 2.0% doses the activity significantly increased on week 2 and 4 but not with 0.5% and 1.0% doses of supplemented diet, as compared with the control (Fig. 4).

3.5 Serum Agglutination Titer Assay

Serum agglutination titer assay was done on 15th day and 30th day of the experimental period. 2.0% *L. indica* added diet fed fishes and highest diluted serum (409600) showed positive agglutination (7±1; 3±2) response (Fig. 5).

3.6 Growth Performance

During the experiment growth of all groups was increased but growth of 2% group increased very rapidly compare to other groups (Table 3).

3. DISCUSSION

In aquaculture, the use of immunostimulants is of increasing interest for boosting the defence mechanisms and conferring protection from infectious diseases against *A. hydrophila*. The effect of *L. indica* on the immune systems in aquatic animal has not been established though it has been used as an important herb for boosting the defence mechanisms. The present study revealed that decreasing cumulative mortality with increasing concentration of *L. indica* against *A. hydrophila* infection in challenge test.

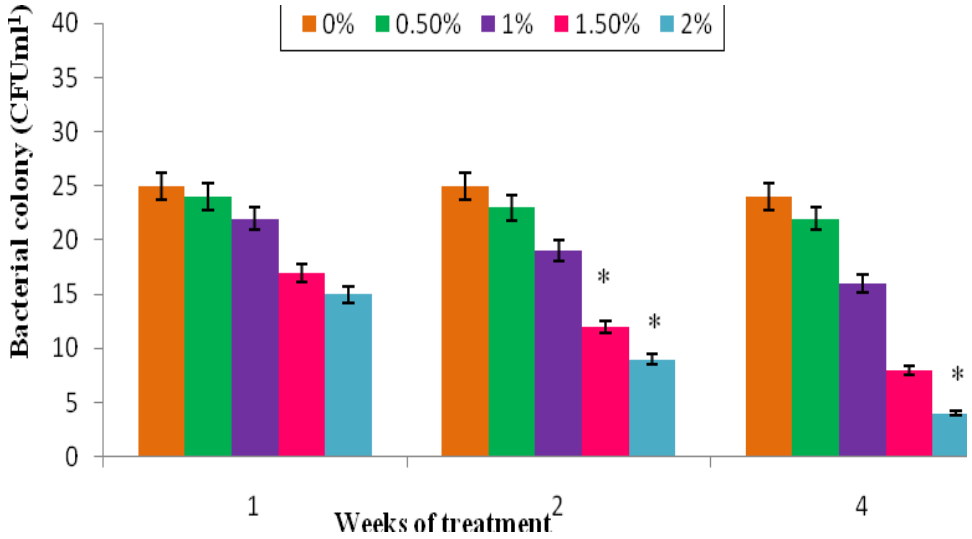

Fig. 3. Bactericidal activity of mucus of mono-sex Nile tilapia fed with different doses of *L. indica* extract supplemented diets against *A. hydrophila*

** indicates relatively significants (P<0.05)*

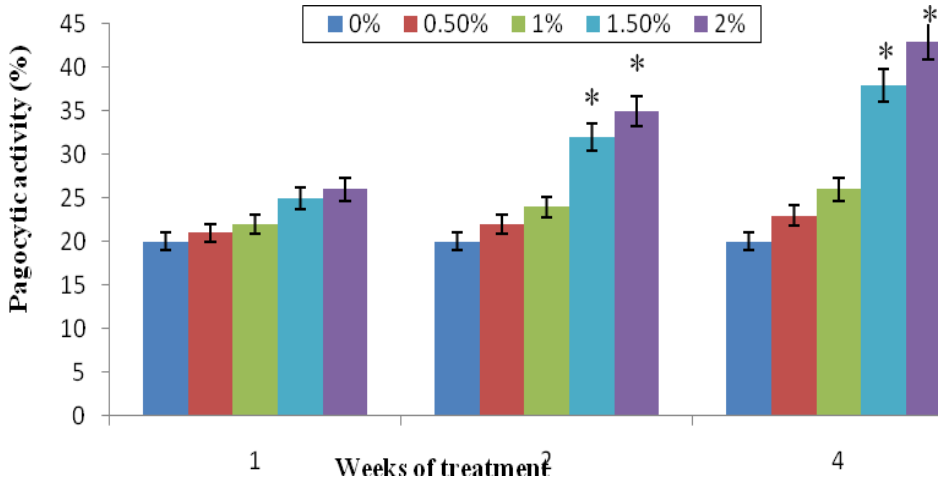

Fig. 4. Phagocytic activity (%) of mono-sex Nile tilapia fed with different doses of *L. indica* supplemented diets against *A. hydrophila*
** indicates relatively significant (P<0.05)*

It showed 72% cumulative mortality when fed 0.5% *L. indica* supplemented diet and 20% cumulative mortality when fed 2% *L. indica* supplemented diet. The decrease in mortality rate with *L. indica* added diet after injection of *A. hydrophila* was similar with previous study conducted in *O. mossambicus* fed with diet containing *Ocimum sanctum* [17] *L. rohita* fed with the diet containing *Achyranthes aspera* [18] *O. mossambicus* treated with *Eclipta alba* leaf extract [19] against *A. hydrophila* infection. *A. hydrophila* infected rainbow trout showed a similar result which was reported when fed with *A. sativum, L. perennis, M. indica*, and *U. dioica* [20].

Bactericidal activity of serum was significantly increased in 1.5% and 2.0% groups on week 2 and 4 compared to control group. Similarly, grouper (*E. tauvina*) juveniles fed with diets containing highest dose of *Ocimum sanctum* and *Withania somnifera* herb extract showed a significant increase of their bactericidal activity of serum [21]. Same like as bactericidal activity of serum the bactericidal activity of mucus also showed efficient result. Fishes fed with 1.5% and 2% *L. indica* added diet dramatically enhanced non-specific immune response.

The present study has revealed that the phagocytic activity has significantly increased when fed with 1.5% and 2.0% *L. indica* supplemented diets on week 2 and 4 but it did not showed any response against the pathogen on the first week. The present findings are in line with the report in grouper *E. bruneus* fed with the diet containing *L. indica* [10] *O. niloticus* fed with chinese herbs (*Lonicera japonica* and *Ganoderma lucidum*) containing diet [22] marine ornamental fish *Amphiprion sebae* with diet containing *Excoecaria agallocha* [23] *Cyprinus carpio* fed with *Eucalyptus sp* and *Plelargonium roseum* herbs containing diet [24] *O. nilotica* fed with *Nigella sativa* and *Bacillus subtilis* herbs containing diet [25]. An increase in phagocytic activity indicated the significant role of *L. indica* enhancing the immune response. Similar finding has been reported in rainbow trout fed with *A. sativum, L. perennis, M. indica,* and *U. dioica* against *A. hydrophila* infection [20].

The groups 1.5% and 2.0% *L. indica* formulated diet feeding mono-sex Nile tilapia showed increased serum protein level than the control. This result is supported by the study of several findings where the serum protein values were always higher in the fish treated with different immunostimulant than those in the control. Increase in the serum protein level is thought to be associated with strong innate immune response in fish [26]. The highest diluted serum (409600) showed significantly positive agglutination (7±1) in experimental group (2%) compared with the control group (18±1).

The specific growth rate exhibited an increased trend in all the five experimental groups; however it was significantly higher in 1.5% and 2.0%

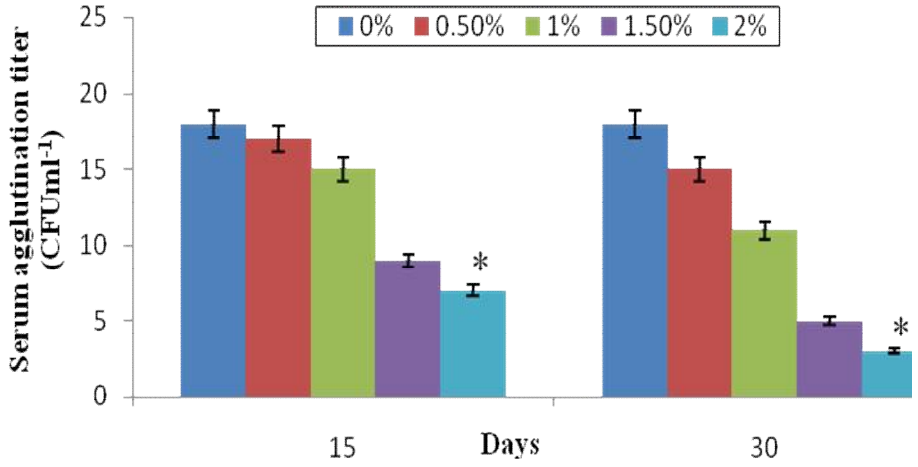

Fig. 5. Serum agglutination titer assay of mono-sex Nile tilapia fed with different doses of *L. indica* supplemented diets against *A. hydrophila*

** indicates relatively significant (P<0.05)*

Table 3. Growth parameters of mono-sex tilapia fed with different doses of *L. indica* supplemented diets against *A. hydrophila*

Growth parameters	Doses	Week-1	Week-2	Week-4
	0%	28±1.2	29±1.3	32±1.5
	0.5%	29±1.4	31±1.6	33±1.7
WG	1.0%	31±1.5	34±1.7	37±1.5
	1.5%	33±1.4	36±1.4	41±1.7
	2.0%	35±1.2	39±1.5	44±1.8*
	0%	1.2±0.4	1.2±0.6	1.3±0.4
SGR	0.5%	1.3±0.2	1.4±0.4	1.6±0.3
	1.0%	1.5±0.4	1.6±0.3	1.7±0.4
	1.5%	1.7±0.3	1.9±0.6	2.2±0.5
	2.0%	2.0±0.4	2.3±0.5	2.5±0.3*
	0%	1.5±0.2	1.6±0.1	1.7±0.3
	0.5%	1.5±0.1	1.6±0.2	1.6±0.3
FCR	1.0%	1.4±0.2	1.5±0.3	1.6±0.1
	1.5%	1.2±0.3	1.3±0.2	1.3±0.3
	2.0%	1.1±0.2	1.2±0.4	1.2±0.7*

*Note: WG = Weight gain, SGR = Specific growth rate, FCR = Food conversion ratio. [*indicates relatively significant (P<0.05)]*

L. indica added diet fed fishes. Thus it evident that dietary supplementation of *L. indica* acted as growth promoter. Nile tilapia *(Oreochromis niloticus*) fed with *Echinacea purpurea* and *Allium sativum* fortified diet exhibited significantly higher specific growth rate [27] significant increase of specific growth rate in nile tilapia fed with green tea (*Camellia sinensis*) incorporated diet even when infected with *A. hydrophila* [28]. Incorporation of *L. indica* in the diet might have improved palatability, digestion and absorption of nutrients.

4. CONCLUSION

L. indica has been shown to contain major antimicrobial compounds such as quinic acid, flavonoids, alkaloids, steroids, phenolics, terpenes which may act as potential immunostimulant. Among these flavonoids were found to have a bacteriostatic effect on fish pathogenic bacteria. In this whole experiment 2.0% *L. indica* enriched diet showed highest positive response against A*. hydrophila* and act as immunostimulants, reduce mortality, growth promoter and disease resistance (survival rate 80%) in *O. niloticus* against *A. hydrophila* infection. The present study opens up new vistas of research to assess the most effective dose under field conditions, experimentation with purified extract of *L. indica*, degree, and duration of the resistance offered, administrative regime for different age group of fish and time of

application to ensure improved harvest in culture ponds.

CONSENT

Not applicable.

ETHICAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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