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## Protection of bacterial infection through dietary administration of *Azadirachta indica* (neem) leaf in Chinese carp after parasitic infestation

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### Abstract

The present study determined the protection afforded by dietary administration with *Azadirachta indica* extract in terms of mortality and innate immune response to *Aeromonas hydrophila* infection in silver carp, *Hypophthalmichthys molitrix* after parasitic infestation in the aquarium. The silver carp was fed with 0% (control), 1.0%, 2.0% and 3.0% supplementation diets at weeks 1, 2, and 4. 2.0% dose diet within all enriched diets showed significantly increased weight gain (WG);  $51.3 \pm 1.0$  g from weeks 1 to 4 while the specific growth rate (SGR), and feed conversion ratio (FCR) did not change significantly when compared to the control. The hematocrit, lymphocytes, and neutrophils significantly increased when fed with 2.0% supplementation diets against pathogen. The phagocytic index, bactericidal activity significantly increased in fish fed with 2.0% dose diet from weeks 1 to 4 as compared to the control, but not with 1.0% and 3.0%. The highest protection by means of lower cumulative mortality was recorded with 2.0% diet when compared to 0%, 1.0% and 3.0% diet. This study suggested that 2.0% supplementation diet significantly influence the growth, hematology, and enhances the innate immune system in silver carp, *H. molitrix* against *A. hydrophila*.

**Keywords:** Disease resistance, *Azadirachta indica* (Neem) leaf, Chinese carp, immune response, bacterial infection, parasitic infestation.

### 1. Introduction

Chinese carp or Silver carp is important candidate species for intensive aquaculture particularly in the Asia region because of high consumer demand, desirable taste, and hardiness in a crowded environment, fast growth, efficient feed conversation, and rapid growth. Recently Silver carp, *Hypophthalmichthys molitrix* has become one of the important species opted for aquaculture. However, large scale production through intensive culture under crowded condition has led to the outbreak of many bacterial diseases, caused by pathogens like *Aeromonas* sp., *Vibrio* sp., *Pseudomonas* sp., *Flexibacter* sp., and *Streptococcus* sp. Recently hemorrhagic septicaemia caused by *Aeromonas caviae*, *A. hydrophila*, and *A. veronii* has become a major problem in aquaculture. Till date, traditional use of large quantities of antimicrobial compounds and to some extent vaccination disease control strategies has been used in aquaculture. However, the use of antimicrobial compounds is less favored due to adverse impacts like bioaccumulation and the emergence of drug resistant strains in the environment while the vaccines are pathogen specific. Recently, attention has been focused on immunostimulants and plant products which could have a beneficial effect in fish disease management. A number of herbs and its products have been tested for enhancing growth, non-specific, and specific immune system in finfish and shellfish<sup>[1]</sup>.

*Azadirachta indica* (Neem) of family Meliaceae is evergreen tree of potential medicinal value found in most tropical countries<sup>[2]</sup>. It is native to India, Pakistan, and Bangladesh growing in tropical and semi-tropical regions. *Azadirachta indica*, also known as Neem, Neem tree, and Indian Lilac.

Neem products are believed by Ayurvedic practitioners to be anthelmintic, anti-fungal, anti-diabetic, anti-bacterial, antiviral, contraceptive and sedative. Neem has been considered to have various activities such as astringent, antiseptic, insecticidal and antiulcer in human [3]. Neem leaf is used for leprosy, eye disorders, bloody nose, intestinal worms, stomach upset, loss of appetite, skin ulcers, diseases of the heart and blood vessels (cardiovascular disease), fever, diabetes, gum disease (gingivitis), and liver problems. The leaf is also used for birth control and to cause abortions. Other than this, the leaf extract of neem showed superior antiviral and antihyperglycemic activity [4]. The bark is used for malaria, stomach and intestinal ulcers, skin diseases, pain, and fever. The flower is used for reducing bile, controlling phlegm, and treating intestinal worms [5]. The fruit is used for hemorrhoids, intestinal worms, urinary tract disorders, bloody nose, phlegm, eye disorders, diabetes, wounds, and leprosy [6]. Neem twigs are used for cough, asthma, hemorrhoids, intestinal worms, low sperm levels, urinary disorders, and diabetes. People in the tropics, sometimes chew neem twigs instead of using toothbrushes. The stem, root bark, and fruit are used as a tonic and astringent. However, there is no report on immune functions of *A. indica* in Silver carp. Therefore, the present study investigated the effects of dietary supplementation of *A. indica* in Silver carp with reference to *A. hydrophila* infection.

## 2. Materials and Methods

### 2.1. Bacterial culture

*A. hydrophila* strain was isolated from moribund silver carp (*H. molitrix*) and the bacterium was confirmed through standard biochemical tests [7]. It was cultured in 1000 ml Brain Heart Infusion (BHI, Difco) broth for 24 h at 37 °C, centrifuged at 5000 × g (5000 rpm) for 15 min at 4 °C. The supernatant fluid was removed and the bacterial pellet was re-suspended in 0.85% NaCl at  $3.1 \times 10^6$  cfu ml<sup>-1</sup> as the stock (at -20 °C) bacterial suspension for the challenge.

### 2.2. Plant extract and feed preparation

*Azadirachta indica* plant (Meliaceae) was collected from a botanical garden in the Jessore University of Science & Technology campus. The leaves were washed in sterile distilled water, dried in shade, coarsely powdered, and extracted by the following method of Dhayanithi *et al.* (8). Five grams of dried powder was dissolved in 50 ml of ethanol, methanol, chloroform and acetone in 500 ml conical flask. The conical flask was tightly covered with aluminum foil, kept for 7 days at room temperature and agitated daily. The extract was then filtered using Whatman No. 1 filter paper (5 µm) to remove debris. The filtrate was collected and the solvent was evaporated during burning with gas burner and stored at -4°C prior to use. The formulated basal feed used as normal diet composed with fish meal, krill meal, fish oil, vitamin and mineral mixture, guar gum, CMC Na, and cellulose (Table 1). After pelletization, the basal diet were sprayed with 0% (control), 1.0%, 2.0%, and 3.0% doses of *A. indica* extract slowly, mixing evenly in a drum mixer; the feed pellets were air dried under sterile conditions for 12 h. The proximate compositions of the diet were: crude protein 43.8%, crude fat 14.2%, crude moisture 7.34%, and crude ash 11.5%. The pellets were dried in an oven at 30 °C for 18 h, packed, and stored in a freezer at -20 °C until used.

**Table 1:** Composition of experimental diets for silver carp.

Ingredients	(%)
Fish meal	50
Krill meal	12
Fish oil	8
Vitamin mixture <sup>a</sup>	5
Mineral mixture <sup>b</sup>	5
Guar gum	2
CMC Na <sup>c</sup>	4
Cellulose	14

- a. **Vitamins:** (mg/100 g dry diet): thiamine HCl, 1.5; pyridoxine HCl, 1.3; nicotinic acid, 5.7; inositol, 110; folic acid, 1.2; choline chloride, 637; calcium ascorbate, 136; menadione-NaHSO<sub>4</sub>, 5.10; riboflavin, 1.90; calcium pantothenate, 8.95; biotin, 3.75; cyanocobalamin, 1.39; vitamin A palmitate, 3.21; α-tocopherol, 118; α-cellulose, 850
- b. **Minerals:** (mg/100 g dry diet): KH<sub>2</sub>PO<sub>4</sub>, 230; Ca(H<sub>2</sub>PO<sub>4</sub>)·4H<sub>2</sub>O, 265; calcium lactate, 120; iron citrate, 65; ZnSO<sub>4</sub>·H<sub>2</sub>O, 6; CuSO<sub>4</sub>·4H<sub>2</sub>O, 4.55; CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.05; KIO<sub>3</sub>, 0.14; α-cellulose, 460; dextrin, 370
- c. Carboxymethyl cellulose sodium salt.

### 2.3. Fish and experimental design

Healthy Silver carp, *H. molitrix* (weight 30 ± 5 g, Ns: 120) obtained from a fish hatchery located in Chanchra, Jessore and were maintained in 12 aquariums (50L) aerated recirculation fresh water system. The health status of the fish was examined immediately upon arrival [9]. The fish were divided into four groups of 30 each kept in triplicate and fed with 0% (control), 1.0%, 2.0%, or 3.0% *A. indica* extract supplementation diets at the rate of 5-3% of their body weight twice a day till the end of experiment. On 30<sup>th</sup> day of feeding, all fish were injected intraperitoneally (i.p.) with 100 µl phosphate buffered saline (PBS) containing *A. hydrophila* at  $3.1 \times 10^7$  cfu ml<sup>-1</sup>. On weeks 1, 2 and 4 post-infection, six fish randomly collected from each experimental tank and to collect blood samples for hematological and immunological assays. For growth performance and cumulative mortality [10] 20 fish were used in each diet and any dead or moribund fish was examined bacteriologically to confirm the presence of *A. hydrophila* (9).

### 2.4. Growth performance

To growth performance of percentage weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) were determined according to Choudhury *et al.* (11).

$$\text{Weight (Wt) gain\%} = \frac{\text{final wt} - \text{initial wt}}{\text{initial wt}} \times 100$$

$$\text{SGR} = \frac{\log \text{ of final wt} - \log \text{ of initial wt}}{\text{no. of days}}$$

$$\text{FCR} = \frac{\text{feed given (dry wt)}}{\text{body wt} \times \text{gain (wet wt)}}$$

### 2.5. Blood and serum collection for hematology and immunology

The head kidney (HK) leucocytes and blood were collected from six fish in each experimental group at weeks 1, 2, and 4 postinfection with *A. hydrophila* for hematological and immunological assays. The blood collected by venepuncture was transferred into vacuette tubes containing heparin as anticoagulant (Greiner) to prevent clotting and used for

determination of haematocrit (Ht), haemoglobin (Hb) content, and total erythrocyte and leucocyte counts (12, 13, 14). Part of the blood was allowed to clot at room temperature for 2 h and stored overnight at 4 °C, before that the clotted blood was centrifuged at 2000 g for 25 min at 4 °C, and the serum collected and stored at -70 °C until use. The blood was diluted to 10<sup>-2</sup> and 10<sup>-3</sup> in PBS and the number of leucocytes and erythrocytes were counted following Sarder *et al.* [15]. The blood was centrifuged at 4000 rpm for 10 min and the serum was collected.

## 2.6. Head kidney macrophages preparation

After blood sampling, head kidneys (HK) removed from each fish was crushed in a tissue grinder (Jencons), diluted 1:10 in RPMI 1640 (Gibco-Invitrogen), filtered with 0.22 µm Millipore Millex porosity filters in RPMI 1640 containing 1 µl of 100 ml of penicillin and streptomycin (Sigma-Aldrich), 0.2 mg of 100 ml of heparin (Sigma-Aldrich) and 0.1% (v/v) foetal calf serum (FCS; Sigma-Aldrich) following Sakai *et al.* [36]. Macrophages were obtained by forcing the suspension through 100 µl nylon mesh (Simon) before layering onto a 34/51% (v/v) Percoll gradient (Sigma-Aldrich) in Hank's balanced salt solution (HBSS; Sigma-Aldrich) and centrifuging at 2000 g for 25 min at 4 °C. The cells were collected from the interface and washed twice with RPMI 1640 and adjusted to 10<sup>5</sup> cells ml<sup>-1</sup> using a haemocytometer slide at a magnification of ×400 on a Kyowa microscope. Cell viability was determined by using trypan blue exclusion in which cell suspensions were stained with 0.4% (w/v) trypan blue (Sigma-Aldrich) and the percentage of viable (unstained) and dead (stained) cells was scored by the method of Sakai *et al.* [36]. The percentage inhibition of trypsin activity was calculated by comparing the average of the absorbance reading with the value for 100% enzyme activity. This allowed the calculation of residual activity of trypsin based upon the amount (µl<sup>-1</sup>) of serum used in terms of percentage trypsin inhibition [37], i.e. trypsin inhibition (%) = % trypsin OD - test sample OD / % trypsin OD × 100. Phagocytic activity, phagocytic index, bactericidal activity, lysozyme, respiratory burst, and serum anti-protease activity were measured following [37] and [38].

## 2.7. Bactericidal activity

*A. hydrophila* was used to examine the effectiveness of supplements to kill the bacterial infection. To prepare stock solutions of experimental bacterial strains in a conical flask containing 100 ml distilled water. Inoculating loop was touched from single bacterial colony of fresh culture. Bacterial suspension was then diluted using serial dilution method. 15 µl of serum was added with 15 µl of bacterial suspension and mixed properly. The serum-bacterial mixture (30 µl) was plated onto the nutrient agar and BHI agar plates and incubated for 24 h at 37 °C before the number of colonies was counted.

## 2.8. Challenge test

For the challenge test *A. hydrophila* strains were prepared from maintaining the serial dilution which LD<sub>50</sub>: 10<sup>-6</sup> cfuml<sup>-1</sup> was used. 2 days after the last bleeding fish from each group were injected intraperitoneally (i.p.) 1ml of 24 hrs culture of *A. hydrophila* with challenge strain LD<sub>50</sub> value: 3.1 × 10<sup>-6</sup> cfuml<sup>-1</sup>. The clinical sign and mortality was recovered upto 7 days post challenge.

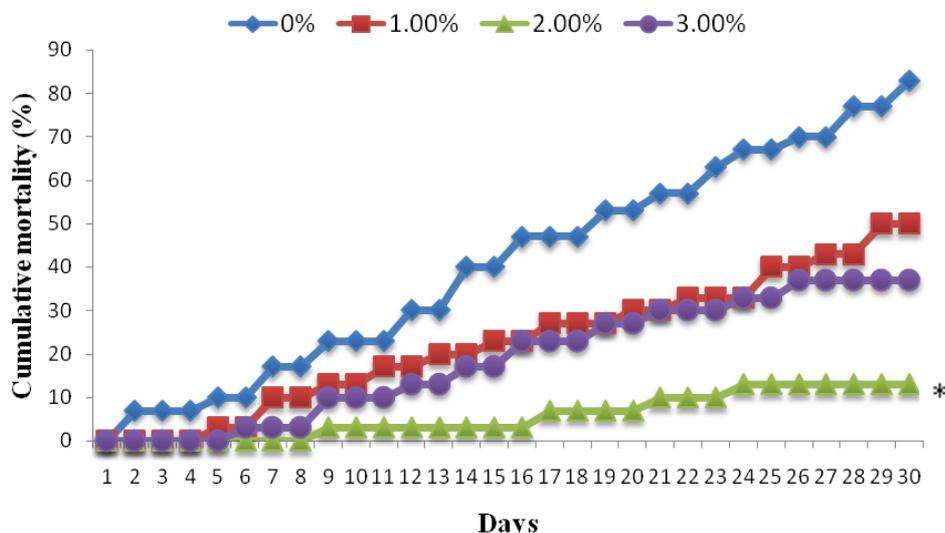
## 2.9. Statistical analysis

Values for each parameter measured were expressed as the arithmetic mean ± standard error (SE) by using Tukey statistical analysis by Statplus 2007 professional. 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 the 5% level of significance [16].

## 3. Results

### 3.1. Disease resistance

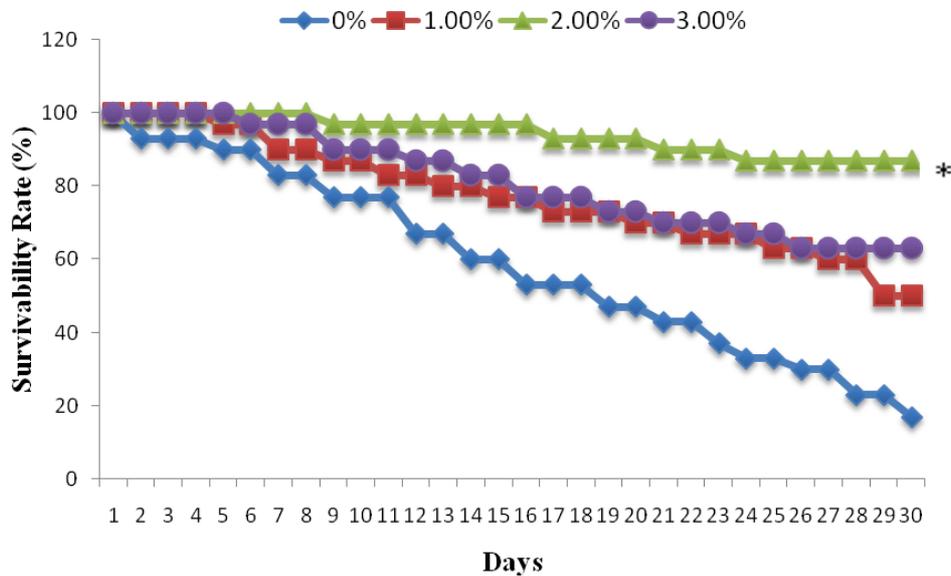
The cumulative mortality was 13% and 37% in fish fed with 2.0% and 3.0% supplementation diet for 30 days against *A. hydrophila*. 2.0% dose diet showed the highest relative percentage of survivability (RPS) 83% compared to other dose diet in the experimental study (Table 2). The mortality increased to 50% with 1.0% dose diet. The lowest mortality of 13% and a higher survivability rate of 87% were observed in fish fed with 2.0% dose diet (Fig 1 and 2).



**Fig 1:** Cumulative mortalities of silver carp fed diets containing *A. indica* and challenged with *A. hydrophila* for 30 days. Data are expressed as the difference in values ( $p < 0.05$ ) between groups in indicated by asterisk (\*).

**Table 2:** Treatment challenge of *A. indica* against *A. hydrophila* injected in silver carp.

Treatment	Challenge dose cfu ml <sup>-1</sup>	Total fish	No. of infected fish	No. of death fish	Mortality (%)	Survivability (%)	RPS (%)
0%	$3.1 \times 10^{-6}$	30	28	25	83	17	—
1.0%	$3.1 \times 10^{-6}$	30	22	15	50	50	40
2.0%	$3.1 \times 10^{-6}$	30	10	4	13	87	83



**Fig 2:** Survivability rates of silver carp fed diets containing *A. indica* and challenged with *A. hydrophila* for 30 days. Data are expressed as the difference in values ( $p < 0.05$ ) between groups in indicated by asterisk (\*).

**3.2. Growth**

In silver carp fed with all doses (1.0%, 2.0%, and 3.0%) of supplementation diet growth rate significantly increased as compared to the control. However, with 1.0% and 3.0% diets the SGR and FCR did not significantly increase with any

supplementation diet at any time. On the other hand, SGR, FCR, hematocrit (Ht), lymphocytes and neutrophils significantly increased in fish on 2.0% supplementation diet during all the weeks against pathogen compared to the control (Table 3).

**Table 3:** Growth parameters and hematological parameters of silver carp fed with different doses of *A. indica* supplementation diet against *A. hydrophila*.

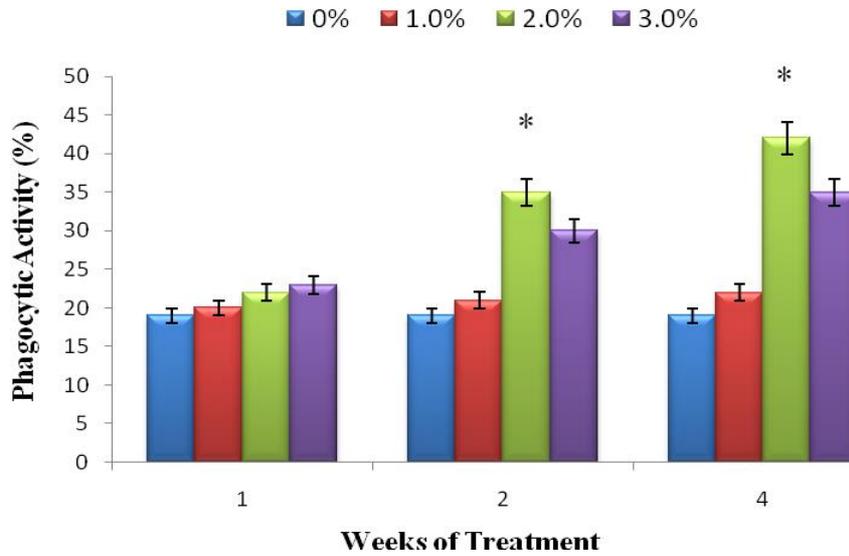
Growth parameters	Doses	Week 1	Week 2	Week 4
WG	0%	28.3 ± 1.3	29.1 ± 1.2	30.4 ± 1.5
	1.0%	30.4 ± 1.4	34.1 ± 1.1	36.4 ± 1.9
	2.0%	41.6 ± 2.0*	46.3 ± 1.3*	51.3 ± 1.0*
	3.0%	36.7 ± 2.2	41.6 ± 1.5	45.8 ± 1.2
SGR	0%	1.3 ± 0.2	1.4 ± 0.2	1.6 ± 0.2
	1.0%	1.4 ± 0.2	1.6 ± 0.2	1.6 ± 0.2
	2.0%	1.5 ± 0.1	1.6 ± 0.2	1.7 ± 0.2
	3.0%	1.4 ± 0.2	1.7 ± 0.1	1.8 ± 0.1
FCR	0%	1.6 ± 0.4	1.8 ± 0.3	2.0 ± 0.4
	1.0%	1.6 ± 0.2	1.7 ± 0.2	1.8 ± 0.2
	2.0%	1.5 ± 0.4	1.7 ± 0.4	1.7 ± 0.3
	3.0%	1.1 ± 0.3	1.3 ± 0.2	1.5 ± 0.2
Haematocrit	0%	22.6 ± 1.2	23.4 ± 1.4	23.9 ± 1.0
	1.0%	24.3 ± 0.9	23.4 ± 1.4	25.7 ± 1.6
	2.0%	26.2 ± 1.0*	27.9 ± 1.5*	29.0 ± 1.1*
	3.0%	24.1 ± 1.5	25.6 ± 1.2	26.2 ± 1.3*
Neutrophils	0%	0.6 ± 0.04	0.6 ± 0.03	0.7 ± 0.04
	1.0%	0.7 ± 0.02	0.8 ± 0.04	0.8 ± 0.06
	2.0%	1.3 ± 0.04*	1.5 ± 0.05*	1.8 ± 0.03*
	3.0%	0.9 ± 0.06	1.2 ± 0.04*	1.4 ± 0.05*
Lymphocytes	0%	21.3 ± 1.2	22.4 ± 1.1	22.6 ± 1.2
	1.0%	22.6 ± 1.0	24.1 ± 1.4	24.1 ± 1.4
	2.0%	27.5 ± 1.5*	31.8 ± 1.2*	35.4 ± 1.2*
	3.0%	24.7 ± 1.4*	27.8 ± 1.3*	29.5 ± 1.4*

Data expressed as mean ± SE, \*significantly different from controls ( $P < 0.05$ ), n=10. WG: Weight gain, SGR: specific growth rate, FCR: feed conversion ratio.

### 3.3. Phagocytic activity

The phagocytic activity of the head kidney macrophages as indicated by the phagocytic ratio and phagocytic index are described in Fig 3. The phagocytic ratio significantly increased

in fish fed with any doses supplementation diet from week 1 to 4 (Fig 3). On the other hand, phagocytic index significantly enhanced on being fed with 2.0% enriched diets from week 1 to 4 but not with 1.0% and 3.0% diet (Fig 3).

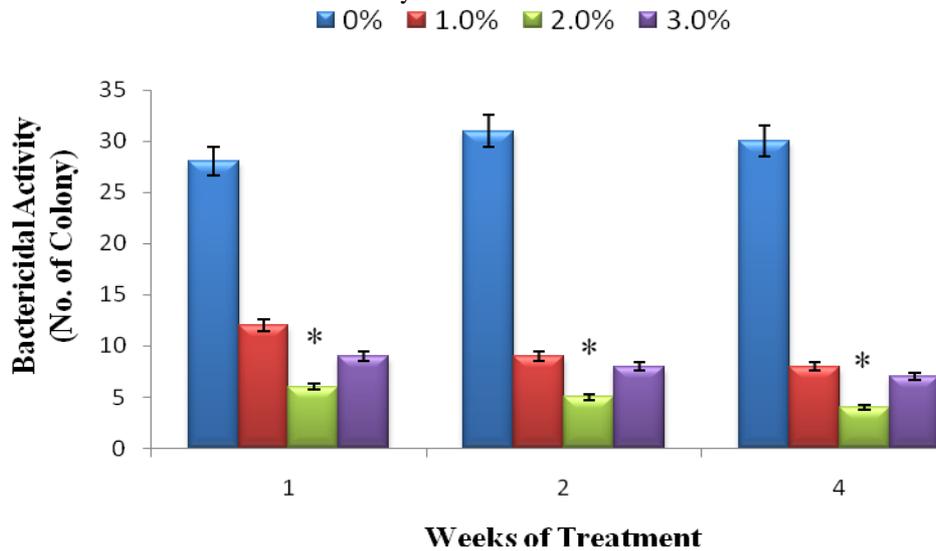


**Fig 3:** Phagocytic activity (%) of *H. molitrix* fed with different doses of *A. indica* extract against *A. hydrophila*. Data are expressed as mean  $\pm$  S.E.; (n=3) and the differences in values ( $p < 0.05$ ) between groups is indicated by asterisks (\*).

### 3.4. Serum bactericidal activity

The serum bactericidal activity was significantly high in fish fed with all doses of *A. indica* supplementation diet against pathogen as compared with the control (Fig 4). The lowest number of bacterial colonies indicated the efficiency of

immune cells in serum to kill the pathogen. With *A. indica* the lowest number of colonies,  $4 \times 10^{-6}$  developing on BHI occurred with the 2% dose (Fig 4), with highly significant differences to the control, ( $30 \times 10^{-6}$ ) ( $p < 0.005$ ), followed by 3% ( $p < 0.005$ ), respectively.



**Fig 4:** Bactericidal activity (CFU/ml) of *H. molitrix* fed with different doses of *A. indica* extract against *A. hydrophila*. Data are expressed as mean  $\pm$  S.E.; (n = 3) and the differences in values ( $p < 0.05$ ) between groups is indicated by asterisks (\*).

### 4. Discussion

In the recent years, there is increasing interest in the use of herbal extracts as dietary and therapeutic supplements indicate that modulate immune function in fish [17]. Immunological approaches to prevent fish diseases have been normally used antibiotics, chemicals or vaccination against specific pathogens, while the use of immune - stimulants is relatively a new and developing area [18].

In the present study silver carp fed with all doses diet had significantly increased growth rate when compared to the

control. However, SGR and FCR did not significantly increase with any diet or time. The Ht, lymphocytes and neutrophils levels significantly increased in 2.0% dose diet fed group. The results are in agreement with *Oncorhynchus mykiss* after dietary administration with garlic against *Aeromonas hydrophila* infection had similar effects [19]. In the present study, the cumulative mortality in 2.0% and 3.0% diet fed groups was 13% and 37% when compared to the 83% in infected untreated group. *Labeo rohita* and *O. mykiss* fed with *Achyranthes aspera* or garlic [19, 20] and *Ocimum sanctum* leaf

extracts administered through intraperitoneally injection in *Oreochromis mossambicus* [21] also reduced the mortality rate against *A. hydrophila* infection. *L. rohita* fed with garlic enriched diet exhibited increased number of serum bactericidal activity [22].

Such enhancement in the serum activity could also be correlated with enhanced phagocytic activity. The phagocytic activity significantly increased with any enriched diet while the superoxide anion level significantly was enhanced in 1.0% and 2.0% doses diet from week 1 to 4 against pathogen. These results indicate that *A. indica* supplementation diets might have induced other antimicrobial mechanisms, which include release of lysosomal enzymes, cationic peptides, complement components, and production of reactive oxygen species [23]. Enhanced or elevated phagocytic activity has been reported in goldfish and rainbow trout after treatment with different herbals against *A. hydrophila* [19, 24, 25, 26, 27]. The enhancement of phagocytosis observed in this study may be due to *A. indica*. In the present study the serum bactericidal activity significantly increased with any enriched diets at any time. Such an increase in the bactericidal properties in fish serum could be attributed to the reduced mortality (%) as was observed for 30 days post-infection particularly in fish fed with 1.0% and 3.0% doses of supplementation diet slicing down the mortality to 50% and 30% respectively when compared to the 90% mortality in the infected and untreated group.

In Asia, mushrooms and medicinal herbs have long been used as dietary supplements and medicinal food, because they contain numerous biologically active compounds, such as polysaccharides demonstrated that immune - modulator activities such as enhance immune function, inhibit cancer growth and metastasis, and increase host resistance to bacterial, viral, and parasitic infections [17, 18, 28, 29, 30, 31, 32, 33]. A number of studies indicate that dietary administrations of herbs enhance the respiratory burst of phagocytes in fish [19, 27, 34]. This study concluded that *A. indica* has a potential value as an immunostimulant for aquaculture both in terms of growth performance and protection from *A. hydrophila* infection in silver carp.

The phagocytic activity significantly increased when fed with 2.0% and 3.0% supplementation diets on weeks 2 and 4 but it did not any diet on the first week against the pathogen. The present findings are in line with the report in *C. carpio* fed with the diet containing oligodeoxynucleotides, greasy groupers *E. tauvina* fed with herbal diet containing purified active principle of *Ocimum sanctum*, *Withania somnifera*, and *Myristica fragrans* and in Chinese sucker treated with TCM extracts [35]. An increase in phagocytic activity indicated in the present study indicates the significant role of *A. indica* in enhancing the specific 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 [24, 27].

The stimulation of specific immune defense observed in the present study might be due to the presence of one or more than one components present in *A. indica*. The present results has confirmed the maximum response with 2.0% and 3.0% enriched diet against *A. hydrophila* which might suffice to activate the receptors and the corresponding genes responsible for the secretion of immune defense factors. The present study opens up new vistas of research to assess the most effective dose under field conditions, experimentation with a purified extract of *A. indica*, degree, and duration of the resistance

offered, an administrative regime for different age group of fish and time of application to ensure improved harvest in culture ponds.

## 5. Conclusion

In the light of the overall results obtained in the current study, it is clearly indicated that extracts of *A. indica* has great potential as antibacterial compound. It is anticipated that this herb can be potentially used in combating fish bacterial diseases especially for haemorrhagic septicaemia infection. However, further study on safety and toxicity are warranted. Now a day, most pathogenic organisms are becoming resistant to antibiotics. The present study also suggested the potential of some herbs as an alternative to commercial and synthetic antibiotics, which could be used in aquaculture industries.

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