

EFFECT OF DIETARY ADMINISTRATION OF *ZINGIBER OFFICINALE* ON GROWTH, SURVIVAL AND IMMUNE RESPONSE OF INDIAN MAJOR CARP, *CATLA CATLA* (HAM.)

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ABSTRACT

A study was conducted to evaluate the efficacy of different dietary doses of *Zingiber officinale* powder for the immune response and the disease resistance of the Indian major carp (*Catla catla*) infected by *Aeromonas hydrophila*. Haematological, biochemical and immunological studies were performed on fish and were analyzed different days of feeding trial. The total erythrocyte, leukocyte count, haemoglobin content and total serum protein were significantly ($P < 0.05$) enhanced in *Z. officinale* supplemented groups. NBT assay and bactericidal activity were significantly stimulated also supplemented groups exhibited the highest value in group D on 30th day. After all the groups had been challenged intra-peritoneally with *A. hydrophila* on 30th day, the Relative Percentage Survival (RPS) was significantly higher in 12, 24, 48 and 72 hrs. Specific Growth Rate (SGR) was the maximum in group C and lowest in control group. The results concluded that the *Z. officinale* powder used in this study can act as immunostimulant, enhance the non-specific immunity and disease resistance of *C. catla* to *A. hydrophila* infection.

Keywords: Immunostimulant, *Zingiber officinale*, *Catla catla*, *Aeromonas hydrophila*

INTRODUCTION

Aquaculture is a fast developing industry. The world's total production of fish and shellfish (including molluscs and crustacea) was 99 million tons (mt) in 1990 and it increased to 122 mt in 1997. According to Food and Agriculture Organization (FAO) of the United Nations, the global aquaculture production has increased from about 28.3 to 40 mt in 2009 [1]. Aquaculture fish production increased significantly over the past few decades necessitating intensive fish culture practices. Due to this practice a number of associated stressors like overcrowding, transport, handling, grading, and poor water quality tends to adversely affect the health of cultured fish. These practices are the major factors that make the fish susceptible to disease. *Aeromonas hydrophila* is the most widespread pathogen and it can be easily spread through accidental abrasions. This bacterium causes hemorrhagic septicemia, characterized by presence of small superficial lesion, focal hemorrhages, particularly in the gills and opercula, ulcers, abscesses, exophthalmia, and abdominal distension [2].

"An immunostimulant is a naturally occurring compound that modulates the immune system by increasing the host's resistance against diseases that in most circumstances are caused by pathogens" [3]. Immunostimulants enhance the humoral and cellular response in both specific and non-specific ways. The use of immunostimulants for the prevention of disease in fishes is considered as an attractive and promising area in the field of aquaculture [4,5]. Immunostimulants are valuable for the prevention and control of fish diseases in aquaculture as they represent an alternative and supplementary treatment to vaccination. The immunostimulants also have additional effects such as growth enhancement and increase in the survival rates of the fishes under stress [6].

Various chemotherapeutics have been used to treat bacterial infections in cultured fish for about the last 20 years. However, the incidence of drug-resistant bacteria has become a major problem in fish culture [7]. Natural products like plant extracts might have beneficial effects but cause no problems [8,9]. Plants are used for many purposes including foods, treating medical ailments. Nowadays, many researchers have focused their interest on medicinal plants due to the increasing demand for health food. Species of *Zingiberaceae*, *Annonaceae*, *Araceae*, *Simaroubaceae* and many others are known as having medicinal values. In India, several herbs are being used as therapeutic agents to treat the infections caused by *Aeromonas* sp. and other fish pathogens. Herbs such as

Eclipta alba [10], *Aloe vera* [11], *Ocimum sanctum* [12], *Solanum trilobatum* [13], *Astragalus radix* and *Scutellari radix* [14] and *Achyranthes aspera* [15,16] have been reported to enhance the immune parameters and elevated resistance against the disease causing agents.

Ginger (*Zingiber officinale* Roscoe, Zingiberaceae) is widely used around the world in foods as a spice. Ginger is a plant which commonly known as "Ingi" in Tamil Nadu, India. For centuries, it has been an important ingredient in Chinese, Ayurvedic and Tibb-Unani herbal medicines for the treatment of catarrh, rheumatism, nervous diseases, gingivitis, toothache, asthma, stroke, constipation and diabetes [17,18,19]. Previously, studies have indicated that ginger is effective for the control of a range of bacterial, fungal and parasitic conditions [20]. Also, ginger has been reported to have anti-inflammatory and anti-oxidative activity [21,22,23] and to be effective as an immune modulatory agent in animals, including fish [24,25]. Thus, the present study is aimed at evaluation of the traditionally used herbal medicine ginger powder as a feed additive on the growth, survival, immune response and disease resistance of fingerlings *Catla catla* against *Aeromonas hydrophila*.

MATERIALS AND METHODS

Animal collection and maintenance

Freshwater *C. catla* fingerlings were obtained from Bharath Fish Seed Farm, Poondi, Tamilnadu, India. The fingerlings were brought to the laboratory in plastic bags in oxygenated habitat water and acclimatized for 14 days in disinfected 1000 L FRP (Fiberglass Reinforced Plastics) tanks. During the acclimatization, the fingerlings were fed with normal diet. Healthy and disease free fingerlings, weighing average body weight of 20.27 ± 1.0 g were selected for further experiments.

Bacterial strain

Aeromonas hydrophila is a gram negative, facultative anaerobic rod shaped bacteria, belonging to the family Vibrionaceae. A pure virulent strain of *A. hydrophila* received from IMTECH (Institute of Microbial Technology, Chandigarh, India) was maintained at 4°C. From this culture, sub-cultures were maintained on Nutrient Agar (NA) slants (Hi-media, Mumbai) at 5°C. A stock culture in Nutrient Broth (NB) was also maintained at -20°C with 0.85% NaCl (w/v) and 20% (v/v) glycerol to provide stable inoculated throughout the study period [26].

Sources of immunostimulant and feed ingredients

Zingiber officinale (Ingi) were obtained from local market, Mylapore, Chennai. The specimen was examined and identified by a botanist from Centre for Advanced Studies (CAS) in Botany, University of Madras, Maraimalai Campus, Guindy, Chennai, Tamil Nadu, India. Raw ginger were washed several times with distilled water, air dried in shaded area. The dried ginger was grinded into fine powder using mixi and stored in glass containers at room temperature for future use as an immunostimulant. Feed ingredients like dry fish, shrimp head, wheat flour, soya bean meal, broken rice, fish oil, vitamins and minerals mixture etc., were purchased from local market.

Diet preparation and experimental design

The preparation of experimental diets, respective ingredients were mixed, other than fish oil, vitamins and minerals in water to make dough followed by cooking in an autoclave at 15 psi for 20 min. After cooling, fish oil, vitamins and minerals along with the precondition *Z. officinale* powder concentrations were added (Table 1). Finally the dough was pressed thoroughly using a hand pelletizer to obtain uniform-sized pellets, and spread on paper to dry under shade. After drying for 4 hrs, the pellets were kept in a hot air oven at $55 \pm 3^\circ\text{C}$ for overnight to dry completely. After drying, the pellets were packed in polythene bags and stored at 4°C until further use.

Table. 1. Composition of basal diet for supplemented with herbal immunostimulant

Ingredients Composition (g kg ⁻¹)	Diet			
	A (Control)	B	C	D
Fish meal	275.00	275.00	275.00	275.00
Shrimp head meal	150.00	150.00	150.00	150.00
Wheat flour	200.00	200.00	200.00	200.00
Gluten mixture	65.00	65.00	65.00	65.00
Soya bean meal	100.00	100.00	100.00	100.00
Broken rice	100.00	100.00	100.00	100.00
Fish oil	20.00	20.00	20.00	20.00
Vitamins & Minerals mixture	40.00	40.00	40.00	40.00
Maida flour	50.00	50.00	50.00	50.00
Herbal immunostimulant	0.00	0.10	0.50	1.00

After acclimatization fingerlings were divided into 4 groups [control (A) and three experimental (B, C and D) groups] with three replicates each in 150 L capacity (80 × 41 × 41 cm) FRP tanks. Totally 30 fingerlings were used in each group for feeding experiments. Control (group A) fingerlings were received normal diet without *Z. officinale*, group B were received 100 mg/kg of *Z. officinale* mixed diet, group C were received 500 mg/kg of *Z. officinale* mixed diet, group D were received 1000 mg/kg of *Z. officinale* mixed diet. During experimental period the fingerlings were fed with 3% of body weight with the following diet twice daily at 10 am and 5 pm. The total experimental period was 30 days.

Collection of blood samples

Each fish were individually caught using a dip net and were bled from common cardinal vein using 1 ml tuberculin syringe fitted with 24 gauge needle. In order to sample the blood for serum separation, 200 µl of blood was drawn and whole bleeding procedure was completed within 1 min. The blood was collected in serological tubes and clot was stored at 20°C overnight. The clot was then spun down at 400 g for 10 min. The serum collected by aspiration was stored in sterile eppendorf tubes at 20°C for further use. After blood collection, spleen was removed from fish for histological studies.

Haematology and biochemical indices

Haematological analyses were carried out by standard methods. Total erythrocyte and leucocytes count were determined by following the method of Russia and Stood [27]. Haemoglobin estimation was done by acid-haematin method using Sahli's haemoglobinometer and the value is expressed in g%. The total serum protein content was estimated by the following method of Bradford [28].

Nitro blue tetrazolium assay (NBT)

For the Nitro blue tetrazolium assay, Secombes's [29] method was followed with the modification described by Stasiak and Baumann [30]. The heparinized blood was collected in silica-coated Eppendorf

tubes and the buffy coat was separated by centrifuging at 10,000 rpm for 10 min. Fifty microlitres of the blood coat was placed in each of the 96 wells of U bottom microtitre plates (Laxbro, Pune, India) and incubated at 37°C for 1 hr, to facilitate adhesion of cells. Then the supernatant was removed and 50 ml of 0.3% NBT was added. After incubation, NBT was removed. The cells were then fixed with 100 % methanol and washed thrice with 70 % methanol. The plates were air-dried. Sixty microlitres of 2N KOH and 70 microlitres dimethyl sulphoxide were added to each well to dissolve the formazan blue precipitate. The turquoise-blue-coloured solution was then read in an ELISA reader at 655 nm.

Bactericidal activity

Serum bactericidal activity was estimated by following the procedure of Kajita [31]. An equal volume (100 µL) of serum and bacterial suspension was mixed and incubated for 1 hr at 25°C . A blank was prepared by replacing serum with sterile PBS buffer. The mixture was then diluted with sterile PBS at a ratio 1:10. The serum-bacterial mixture (100 µL) was pour-plated in nutrient agar and the plates were incubated for 24 hrs, at 37°C . The number of viable bacteria was determined by counting the colonies grown on nutrient agar plates. Data on bactericidal activity were calculated as follows

$$\text{Positive control CFU} - \text{Sample CFU} / (\text{Positive control CFU} / 100)$$

Growth parameters

Growth measurements such as weight and length of the fingerlings were recorded individually. Sample size and total biomass were optimized in the tank to adjust the amount of feed for each experimental group. At the end of 30 days experimental period, specific growth rate [32] of fingerlings fed with different diets were calculated as given below

$$\text{SGR} = [\text{Ln}(\text{Final weight}) - \text{Ln}(\text{Initial weight})] / t (\text{time interval in days}) \times 100$$

Where, Ln – logarithm

Challenge study

Challenge study was performed by infecting *A. hydrophila* in the fingerlings of *C. catla* of post dietary experimental groups. Approximately 10 fingerlings of each dietary experimental group were challenged with 100 µl of *A. hydrophila* by injecting intraperitoneally. The pathogen injected fingerlings were maintained in separate tanks up to 72 hrs. Relative percentage survival (RPS) was calculated by following formula

$$RPS = 1 - \left\{ \frac{\text{percent mortality in treated group}}{\text{percent mortality in control group}} \right\}$$

Statistical analysis

Data were subjected to standard statistical analysis by using statistical package for social sciences (SPSS, version 17.0 for Windows, SPSS Inc., Chicago, USA). The level of significance was chosen at P<0.05 and the results are presented as means ± SD.

RESULTS

Haematological parameters

In the present investigation, the efficacy of *Z. officinale* as feed additive supplemented diets on the haematological changes of fish fingerlings *C. catla* was studied. Erythrocytes count has been showed an increasing trend in all experimental groups when compared to control. This increasing trend in statistically significant (p<0.05). The highest mean value of (2.01 ± 0.30 × 10⁶ cells mm⁻³) 30th day and the lowest value (1.37 ± 0.00 × 10⁶ cells mm⁻³) in initial stage group B. The group D on the 30th day has showed significant increasing

when compared to groups (A, B and C) on the 10th and 20th day. The increasing concentration of *Z. officinale* in the feed shows the increasing of RBC count in the groups B, C and D highly than the control group (Fig.1).

The leucocytes count in groups (B, C and D) was significantly higher than those of control group A (p<0.05). The maximum mean value was observed in group D (30.32 ± 0.06 × 10³ cells mm⁻³) on the 30th day and minimum mean value, was observed in control group A (25.28 ± 0.16 × 10³ cells mm⁻³) on the initial stage. The increasing concentration of *Z. officinale* in the feed shows the leucocytes count was increased in groups (B, C and D) on the 30th day highly than the control group A on the initial stage. According to the results, herbal diet could increase leucocytes count of fish in experimental groups compared to control group (Fig.2).

The Hb content was significantly increased (p<0.05) in experimental groups compared to control group (Fig.3). This graph also indicates that the Hb content was gradually increased throughout the experimental period compared to initial value. The highest mean value of Hb content (8.46 ± 0.05 g dl⁻¹) was observed in group D on the 30th day and the lowest mean value of (5.63 ± 0.05 g dl⁻¹) was observed in initial stage (control group A). The groups B, C and D shows the huge increase in the Hb content than the group A (control) due to the addition of different concentration of *Z. officinale* in the feed the highest amount of Hb content was recorded in the 30th day in the groups C and D (8.2 ± 0.10 g dl⁻¹; 8.46 ± 0.05 g dl⁻¹). According to these results, the total haemoglobin level increased from initial stage (0) to 30 days of feeding in all the experimental diet groups than in the control.

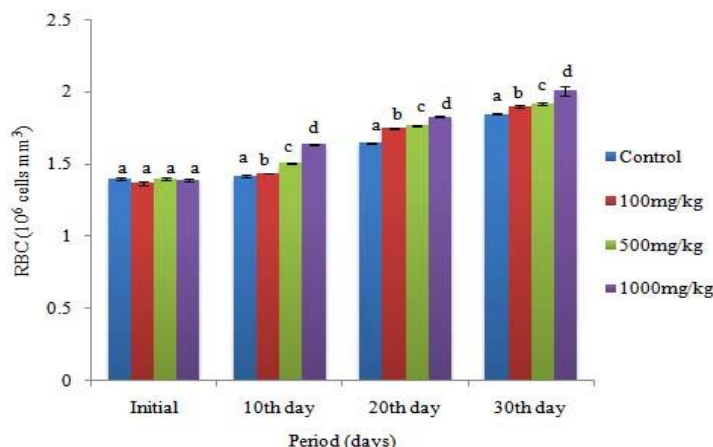


Fig. 1: Difference in RBC count in common carp, *C. catla*, fed on herbal immunostimulants supplemented experimental diets and control. Each value represented were the arithmetic mean ± SD (P<0.05).

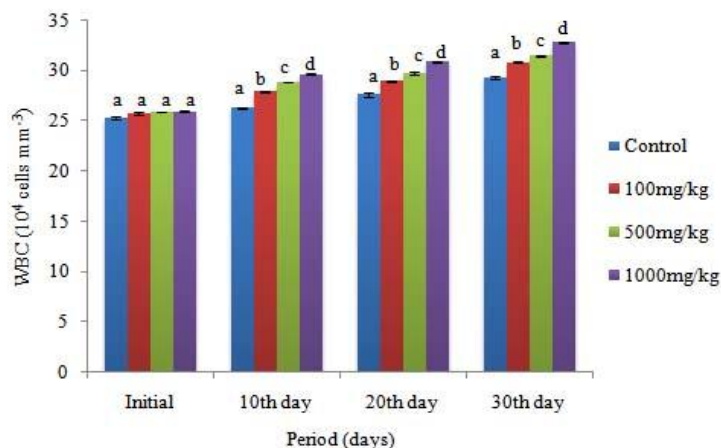


Fig. 2: Leucocytes count of common carp, *C. catla* fed on herbal immunostimulants supplemented experimental diets and control. Each value represented were the arithmetic mean ± SD (P<0.05).

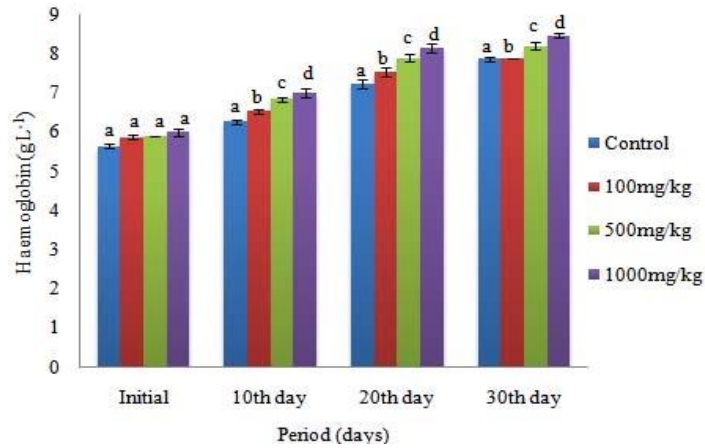


Fig. 3: Effect of supplemented feed of *Z. officinale* mixed diet on haemoglobin content (g/L⁻¹) in *C. catla*. Each value represented were the arithmetic mean \pm SD (P<0.05).

Total serum protein

Total serum protein level was shown in Fig.4. The result indicates that the total serum protein level was significantly increased (p<0.05) in experimental groups compared to control group. This graph also indicates that the total serum protein level in serum was gradually increased throughout the experimental period, compared to initial value. The groups B, C and D shows the increasing trend in the total protein level in serum than the group A (control) due to the addition of different concentration of *Z. officinale* in the feed. The highest amount of total serum protein level was recorded in the 30th day in the groups

C and D (4.56 ± 0.10 g dl⁻¹; 4.95 ± 0.05 g dl⁻¹) and lowest level was recorded in the initial in group A (control) (1.48 ± 0.1 g dl⁻¹).

Nitro blue tetrazolium assay

Nitro Blue Tetrazolium results were showed in Fig.5. The results of respiratory burst activity were increased in experimental groups when compared to initial value. The highest range of respiratory burst was recorded at 30th day in group C and D (0.868 ± 0.00 ; 1.002 ± 0.00) and the lowest range of was recorded at initial stage in group A (control) (0.321 ± 0.00).

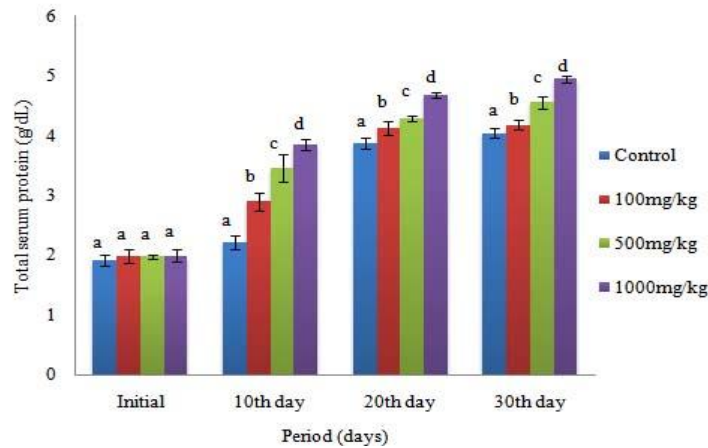


Fig. 4: Total serum protein concentration in *C. catla* fish fed with food added different doses of crude powder. The values in a bar with different letters are significantly different (p<0.05), data are expressed in as mean \pm SD

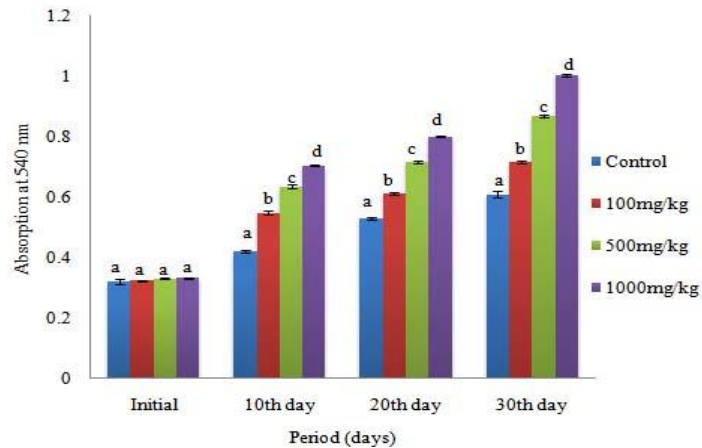


Fig. 5: Respiratory burst activity of Indian major carp, *C. catla* fed on herbal immunostimulant supplemented diets (B, C and D) and control diet (A). Data represented as mean \pm SD (P<0.05).

Serum bactericidal activity

Serum bactericidal activity was showed in Fig.6. The result shows that the serum bactericidal activity was significantly increased in experimental groups compared to control group. The serum bactericidal activity was gradually increased throughout the experimental period compared to initial value. The highest amount of serum bactericidal activity was recorded on 30th day in the groups C and D (72.32 ± 2.88 CFU/control %; 81.15 ± 1.9 CFU/control %) and lowest amount activity was recorded on initial stage in group A (control) (26.41 ± 3.2 CFU/control %). The groups B,C and D shows the huge increase in the serum bactericidal activity that the control

group due to the addition of the different concentration of *Z. officinale* in the feed.

Growth parameters

Specific growth rate results on growth of the fishes are given in Fig.7. Generally there is an increase in body weight of the fishes in control and experimental groups. Fish fingerlings weight gain has showed a significantly ($p < 0.05$) increased in experimental groups when compared to control. The highest SGR weight value of 0.651 ± 0.075 %/day was observed in group D and the lowest value of 0.439 ± 0.44 %/day was observed in group A (control).

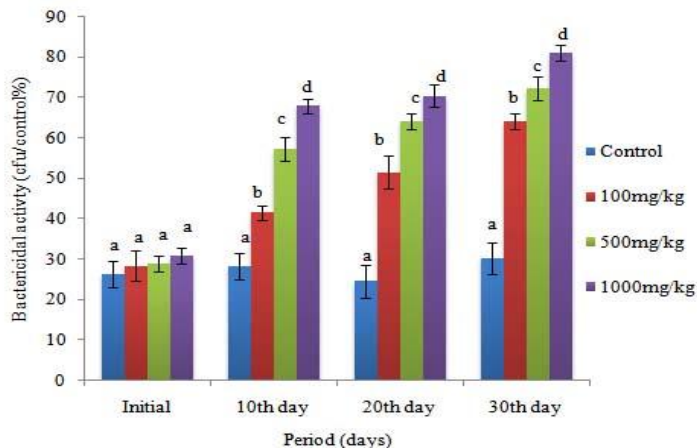


Fig. 6: Effect of *Z. officinale* mixed diet on serum bactericidal activity in *C. catla*. Each value represented were the arithmetic mean ± SD ($P < 0.05$).

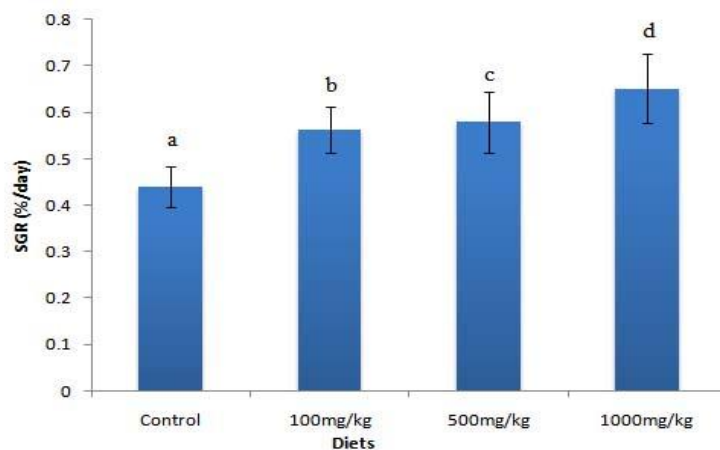


Fig. 7: Specific growth rate of weight (%/day) of *C. catla* feed on *Z. officinale* immunostimulant supplemented diets and control. Each value represented were the arithmetic mean ± SD ($P < 0.05$).

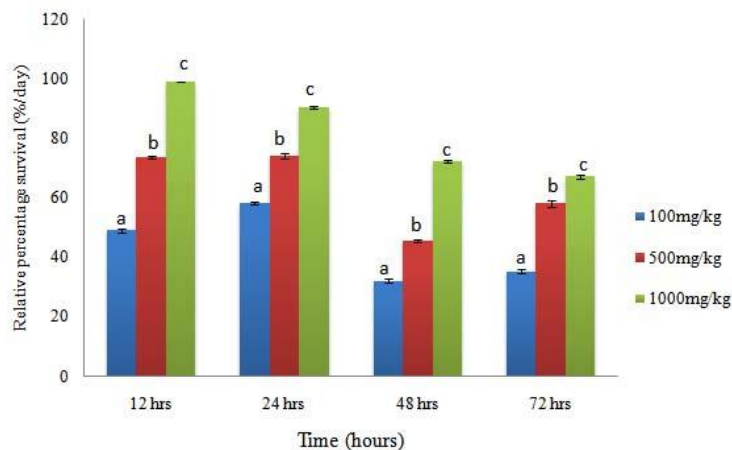


Fig. 8: Relative percentage survival of *C. catla* fed with *Z. officinale* powder supplemented after challenging with *Aeromonas hydrophila*. Data are expressed in as mean ± SD ($P < 0.05$).

Relative percentage survival

The Relative Percentage Survival was found highest (100 %) in the group D (12 hrs) and lowest (33.2 %) in the group B (48 hrs). The RPS rate was diminished after the injection of *A. hydrophila* with regarding time. But the RPS rate was increased in the group C and D when compared to the group B (Fig.8). The result showed a significantly ($p < 0.05$) increased in experimental groups when compared to control.

DISCUSSION

To develop alternative practices for growth promotion and disease management in aquaculture, attention has also been focused in identifying novel drugs, especially from plant source. These drugs may be delivered to the cultivable organisms either through feed supplementation or oral delivery through diets mode. Several herbs have been tested for the growth promoting activity in aquatic animals [33,34]. Immunostimulants have the competence to promote the nonspecific resistance of fish before infection of pathogens. In recent decades, many substances have been shown to enhance the nonspecific immunity of fish and the route of their administration has differential effects on the immune system.

Erythrocytes count has been showed an increasing trend in all experimental groups when compared to control. The leukocyte counts in groups (B, C and D) were significantly higher than those of control group (A) ($p < 0.05$). Similar results are indicated by Sahu *et al*, [35] who reported that WBC counts were higher in *Labeo rohita* fingerlings fed *mangifera indica* kernel when compared to control. Gopalakannan and arul [36] also reported that there was an increase in the WBC count after feeding the common carp with immunostimulants like chitin.

The Hb content was significantly increased ($p < 0.05$) in experimental groups compared with the control group. The results also indicate that the Hb content was gradually increased throughout the experimental period compared with the initial value. Similarly, the results findings Sahu *et al*, [35] and Harikrishnan *et al*, [37] also reported increasing level of haemoglobin content were observed in *A. hydrophila* infected *C. carpio* treated with *Azadiracta indica*.

The immunostimulant herbal incorporated diets helped to increase the humoral elements in the serum. The total serum protein level was significantly increased ($p < 0.05$) in experimental groups compared with the control group. The results indicate that the total serum protein level in serum was gradually increased throughout the experimental period, compared with the initial value. The groups B, C and D shows the increasing in the total serum protein level in serum than the group A (control) due to the addition of different concentration of *Z. officinale* in the feed. This result was supported by the study of founding that serum protein values were always higher in the fish treated with different immunostimulant than those in the control. Increase in the serum protein levels is thought to be associated with a stronger innate immune response in fish [38].

The result of respiratory burst activity was significantly increased ($p < 0.05$) in experimental groups when compared to initial value. Phagocytosis and killing activity by phagocytic cells is an important defense mechanism against pathogenic bacteria [33]. Fish phagocytes are able to produce Superoxide anion (O_2^-) during a process called respiratory burst [29]. It is considered that these oxygen forms are toxic for bacterial pathogens [35]. The respiratory burst activity can be quantified by the nitro blue tetrazolium assay which measures the quantity of intracellular superoxide radicals produced by leukocytes [39]. Herbal based immuostimulants can enhance the respiratory burst activity of fish. For instance, Rao *et al*, [33] reported that superoxide anion production by the blood leucocytes was enhanced in *Labeo rohita* after feeding the fish with *Achyranthes aspera* seed. Ardo *et al*, [39] also reported that feeding Nile tilapia (*Oreochromis niloticus*) with two herbal extracts (*Astragalus membranaceus* and *Lonicera japonica*) alone or in combination significantly enhanced phagocytic and respiratory burst activity of blood phagocytic cells. Similarly, the plant extracts we used in this study could enhance respiratory burst activity in treatment groups compared to control group.

Serum bactericidal activity was significantly increased in experimental groups compared to control group. This also showed that the serum bactericidal activity was gradually increased throughout the experimental period compared to initial value. The plant extracts we used in this study could enhance serum bactericidal activity in all experimental groups. According to Sivaram *et al*, [40] reported that serum bactericidal activity was enhanced in juvenile greasy groupers (*Epinphelus tauvina*) fed antibacterial active principles of *Ocimum sanctum* and *Withania somnifera*. Similarly, grouper (*E. tauvina*) juveniles fed with diets containing different doses of extract mixture of some herbs showed a significant increase in their serum bactericidal activity [41].

The present study demonstrated that diets supplemented with *Z. officinale* powder enhanced growth and immunity in *C. catla* fed with 30 days. There is an increase in specific growth rate of weight of the fishes in control and experimental groups. Fish fingerlings weight gain has showed a significantly ($p < 0.05$) increased in experimental groups when compared to control. Similar results are obtained by Mathivanan *et al*, [42]. Who reported that the growth of the fish has been increased in all experimental groups when compared to control except in D4. Among the tested diets, D2 showed highest rate of growth and the enhanced growth rates could be due to the growth promoting effect of *A. paniculata* extract.

The relative percentage rate was increased in the group C and D when compared to group B. Similar findings were reported by Gopalakrishnan and Venkatesan [43] while challenging *Cyprinus carpio* with *A. hydrophila*. Baba *et al*, [44] also reported that survival rate after challenging the fish with *A. hydrophila* was enhanced in common carp treated with lavamisole. Dina Rairakhwada *et al*, [45] also reported highest RPS (100%) was recorded in 0.5% levan fed and the least RPS was recorded in 1% levan fed fish.

The overall results of the present study proved that the powder of *Z. officinale* induced the innate immunity of the fish in all treated groups. A critical examination of the parameters of the various experimental groups shows that fishes treated with *Z. officinale* of 1000 mg/kg feed (group D) exhibited higher growth rate and at the same time this dose seems to be sufficient to swell the innate immunity by way of enhancing the major haematological parameters and immunological parameters and immunological assays. Hence, from the present results it is recommended a dose of 1000 mg/kg feed of *Z. officinale* for this species of fish culture conditions.

Our results suggest the protective ability of *Z. officinale* powder mediated through cellular and may be non cellular immune mechanism, as evident from the enhanced haematological parameters such as erythrocytes, leucocytes and haemoglobin, total serum protein, immunological parameters such as respiratory burst activity, bactericidal activity, growth and survival rate against *A. hydrophila*. The *Z. officinale* (ginger) has been shown to contain major chemical constituents such as gingerol, shogaol, paradol, methyl [6] - isogingerol, isoshogaol, gingerdione, (3S,5S) - 3,5 - diacetoxy - 1,7 - bis (4-hydroxy-3-methoxy) - heptanes [46] that might act as potential immunostimulant. However, the active principle responsible for the immunostimulatory property observed in the present study has to be identified. Similar immunostimulatory effect has been observed in *O. mossambicus* administered with azadirachtin, a tetropenoid derived from the seek kernel of *Azadirachta indica*. The results of the present investigation show that *Zingiber officinale* can act as an immunostimulant even in low concentrations. Even though the exact modulation of immune response elicited by the natural compound in fish will function as an immunostimulant and might act directly.

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