



Holy Basil: A Potent Growth and Immunity Promoting Herb For Sustainable Culture of *Cirrhinus mrigala* (HAMILTON, 1822)

Anita Bhatnagar* and Deepika Mann

Department of Zoology, Kurukshetra University Kurukshetra-136119, Haryana, India

*Corresponding Author: anitabhatnagar@gmail.com

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ABSTRACT

The immunostimulants have proven efficacy in aquaculture, enhancing survival, growth, and immune responses. This research aimed to determine the optimal dietary level of the plant-based immunostimulant, holy basil (*Ocimum sanctum*), for maximizing the growth performance and non-specific immunological response of *Cirrhinus mrigala* fingerlings. Five experimental diets (C, OS1, OS2, OS3, and OS4) were prepared, incorporating holy basil powder at rates of 0, 25, 30, 35, and 40g kg⁻¹, respectively. The fingerlings were fed these diets at 4% of their body weight over a 90-day period. Analysis of the collected data revealed significant improvements in growth parameters within the OS3 (35g kg⁻¹) treatment group. This group exhibited enhanced live weight gain, growth percent gain in body weight, daily growth in percent body weight, specific growth rate, gross conversion efficiency, protein efficiency ratio, apparent protein digestibility, and intestinal enzymatic activity. Furthermore, certain hematological and immunological indicators, including total erythrocyte count, total leukocyte count, serum protein levels, respiratory burst activity, phagocytic activity, lysozyme activity, and carcass composition (crude protein and fat), displayed remarkable results in the OS3 group compared to other treatments. Minor changes were observed in water quality metrics across all dietary groups, except for ammonia and phosphate levels, which were significantly lower in the OS3 (35g kg⁻¹) group compared to others. In a separate 10-day experiment, fingerlings were exposed to the pathogenic bacteria *Aeromonas hydrophila* (MTCC-1739), with the OS3 (35g kg⁻¹) group displaying the highest survival rate. In summary, this investigation demonstrated that fingerlings exhibited optimal growth, non-specific immunological responses, and the highest survival rate when fed a diet containing 35g kg⁻¹ of holy basil powder. These findings emphasize the effectiveness of holy basil as an immunostimulant in aquaculture.

INTRODUCTION

Fish is one of the crucial protein rich staple food due to its high assimilability and enrichment essential amino acids (like methionine, lysine, among others), which are often deficient in terrestrial meat (Tacon & Metian, 2013). Hence, the global fish output is steadily increasing (FAO, 2016). The rapid growth of aquaculture has led to the intensification of fish culture systems (Naylor *et al.*, 2000; Easterling, 2007; Smith *et*

al., 2011). Intensification in aquatic culture systems invariably leads to overpopulation and adversely affects the well-being of fish due to compromised physiological conditions and increased susceptibility to diseases. To restrain such infectious bacterial diseases, immunostimulants can be used as an important alternative. Immunostimulants are those chemicals, which stimulate immune system either by triggering any of its components or by directly enhancing their activity. Synthetic immunostimulants may linger in the atmosphere. Therefore, natural immunostimulants are preferred over synthetic ones due to their certain characteristics, such as broad spectrum efficacy, economical viability, and environmental friendly nature. Certain phytochemicals, such as alkaloids, terpenoids, flavanoids, quinine, and phenolics possess antimicrobial, growth promoting, and immunostimulating property. Holy basil, known as the "queen of herbs", is considered an extremely potent immunostimulant even at low concentrations (Logambal *et al.*, 2000). It was observed that incorporation of holy basil in diet can promote growth and immunity of fish leading to its sustainable aquaculture (Bhatnagar & Lamba, 2018). Increasing the herb dose can lead to additional improvements in immunity and growth performance. Thus, it is necessary to calculate the optimum herb dose at which excellent growth performance of fish may be acquired.

C. mrigala, the IMC, is a commercially significant fish species in the Indian subcontinent, and there is a need to explore acceptable, economically feasible, environmentally friendly, growth and immunity stimulating dietary components for this fish species. Therefore, the current study was designed to investigate the optimal incorporation concentration of natural immunostimulant (holy basil powder) for high growth potential, balanced nutrient retention, and immunity of *C. mrigala*.

MATERIALS AND METHODS

Holy basil powder preparation

Holy basil leaves were appropriately recognized and then picked from a nearby field. Subsequently, the leaves were thoroughly washed and initially dried in the shade for 4- 5 days before being dried in the oven (60°C) until they reached a uniform weight. Leaves were reduced to fine powder by crushing them manually using pestle mortar. The powder was then sieved through muslin cloth to ensure uniformity.

Experimental feed preparation

The dried holy basil powder was added to the prepared experimental diet at rates of 25, 30, 35, and 40g kg⁻¹ of feed for dietary treatment groups of OS1, OS2, OS3, and OS4 (Table 1). Groundnut oil cake powder (650g kg⁻¹), duckweed powder (266g kg⁻¹), rice bran powder (32g kg⁻¹), wheat flour (32g kg⁻¹), chelated mineral mixture (10g kg⁻¹), and chromium oxide (10g kg⁻¹) were used to produce the control (C) diet of approximately 40% protein (Kalla *et al.*, 2004).

Table 1. Ingredient composition (g kg^{-1}) of experimental diets

Ingredient	Control	OS1	OS2	OS3	OS4
Groundnut oil cake powder	650	650	650	650	650
Rice bran powder	32	19.5	17	14.5	12
Duckweed powder	266	266	266	266	266
Wheat flour	32	19.5	17	14.5	12
Chelated mineral mixture	10	10	10	10	10
Chromium oxide (green)	10	10	10	10	10
Holy basil powder	-	25	30	35	40

All dietary elements were evenly combined, and dough was kneaded using lukewarm distilled water. Then, the dough was compressed and moulded into uniform feed pellets (diameter = 0.5mm) with the help of manual pelletizer machine. Pellets were dried in an oven at 60°C for 24hr and then stored in air- tight boxes to avoid any contamination and moisture. All the five experimental diets were isocaloric and isonitrogenous with approximately 40% protein content (Table 2).

Table 2. Proximate analysis of holy basil (*O. sanctum*) formulated diet at varied inclusion level

Note: All the values are mean \pm S.E. of mean.

Proximate analysis	Dietary treatment				
	C	OS1	OS2	OS3	OS
Crude protein (%)	39.92 \pm 0.01 ^A	39.87 \pm 0.01 ^A	39.90 \pm 0.01 ^A	39.85 \pm 0.01 ^A	39.81 \pm 0.00 ^A
Crude fat (%)	9.16 \pm 0.01 ^B	9.09 \pm 0.01 ^D	9.12 \pm 0.02 ^A	9.17 \pm 0.01 ^{AB}	9.19 \pm 0.01 ^A
Total ash (%)	6.65 \pm 0.01 ^A	6.69 \pm 0.01 ^A	6.70 \pm 0.01 ^A	6.73 \pm 0.01 ^A	7.08 \pm 0.34 ^A
Crude fiber (%)	6.17 \pm 0.01 ^C	6.21 \pm 0.01 ^B	6.21 \pm 0.01 ^B	6.23 \pm 0.01 ^{AB}	6.25 \pm 0.01 ^A
Moisture (%)	7.41 \pm 0.01 ^{BC}	7.43 \pm 0.01 ^{AB}	7.40 \pm 0.01 ^C	7.44 \pm 0.01 ^{AB}	7.46 \pm 0.01 ^A
Nitrogen free extract (%)	37.33 \pm 0.00 ^B	37.38 \pm 0.01 ^A	37.40 \pm 0.00 ^A	37.33 \pm 0.01 ^B	37.19 \pm 0.01 ^C
Gross energy (kJ g ⁻¹)	19.46 \pm 0.00 ^A	19.43 \pm 0.00 ^A	19.44 \pm 0.00 ^A	19.44 \pm 0.00 ^A	19.44 \pm 0.00 ^A

Means with different letters in the same row are significantly ($P < 0.05$) different (Tuckey's test)

Procurement and acclimatization

Experimental fingerlings of weight (1- 2g) and length (2- 3cm) were procured from Sushil fish seed farm Kamalpur roran, Karnal (29.686°N 76.989°E). A short term bath in potassium permanganate solution was administered to treat fingerlings from parasites. Furthermore, fingerlings were acclimatized for 15 days prior to experiment in FRP tanks (capacity 300 liters) along with proper aeration in Aquaculture Research Unit of the Department of Zoology, Kurukshetra University, Kurukshetra (29°58' N, 76°51' E).

Experimental setup

The research experiment was carried out in plastic tubs (capacity = 50L) under laboratory setting (26±2°C). All 5 dietary treatments (C, OS1, OS2, OS3 and OS4) were performed with three replicates for each. Experimental tubs (15) were filled with previously stored tap water and seeded with 10 fish fingerlings (1.45±0.03g). The water in all tubs was replaced on a regular basis (60- 70 percent), and adequate aeration was maintained. For 90 days, all treatment group fingerlings were fed at 4% of their body weight (BW) at 08:00 and 17:00h. After 6hrs of feeding, the feces were siphoned out and dried for further digestibility analysis. With the help of electronic top pan balance (Make, AFCOSET FX-1200), each individual fingerling was weighed every 15 days.

Growth performance

To assess the dietary performance of fingerlings, the following growth metrics by **Garg *et al.* (2002)** were used, such as live weight gain, growth % gain in BW, growth per day BW, specific growth rate (SGR), gross conversion efficiency (GCE), feed conversion ratio (FCR), and protein efficiency ratio (PER), all of which were calculated. Apparent protein digestibility (APD) was computed following the method of **Cho *et al.* (1982)**. Feed was subjected to a proximate analysis in accordance with the standards of **AOAC (1995)**.

Water quality parameters

A variety of water quality parameters, such as temperature, pH, electrical conductivity, calcium, chloride, total dissolved solids (TDS), and total alkalinity were performed by following the **APHA (2005)**, on every 15th day to assess the influence of tailored food on holding water. At the end of feeding trial, the total ammonia (mg kg⁻¹ BW d⁻¹) and reactive orthophosphate (mg kg⁻¹ BW d⁻¹) were analyzed following **APHA (2005)**. The nitrogen and phosphorus concentrations excreted by fingerlings in holding water were calculated as follows:

$$\text{Total N-NH}_4/\text{o-PO}_4 \text{ excretion} = \frac{[(\text{N-NH}_4/\text{o-PO}_4)_{120} - (\text{N-NH}_4/\text{o-PO}_4)_0] \times a}{\text{Fish biomass/ kg} \quad (\text{mg kg}^{-1} \text{BW} 2\text{h}^{-1})}$$

Where,

(N-NH₄/o-PO₄)₀ and (N-NH₄/o-PO₄)₁₂₀ = values at intervals 0 and 2h following feeding.
a = the volume (L) of holding water used to keep the fingerlings.

Intestinal enzyme activity

Following the end of the 90-day feeding trial, 3-4 fingerlings from each dietary group were retrieved and kept on polar brick (-20°C). Then, intestinal enzymes such as protease (Walter, 1984), cellulase (Sadasivam & Manickam, 1996), and amylase (Sawhney & Singh, 2000) activities were tested by extirpating the tissue.

Hematological and immunological assessment

Total erythrocyte and leukocyte counts

At the end of feeding trial, 5-6 fingerlings from each treatment group were anesthetized by Tricaine methanesulfonate (MS_{222}) before pooling out blood. Blood samples were drawn by inserting an insulin syringe (0.5mL) into the heart of fingerlings. It was then stocked up in a vacutainer tube coated with EDTA. Then, total erythrocyte count and total leukocyte count were estimated by using hemocytometer and Neubauer counting chamber, as followed in the study of Dacie and Lewis (1963).

Phagocytic assay

The phagocytic assay was carried out in accordance with the conventional techniques of Siwicki *et al.* (1994) and Park and Jeong (1996), with minor modifications. In an aseptic microplate, cells of freshly developed contagious bacteria i.e. *Aeromonas hydrophila* (procured from MTCC, Chandigarh) in 0.1mL of PBS were stirred with 0.1mL of anticoagulated blood sample of fingerlings from each treatment group. Then, the sample was kept in incubator universal (Make, NSW-151) at 27°C for 30min. After incubation, microplates were taken out, and then 50 μL of this suspended liquid was shifted on three glass slides to prepare smears. After dehumidifying, smear was settled in 92% ethanol, dehumidified and then tinted with May-Grunwald Giemsa stain. The procedure was carried out in laminar airflow (Make, RESCHOLAR) under aseptic settings. After that, computation of phagocytic cells and phagocytosed bacteria was done under compound microscope (Make, Magnus MX21iLED). Phagocytic ratio (PR) and phagocytic index (PI) were calculated by using the following formula:

Phagocytic ratio (PR) = $\frac{\text{No. of phagocytic cells with phagocytosed bacteria}}{\text{No. of phagocytic cells}} \times 100$.

Phagocytic index (PI) = $\frac{\text{No. of phagocytosed bacteria}}{\text{No. of phagocytic cells}}$.

Nitroblue tetrazolium (NBT) assay

Nitroblue tetrazolium (NBT) test was used to evaluate the respiratory burst activity of blood phagocytes as reported by Anderson and Siwicki (1996). By puncturing the heart of the fingerlings, anticoagulated blood was collected in a vacutainer coated with EDTA. Then, in a vial with 0.1mL of this blood was extracted and 0.1ml of 0.2% NBT solution was poured over it. This blend was kept in incubator universal (Make, NSW-151) for 20- 30min at 27°C . Subsequently, 50 μL of the resulting blend was put into a glass tube, to which 1.0ml of N, N-dimethyl formamide was added and centrifuged at $3000 \times g$ for 5min in cold centrifuge (Make, REMI CM-12 PLUS). The optical density of the supernatant was estimated at 540nm in UV-visible spectrophotometer (Make, SHIMADZU).

Serum protein determination

The serum protein was evaluated by following Gornall biuret method (Kulow, 1967). The non-heparinized blood sample was poured in a glass test tube, and clot

formation was allowed. The blood was then drained into other glass test tube for centrifugation at 3000 x g for 10min in cold centrifuge (Make, REMI CM-12 PLUS). Later 0.1mL of supernatant was taken and 5mL of Gornall reagent (1.5g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ + 6.0 g $\text{KNaC}_4\text{H}_4\text{O}_6$ + 500 mL H_2O + 300 mL 10% NaOH) was added to it, and reaction was allowed. OD of blend was spectrophotometrically evaluated with the help of UV-visible spectrophotometer (Make, SHIMADZU) at 540nm.

Lysozyme activity

The lysozyme activity was determined using turbidimetric assay focusing on the lysis of *Micrococcus lysodeikticus*. Serum lysozyme activity was spectrophotometrically calculated using the standard methodology described by Parry *et al.* (1965). 2.5mL suspension of lyophilized *Micrococcus lysodeikticus* in 0.067m phosphate buffer (pH 6.2) was taken as substrate. After 0.5 and 4.5min., 100 μL of fish serum was poured into 250mL of bacterial suspension, and reduction in absorbance was determined at 490nm.

Carcass composition

Fingerlings from each dietary treatment group were refrigerated at -20°C for further proximate carcass evaluation. Evaluation of moisture (%), crude protein (%), crude fat (%), and ash (%) was performed by following the AOAC (1995).

Challenge trial

After 90 days of feeding trial, 10 fingerlings from each treatment were subjected to infection with *Aeromonas hydrophila* (MTCC-1739), which was then grown and maintained in the selected media. Fish were immersed in pathogenic *A. hydrophila* (MTCC-1739) culture for 10^5 CFU mL^{-1} for one week before being immersed again for 10^7 CFU mL^{-1} (Austin *et al.*, 1995). Based on the discovery that death peaked after one week, percent survival for ten days was computed (Sahoo *et al.*, 1998).

Statistical analysis

Data for all the indices was represented as mean \pm S.E. of mean. To assess if there was a significant difference among different dietary treatment groups, complete data were subjected to one-way ANOVA, followed by Tuckey's test. Statistical magnitude was calibrated at a probability scale of $P < 0.05$. All statistics were performed by operating SPSS version 16.0. A Kaplan-Meier survivorship plot was also constructed in MS Excel to demonstrate the trend of survival of fingerlings, given varied dosages of *Ocimum sanctum* after a 10-day challenge trial with the fish pathogenic bacteria, *A. hydrophila* (MTCC-1739).

Ethical considerations

All the methods and experimental protocols used in this study were approved by the Institute Animal Ethics Committee (IAEC) of Kurukshetra University, Kurukshetra India with Project No. IPS/IAES/313/22. Tests were carried out in accordance with ethical standards.

RESULTS

1. Growth performance

The outcome of using various concentrations of holy basil (*O. sanctum*) powder in formulated diets (0, 25, 30, 35, 40g kg⁻¹) on various growth indices of *C. mrigala* fingerlings is represented in Table (3). One way ANOVA (analysis of variance) followed by Tuckey's test distinctly showed that the fingerlings fed on formulated diets containing 35g kg⁻¹ *O. sanctum* (treatment OS3) had a significant ($P < 0.05$) rise in live weight gain, growth % gain in BW, growth per day in BW, SGR, PER, GCE, and APD values in comparison with other treatment groups. Feed conversion ratio was significantly low in dietary treatment OS3 (Fig. 1). After completion of feeding trial, certain intestinal enzyme such as protease, amylase, and cellulase activities were investigated, and notable increases were seen in fingerlings of dietary treatment OS3 compared to other dietary treatment groups (Fig. 2).

Table 3. Effect of holy basil (*O. sanctum*) in formulated diet at varied inclusion levels on growth performance of *C. mrigala*

Growth parameter	Dietary treatment				
	C	OS1	OS2	OS3	OS4
Initial weight (g)	1.46±0.01 ^A	1.45±0.01 ^A	1.46±0.02 ^A	1.46±0.01 ^A	1.45±0.01 ^A
Final weight (g)	4.19±0.02 ^D	4.35±0.02 ^C	4.53±0.03 ^B	4.79±0.03 ^A	4.36±0.03 ^C
Growth % gain in BW	186.67±3.89 ^C	200.01±2.96 ^B	209.69±4.41 ^B	228.38±4.38 ^A	199.49±3.20 ^B
Growth day ⁻¹ in BW	1.07±0.01 ^C	1.11±0.01 ^B	1.14±0.01 ^B	1.05±0.01 ^A	1.07±0.01 ^B
Gross conversion ratio	0.40±0.00 ^C	0.42±0.00 ^{BC}	0.44±0.00 ^B	0.46±0.00 ^A	0.43±0.01 ^B
Protein efficiency Ratio	0.69±0.02 ^C	0.74±0.00 ^{BC}	0.77±0.01 ^B	0.85±0.02 ^A	0.76±0.01 ^B

Note: All values are Mean ± S.E. of mean.

Means with different letters in the same row are significantly ($P < 0.05$) different (Tuckey's test).

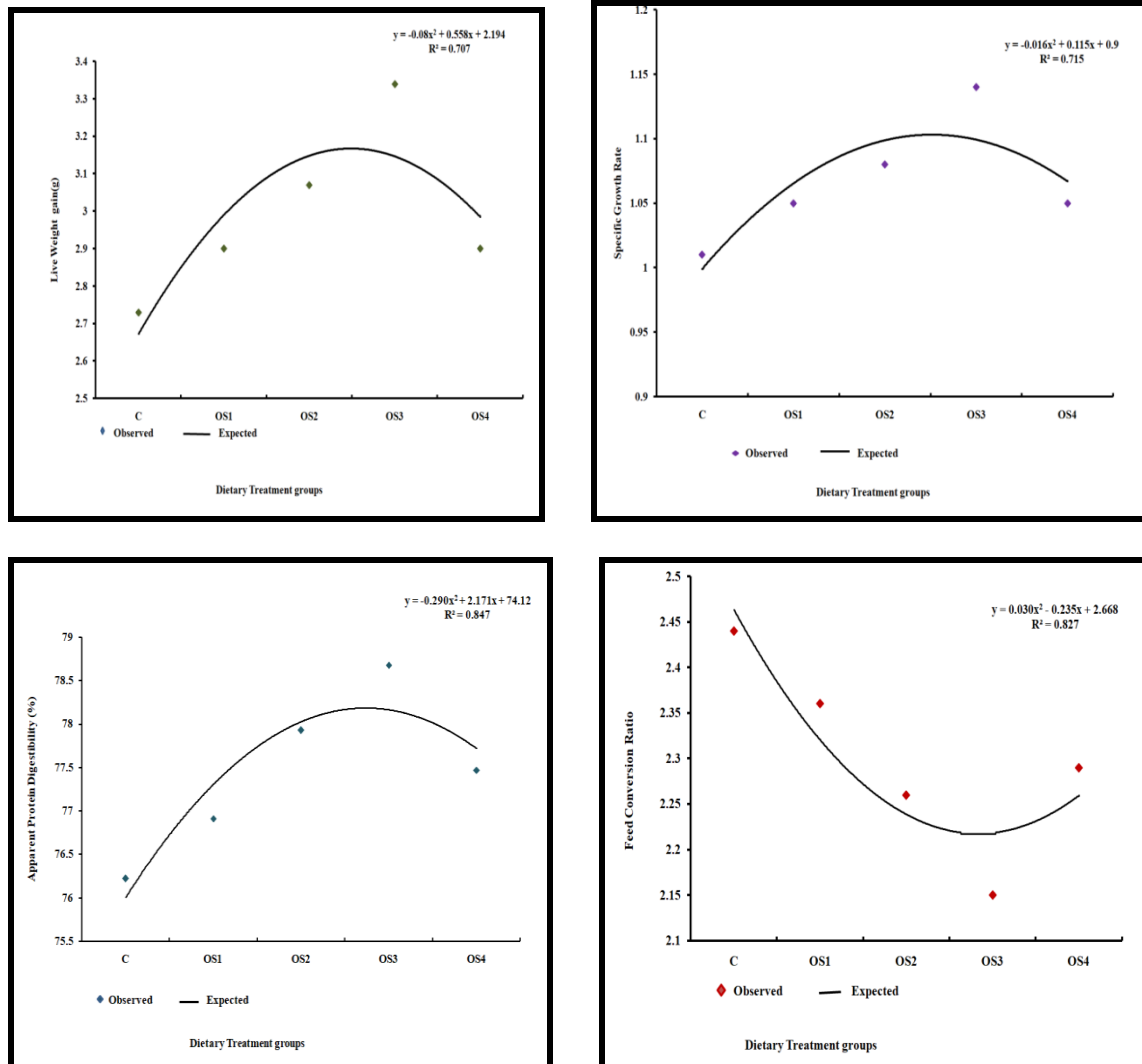
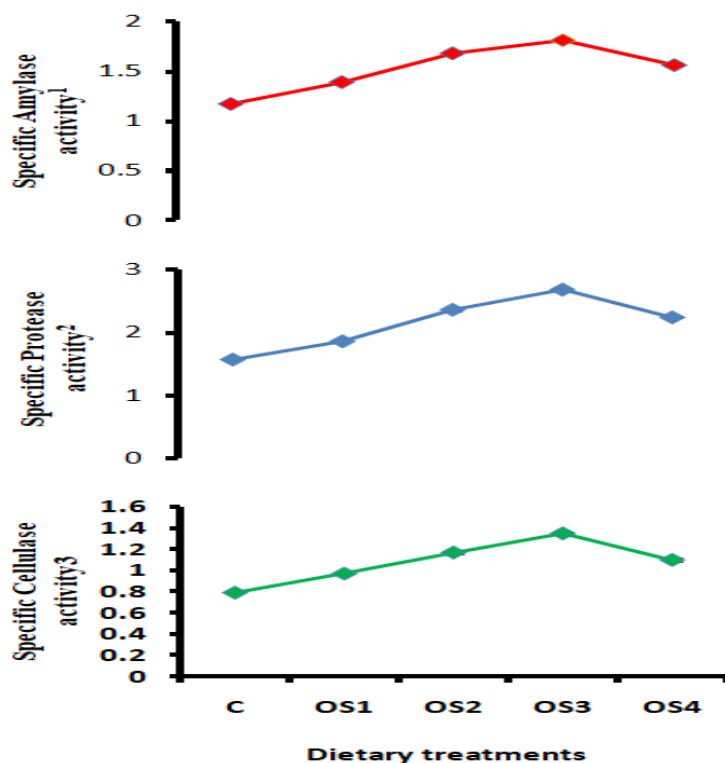


Fig. 1. Polynomial fit curve showing the effect of dietary supplementation of *O. sanctum* at different inclusion levels fitting to the data of growth performance in the fingerlings of *C. mrigala*



¹ mg of maltose liberated mg of protein⁻¹ h⁻¹

² mg of tyrosine liberated mg of protein⁻¹ h⁻¹

³ mg of glucose liberated mg of protein

Fig. 2. Intestinal enzymatic activity of *C. mrigala* fingerlings fed on varying concentration of holy basil powder

2. Water quality parameters

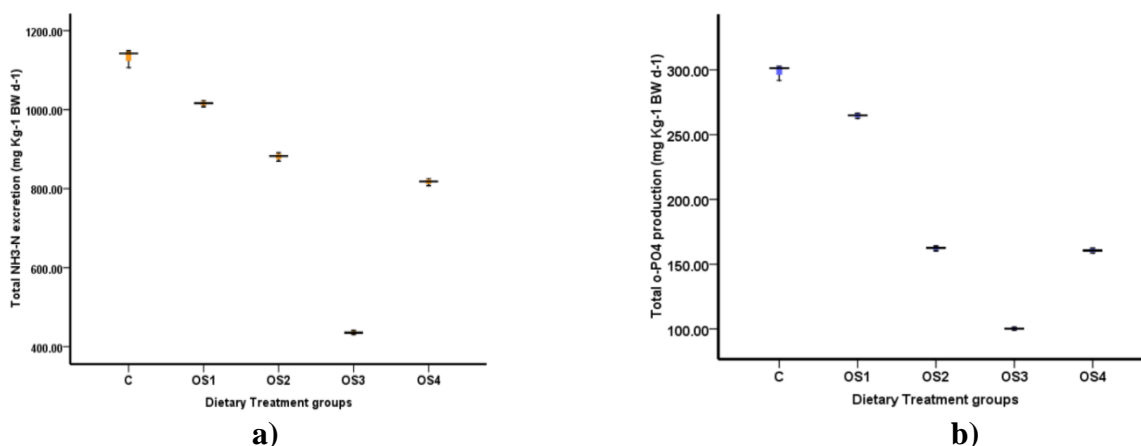
Not many changes were identified in various water quality parameters, and values appeared in permissible limits for fish culture (Table 4). Nevertheless, dietary treatment OS3 (35g kg⁻¹ of *O. sanctum*) displayed subsequent reduction in the amount of ammonia excretion and reactive orthophosphate generation compared to other treatment groups (Fig. 3).

Table 4. Effect of holy basil (*O. sanctum*) in formulated diet at varied inclusion levels on water quality characteristics

Physico-chemical parameter	Dietary Treatment					Desirable standard values for fish culture
	C	OS1	OS2	OS3	OS4	
Dissolved oxygen (DO) mg L ⁻¹	7.00±0.11 ^A	6.26±0.12 ^B	6.40±0.23 ^B	6.13±0.08 ^B	6.50±0.11 ^B	4.0-5.0
pH	8.27±0.02 ^D	8.50±0.00 ^C	8.74±0.02 ^A	8.58±0.04 ^B	8.69±0.01 ^A	7.0-9.5
Temperature (°C)	26.96±0.14 ^A	27.30±0.17 ^A	27.23±0.08 ^A	27.30±0.15 ^A	27.34±0.14 ^A	
Conductivity (µ mhos cm ⁻¹)	679.33±2.185 ^D	695.33±3.18 ^C	661.33±3.75 ^E	715.33±2.72 ^B	726.33±3.52 ^A	
Alkalinity (mg L ⁻¹)	231.00±1.52 ^A	216.67±2.40 ^B	209.67±0.88 ^C	229.33±1.20 ^A	214.00±2.08 ^C	50-200
Chloride (mg L ⁻¹)	37.56±0.15 ^C	38.06±0.145 ^B	37.43±0.12 ^{CD}	38.70±0.12 ^A	37.06±0.088 ^D	
Calcium (mg L ⁻¹)	23.70±0.12 ^C	24.30±0.12 ^B	23.50±0.21 ^C	24.80±0.057 ^A	23.40±0.115 ^C	4.0-160.0
Total Dissolved Solids (mg L ⁻¹)	545.33±3.17 ^B	617.33±1.45 ^A	555.67±8.08 ^B	455.00±14.43 ^C	411.00±5.19 ^D	

Note: All the values are Mean ± S.E. of mean.

Means with different letters in the same row are significantly ($P<0.05$) different (Tuckey's test).

**Fig. 3.** The effect of holy basil (*O. sanctum*) in formulated diet at varied inclusion levels on: (a) Total ammonia excretion (mg kg⁻¹BW day⁻¹) and (b) Total orthophosphate production (mg kg⁻¹BW day⁻¹).

3. Carcass analysis

The results in Table (5) show the carcass constitution of experimental fingerlings fed on varied levels of *O. sanctum*. The crude protein and crude fat content in fingerlings of treatment group OS3 (35g kg⁻¹) was considerably ($P < 0.05$) greater than other treatment groups. Gross energy was also observed to be significantly higher in the OS3 treatment group (35g kg⁻¹) and lower in the dietary treatment control (C).

Table 5. Proximate carcass constitution of *C. mrigala* fed on formulated diet with *Ocimum sanctum* at varying inclusion levels

Proximate analysis	Dietary Treatment				
	C	OS1	OS2	OS3	OS4
Crude protein (%)	12.21±0.04 ^D	14.61±0.13 ^C	15.29±0.02 ^B	16.07±0.11 ^A	15.55±0.03 ^B
Crude fat (%)	5.65±0.06 ^D	6.59±0.15 ^C	7.28±0.04 ^B	7.76±0.03 ^A	7.30±0.01 ^B
Total ash (%)	2.34±0.10 ^D	3.15±0.04 ^A	2.83±0.04 ^B	2.60±0.02 ^B	2.84±0.05 ^B
Moisture (%)	70.94±0.15 ^A	67.15±0.20 ^B	65.32±0.06 ^C	64.45±0.06 ^D	65.66±0.26 ^C
Gross energy (kJ g ⁻¹)	7.04±0.04 ^D	7.99±0.06 ^C	8.57±0.01 ^B	8.87±0.00 ^A	8.53±0.04 ^B

Note: All the values are Mean ± S.E. of mean.

Means with different letters in the same row are significantly ($P < 0.05$) different (Tuckey's test)

4. Hematological and immunological parameters

Certain hematological and immunological parameters of the blood from experimental fingerlings fed on various incorporation level of holy basil are presented in Table (6). It was observed that the level of TEC and TLC was significantly ($P < 0.05$) high in treatment group OS3 (35g kg⁻¹) fingerlings. Similarly, remarkable rise was observed in phagocytic activity of blood phagocytes of *C. mrigala* fingerlings of same treatment group. Respiratory burst activity of experimental fingerlings of dietary treatment group OS3 (35g kg⁻¹) conceded relevant ($P < 0.05$) increase when compared to the fingerlings of other dietary treatments. Total serum protein was subsequently ($P < 0.05$) high in the OS3 group fingerlings fed on diet containing 35g kg⁻¹ of *O. sanctum*. Collation of data of lysozyme activity in blood of fingerlings of different treatment groups showed significant increase in treatment group OS3 (35g kg⁻¹) in comparison with other dietary groups (Fig. 4).

Table 6. Effect of holy basil (*O. sanctum*) in formulated diet at varied inclusion levels on hematological and immunological parameters

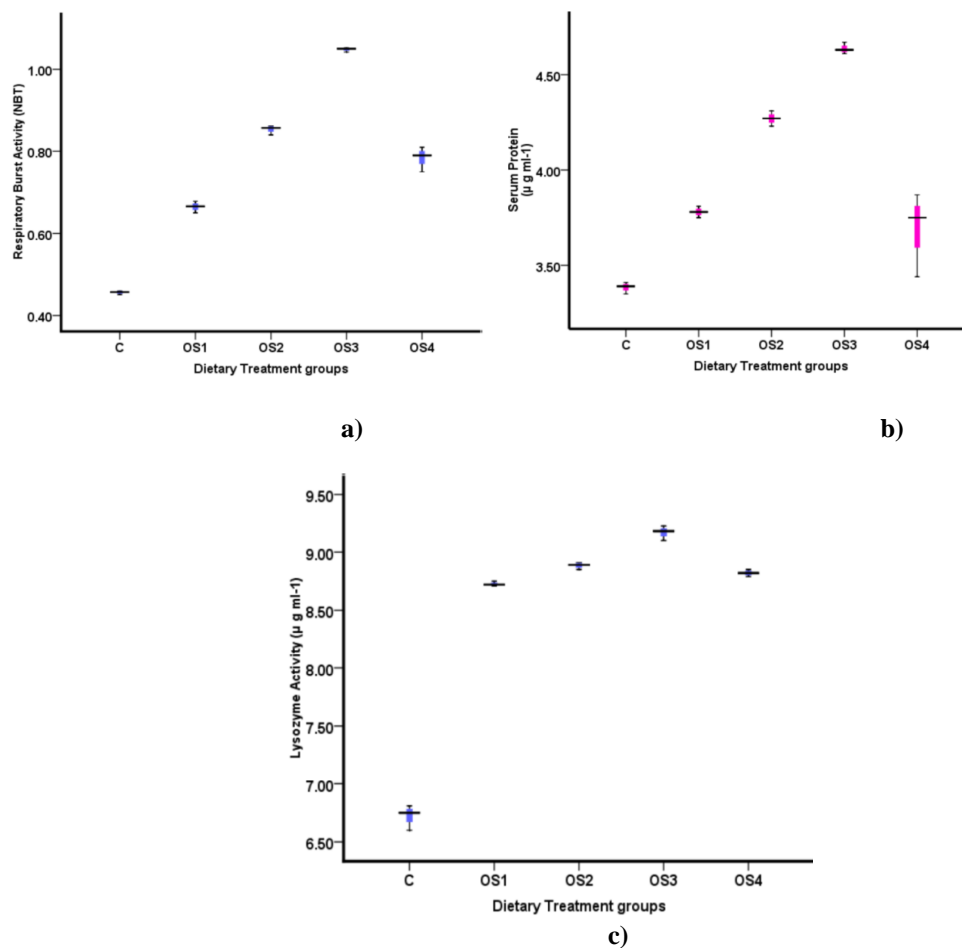
	Dietary Treatment				
	C	OS1	OS2	OS3	OS4
TLC¹⁾ (10^3 mm^{-1})	23.43±0.17 ^E	31.96±0.06 ^D	38.73±0.10 ^B	45.52±0.24 ^A	35.55±0.26 ^C
TEC²⁾ (10^6 mm^{-1})	1.32±0.03 ^E	1.49±0.01 ^D	1.79±0.01 ^B	2.14±0.02 ^A	1.71±0.02 ^C
Phagocytic Ratio (PR)	59.14±0.41 ^C	66.92±3.86 ^B	73.82±1.03 ^{AB}	80.25±1.01 ^A	72.15±2.54 ^B
Phagocytic Index (PI)	1.43±0.01 ^C	1.70±0.08 ^B	1.88±0.02 ^{AB}	2.05±0.07 ^A	1.81±0.06 ^B

¹⁾ TLC-Total Leucocyte Count

²⁾ TEC-Total Erythrocyte Count

Note: All the values are Mean ± S.E. of mean.

Means with different letters in the same row are significantly ($P<0.05$) different (Tuckey's test)

**Fig. 4.** Effect of holy basil (*O. sanctum*) in formulated diet at varied inclusion levels on: (a) Respiratory burst activity (NBT), (b) Serum protein ($\mu\text{g mL}^{-1}$), and (c) Lysozyme activity ($\mu\text{g mL}^{-1}$)

5. Survival after challenge Trial

The results of the challenge test indicate that optimum supplementation of basil powder (35g kg^{-1}) in the diet enhanced tolerance to pathogenic bacterial infection (Fig. 5).

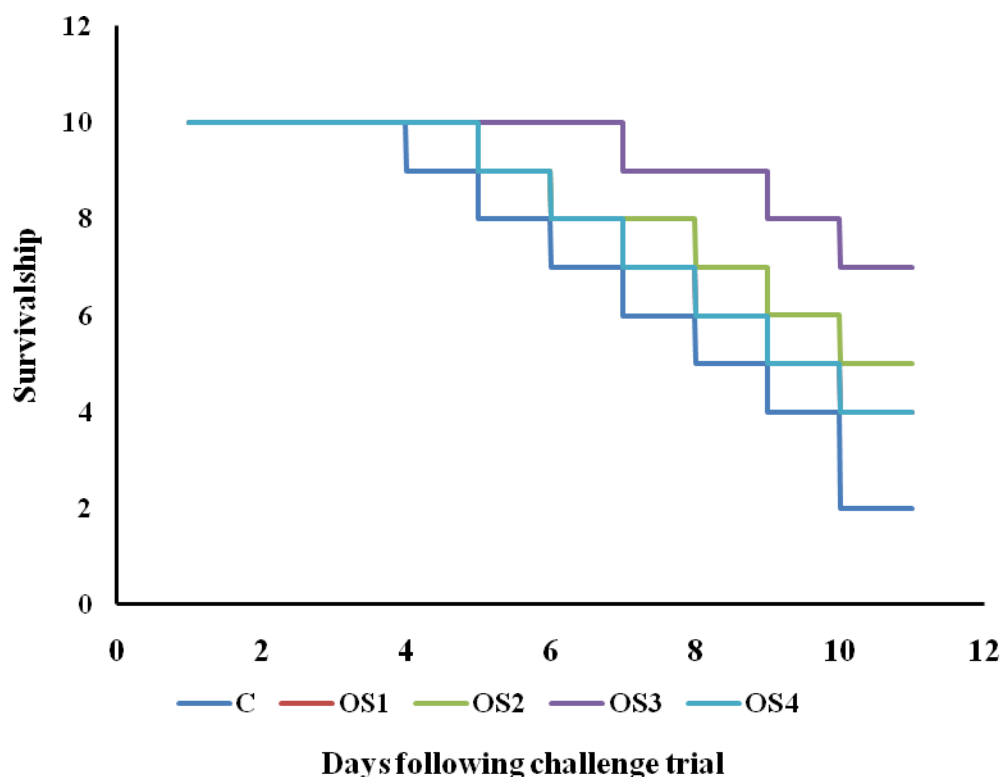


Fig. 5. Plots of Kaplan-Meier estimates of survival of *C. mrigala* fingerlings fed on varying concentration of *Ocimum sanctum* after challenge trial

DISCUSSION

In aquaculture, there has been a transition towards the usage of natural immunostimulant over synthetic immunostimulants during the previous several decades. Hence, the present research was purposed to investigate the effect of natural immunostimulant (holy basil) on growth performance and immune response of *C. mrigala* fingerlings at various inclusion dosages, and its optimum incorporation level was also identified. In earlier studies, *O. sanctum* supplemented diet was proved to have immunomodulatory and growth promoting effect on *C. mrigala* fingerlings (**Bhatnagar & Lamba, 2018**). With an increase in *O. sanctum* incorporation dose, there is a greater likelihood of growth and immunity enhancement. Thus, it is vital to determine the ideal dose of holy basil for maximum growth performance. Consequently, in the present research, an optimum dose of holy basil was determined, and it was discovered that dietary treatment group OS3 (35g kg^{-1}) fingerlings exhibited a substantial improvement in growth performance, live weight gain, SGR, PER, GCE, growth % gain and APD values in comparison with other treatment groups. FCR (2.15 ± 0.02) was considerably low in dietary treatment group OS3 (35 g kg^{-1}). The results of this study are consistent

with the findings of **Panprommin *et al.* (2015)**, who assessed the high growth rate in *Oreochromis niloticus* fed with tulsi supplemented diet. Low FCR in treatment group OS3 (35g kg⁻¹) clearly demonstrates the role of holy basil as a productive appetizer in the diet. Intestinal enzymatic activity determines the level of food recession, or the digestibility of the variety of formulated feed supplied to the aquatic animals (**Ziaei-Nejad *et al.*, 2006**). The significance of holy basil in assessing the amount of intestinal enzymatic activity is clearly seen in Fig. (1). In dietary treatment group OS3, fingerlings showed a significant ($P < 0.05$) rise in intestinal enzyme activities compared to other dietary treatments. The current study found that holy basil can increase the activity of enzymes present in the intestinal tract due to the presence of a high concentration of eugenol, thereby increasing nutrient utilization. Fingerling growth increases up to a particular dose, i.e. 35g kg⁻¹ after which a reduction in growth performance is noted. Consequently, 35g kg⁻¹ of *O. sanctum* is the optimal dosage for *C. mrigala* fingerlings. Inconsequential variations in key water quality metrics of different dietary treatment groups were detected. Despite the fact that, treatment group OS3 (35g kg⁻¹) had the lowest ammonia excretion and reactive orthophosphate generation, this represents improved consumption of dietary proteins by fingerlings, which further reduces deamination and hence ammonia excretion in the holding water. Parallel results were estimated by **Bhatnagar and Lamba (2018)** for *C. mrigala* fingerlings fed on 30g kg⁻¹ *O. sanctum* for 60 days. In an earlier investigation, it was also stated by **Jana *et al.* (2006)**, that the water quality metrics are equally important in contributing towards fish growth even in saline waters. In general, a fingerling's physiological health is determined by the number of erythrocytes and leukocytes and leucocytes are the body's initial line of defense against any kind of infection. As a result, hematological and immunological evaluations of different dietary treatment group were performed, and it was discovered that group OS3 (35g kg⁻¹) showed significantly ($P < 0.05$) higher TLC and TEC when compared to other dietary treatment groups. This might be due to the holy basil leaf possessing certain bioactive components, such as eugenol (1-hydroxy-2-methoxy-4-allylbenzene), benzene, 1, 2-dimethoxy- 4- (2- propenyl), α -farnesene and cyclohexane, 1, 2, 4-triethenyl.

Immunostimulants triggers innate immunity by boosting phagocytosis as well as respiratory burst activity (**Shoemaker *et al.*, 1997**). Holy basil is one of the competent natural immunostimulant since it contains water-soluble phenolic compounds and a variety of other ingredients, such as eugenol, methyl eugenol, and caryophyllene. *O. sanctum* leaf extract improves both specific and non-specific immune responses (**Santra *et al.*, 2017**). After completion of 90 days of the feeding trial, it was observed that phagocytic activity was increased in all dietary treatment groups, and highest phagocytic activity was observed in OS3 (35g kg⁻¹). It might be due to participatory role of active constituents of holy basil in immune functions (**Kollner *et al.*, 2002**). Parallel results in holy basil fed diet were also evaluated by **Pavaraj *et al.* (2011)** in *Cyprinus carpio* and **Nahak and Sahu (2014)** in *C. batrachus*. NBT assay was performed to determine the respiratory burst activity of phagocytes, and it was observed that respiratory burst activity was significantly high in OS3 (35g kg⁻¹) in comparison with other treatment groups. The reason behind high NBT activity in treatment groups might be due to the presence of certain antioxidants, such as polyphenol rosmarinic acid, eugenol (1-hydroxy-2-methoxy-4-allylbenzene), methyl eugenol, certain flavonoids, such as orientin (8-C-beta-

glucopyranosyl-3',4',5,7-tetrahydroxyflav-2-en-3-one) and vicenin (6-C-beta-D-xylopyranosyl-8-C-beta-D-glucopyranosyl apigenin) in holy basil leaf. Serum proteins are humoral aspects of the non-specific immune system; their high concentrations in treatment group OS3 are most likely due to an increase in the non-specific immune response of experimental fish. Lysozyme inhibits bacterial growth by breaking α -1, 4 glycosidic linkages in peptidoglycan present in the bacterial cell wall. Hence, it is an antimicrobial agent and a cornerstone of innate immunity. As a result, a turbidimetric test was used to assess lysozyme activity. The dietary treatment group OS3 (35g kg⁻¹) showed considerably higher lysozyme activity, which is consistent with numerous publications suggesting the involvement of herbal immunostimulants in increasing lysozyme activity (**Rao et al., 2006; Choi et al., 2008**). High values of crude protein (%) and crude fat (%) were observed in dietary treatment group OS3 (35g kg⁻¹) fingerling carcass; this might be due to appraised level of intestinal enzyme activity and thereby better assimilation of feed as compared to other treatment groups. Concurrent results were also demonstrated by **Bhatnagar and Saluja (2019; 2021)** for *Catla catla* fed on *Mentha piperita* and *Zingiber officinale* supplemented diet, respectively.

Challenge tests are frequently used to assess the biological and physical stress responses of fish (**Wendelaar Bonga, 1997**). The results of a challenge trial with the pathogenic bacterium *A. hydrophila* (MTCC-1739) revealed that all treatment groups experienced mortality at first. However, OS3 treated groups achieved higher survival rates, whereas the number of fish in the control diet group was steadily declining, demonstrating the immunostimulating effects of potent immunostimulant (Basil) supplementation. The Kaplan-Meier analysis is the most basic approach to assess survival across time. The Kaplan-Meier survival plots show that the OS3 dietary therapy has a higher survival rate than the control (C), highlighting the immunostimulatory properties of basil powder. Concurrent reports of high amplitude survival for *C. mrigala* fingerlings administered with basil and ginger support our investigation (**Bhatnagar & Lamba, 2018**).

CONCLUSION

The incorporation of holy basil in the formulated diet not only enhanced the growth performance but also led to notable improvements in hematological and nonspecific immunological markers in *C. mrigala* fingerlings. Based on this research, it has been established that *C. mrigala* exhibits optimal growth, immunity, and survival at the incorporation level of 35g kg⁻¹ of the herb *Ocimum sanctum*. These findings are crucial for promoting sustainable culture practices for *C. mrigala*, emphasizing the importance of incorporating specific herbal supplements for enhanced aquaculture outcomes

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