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IMMUNOMODULATORY AND GROWTH PROMOTING EFFECT OF DIETARY ADMINISTRATION OF INDIAN HERBS *ALLIUM SATIVUM* (GARLIC) AND OCIMUM *SANCTUM* (TULSI) ON *CIRRHINUS MRIGALA*

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ABSTRACT

The present study was designed to monitor the effect of herbs garlic and tulsi. *Cirrhinus mrigala* (mrigal) fish (0.99-1.04 g) were fed on diets supplemented with dried powder of garlic/tulsi at 3 different inclusion level viz., 10, 20 and 30 g Kg^{-1} of feed for 60 days while control treatment (CC) was not supplemented with any herb. Growth performance, intestinal enzyme activities, nutritional physiological parameters, non-specific immune response and post challenge survival were surveyed. Significantly ($P<0.05$) higher growth of fish in terms of live weight gain (g), specific growth rate, Apparent protein digestibility (APD), Gross conversion efficiency (GCE) and Protein efficiency ratio (PER) while lower FCR were observed in diets supplemented with herbs when compared to control. Significantly ($P<0.05$) high values of digestive enzyme activities (protease, amylase and cellulase), carcass protein coupled with low excretion of metabolites (ammonia and phosphates) were also obtained from herbal supplemented diets. Erythrocyte and leucocyte count; phagocytic ratio and index as well as NBT value were enhanced in fish fed on the plant extract-supplemented diets. A challenge test with *Aeromonas hydrophila* showed that the fish fed on the garlic/tulsi supplemented diets registered high survival rate. Garlic supplemented diets resulted in better growth and immunomodulatory response as compared to tulsi supplemented diets with feed G3 containing garlic in the proportion of 30 g kg^{-1} of diet showing best results among all diets indicating that incorporation of herbs *A. sativum* (*garlic*) and *O. sanctum* (*tulsi*) in optimum dose can promote growth and improve immunity leading to sustainable aquaculture.

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INTRODUCTION

The new trend of improving food security and use of natural products will drive the chemically synthesized antibiotics and growth promoters out of use. Various antibiotics and chemotheraputics have been extensively used as growth promoters and antibacterial agent. However, extensive use of antibiotics to control diseases without knowledge of dose and pharmacokinetic data on the fish poses serious environmental hazards (Baticados and Paclibare, 1992). Alongside, several studies have indicated antibiotics such as oxytetracycline often cause immunosuppression in many fish (Lunden *et al*., 1998). Hence, to replace antibiotics, the attention is now being focused on various animal and plant derived immunostimulants. One such strong candidate to replace antibiotics is phytoadditive. Phytoadditives are fodder additives obtained from medicinal plants or plants extracts (Gabor *et al.,* 2010). The use of herbs in aqua farming have received more attention recently not only for their immune stimulating functions but also for their influence on growth promotion. With their use it is expected to achieve the identical results as in the use of antibiotics with none or minimal side effects. *Allium sativum* (Garlic), an important medicinal plant provide a wholesome effect on the immune and cardiovascular systems besides having antiviral, antibacterial, antiprotozoal, and antifungal actions (Harris *et al.,* 2001). *A. sativum* (garlic) is known to be a rich source of calcium, phosphorus and vitamins; with high content of carbohydrates giving a remarkable nutritive value. Its antibacterial action is thought to be due to multiple inhibitory effects on various thiol-dependent enzymatic systems by allicin (Ankri and Mirelman, 1999). Allicin is formed catalytically by crushing raw garlic or adding water to dried garlic when enzyme alliinase convert allin into allicin (Kourounakis and Rekka, 1991). Additionally, direct intragastric effects are achievable because *A. sativum* antimicrobials are not affected by acid environments (Lawson, 1996); rather the gastric juice enhances the antimicrobial

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activity of its constituents (Fortunatov, 1995). Among the phytoadditives, the plants of genus *Ocimum* belonging to family Labiatae are also known for their therapeutic potentials (Gupta *et al*., 2002). *Ocimum sanctum* (tulsi) may act at various levels in the immune mechanism (Mediratta *et al*., 2002). Its plant extracts are effective as immunostimulant even at low concentrate making it a very cost effective additive (Logambal *et al*., 2000). However studies on use of *O. Sanctum* on growth, nutrient utilization, immune response and survival are scant and scattered. *Cirrhinus mrigala* (mrigal) is one of the most important Indian Major Carps and is an integral part of the inland fisheries. In fact, it is an important component of sustainable food security in India. Despite severe economic losses due to disease outbreak in mrigal, very few attempts have been made to find suitable immunostimulants for effective improvement of health condition of this IMC. Consequently, the aim of present study is to determine the influence of dietary intake of *A. sativum* (garlic) and *O. sanctum* (tulsi) by monitoring various growth parameters, nutrient retention and survival of *C. mrigala*.

MATERIALS AND METHODS

Sample collection

The experimental fish *Cirrhinus mrigala* were collected from a local fish farm (Jasbeer fish seed farm, Adon, Kurukshetra, India). The fish were stocked in re-circulating water plastic tubs (30 L capacity) and acclimatized to the ambient laboratory condition. The fish were fed with basal diet for 10 days, prior to starting the experiment.

Feed preparation

Fresh garlic cloves were removed from the garlic bulb. The parchment skin was removed from the cloves and the cloves were chopped, sun dried followed by oven drying and later crushed with mortar and pestle. For preparing fish diet with tulsi, green tulsi leaves were dried for three to four days initially and then in oven at 55°C until a constant weight were obtained. Then the leaves were crushed manually to make it fine. It was passed through fine meshed wire sieve to obtain uniform powder. A basic diet was prepared that contained ground nut oil cake, 650; rice bran 42; hydrothermically processed soybean 266; wheat flour 32; mineral mixture 10 (in kg g^{-1}) which was considered the control diet. Three treatments G1, G2 and G3 were supplemented with garlic @ 10, 20 and 30 g Kg^{-1} of feed simultaneously three other treatments O1, O2 and O3 supplemented with tulsi @ 10, 20 and 30 g Kg^{-1} of feed, respectively.

All dietary ingredients were mixed thoroughly by adding warm distilled water and homogeneous dough was prepared. The resulting dough was passed through a mincer to produce feed pellets of 0.5 mm in diameter. Then, pellets were first air and packed in airtight containers. The diets were kept in a refrigerator at 4ºC until use. All these diets were isocaloric and isoproteic with approximately 40% proteins (Table 1).

Experimental design

All dietary treatments were performed with three replicates of each. The experiment was conducted under laboratory conditions (25±1°C) in plastic tubs (30 L capacity). Each tub was filled with de-chlorinated tap water and then stocked with 20 advanced fish fry. All groups of fish were fed daily at 4% BW in 2 instalments at 08:00 and 16:30 hours for 60 days with constant aeration and daily two-thirds water exchange. Individual weights of fish were recorded at the beginning and end of experiment and at every 15^{th} day of interval with the help of top pan balance. Length of fish was measured using a simple centimeter scale. Initial and final experiment fish were processed for proximate analysis following AOAC (1995).

At the beginning of the experiment some fishes were taken from the reserve and their weight and length was recorded. After evisceration the fishes were cut into pieces. These pieces were weighed and put in hot air oven (60°C for 12 h) for drying to determine the moisture content. The dried sample in aluminium foil was stored in desiccators for proximate analysis of crude protein, fat, protein, ash, nitrogen free extract, gross energy and phosphorus in it. The same protocol was followed at the end of each experiment to estimate final carcass quality following the standard methods of the Association of Official Analytical Chemists (1995). Chromic oxide levels in the diets as well as in the faecal samples were estimated spectrophotometrically (Furukawa and Tuskahara, 1966). The intact pellets were picked up by tube dropper or siphon pipe and dried in oven in a petridish.

Apparent protein digestibility (APD) was determined by an indirect method, using chromic oxide as a dietary inert external marker. As a dietary inert substance, chromic oxide (Cr_2O_3) is excreted without digestion or loss. Chromic oxide was incorporated into the diet and faecal samples were collected after a minimum period of feeding, normally 14 days. Faecal samples and diet were assayed for Cr_2O_3 and the percentage dry matter digestibility was calculated according to Cho *et al*. (1982) as follows.

Table 1 Proximate composition (% dry weight basis) of experimental diet

Proximate Analysis	Dietary Treatments						
	CC (control)	G1 (10 g)	G2 (20 g)	G3 (30 g)	O1 (10 g)	O2 (20 g)	O3 (30 g)
Crude protein (%)	39.7±2.13	38.7±1.32	39.1±0.9	39.5±0.81	39.00±1.01	39.20±1.06	38.10±0.05
Crude fat (%)	9.0±0.31	9.18±0.16	9.25±0.17	9.3±0.11	9.03±0.04	9.15±0.12	9.26±0.21
Crude fiber (%)	6.18±0.58	6.25±0.15	6.08±0.17	6.16±0.16	6.15±0.25	6.10±0.34	6.23±0.26
Total ash (%)	6.71±0.52	6.9±0.24	6.85±0.16	6.86±0.18	6.80±0.17	6.90±0.18	6.75±0.22
Moisture (%)	7.39±1.0	7.41±0.69	7.4±0.25	7.31±0.5	7.50±0.74	7.48±0.16	7.41±0.47
Nitrogen free extract (%)	30.95±2.32	31.5±1.35	31.31±1.08	30.85±1.08	31.50±1.89	31.10±1.45	32.20±0.53
Gross energy (kJ g^{-1})	18.28±0.2	18.19±0.13	18.27±0.08	18.3±0.08	18.17±0.12	18.23±0.04	18.19±0.08
Feed phosphorus (%)	1.34±0.07	1.39±0.05	1.3±0.04	1.36±0.02	1.35±0.05	1.37±0.11	1.40±0.02

All values are Mean ± S.E of mean.

$$\text{Apparent protein digestibility} = 100 - \frac{100\% \text{ marker in diet}}{\%\text{marker in faeces}} \times \frac{\%\text{ nutrient in faeces}}{\%\text{nutrient in diet}}$$

Live weight gain (g), percent weight gain, specific growth rate, feed consumption per day in percentage of body weight, feed conversion ratio (FCR), gross conversion efficiency (GCE), and protein efficiency ratio (PER) were calculated using standard method (Steffens, 1989).

Weight gain (g) = W_2-W_1

$$\text{Growth \% gain in body weight} = \frac{W_2-W_1 \times 100}{W_1}$$

$$\text{Growth per day in \% body weight} = \frac{2(W_2-W_1) \times 100}{t\,(W_1+W_2)}$$

$$SGR = \frac{\text{In } W_2 - \text{In } W_1 \times 100}{t}$$

$$FCR = \frac{\text{Feed Offered (Dry wt.) (g)}}{\text{Body weight gain (Wet wt.) (g)}}$$

$$GCE = \frac{\text{Body weight gain (Wet wt.) (g)}}{\text{Feed Offered (Dry wt.) (g)}}$$

$$PER = \frac{\text{Wet Weight Gain (g)}}{\text{Crude protein fed (\%)}}$$

Where,

W_1 = Initial Weight (g)
W_2 = Final Weight (g)
t = Duration of experiment (No. of days)

At the termination of experiment, from each treatment, six fish were randomly sampled and kept on ice to remove the intestines which were processed for the determination of enzyme activity of protease (Walter, 1984), amylase (Sawhney and Singh, 2000), and cellulase (Sadasivam and Manickam, 1996).

During experimentation, the water samples from all the experimental tubs were collected fortnightly and analyzed for temperature, dissolved oxygen (DO), pH, electrical conductivity, calcium, chlorides and total alkalinity following American Public Health Association (1998) to investigate the influence of compounded feeds on quality of holding water. At the end of feeding trials, water samples from each tub were collected at two- hour intervals for the estimation of excretory levels of total ammonia and reactive orthophosphate following the APHA (1998). The ammonium ions react with alkaline solution of Nessler's reagent to form a yellow brown colored complex of ammonium mercury iodine and absorption was measured at wavelength of 425 nm. The orthophosphate (o-PO_4) react with an acidified ammonium molybdate solution and form molybdophosphoric acid, which was then reduced to a blue complex in the presence of stannous chloride. The intensity of blue color was noted at 690 nm on spectrophotometer. The levels of ammonia and orthophosphate were then calculated following Sumagaysay-Chavoso (2003):

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

Fish biomass/ kg

$(N\text{-}NH_4/o\text{ -}PO_4)_0$ and $(N\text{-}NH_4/o\text{ -}PO_4)_{120}$ =concentration at times 0 and 120 min (2h) post feeding.

a = amount of holding water (L) in which fishes were kept.

Collection of blood

Blood samples were collected for hematological diagnosis by syringe from caudal vein. EDTA was used as anticoagulant. 1 mg EDTA ml^{-1} of blood was used as anticoagulant in blood.

Haematological measures

The blood samples were used to estimate the total erythrocyte and leucocyte count with the help of haemocytometer using a Neubaur's counting chamber following the methods given by Dacie and Lewis (1971).

Phagocytic assay and phagocytic index

On the day 60, blood was collected from fish of each group. The phagocytic cells and phagocytosed bacteria were enumerated. Phagocytic ratio (PR) and phagocytic index (PI) were determined by enumerating 100 phagocytes per slide under a microscope.

Phagocytic ratio (PR; *i.e.* percentage of cell with engulfed bacteria) = (No. of phagocytic cells with engulfed bacteria/No. of phagocytic cells) × 100.

Phagocytic index (PI; *i.e.* number of engulfed bacteria per cell) = No. of engulfed bacteria/No. of phagocytic cells.

Nitroblue tetrazolium (NBT) assay

The oxygen radical production by blood phagocytes during respiratory burst activity was measured through nitroblue tetrazolium (NBT) assay as described by Anderson and Siwicki (1995).

Challenge test

After feeding for 60 days, 10 fish from each treatment were challenged with *Aeromonas hydrohila* which has been cultured and maintained in the selective medium. Fishes in all replicates immersed in a suspension of *A. hydrophila* ~ 10^5 CFU ml^{-1} followed by a second immersion ~10^7 CFU ml^{-1} after 7 days (Austin *et al*., 1995). Per cent survival was measured for 10 days based on observation that mortality reached its plateau after 1 week (Sahoo *et al*., 1998) and relative percentage survival was calculated by the following formula (Ellis, 1988):

RPS = 1- (Percent mortality in treated group/ Percent mortality in control group) × 100

Statistical assay

One-way ANOVA followed by Duncan's multiple range test (Duncan, 1955) was applied to find out significant differences among dietary treatments Statistical significance was tested at a probability value of $P<0.05$. The statistical tests were performed using SPSS Version 11.5 for Windows.

RESULTS

Effect of herbs on growth and digestibility

At the end of both experiments all groups receiving herb supplemented diets revealed significant increase in the body weight gain, specific growth rate (SGR), protein efficiency ratio (PER), gross conversion efficiency (GCE) and apparent protein digestibility (APD). A significant decrease in feed conversion ratio (FCR) in comparison with control group was

found. These results are demonstrated in Table (2) for fish fed on garlic supplemented diets and Table (3) for group of fish fed on diet incorporated with tulsi and figure 1 and 2 respectively. In general, among two herbs, garlic supplemented diets resulted in better growth. The feed G3 containing garlic in the proportion of 30 g kg^{-1} of diet showed significant highest growth response among all diets.

After 60 days, mean digestive enzyme activities of all treated groups were significantly different (P<0.05) with that of the control. The protease activity was remarkably higher (P<0.05) in G3 (2.36±0.06) compared with control and other treatments.

Table 2 Growth performances and intestinal enzyme activities of *Cirrhinus mrigala* fed on diets containing varying proportions of *A. sativum* (garlic)

Growth parameters	Dietary treatments			
	CC (control)	G1 (10 g)	G2 (20 g)	G3 (30 g)
Initial weight (g)	0.99 ± 0.05^{A}	0.98 ± 0.02^{A}	1.01 ± 0.02^{A}	1.02 ± 0.04^{A}
Final weight (g)	3.02 ± 0.03^{C}	3.27 ± 0.09^{C}	3.70 ± 0.11^{B}	4.26 ± 0.08^{A}
Live weight gain (g)	2.03 ± 0.03^{C}	2.29 ± 0.07^{C}	2.69 ± 0.13^{B}	3.24 ± 0.05^{A}
Survival rate (%)	98.40±1.8	100	100	100
Growth (%) gain in BW	205.70 ± 2.60^{C}	232.80 ± 2.90^{C}	267.70 ± 18.79^{B}	319.00 ± 9.56^{A}
Growth day^{-1} (%) in BW	1.69 ± 0.01^{C}	1.79 ± 0.01^{C}	1.90 ± 0.05^{B}	2.04 ± 0.02^{A}
Specific growth rate (% BW d^{-1})	0.80 ± 0.006^{C}	0.87 ± 0.006^{C}	0.94 ± 0.03^{B}	1.03 ± 0.016^{A}
Feed conversion ratio (FCR)	2.95 ± 0.05^{A}	2.60 ± 0.03^{B}	2.57 ± 0.09^{B}	2.38 ± 0.03^{C}
Gross conversion efficiency (GCE)	0.33 ± 0.005^{C}	0.38 ± 0.004^{B}	0.39 ± 0.01^{B}	0.42 ± 0.005^{A}
Protein efficiency ratio (PER)	0.84 ± 0.01^{C}	0.95 ± 0.01^{B}	0.97 ± 0.03^{B}	1.04 ± 0.01^{A}
Apparent Protein Digestibility (%)	72.60 ± 0.87^{C}	75.83 ± 0.92^{B}	77.00 ± 0.58^{B}	80.13 ± 0.46^{A}
Specific protease activity[1]	1.57 ± 0.06^{C}	1.72 ± 0.08^{C}	2.00 ± 0.05^{B}	2.36 ± 0.06^{A}
Specific amylase activity[2]	1.19 ± 0.04^{D}	1.27 ± 0.02^{C}	1.44 ± 0.04^{B}	1.63 ± 0.02^{A}
Specific cellulase activity[3]	0.81 ± 0.06^{B}	0.87 ± 0.04^{B}	0.95 ± 0.04^{AB}	1.05 ± 0.07^{A}

All values are Mean ± S.E of mean.
[1]mg of tyrosine liberated mg of $protein^{-1}$ h^{-1}
[2]mg of maltose liberated mg of $protein^{-1}$ h^{-1}
[3]mg of glucose liberated mg of protein $^{-1}$ h^{-1}
Means with different letters in the same row are significantly (P<0.05) different
Duncan's Multiple Range test

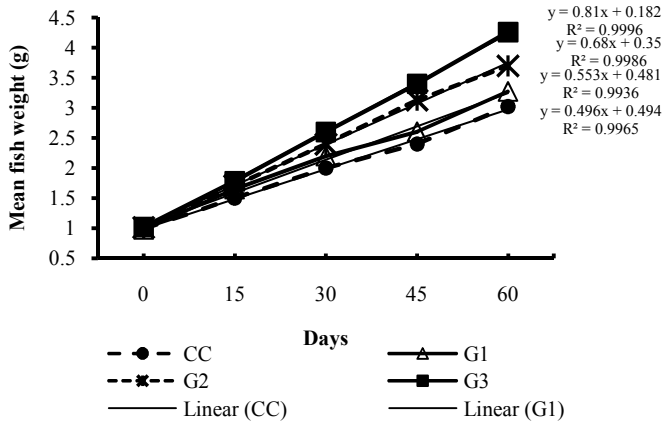

Figure 1 Linear fit curve to show increase in mean fish weight (g) of *C. mrigala* fed on diet supplemented with varying proportions of *Allium sativum* (CC = control, G1 = 10 g, G2 = 20 g and G3 = 30 g) from day 15 to 60.

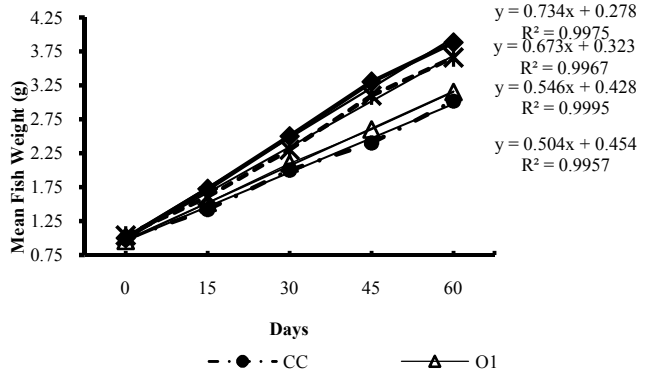

Figure 2 Linear fit curve to show increase in mean fish weight (g) of *C. mrigala* fed on diet supplemented with varying proportions of *Ocimum sanctum* (CC = control, O1 = 10 g, O2 = 20 g and O3= 30 g) from day 15 to 60.

As for amylase and cellulase, assays showed significantly higher (P<0.05) activity in treatment G3 fed fishes as compared to the rest. Same trend was observed with dietary treatments of tulsi.

Carcass composition

After 60 days feeding trial initial and final carcass composition of *Cirrhinus mrigala* in relation to various feeds supplemented with garlic is presented in (Table 4). The carcass composition of the test animals revealed a significant (P<0.05) increase in the final carcass protein and lipid over the initial carcass protein and lipid. Significantly highest carcass protein (15.97±0.07) and lipid (7.33±0.19) was recorded in treatment G3 fed fishes as compare to other experimental and control feeds. However, no significant variation was found in nitrogen free extract (NFE), moisture (%) and gross energy (kJ g^{-1}) between treatments. For tulsi, Crude protein (%) was found to be significantly, (P<0.05) higher (15.41 ± 0.05) in fish fed with O3 treatment (Table 5).

Effects of experimental diets on water quality characteristics

The relation of most water quality parameters do not followed any specific trend. No significant variations were observed in these parameters with respect to dietary treatments and were found to be in favourable ranges. However, significantly (P<0.05) low values in total ammonia excretion and reactive phosphate production (mg Kg^{-1} BW d^{-1}) were recorded in fish fed on both tulsi and garlic diet supplemented with 30 g kg^{-1} of feed (Table 6).

Haematological parameters (Total erythrocyte and leucocyte count)

The Total erythrocyte count was significantly higher (P<0.05) in fishes fed on diet G3 (2.06 ±0.07) than in the control treatment GC (1.28±0.03).

Table 3 Growth performances and intestinal enzyme activities of *Cirrhinus mrigala* fed on diets containing varying proportions of *O. sanctum* (tulsi).

Growth parameters	Dietary treatments			
	CC (control)	O1 (10 g)	O2 (20 g)	O3 (30 g)
Initial weight (g)	0.99±0.05^{A}	0.96±0.08^{A}	1.04±0.08^{A}	1.00±0.05^{A}
Final weight (g)	3.02±0.03^{B}	3.10±0.07^{B}	3.66±0.14^{A}	3.88±0.11^{A}
Live weight gain (g)	2.03±0.03^{B}	2.14±0.02^{B}	2.62±0.13^{A}	2.88±0.16^{A}
Survival rate (%)	98.40±1.80^{B}	97.20±3.70^{B}	100^{A}	100^{A}
Growth (%) gain in BW	205.70±2.60^{B}	225.50±22.9^{AB}	255.40±27.9^{AB}	292.15±33.6^{A}
Growth day^{-1} (%) in BW	1.68±0.01^{B}	1.75±0.08^{B}	1.85±0.08^{AB}	1.96±0.09^{A}
Specific growth rate (% BW d^{-1})	0.80±0.06^{B}	0.85±0.05^{AB}	0.91±0.05^{AB}	0.98±0.06^{A}
Feed conversion ratio (FCR)	2.95 ± 0.05^{A}	2.92 ± 0.11^{A}	2.73 ± 0.12^{AB}	2.51 ± 0.07^{B}
Gross conversion efficiency (GCE)	0.33 ± 0.05^{B}	0.34 ± 0.01^{B}	0.36 ±0.01^{AB}	0.39± 0.01^{A}
Protein efficiency ratio (PER)	0.84± 0.01^{B}	0.85± 0.03^{B}	0.91 ± 0.04^{AB}	0.99 ± 0.03^{A}
Apparent Protein Digestibility (%)	72.60±0.87^{C}	74.60±0.80^{B}	76.00±1.00^{B}	79.00±0.57^{A}
Specific protease activity[1]	1.57±0.02^{C}	1.67±0.05^{C}	1.89±0.03^{B}	2.10±0.03^{A}
Specific amylase activity[2]	1.19±0.04^{C}	1.25±0.02^{C}	1.37±0.04^{B}	1.60±0.04^{A}
Specific cellulase activity[3]	0.81±0.06^{C}	0.84±0.02^{B}	0.91±0.03^{AB}	1.01±0.03^{A}

All values are Mean ± S.E of mean.
[1]mg of tyrosine liberated mg of protein^{-1} h^{-1}
[2]mg of maltose liberated mg of protein^{-1} h^{-1}
[3]mg of glucose liberated mg of protein $^{-1}$ h^{-1}
Means with different letters in the same row are significantly (P<0.05) different
Duncan's Multiple Range test

Table 4 Proximate carcass composition of *Cirrhinus mrigala* fed on diets containing varying proportions of *A. sativum* (garlic)

Proximate Composition	Initial value	Dietary Treatments			
		CC (control)	G1 (10 g)	G2 (20 g)	G3 (30 g)
Moisture (%)	70.00±0.53	66.00±0.25^{AB}	65.70±0.20^{B}	66.80±0.20^{A}	63.40±0.30^{C}
Crude protein (%)	10.40±0.38	12.01±0.16^{C}	12.47±0.37^{C}	14.01±0.06^{B}	15.97±0.08^{A}
Crude fat (%)	5.31±0.18	6.08±0.28^{B}	6.45±0.17^{B}	7.21±0.08^{A}	7.33±0.19^{A}
Crude ash (%)	2.25±0.14	3.08±0.17^{B}	2.8±0.07^{B}	2.66±0.14^{A}	2.46±0.3^{AB}
Nitrogen free extract (%)	12.03±0.22	12.82±0.08^{A}	12.51±0.6^{A}	9.30±0.18^{B}	10.83±0.4^{B}
Gross energy (kJ/g)	6.62±0.12	7.44±0.13^{B}	7.64±0.01^{B}	7.76±0.01^{B}	8.53±0.14^{A}
Phosphorous (%)	0.52±0.01	0.50±0.01^{C}	0.62±0.06^{B}	0.7±0.04^{A}	0.76±0.02^{A}

All values are Mean ± S.E of mean
Means with different letters in the same row are significantly (P<0.05) different
Duncan's Multiple Range test

Table 5 Proximate carcass composition of *Cirrhinus mrigala* fed on diets containing varying proportions of *O. sanctum* (tulsi).

Proximate Composition	Initial value	Dietary Treatments			
		CC (control)	O1 (10 g)	O2 (20 g)	O3 (30 g)
Moisture (%)	70.00±0.53	66.00±0.25^{A}	62.40±0.20^{D}	64.20±0.17^{C}	65.00±0.15^{B}
Crude protein (%)	10.40±0.38	12.01±0.16^{D}	13.11±0.11^{C}	13.57±0.12^{B}	15.41±0.05^{A}
Crude fat (%)	5.31±0.18	6.08±0.28^{B}	7.23±0.13^{A}	7.08±0.10^{A}	6.41±0.20^{B}
Crude ash (%)	2.25±0.14	3.08±0.17^{A}	2.91±0.12^{AB}	2.71±0.14^{AB}	2.60±0.05^{B}
Nitrogen free extract (%)	12.03±0.22	12.82±0.08^{B}	14.35±0.17^{A}	12.43±0.44^{B}	10.57±0.17^{C}
Gross energy (kJ/g)	6.62±0.12	7.44±0.13^{C}	8.42±0.04^{A}	8.14±0.02^{B}	7.99±0.07^{B}
Phosphorous (%)	0.52±0.02	0.50±0.01^{C}	0.55±0.06^{B}	0.60±0.03^{B}	0.72±0.01^{A}

All values are Mean ± S.E of mean
Means with different letters in the same row are significantly (P<0.05) different
Duncan's Multiple Range test

Table 6 Total orthophosphate and ammonia (mg Kg^{-1} Body weight day^{-1} of fish) in holding water for fish fed on diets supplemented with varying proportions of *A. sativum* (group 1) and *O. sanctum* (group 2) after 60 days of feeding trial

Parameters	Dietary Treatments (*A. sativum*)				Dietary Treatments (*O. sanctum*)			
	CC (control)	G1 (10 g)	G2 (20 g)	G3 (30 g)	CC (control)	O1 (10 g)	O2 (20 g)	O3 (30 g)
Total Ammonia excretion (mg Kg^{-1} BW day^{-1})	1653.21±11.09^{A}	1328.60±5.60^{B}	1156.00±7.25^{C}	1074.16±8.74^{D}	1653.21±11.09^{A}	1492.01±15.46^{B}	1248.00±13.76^{C}	1105.00±22.90^{D}
Total phosphate production (mg Kg^{-1} BW day^{-1})	743.16±5.60^{A}	618.00±10.97^{B}	593.00±13.06^{B}	530.60±5.74^{C}	743.16±5.60^{A}	664.00±13.41^{B}	602.00±14.75^{C}	568.01±10.99^{C}

All values are Mean ± S.E of mean
Means with different letters in the same row are significantly (P<0.05) different
Duncan's Multiple Range test

Also, significant increase (P<0.05) in WBC count was observed in fishes of treatment G3 (49.16 ± 0.44) than in control treatment GC (22.23± 0.53). Similar results for haematological parameters were observed in tulsi treated diet. Post-challenge, the value of TEC observed was lower than previous but followed the same trend among groups (Table 7).

For tulsi, maximum values of phagocytic ratio (78.41±0.91) and phagocytic index (2.18±0.06) were observed in dietary treatment O3 (containing tulsi @ 30 g kg^{-1} of diet) than those fed on the control diet (59.63±0.51 and 1.48±0.015 respectively) for 60 days.

Table 7 Hematological Values of *Cirrhinus mrigala* fed on diets containing varying proportions of *A. sativum* (garlic) and *O. sanctum* (tulsi).

Haematological Parameters		Dietary Treatments (*A. sativum*)				Dietary Treatments (*O. sanctum*)			
		CC (control)	G1 (10 g)	G2 (20 g)	G3 (30 g)	CC (control)	O1 (10 g)	O2 (20 g)	O3 (30 g)
TEC ($10^6 mm^{-3}$)	Pre Challenge*	1.28±0.03^{D}	1.45±0.02^{C}	1.76±0.53^{B}	2.06±0.07^{A}	1.28±0.03^{C}	1.33±0.03^{C}	1.56±0.02^{B}	1.86±0.02^{A}
	Post Challenge*	1.14±0.02^{D}	1.33±0.01^{C}	1.54±0.02^{B}	1.78±0.02^{A}	1.14±0.02^{D}	1.25±0.01^{C}	1.40±0.01^{B}	1.74±0.02^{A}
TLC ($10^3 mm^{-3}$)	Pre Challenge**	22.23±0.53^{D}	31.60±0.70^{C}	38.4±0.87^{B}	49.16±0.44^{A}	22.23±0.53^{D}	29.03±0.53^{C}	35.40±0.83^{B}	44.13±0.64^{A}
	Post Challenge**	24.13±1.12^{D}	36.0±0.60^{C}	42.5±0.67^{B}	53.5±0.83^{A}	24.13±1.12^{C}	34.63±1.35^{B}	36.70±0.90^{B}	46.70±0.83^{A}

Means with different letters in the same row are significantly (P<0.05) different.
Duncan's Multiple Range test.
*Significantly (P<0.05) different student's t test.

Table 8 Effect of herbs and their interaction on Phagocytic ratio and Phagocytic index of *Cirrhinus mrigala*.

Peripheral blood monicytes	Dietary Treatments (*A. sativum*)				Dietary Treatments (*O. sanctum*)			
	CC (control)	G1 (10 g)	G2 (20 g)	G3 (30 g)	CC (control)	O1 (10 g)	O2 (20 g)	O3 (30 g)
Total no. of phagocytes	66.00±2.89^{B}	78.60±1.76^{B}	85.00±3.20^{A}	87.30±4.10^{A}	66.00±2.89^{B}	69.60±1.85^{B}	76.30±2.60^{AB}	81.60±3.76^{A}
No. of ingested phagocytes	39.30±1.45^{C}	52.60±2.70^{BC}	63.30±2.40^{A}	70.60±2.03^{A}	39.30±1.45^{C}	45.00±3.21^{C}	54.60±5.81^{B}	64.00±2.04^{A}
Blatopores within phagocytes	98.00±4.16^{C}	130.00±7.00^{B}	153.30±10.70^{B}	216.00±10.80^{A}	98.00±4.16^{C}	116.00±3.08^{B}	132.00±11.02^{B}	178.6±13.05^{A}
Phagocytic ratio (%)	59.63±0.50^{D}	66.80±1.95^{C}	74.50±0.36^{B}	81.46±1.83^{A}	59.63±0.50^{C}	64.40±3.20^{BC}	71.20±5.31^{AB}	78.41±0.91^{A}
Phagocytic index	1.48±0.01^{C}	1.65±0.05^{BC}	1.80±0.08^{B}	2.47±0.01^{A}	1.48±0.01^{C}	1.66±0.01^{B}	1.72±0.08^{B}	2.18±0.06^{A}

Means with different letters in the same row are significantly (P<0.05) different.
Duncan's Multiple Range test.

Phagocytic responses

Phagocytic activity (PA) and phagocytic index (PI) of fish fed garlic diets were significantly higher than those of fish fed the control diet for 60 days (Table 8). The relative PA and PI levels (compared to the control group) of fish fed diets G1, G2 and G3 for 60 days increased by 66.8±1.95% and 1.65±0.05%; 74.5±0.36% and 1.8±0.08%; 81.46±1.83% and 2.47±0.08%, respectively.

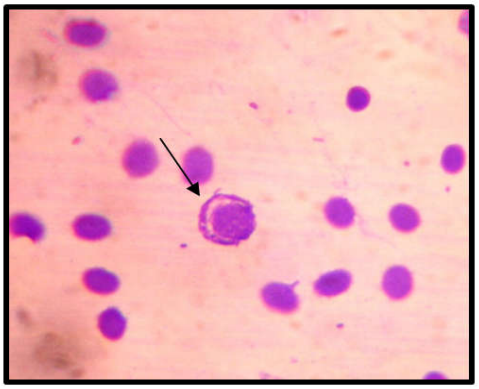

Figure 3 Mature phagocytic cell observed in *A. sativum* (garlic) fed *Cirrhinus mrigala* after a feeding trial of 60 days (1000X).

Photographs of phagocytic cells observed in blood of treated fish are presented in Figure 3 and 4.

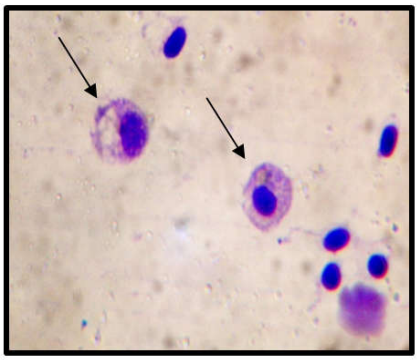

Figure 4 Phagocytic cells of *Cirrhinus mrigala* in O3 treatment after 60 days of feeding trial (1000X).

NBT assay or respiratory burst activity

The respiratory burst activities of mrigal blood cell in treated group increased significantly (P<0.05) compared with the control fish (Table 9). Maximum increase in the NBT reduction value was observed in Treatment G3 and O3 containing garlic and tulsi respectively @ 30 g kg^{-1} of diet.

Table 9 NBT activity of *Cirrhinus mrigala* fed on diet containing various proportions of herbs *A. sativum* (garlic) and *O. sanctum* (tulsi).

Treatments (Group 1 Garlic)	NBT activity (O.D. at 450 nm)	Treatments (Group 2 Tulsi)	NBT activity (O.D. at 450 nm)
CC (control)	0.42 ± 0.011^{D}	CC (control)	0.42 ± 0.01^{C}
G1 (10 g)	0.50 ± 0.010^{C}	O1 (10 g)	0.44 ± 0.02^{C}
G2 (20 g)	0.63 ± 0.014^{B}	O2 (20 g)	0.62 ± 0.01^{B}
G3 (30 g)	0.74 ± 0.012^{A}	O3 (30 g)	0.72 ± 0.01^{A}

Means with different letters in the same column are significantly (P<0.05) different
Duncan's Multiple Range test

Table 10 Per cent mortality and relative percent survival (RPS) of *Cirrhinus mrigala* fed on diets containing *A. sativum* (group 1) and *O. sanctum* (group 2) in a challenge trial with *Aeromonas hydrophila* for 10 days

Treatments (Group 1 Garlic)	Percentage Mortality (%)	Relative Percent Survival (%)	Treatments (Group 2 Tulsi)	Percentage Mortality (%)	Relative Percent Survival (%)
CC (control)	80.0 ± 5.70^{A}	-	CC (control)	80.0 ± 5.70^{A}	-
G1 (10 g)	46.6 ± 6.77^{B}	41.75	O1 (10 g)	60.0 ± 5.78^{AB}	25.00
G2 (20 g)	33.3 ± 3.34^{B}	58.37	O2 (20 g)	43.3 ± 8.83^{BC}	45.88
G3 (30 g)	6.6 ± 3.33^{C}	91.62	O3 (30 g)	23.3 ± 1.45^{C}	72.13

Means with different letters in the same column are significantly (P<0.05) different
Duncan's Multiple Range test

Relative percentage survival after challenge test

The challenge test revealed that long-term oral administration of herb-supplemented feed enhanced the resistance of *Cirrhinus mrigala* to bacterial infection (Table 10). Significantly higher (P<0.05) post-challenge survival rates were observed in the fish groups fed diets containing garlic @ 30 g kg^{-1} of feed (91.62%) followed by G2 (58.37%) and G1 (41.75%) while the fish fed with control diet exhibited the highest mortality (80±5.7%). In tulsi, treatment O3 fed groups showed significantly (P<0.05) lower mortality, *i.e.,* 23.3±1.45 than control group.

Among the two herbs *A. sativum* (garlic) and *O. sanctum* (tulsi), *C. mrigala* fed on diets containing garlic showed better haematological count and other immunomodulatory response after 60 days of feeding trial.

DISCUSSION

The group of fish fed on herbal diet containing 30 g kg^{-1} *Allium sativum* (garlic) and same concentration of *Ocimum sanctum* (tulsi) had significantly (P<0.05) better growth performance as compared to fish fed on control diet. Khalil *et al*. (2001) reported that compound allicin present in *A. sativum* (garlic) improves the performance of intestinal flora, boosting digestion which enhances the utilization of energy hence resulting in improved growth. A number of studies have reported a positive improvement in biomass and specific growth rate as well improvement in other growth parameters in *Oreochromis niloticus* (Nile Tilapia) on garlic supplementation (Abou-Zeid, 2002; Diab *et al*., 2002; Shalaby *et al*., 2006; Abdel-Hakim *et al*., 2010). The sulfur compounds in garlic are believed to be as active antimicrobial agents, enhancing the immune response and therefore stimulate growth having a mode of action similar to antibiotics (El-Afify, 1997). According to a study by Aly *et al*. (2008) unpleasant odour may reduce feed intake in fish when given in high doses. However, no such behaviour of mrigal was observed during our feeding trial suggesting that *A. sativum* supplementation was at optimum level.

In another feeding trail, the enhanced growth performance and nutrient retention in fingerlings fed on tulsi could be due to the growth promoting effect of *Ocimum sanctum* leaf extracts. A similar observation with tulsi fed diet has been reported by Pavaraj *et al*. (2011) in Common carp and Nahak and Sahu (2014) in *Clarias batrachus.*

The high values of fish carcass nutrient observed in herbal fed fishes were perhaps due to the increased enzymatic activity in the gut; increasing the digestibility and hence nutrients were utilized properly. These results are in accordance with the findings of Soltan and El-Laithy (2008) who reported that incorporation of garlic into *O. niloticus* (Nile tilapia) diets resulted in significant effects in dry matter, crude protein and crude fat contents of tilapia. In this respect, Barros *et al*. (2000) and Yildirim *et al*. (2003) reported that body fat content is closely related to weight gain and inversely related to body moisture content and this agreed with the obtained results of the present study. Anyanwu *et al*. (2012) reported that up to 15% dietary inclusion level of *Ocimum gratissimum* Leaf Meal could support optimal carcass composition of *Clarias gariepinus* without grossly affecting the quality of the fish. The low excretion of metabolites for each feeding trial coincided with high digestibility of respective group. This may be attributed to more feed utilization due to high enzymatic activity hence better utilization of proteins and correspondingly lower excretion of metabolites. Kumar *et al*. (2009) and Jana *et al*. (2012) for brackishwater fish species whereas Bhatnagar *et al*. (2012); Bhatnagar and Raparia (2014) and Bhatnagar and Lamba (2015, 2016) for freshwater carp species have also reported low excretion of metabolites with better feed utilization.

On encounter of foreign microorganisms, the number of leucocytes increases sharply as one of the first lines of body defence. Such increase in total leucocyte count was observed in garlic fed *C. mrigala* fingerlings in present study. The increase in total white blood cell counts, and red blood cells counts following 60-day garlic feeding supports the anti-infection

properties of garlic (Iranloye, 2002). Fazlolahzadeh *et al*. (2011) in a study on garlic supplemented diet for *Oncorhynchus mykiss* (Rainbow trout) reported that lymphocyte and erythrocytes count increased significantly ($P<0.05$) in fish fed on diets containing 0.45, and 0.6 g kg^{-1} doses compared with control. Similar results were obtained by Sahu *et al*. (2007) for *Labeo rohita* (rohu) fed on garlic, demonstrating the anti-infection characteristics of garlic. Invariably increased RBC and WBC counts of *C. mrigala* treated with leaf extracts of *Ocimum sanctum* observed in present study may be due to the effect of bioactive principle of this herb to protect murine peritoneal macrophage and help to restore their normal functions (Nahak and Sahu, 2014). A significant decrease in RBC count during post challenge was also reported which are in in agreement with findings of Ranzani- Paiva *et al*. (2004) who showed decreased RBC number in Nile Tialpia after bacterial inoculation. Since WBC is known as first line of defence playing a major role in innate immunity; hence, increase in their numbers in the present study indicate the role of herbs in stimulating innate immunity (Menezes *et al*., 2006).

Immunostimulants act to enhance the non-specific immune response by either increasing the number of phagocytes or activating phagocytosis and respiratory burst (Shoemaker *et al.*, 1997). Garlic quickens macrophage phagocytosis; hence cause increased engulfing and destroying microorganisms and cellular debris (Lau *et al*., 1991). Germanium, a therapeutic factors contained in garlic, has been shown to enhance natural killer cell activity and macrophage activity in experimental animals (Aso, 1985). The present study indicated that both phagocytic activity and phagocytic index increased significantly in *Cirrhinus mrigala* fed with garlic. Studies shows that the aqueous extract of raw garlic as well dried powder scavenge hydroxyl radcals (Yang *et al.*, 1993; Kim *et al.*, 2001), and superoxide anions (Kim *et al*., 2001). Similar phagocytic activities may have occurred in the present work. In our 60 days of *Ocimum sanctum* extract treatment, the phagocyte counts were increased in all experimental groups as compared to control group. The major reason for this enhanced concentration of phagocytes in the experimental groups may be due to their participatory role in immune functions as observed by Kollner *et al*. (2002). Similar results with tulsi fed diet were also observed by Pavaraj *et al*. (2011) in *Cyprinus carpio* and Nahak and Sahu (2014) in *C. batrachus*. Durgadevi and Balasubramanian (2009) also reported maximum phagocytic activity in plant extracts treated groups of *Cyprinus capio* than the control groups. Reduced mortalities following pathogenic challenges in the presence of a low dose of herbal supplements have been reported by Kim *et al*. (2001) and Jain and Wu (2003). The results of challenge trial are in agreement with previous study conducted by Logambal *et al.* (2000) in *O. mosambicus* fed with diet containing *O. Sanctum*; Christybapita *et al*. (2007) in *O. mossambicus* treated with *Eclipta alba* leaf extract and Pavaraj *et al*. (2011) in Common cap fed with tulsi treated diet.

CONCLUSIONS

In conclusion, utilization of herbs such as garlic and tulsi, balanced and formulated in a suitable dose can have positive effect on growth, nutrient utilization and immune response with reduced metabolite excretion in holding water and decreased mortality rate which have the potential for achieving sustainable aquaculture. However, further studies should be done to establish the relationship between the dose used, period of application and time of withdrawal to obtain the best evaluation for the fish quality and shelf-life.

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Conflict of interest

The authors have no conflict of interest to declare.

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