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Growth response, feed conversion ratio and antiprotease activity of *Cynodon dactylon* (L.) mixed diet in *Catla catla* (Ham.)

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ABSTRACT

The effect of C. dactylon incorporated into diet formulations on the growth and body composition of Indian major carp, Catla (Catla catla) were investigated in a 45 days feeding trial. Different concentration (0%, 0.05%, 0.5% and 5% with the total fish feed) of C. dactylon ethanolic extract was mixed with feed ingredient. The incorporation of C. dactylon mixed diet improved the feed conversion ratio and body composition in C. catla. The elevated levels of amylase and protease activity were found in hepatopancreas at 30th days of feeding trial in 0.5% and 5% level of mixed diet groups. Non-specific immune parameter of serum antiprotease activity was higher in 5% level of experimental group. The results indicate that 5% inclusion of C. dactylon mixed diet improves the growth performance, feed efficiency, body composition, digestive enzyme and antiprotease activity in C. catla. In general, this study demonstrated the benefits of incorporating C. dactylon into fish feeds.

Key words: Cynodon, catla, feed conversion ratio, amylase, protease, antiprotease

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INTRODUCTION

India is a biodiversity nation and it has a rich background in medicinal herbs, most of which have been used to treat human and animal diseases. Aquaculture is a fast developing industry in India. Fish farming and aquaculture industries take part in contributing fish protein to large Asian population [1]. Fish is a good source of protein and also has essential amino acids with minerals like zinc, magnesium, sodium, etc. [2]. Development of Aquaculture is mainly depended on availability of compatible and suitable diet. For the formation of fish diet, feed conversion ratio (FCR) and Specific Growth Rate (SGR) are good tools to compute the acceptability and suitability of artificial diet. Normally, balanced fish feeds contain fishmeal, deoiled cakes and rice bran. Our research mainly focuses on alternative sources of feed ingredients, the main reasons being escalating cost and uncertainty of constant supply and improves the disease resistant capability through prophylactic treatment of normal feed ingredients. Already different plant materials have been studied widely for their nutritive values [3]. Many of medicinal herbs and their chemical components are used as an Immunostimulants, which are used in artificial diet

preparation, aquaculture research and their practices. Many of the herbal plants have the ability to inhibit the microbial pathogens and activate the immunity [4]. Immunostimulation is an alternative effective method against vaccination. It may be achieved through only feed supplement. Several ayurvedic medicinal plants are acting as a powerful immunomodulators. Nowadays, supplemental treatment is popular for preventing the diseases in aquatic animals. Moreover, they are cheaper, safer and biocompatible. Respiratory burst activity of phagocytic cells and plasma lysozyme activity have been significantly increased in common carp (*Cyprinus carpio*) and large yellow croaker (*Pseudosciena crocea*) after feeding with *Astragalus membranaceus* and *Angelica sinensis* mixed diet [5]. The present study was conducted to evaluate the effects of *Cynodon dactylon* L. mixed artificial diet on growth, survival, body composition, digestive enzyme activity and antiprotease activity of experimental fish *Catla catla*.

MATERIALS AND METHODS

Preparation of ethanolic extract of *Cynodon dactylon* L.

Cynodon dactylon L. was collected from Madurai, Tamil Nadu, India and then transferred to PRIST University, Thanjavur, Tamil Nadu, India. It was taxonomically identified and authenticated by Rev Dr. S. John Britto SJ, Director, The Rapinat Herbarium and Centre for Molecular Systematics, St. Joseph College (Autonomous), Thiruchirapalli, Tamilnadu, India. The voucher specimen was deposited at the Rapinat herbarium and the voucher number is RHCD BP18.

Fresh plant of *C. dactylon* L. was cleaned and shade dried. The dried plants were pulverized by an electrical blender and passed through 20 µ mesh sieve. A powdered plant (550 gm) was extracted successfully with 1:2 w/v in 70% ethanol by using soxhlet apparatus. The extraction was carried out in 24 hrs at room temperature with mild shaking [6]. The extract was filtered and concentrated at 45 °C using rotary vacuum evaporator under reduced pressure. The extract was stored in tight containers in desiccators.

Experimental diet preparation

Artificial balanced diet was prepared by using fish meal, fish grower, wheat flour, cod liver oil (Universal medicare Pvt. Ltd.), vitamin premix (Vetsfarma Ltd.) as feed ingredients, which contain 20% Carbohydrate; 41% Protein; 15% Lipid and 9% ash (Table 1). Diet groups were designed to provide with different concentrations of 0.05%, 0.5% and 5 % ethanolic extract of *C. dactylon* mixed with the normal diet before pelletization. All dietary ingredients were mixed thoroughly, moistened, cold-pelleted with a pelletizer and dried at 40°C for 24 hrs. Diets were stored at -20°C. The dried pellets were hand crumbled into small pieces and stored in airtight PVP containers.

Proximate composition analysis

Proximate composition of feed ingredients were analysed by the following method of AOAC (2003) [7]. Moisture was determined by oven drying method. Crude protein and fat content were analysed by Kjeldahl and Soxhlet apparatus (Tempo, Bombay, India). Total ash content was determined by burning the sample at 500 °C for 10 hrs in a muffle furnace.

Experimental animal and their maintenance

Similar age groups of Indian major carp, *Catla catla* was obtained from Golden fish farm Karandhai, Thanjavur, Tamil Nadu, India. Average weight of fishes used for the experiment was 88.05 ± 4.75 gm. All fishes were maintained in fiber reinforced plastic (FRP) tanks. The water

was replaced once in two days to maintain the water quality. Bore well water was used to rear the fish with 2 hrs aeration daily. Dissolved oxygen, dissolved carbon dioxide, pH and total alkalinity of water were monitored at weekly intervals [8]. Fishes were kept at the ambient, uncontrolled temperature of 28 ± 2 °C under the natural photoperiod. Fishes were acclimated for 15 days and fed *ad libitum* with balanced fish diet prepared in the laboratory.

Feeding trial

The feeding trial was conducted over a period of 45 days. The test diets were fed with apparent sanitation twice a day at 09.00 and 18.00 hrs during 45 days of experimental period. Experimental fishes were fed with supplemental diet at the rate of 2 % of their body weight per day. Fishes were randomly divided into four groups, namely, diet group I (DG1), diet group II (DG2), diet group III (DG3) and diet group IV (DG4) and they were fed with 0%, 0.05%, 0.5% and 5 % plant extract mixed diet respectively.

The initial body weights of each fishes were recorded. Fishes from each tank were weighed randomly at 10 days of interval. The experiment was conducted in triplicate to determine average readings.

The morphological growth parameters were calculated as follows:

1. Specific growth rate (SGR) (% day^{-1}) = $\frac{\text{Log Wt} - \text{Log Wo} \times 10}{\text{Feeding days}}$

where, Log *Wo*: weight of fish on the first day of trial, Log *Wt*: weight of fish on the last day of trial.

2. Feed conversion ratio (FCR) = $\frac{\text{Dry weight of feed (g)}}{\text{Live weight gain (g)}}$

3. Average daily growth (ADG) = $\frac{\text{Final body weight - Initial body weight}}{\text{No. of feeding days}}$

On termination of the experiments, all the surviving fishes were harvested, weighed individually and a portion of the dermal muscle was dissected for proximate analysis of protein, carbohydrate, moisture, ash and fat content of the muscle as per the methods of AOAC (2003) [7].

Digestive enzyme activity

Hepatopancreas of fishes were collected every 10 days of experimental period for measure the digestive enzyme activity. Fishes were taken at morning 9 am to 11 am for the removal of hepatopancreas, which were kept in frozen condition at -70 °C until further use.

Approximately 1.0 gm of hepatopancreas were homogenized in chilled 10 mM Tris–HCl buffer at pH 7.5 and enzyme extracts were obtained after centrifugation at 10,000 g for 30 min at 4 °C. The supernatant of each sample was assayed in triplicate.

Total soluble protein was measured by the method of Bradford (1976) [9], using bovine serum albumin as a standard. Amylase activity was assayed by the Bernfeld method (1955) [10], using soluble starch as the substrate and react with 3, 5-dinitrosalicylic acid. Total protease activity was assayed by the method of Anson (1938) [11] with slight modification. Casein used as the substrate and react with Folin reagent. The absorbance of enzyme activities was measured by using a Techcomp UV-2310 spectrophotometer at 630 nm.

Immunization of fish:
Another set of experimental diet groups were fed with the supplemental diet at the rate of 2 % of the body weight for 30 days. On 5th day, 500 µl of 20 % suspension of RaRBC in phosphate buffered saline were injected intraperitoneally to the fishes using 1 ml tuberculin syringe fitted with 28 G needle.

Collection of blood samples
After immunization, blood was collected at 7th, 14th, 21st and 28th day of experimental period. For bleeding, each fishes were individually caught using a dip net and were bled from common cardinal vein using 1 ml tuberculin syringe fitted with 24 gauge needle [12]. 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 allowed to clot at room temperature. 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.

Assay of serum anti-trypsin activity
Serum anti-protease activity was performed by incubating 10 µl of serum with 20 µg of trypsin dissolved in 100 µl of Tris-HCl (50 mM, pH 8.2). In serum blank, 100 µl of Tris-HCl was added to 10 µl of serum, instead of trypsin in Tris-HCl, and in the positive control, no serum was added to trypsin. All tubes were filled with 200 µl of Tris- HCl and incubated for 1 hour at room temperature. After the incubation, 2 ml of 0.1 mM substrate BAPNA (Na-benzoyl-DL-arginine-*p*-nitroanilide HCl, Sigma chemicals), was dissolved in Tris-HCl (containing 20 mM calcium chloride), which was added to all tubes and again incubated for further 15 minutes. At the end of incubation, the reaction was stopped by adding 500 µl of 30% acetic acid. The optical density was measured at 410 nm by using UV-Visible spectrophotometer (Techcomp UV-2310). The percentage of trypsin inhibition was calculated as described by Rao and Chakrabarti (2004) [13]:

$$\text{Trypsin inhibition (\%)} = (A1\text{-}A2/A1) \times 100$$

where,
A1 = control trypsin activity (without serum); A2 = activity of trypsin remained after addition of serum.
Statistical analyses to compare the mean differences among each diet groups for all the parameters was computed by student T-test at $P < 0.05$ level. All treatments were assayed in triplicate.

RESULTS

Preparations of diet for the experiments were given in Table 1 and the percentage proximate composition of the feed ingredients used in the trial (DG1, DG2, DG3 and DG4) is summarised in Table 2. The nutritional profile of *C. dactylon* mixed diet is almost equal to the control feed ingredients. Lipid level of the control and experimental diets do not differ largely, but crude protein content in the DG4 diet increased marginally. Ash content in the DG1 diet was lower than DG4, probably due to reduction of percentage of *C. dactylon* extract mixed in the content of diet. Various feed conversion parameters were studied and described in Table 3. When compared to other diet groups, a significant difference ($P < 0.05$) was observed in final weight of DG3 and DG4. However, there were no significant difference found among DG1 and DG2. Net and average weight gains are higher in DG3 and DG4 than other groups. Specific growth rate linearly increased in the *C. dactylon* mixed diet groups. Even though no significant difference ($P < 0.05$) was found among the four groups, noticeable survival rate of fish (SR 100%) in all experimental

groups indicated that the culture condition and composition diets provided to them were acceptable. According to the results of proximate composition of body moisture and ash, significant ($P < 0.05$) differences were not found among the experimental groups (Table 4). Increased level of protein and lipid were found in all *C. dactylon* incorporated diet groups than DG1, while slight variations observed in the moisture and ash contents. However, crude protein and lipid content were found to be significantly ($P < 0.05$) different among the experimental groups.

Specific enzyme activities of amylase and protease activity were recorded at every 10 days of interval in *C. dactylon* mixed diet treated *C. catla* (Fig. 1a and b respectively). Specific activities of amylase and protease activity were significantly ($P < 0.05$) higher in all experimental diet group than the control diet group throughout the experimental period. In DG4, both amylase and protease activity were significantly ($P < 0.05$) higher at 30 days of feeding than DG1, DG2 and DG3. Whereas, at the end of 40 days of experimental period, all groups of enzyme activity were reduced. Even though, experimental group of DG2, DG3 and DG4 were slightly higher than DG1. The trend of dominating specific amylase and protease activity in *C. catla* fed *C. dactylon* mixed feed was continued until the end of the 40 days.

The level of antiprotease activity in serum of test groups exhibited more than the control group throughout the study period (Fig 2). Antiprotease activity of DG1, DG2, DG3 and DG4 were significantly ($P < 0.05$) higher during all experimental days. From first and second collection (7^{th} and 14^{th} day respectively) of blood, the serum antiprotease activity was slightly higher than third and fourth collection (21^{st} and 28^{th} day respectively). DG3 and DG4 maintained their antiprotease activity in almost all experimental days. Present study revealed that the experimental diet of *C. dactylon* play a vital role in enhancement of serum antiprotease activity.

Table 1 Composition of diet Ingredients used for experiments.

Ingredients/100 g	Experimental diet (g)	Control diet (g)
Wheat flour	45	45
Dry fish meal	32	32
Fish grower	11	11
Cod liver oil	10	10
Vitamin and mineral premix	2	2
Cynodon dactylon extract (0.05%, 0.5%, 5% level)	Present	Absent

Table 2 Proximate Composition of the experimental diets (%).

Composition (%)	DG1	DG2	DG3	DG4
Moisture	6.92	7.03	7.06	7.08
Crude protein	41.42	41.86	42.85	44.15
Lipid	5.99	6.32	6.45	6.48
Ash	12.42	12.63	12.84	13.04

Values represent means of duplicate samples of DG1, DG2, DG3 and DG4 are represented as diet group I, II, III and IV respectively.

Table 3 Feed conversion and morphological parameters in *C. catla* after feeding with *C. dactylon* supplemented feeds for 45 days.

Parameters	DG1	DG2	DG3	DG4
Fish no.	20	20	20	20
IBW	88.05 ± 4.75	88.05 ± 4.75	88.05 ± 4.75	88.05 ± 4.75
FBW	94 ± 4.69	93.95 ± 4.67	100.15 ± 2.4	102.7 ± 2.51
NWG	5.95 ± 0.06	5.9 ± 0.11	12.1 ± 0.58	12.65 ± 0.43
ADG	0.12 ± 0.001 [a]	0.12 ± 0.001 [a]	0.25 ± 0.04 [b]	0.29 ± 0.04 [b]
SGR	0.132 ± 0.02 [a]	0.131 ± 0.02 [a]	0.268 ± 0.5 [b]	0.325 ± 0.46 [c]
FCR	0.51 ± 0.04 [a]	0.58 ± 0.02 [b]	0.66 ± 0.07 [c]	0.68 ± 0.07 [c]
SR	100	100	100	100

Values with different superscripts are significantly different (P < 0.05). DG1, DG2, DG3 and DG4 are represented as diet group I, II, III and IV respectively. IBW—initial body weight (g), FBW—final body weight (g), NWG—net weight gain (g), ADG—average daily growth (g), SGR—specific growth rate, FCR—feed conversion ratio, SR—survival ratio.

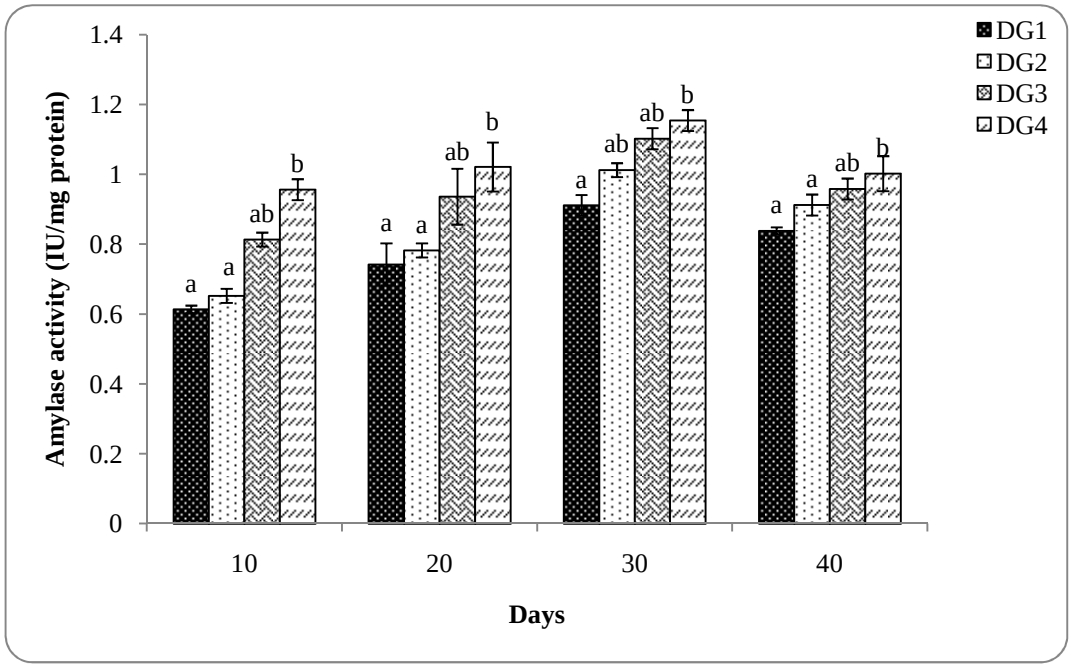
Table 4 Proximate body composition of *C. catla* fed with different experimental diets (%)

Composition (%)	DG1	DG2	DG3	DG4
Moisture	75.44 ± 0.10	75.34 ± 0.12	75.95 ± 0.16	75.96 ± 0.26
Crude protein	68.65 ± 0.25 [a]	71.56 ± 0.12 [b]	72.68 ± 0.18 [c]	73.44 ± 0.18 [d]
Lipid	5.74 ± 0.12 [a]	6.32 ± 0.05 [b]	6.66 ± 0.12 [b]	7.21 ± 0.12 [c]
Ash	13.86 ± 0.12	14.36 ± 0.10	14.05 ± 0.12	14.68 ± 0.10

Values with different superscripts are significantly different (P < 0.05).DG1, DG2, DG3 and DG4 are represented as diet group I, II, III and IV respectively.

Fig: 1 Specific activity of enzymes in digestive gland of *C. catla* fed with different experimental diets. Statistical differences (*P* < 0.05) among groups are indicated by different letters. No significant differences appear among the groups marked with the same letter. DG1, DG2, DG3 and DG4 are represented diet group I, II, III and IV respectively.

a.



b.

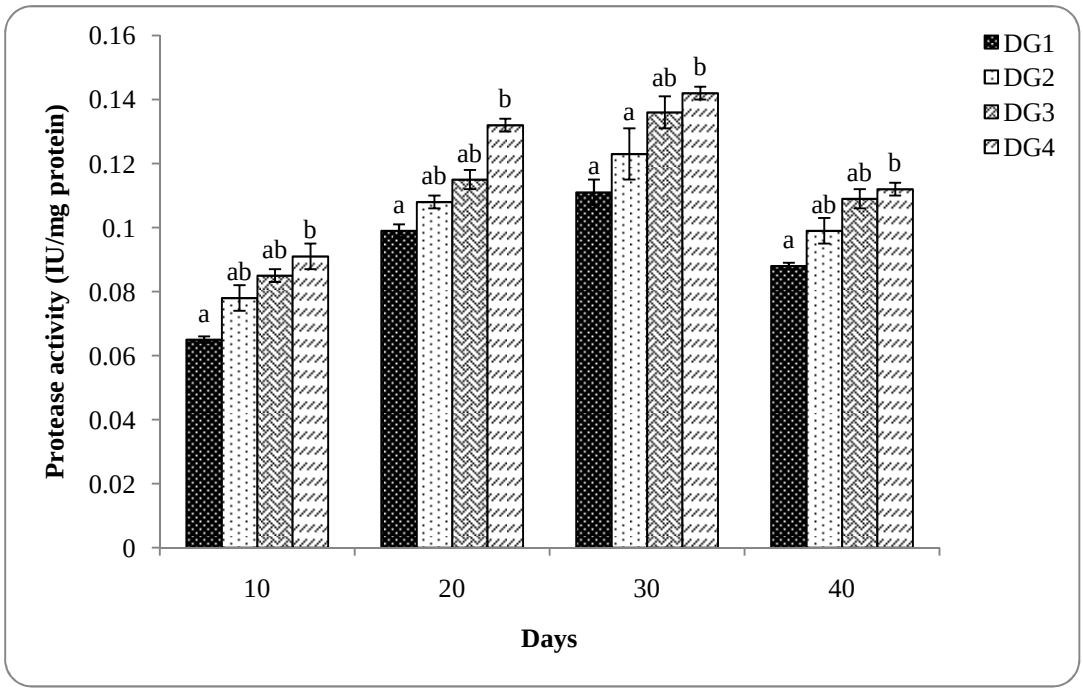
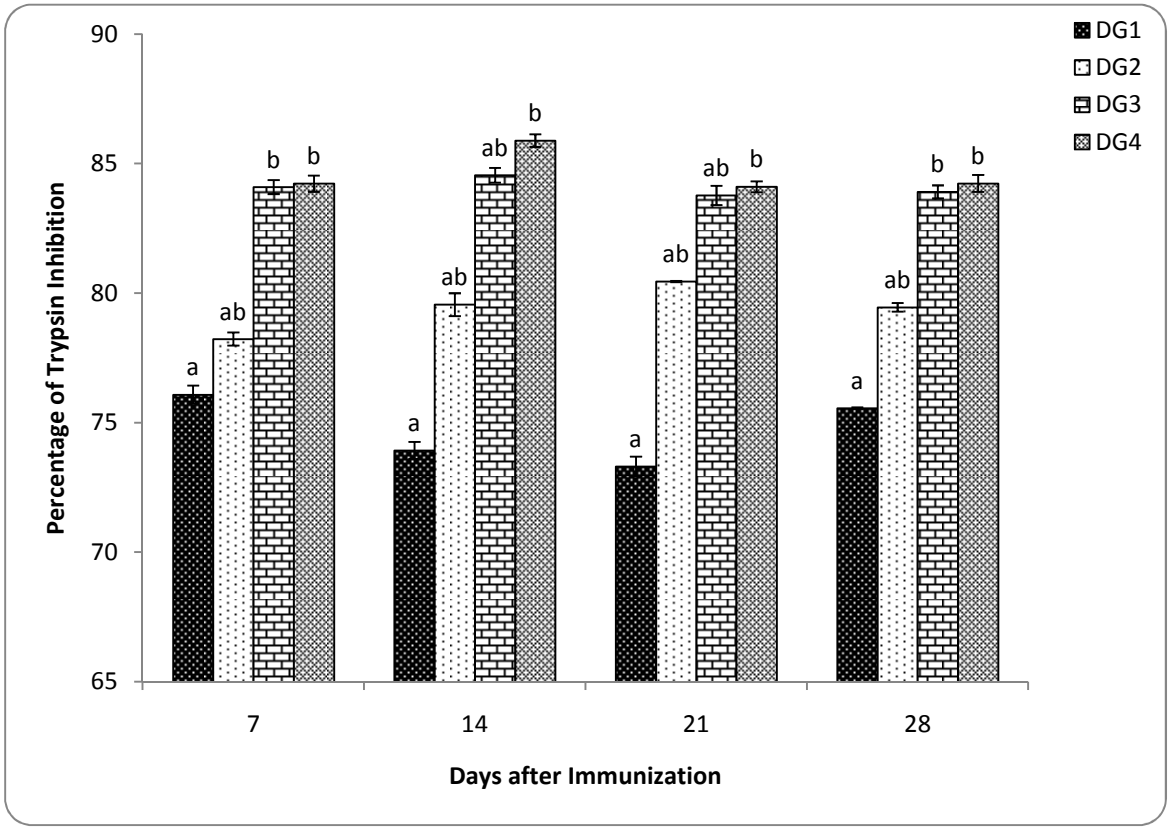


Fig: 2 Fish were immunized with rabbit RBC. Control groups were fed with normal diet and the experimental groups were fed with experimental diet. Four weeks prior to Immunization till the end of the experiment. Serum was collected from individual fishes for four weeks after immunization. The percentage of trypsin inhibition for each individual serum was determined. The values represented were the mean ± SE of four fishes ($P < 0.05$). DG1, DG2, DG3 and DG4 are represented diet group I, II, III and IV respectively.



DISCUSSION

The results of the present study clearly shows that dietary *C. dactylon* extract supplementation enhances the growth of *C. catla*. Kono et al. (1987) [14] experimented that the feeding of supplemented diet containing 10% chitin, chitosan or cellulose had not affected the growth of red sea bream, Japanese eel and yellow tail. Dietary chitosan and levamisole supplementation enhances the growth of common carp [15]. On the contrary, depressed growth in tilapia after feeding with chitin and chitosan at 2%, 5%, and 10% level were observed by Shiau and Yu (1999) [16]. In our results, net weight, average weight and specific growth rate were increased in the *C. dactylon* mixed diet groups. Similarly, Sandbrook and Hepher (1978) [17] proved that the better growth of fish fed on algae enriched diets than conventional diets. Possibly, the presence of herb *C. dactylon*, FCR value was also increased significantly ($P < 0.05\%$) in *C. catla* between the control and experimental diets. Likewise, increased FCR value found in *Sarathrodon niloticus* tilapia fingerlings fed with *Caldophora glomerata* incorporated experimental diet, which may due to the higher lysine content in the alga [18]. The high FCR in fish fed with DG3 and DG4 diets could also activate the absorption of diet ingredient in *C. catla* by fibre rich herbs. Fibre fraction defines extent and rate of feed digestibility [19]. SGR and FCR were significantly increased in DG3 and DG4 of experimental diet groups and attained 100% of SR in all experimental diet groups, which proved that the composition of diet is suitable for this experiment and the fish. The significant increase in SGR, FCR and 100% survival rate in the experimental groups can be attributed to the presence of higher essential amino acids in *C. dactylon* [20]. Significant responses were found in survival, growth, body composition, and digestive enzyme activity of white shrimp *Litopenaeus vannamei* by treating medicinal herbs and Bacillus [21]. High body condition scores and average daily growth observed in supplemented steers could also be due to high dietary protein and energy intake [19]. In our study, the diet composition of crude protein and lipid levels in all experimental diet groups were relatively similar, there is no significant variation found in the diet group. In the proximate value of crude protein and lipid composition were higher in DG3 and DG4 than other diet groups. Likewise, direct relationship between the amount of Spirogyra incorporated diet, and muscle protein and fat contents in *C. catla* were demonstrated by Harish (2004) [22]. Dietary lipids in aquafeeds are an important source of energy and essential fatty acids [23]. Inclusion of 5% Ulva meal at low and high lipid levels significantly improves the growth performance, feed efficiency, nutrient utilization, and body composition of Nile tilapia [24]. Optimum lipid levels results in improved growth rates, feed conversion ratios, nutrient utilization and reduced nitrogen excretion [25].

C. dactylon mixed diet treated *C. catla* were exhibited the increased level of specific activity of amylase and protease. Administration of MH and/or Bacillus bacteria to shrimps resulted in an increase in the specific activity of amylase and protease in the shrimps' digestive gland [21]. Our results were also found that the enhancement in growth parameters and specific activities of digestive enzymes of fish. The noticed changes in digestive enzymes may have led to enhanced digestion and increased absorption of feed, which in turn contributed to the progression in growth of fish. Medicinal herbs contained potent bioactive substances, which may influence digestive processes by enhancing or impairing enzyme activity and improving or diminishing digestibility of nutrients [26].

Principally, α1 protease inhibitor and α2 macroglobulin play a role in restricting the ability of bacteria to invade and grow in fish by acting against proteases from pathogenic organisms [27]. The present study revealed that the experimental diet containing *C. dactylon* has to develop the non specific immunity in *C. catla*. *C. dactylon* play a vital role in enhancement of serum antiprotease activity in all experimental days. This is in agreement with the observation of Rao

and Chakrabarti (2004) [13] that the feeding of *Catla catla* with *Achyranthes aspera* (0.5 %) mixed diet for 4 weeks enhanced the level of serum antiprotease, which might provide the resistance against the bacterial pathogens. Experimental result of Tafalla et al. (1999) [28], plasma bactericidal activity and total protein concentrations were considerably increased by oral administration of oxytetracycline to turbot *Scophthalmus maximus*. Magnadottir et al. (2006) [29] stated that the antiprotease activity was high in serum by immunization or infection. Similar result was also found in aqueous extract of *Eclipta alba* increased the serum antiprotease activity in *Oreochromis mossambicus* after 2 or 3 weeks of feeding [30]. Similarly, the antiprotease activity enhanced in rainbow trout by experimental diet containing taxanthin, a carotenoid from natural source (Carophyll pink) supplemented for 4 months [31]. When fishes were fed with *C. dactylon* mixed diet, the protease inhibitor levels were enhanced in *C. catla*. Thus, the results revealed that the fish can defend more strongly against invading pathogens.

The results of the present study clearly demonstrate that *C. dactylon*, a natural weed, can serve as an alternative replacement to the costly feed ingredients like deoiled groundnut cake, jowar powder and reduce the additional expenditure in disease management for the vaccine or immunomodulator/immunostimulator in the feed for *C. catla*. However, complete investigation is required in long-term feeding trials to assess the potential of *C. dactylon* and optimum dietary inclusion levels.

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