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Evaluation of *Ipomoea aquatica* Supplemented Feed on the Growth and Digestive Enzyme Activities of the Juvenile Climbing Perch, *Anabas testudineus* (Bloch, 1792)

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ABSTRACT

The potential of *Ipomoea aquatica*, a semi-aquatic weed, as a sustainable and cost-effective alternative to animal-based protein in the diet of *Anabas testudineus* is being evaluated in this study. Five isonitrogenous diets with varying percentage incorporation of *I. aquatica* (0%, 5%, 10%, 15%, and 20%) were fed to *A. testudineus* juveniles (mean initial weight 0.75 g ± 0.01 g) for 60 days to evaluate their effects on the growth performance and digestive enzyme activities of the fish. The results demonstrated that a 15% inclusion of *I. aquatica* (IA15) significantly enhanced growth parameters such as body mass gain, specific growth rate, feed conversion ratio, and feed efficiency compared to other diets. The optimal dose of *I. aquatica* inclusion levels ranged from 16.64 to 17.50%, as indicated by regression analysis. The digestive enzymes, viz. amylase, trypsin, total protease, pepsin, and lipase, showed significantly (P< 0.05) higher activities in fish fed with IA15 diet. These findings suggest that *I. aquatica* may be included in the feed of *A. testudineus* up to 15%, replacing the costly animal-based protein. *I. aquatica* enriched diets can lead to an optimal growth and improved feed utilization in the fish without adversely affecting the nutrient composition of the fish, providing a feasible alternative source of protein to enhance sustainability and economic viability in the aquaculture of the species.

INTRODUCTION

Anabas testudineus (Bloch, 1792), or the 'climbing perch,' is a commercially important and highly nutritious fish in the family Anabantidae. It is widely distributed in Asia, including India, and is known for its hardiness and tolerance to low-lying aquatic habitats such as marshlands, swamps, lakes, puddles, canals, and small pits **(Talwar & Jhingran, 1992)**. According to the IUCN Red List, *A. testudineus* is classified as 'Least Concern' due to its wide distribution and adaptability to various habitats **(Ahmad *et al.*, 2019)**. Known locally as *Kawai* (in Bodo), it is a highly preferred fish species in Assam in Northeast India for its superior taste and cultural significance **(Devi *et al.*, 2022)**. This fish is rich in digestible protein, amino acids, and omega-3 fatty acids. Its unique ability to breathe air allows it to thrive in these habitats, making it an important and essential species for local aquaculture. However, a significant obstacle to sustainable aquaculture is the high cost of fish feed production, which makes up 50-70% of the production cost **(Mukherjee *et al.*, 2010; Iskandar *et al.*, 2019)**. Its culture and production in the region remain significantly low compared to carp, primarily because of the expensive cost of producing feed, which relies on animal protein sources (**Devi *et al.*, 2022**). This financial strain has led to studies exploring alternative protein sources that are sustainable to substitute fishmeal in the diet of this particular species.

Fishmeal has traditionally been a common source of protein because of its high essential nutrient quality and digestibility. However, rising prices and diminishing supply owing to the increased demand by the ever-growing aquaculture industry have compelled aquaculture nutritionists to look for sustainable and cost-friendly alternatives (**Ali & Kaviraj, 2018**). Aquatic macrophytes are considered a viable substitute for fish meal in aquafeeds. The superior nutrient composition of aquatic weeds has resulted in partially or even wholly replacing fish meal in recent studies (**Debnath *et al.*, 2018; Ghosh *et al.*, 2021; Shrivastav *et al*., 2022**). *I. aquatica* is a semi-aquatic macrophyte with a potential application in fish feed.

I. aquatica is a tropical semi-aquatic plant that grows naturally in ponds, rivers, and lowlands across Asia. Often considered as a weed, this plant may be one of the possible substitutes in fish feed since it is high in minerals, vitamins, and trace elements Zn, Fe, Mn, P, Na, K, Ca, Mg, Cu, etc.), both essential and non-essential amino acids **(Austin, 2007; Adedokun *et al*., 2019; Ramzy *et al*., 2019)**. **Saikia *et al.* (2023)** reported that essential amino acids contribute about 60.4% of the total amino acids in *I. aquatica*, and lysine reached the highest value (2.141 g/100 g), followed by phenylalanine (1.891/100 g) and isoleucine (1.674 g/100 g). The bioactive phytochemicals present in the plant may provide optimal growth and health benefits to aquatic animals by enhancing their defence system **(Roy *et al*., 2022a)**.

Several studies have shown that incorporating *I. aquatica* into the diet positively affects fish growth. For instance, **Odulate *et al.* (2013)** reported better growth in *Clarias gariepinus*, and **Yousif *et al*. (2019)** observed positive outcomes in *Oreochromis niloticus* when fed with the plant-enriched diet. However, despite its high crude protein and lipid content, *I. aquatica* has been underutilized in fish feed formulations **(Mandal *et al.*, 2010)**. To date, most of the studies on *I. aquatica* are on cyprinids **(Ali & Kaviraj, 2018; Baruah *et al*., 2018)** and tilapia (**Manuel *et al*., 2020**; **Chepkirui *et al*., 2022)**, while limited works are available on carnivorous fish species (**Nandi *et al*., 2023**). This study is the first attempt to explore the potential for replacing animal-based protein in the feed of *A. testudineus* with this freely available semi-aquatic weed.

Few studies have explored some plant-based proteins as alternatives to fishmeal in the diets of *A. testudineus*. **Panchan *et al.* (2024)** reported successful replacement of up to 20% with soybean meal, while **Mishra (2013)** reported that *Azolla pinnata* protein positively impacted the growth of *A. testudineus*. These findings highlight the potential of plant proteins to reduce feed costs and support sustainable fish farming practices **(Naseem *et al.*, 2021)**. The *in vitro* digestibility of *I. aquatica* was reported the highest among four different plant proteins in *A. testudineus*, indicating its potential application in the diet of *A. testudineus* **(Devi *et al.*, 2022)**. For better aquacultural production of this species, developing cost-effective, nutritious, and digestible feed is crucial. This study, therefore, aimed to assess the impact of a diet supplemented with *I. aquatica* on the growth and digestive enzyme activities of *A. testudineus*. By providing comprehensive insights into the nutritional and economic benefits of using *I. aquatica* as a feed ingredient, this research seeks to enhance the sustainability and cost-efficiency of *A. testudineus* aquaculture, potentially benefiting farmers and the broader aquaculture industry.

MATERIALS AND METHODS

1. Feeding experimental unit

The feeding trial was performed at the wet laboratory facility of Bodoland University, Kokrajhar, Assam, India, for 60 days. A batch of 450 *A. testudineus* juveniles, with an average weight of 0.75 ± 0.01 g and a length of 4.1 ± 0.03 cm, was procured from the Bijni fish farm in Chirang, Assam, India. These were randomly distributed among 15 aquaria, each with a 50-liter capacity. For each treatment, 30 fish were stocked (in triplicate) within each aquarium. An inlet and outlet system were established in every tank to facilitate water aeration and renewal. Regular water temperature, dissolved oxygen levels, and pH assessments were made following the standardized procedures outlined in the 2017 APHA guidelines. Throughout the study period, the recorded temperature, dissolved oxygen, and pH values ranged from 26.4 to 27.7 °C, 6.50 to 7.42 mg L^{-1}, and 6.83 to 7.12, respectively.

2. Experimental diet

Samples of *I. aquatica*, collected from Kokrajhar, Assam, India, were initially air-dried in a shed until their moisture content was below 50%. Afterward, they were dried at 50°C in an oven, then crushed and filtered through a 1mm wire mesh. The level of *I. aquatica* incorporation in the diet was based on studies showing positive growth effects in various fish species, such as *Clarias gariepinus* **(Odulate *et al.*, 2013)**, *Oreochromis niloticus* **(Yousif *et al.*, 2019)**, and *Labeo rohita* **(Ali & Kaviraj, 2018)**. Five diets that are isonitrogenous (having 40% crude protein) were prepared in which varying percentages of *I. aquatica* were incorporated. These diets were designated IA0 (0%), IA5 (5%), IA10 (10%), IA15 (15%), and IA20 (20%) with 0, 5, 10, 15 and 20% *I. aquatica* incorporation, respectively, as shown in Table (1). The leaves and stems were used in this study due to their comprehensive nutritional profile, which includes essential minerals, vitamins, and amino acids **(Austin, 2007; Adedokun *et al.*, 2019)**. The feed ingredients were combined thoroughly, made into a homogenous dough by the addition of adequate water, and then forced through a 1mm mesh to form the pelleted diet. These extruded diets were then placed at 50 °C in an oven for drying and stored separately for feeding the fish. The fish were fed daily for 60 days @ 5% body weight twice daily, once at 09:00 a.m. and 04:00 p.m. To measure feed intake, any uneaten feed was collected and oven-dried at 50°C for one hour after feeding.

Table 1. The test diet's composition and primary components (as a percentage of dry weight)

Ingredients (%)	**IA0**	**IA5**	**IA10**	**IA15**	**IA20**
Wheat flour*	51.33	47.27	43.22	39.16	35.1
I. aquatica	0	5	10	15	20
Dry fish powder*	47.27	46.33	45.38	44.44	43.5
Cod liver oil[ψ]	1	1	1	1	1
Vitamin & mineral premix[ϕ]	0.4	0.4	0.4	0.4	0.4
Proximate analysis (%)					
Protein	39.94	39.98	39.57	39.85	39.95
Moisture	5.60	5.49	5.43	5.55	5.61
Ash	7.34	7.37	7.36	7.38	7.36
Fibre	1.86	1.85	1.91	1.95	1.94
Lipid	4.93	4.96	5.04	5.04	5.13
Carbohydrate	40.33	40.35	40.69	40.23	40.01
Energy (Kcal 100g^{-1})	365.45	365.96	366.4	365.68	366.01

*Local Market, Kokrajhar, Assam.

[ϕ]Vitamins: 5000IU Vitamin A, 500mcg Methylcobalamin, 400IU Vitamin D3, 150mcg D – Biotin USP, 75mg Ascorbic acid, 50mg Vitamin B3, 25mg Tocopheryl Acetate, 10mg Calcium D-Pantothenate, 5mg Vitamin B2, 5mg Vitamin B1, 1.5mg Folic Acid, 1.5mg Vitamin B6. Trace Elements: 2mg Copper Sulphate, 250mcg Chromium Picolinate, 70mcg Selenium, 25mcg Sodium Molybdate, 5mg Manganese Sulphate Monohydrate. Amino acid: 50mg L- Glutamic acid

[ψ]SEACOD, Cod Liver Oil (Type B) BP Universal Medicare, Mumbai, India.

3. Sampling and growth parameters

Throughout the experimental period, the fish length and weight were recorded weekly. After completing the 60-day trial, the fish underwent a 24-hour fasting period. Anesthetisation was performed using phenoxyethanol (Sigma, USA) at a concentration of 0.5 mL per liter **(Sankian *et al.*, 2018)**. Their final length (cm) and weight (g) were then measured. Several parameters related to growth were evaluated, such as body mass gain (BMG), specific growth rate (SGR), protein efficiency ratio (PER), survival rate (SR), feed utilization efficiency (FE), and feed conversion ratio (FCR) following the standard protocol **(Castell & Tiews, 1980)**.

4. Proximate composition

The diets and dry muscle tissue's proximate composition from all treatments were assessed using standard methods specified by **AOAC (2000)**. The total nitrogen was evaluated using the micro Kjeldahl method. Specimens were air-dried to measure their moisture levels using a hot oven set to 135°C for two hours. The extraction of crude fats was carried out utilizing petroleum ether, which was then succeeded by a Soxhlet extraction process. To assess the quantity of ash content, the specimens underwent incineration for 16 hours within a muffle furnace at 550°C. The crude fiber was quantified gravimetrically following chemical digestion and the removal of soluble components.

5. Digestive enzyme activity

5.1. Preparation of crude enzyme extract

After the 60-day trial period, entire digestive tracts were collected from fish after they were subjected to a 24-hour fast and anaesthetization. Fifteen fish per treatment were included by randomly selecting five fish from each of the three replicates. Dissections were performed in a chilled environment, and samples were homogenized (1:10 w/v, tissue: distilled water) using a mechanical tissue homogeniser. Following 10,000 × g centrifugation, the supernatants were collected and stored separately for further studies at -20°C.

5.2. Digestive enzyme assays

Digestive enzyme activities of a carbohydrase (amylase), four proteases (trypsin, chymotrypsin, total protease and pepsin) and lipase were evaluated following standard protocols. Amylase activity was determined following **Bernfeld's (1955)** method, utilizing 3, 5-dinitrosalicylic acid. The absorbance was measured at 540nm (Shimadzu 1900i, Japan) to estimate the specific amylase activity (mg maltose mg protein^{-1} h^{-1}). The activities of trypsin and chymotrypsin were analyzed using Nα-Benzoyl-L-arginine ethyl ester (BAEE) and N-Benzoyl-L-tyrosine ethyl ester (BTEE) as substrates respectively **(Bergmeyer, 1974)**. The activities of both enzymes were expressed as Units ml^{-1}. Total protease activity was estimated using azocasein as a substrate, following the method described by **Garcia-Carreno (1992)**, and the specific total protease activity was expressed as Abs (test-control)/mg protein in the reaction mixture per minute. Pepsin activity was determined using Anson's (1938) method of using haemoglobin as a substrate. Specific pepsin activity was calculated as Activity (Units/mg protein/min) = Abs (test-blank) ×1000/mg protein/min. Lipase activity was determined using **Winkler and Stuckman's method (1979)**, with p-nitrophenyl palmitate as the substrate. One enzyme unit was defined as one µM of p-nitrophenol released per minute from the substrate per mL of the crude extract. Total soluble protein was quantified using **Bradford**'s **(1976)** method, using bovine serum albumin as the standard.

6. Statistical analysis

Normality was evaluated using the Shapiro-Wilk test, and consistency of variances was investigated with Levene's test. Subsequently, differences among group averages were evaluated utilizing one-way ANOVA, with further analysis through Tukey's post hoc tests for significant differences, considering a *P*-value of less than 0.05 as substantial, all conducted in SPSS version 23. Regression analysis was done with a quadratic polynomial to determine the ideal level of *I. aquatica* in the diet for FCR and SGR. Data were presented as mean ± standard deviation.

RESULTS

1. Growth performance

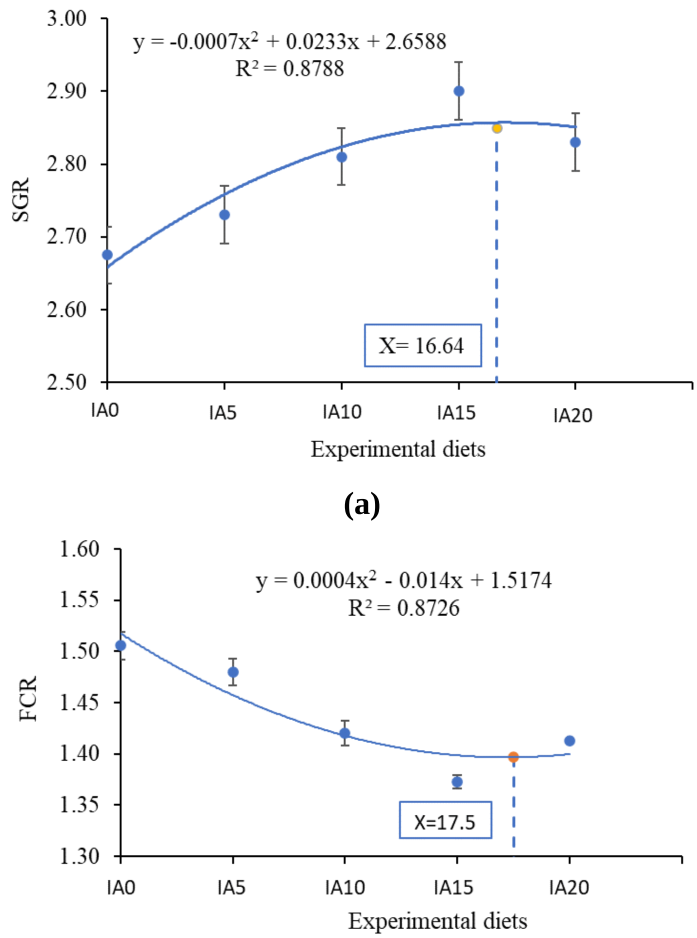
This study showed that fish fed with an *I. aquatica* incorporated diet showed enhanced growth performance than the control group. The IA15 diet-fed fish showed noticeably higher ($P< 0.05$) FW (4.27 ± 0.03g), BMG (469.78 ± 3.36%) and SGR (2.90 ± 0.01% day^{-1}) than other groups (Table 2). Throughout the experimental period, no mortality was observed in any of the groups. Additionally, the IA15 diet-fed fish also showed notably ($P< 0.05$) higher FE (%), whereas PER was significantly ($P< 0.05$) increased in IA15 and IA20 groups. The IA15 group had a significantly ($P< 0.05$) lower FCR than the other diet groups. The SGR and FCR polynomial regression analysis indicated that optimal fish growth occurs within the range of 16.64 to 17.50 (Fig. 1).

Table 2. The nutritional efficiency and growth performance of *A. testudineus* fed with varying percentages of *I. aquatica* diets. All data are represented as mean ± sd.

Parameter	IA0	IA5	IA10	IA15	IA20	P value
IW (g)	0.75 ± 0.01	0.75 ± 0.02	0.75 ± 0.01	0.75 ± 0.02	0.75 ± 0.01	
FW (g)	3.72 ± 0.02^{e}	3.86 ± 0.01^{d}	4.04 ± 0.01^{c}	4.27 ± 0.03^{a}	4.10 ± 0.01^{b}	< 0.001
BMG (%)	397.78± 2.04^{e}	414.67±1.33^{d}	439.11±1.54^{c}	469.78±3.36^{a}	446.67±1.33^{b}	< 0.001
SGR (% day^{-1})	2.67 ± 0.01^{e}	2.73 ± 0.00^{d}	2.81 ± 0.00^{c}	2.90 ± 0.01^{a}	2.83 ± 0.00^{b}	< 0.001

FCR	1.51 ± 0.01^{a}	1.48 ± 0.01^{b}	1.42 ± 0.01^{c}	1.37 ± 0.02^{e}	1.41 ± 0.01^{d}	< 0.001
Survival (%)	100	100	100	100	100	
FE (%)	66.42 ± 0.60^{d}	67.57 ± 0.58^{cd}	70.42 ± 0.58^{c}	72.84 ± 0.33^{a}	70.80 ± 0.09^{b}	< 0.001
PER	1.66 ± 0.02^{c}	1.69 ± 0.01^{bc}	1.76 ± 0.01^{b}	1.82 ± 0.01^{a}	1.77 ± 0.00^{a}	< 0.001

Note: Superscript letters denote statistically significant differences within a common row (n= 30; *P*< 0.05).



(b)

Fig. 1. Polynomial regression analysis based on (a) SGR and (b) FCR of *A. testudineus* fed with different % inclusion of *I. aquatica* in the diet

2. Proximate composition

The proximate composition analysis of dry muscle tissue of all the different trial groups is shown in Table (3). Crude protein ranged from 65.24 ± 0.01 to 65.92 ± 0.02% dry weight in all the groups. Lipid ranged from 8.22 ± 0.02^{d} to 8.56 ± 0.01%, while carbohydrate ranged from 1.12 ± 0.01 to 1.5 ± 0.02 % dry weight among all the groups. The IA15 diet-fed fish showed significantly (*P*< 0.05) higher protein (65.92 ± 0.02%), lipids (8.56 ± 0.01%), and ash content (10.06 ± 0.01%) in the study. However, no notable differences ($P < 0.05$) in the amount of fiber in any of the diets were observed. Compared to the other dietary groups, the moisture content of the IA0 group was considerably ($P < 0.05$) higher.

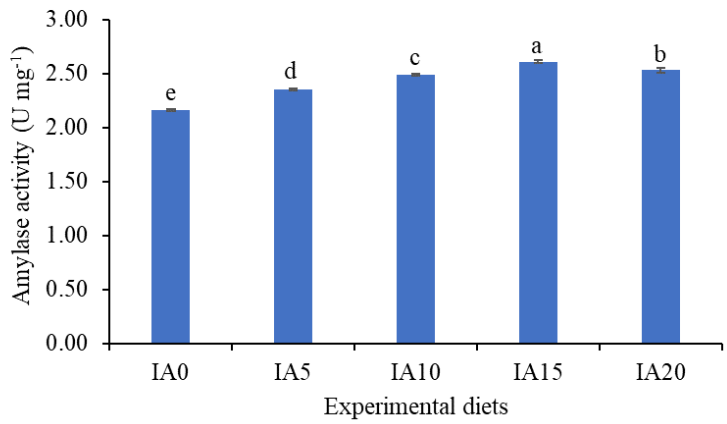
Table 3. Proximate analysis of *A. testudineus* (% dry weight basis) fed varying levels of *I. aquatica*-incorporated diet for 60 days (n=3).

Parameter	IA0	IA5	IA10	IA15	IA20	P value
Moisture	15.42 ± 0.02^{a}	15.15 ± 0.02^{b}	14.80 ± 0.01^{c}	14.23 ± 0.01^{d}	14.57 ± 0.02^{e}	< 0.001
Protein	65.24 ± 0.01^{e}	65.35 ± 0.02^{d}	65.56 ± 0.02^{c}	65.92 ± 0.02^{a}	65.67 ± 0.02^{b}	< 0.001
Lipid	8.22 ± 0.02^{d}	8.35 ± 0.02^{c}	8.42 ± 0.01^{c}	8.56 ± 0.01^{a}	8.46 ± 0.01^{b}	< 0.001
Ash	9.89 ± 0.02^{d}	9.89 ± 0.01^{d}	9.97 ± 0.01^{c}	10.06 ± 0.01^{a}	10.02 ± 0.01^{b}	< 0.001
Fibre	0.10 ± 0.01^{a}	0.10 ± 0.01^{a}	0.10 ± 0.01^{a}	0.11 ± 0.01^{a}	0.11 ± 0.01^{a}	0.737
Carbohydrate	1.13 ± 0.01^{bc}	1.15 ± 0.02^{ab}	1.5 ± 0.02^{ab}	1.12 ± 0.01^{bc}	1.17 ± 0.01^{a}	0.003

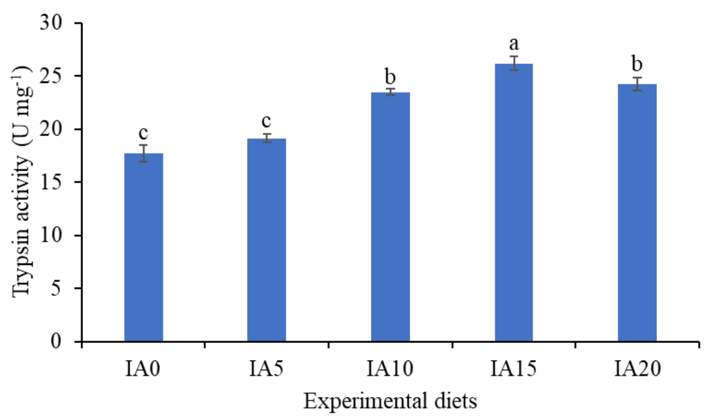
Note: Superscript letters denote statistically significant differences within a common row (n= 3, $P < 0.05$).

3. Digestive enzyme activity

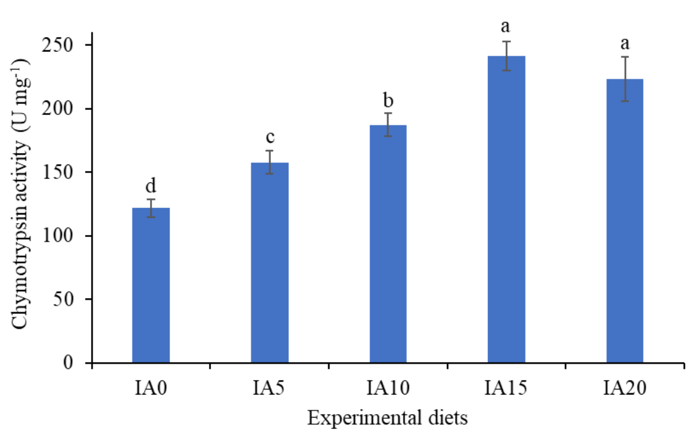
The digestive enzyme activities of *A. testudineus* fed with *I. aquatica* at different concentrations are shown in Fig. (2a- f). The activities of amylase, trypsin, chymotrypsin, pepsin, total protease and lipase were notably ($P < 0.05$) higher in all the plant-incorporated diet-fed fish compared to the control (IA0). Amylase activity ranged between 2.35 ± 0.01 to 2.61 ± 0.01 U mg^{-1} in the fish fed with *I. aquatica* supplemented diets (IA5, IA10, IA15, IA20), whereas the activity was 2.16 ± 0.01 U mg^{-1} in the control group (IA0). Trypsin activity was at its highest value in the IA15 group (26.16 ± 0.66 U mg^{-1}), while its lowest was recorded in IA0 (17.72 ± 0.77 U mg^{-1}). In addition, the same pattern was noticed in the chymotrypsin, pepsin, and total protease activity. Chymotrypsin activity ranged from 121.69 ± 6.80 U mg^{-1} (IA0) to 241.46 ± 11.54 U mg^{-1} (IA15). However, chymotrypsin activity between IA15 and IA20 groups showed no significant difference ($P > 0.05$). The pepsin activity was at its highest in IA15 (1233.95 ± 4.18 U mg^{-1}), while the IA0 group (1060.95 ± 8.46 U mg^{-1}) showed the lowest activity. The total protease activity was significantly higher among all plant-supplemented diet-fed fish, with the highest activity recorded in the IA15 group (1.93 ± 0.02 U mg^{-1}). Lipase activity was at its lowest in the IA0 group (33.35 ± 0.27 U ml^{-1}) compared to all the other treatments. Notably, the IA15-fed diet fish recorded higher amylase, trypsin, total protease, pepsin and lipase ($P < 0.05$) among all the treatments.


Amylase activity (U mg^{-1})
3.00
2.50
2.00
1.50
1.00
0.50
0.00
e
d
c
a
b
IA0
IA5
IA10
IA15
IA20
Experimental diets

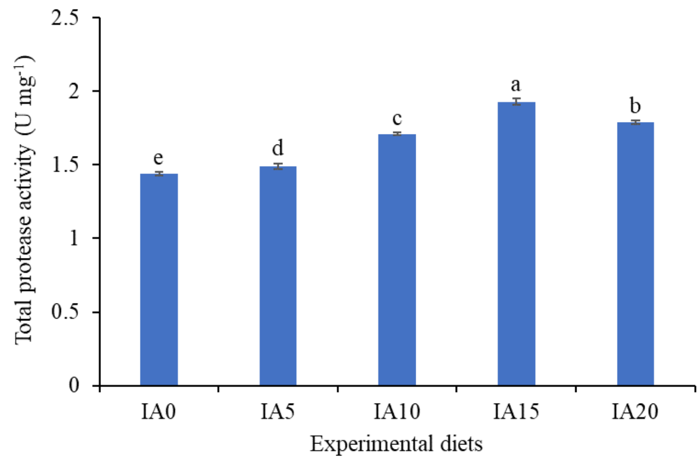
(a)


Trypsin activity (U mg^{-1})
30
25
20
15
10
5
0
c
c
b
a
b
IA0
IA5
IA10
IA15
IA20
Experimental diets

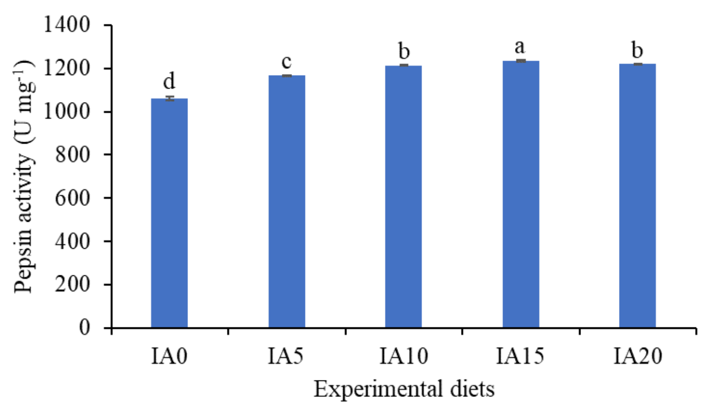
(b)


Chymotrypsin activity (U mg^{-1})
250
200
150
100
50
0
d
c
b
a
a
IA0
IA5
IA10
IA15
IA20
Experimental diets

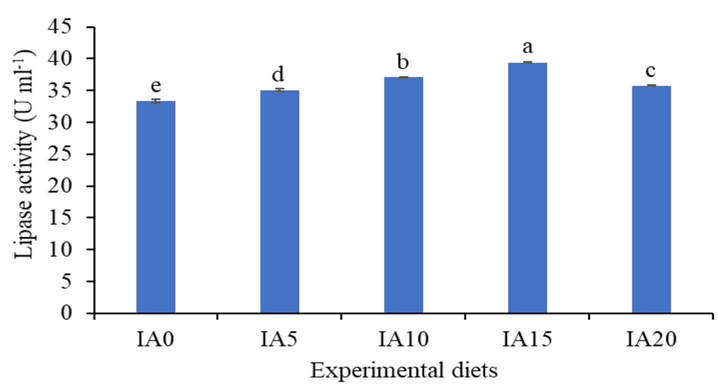
(c)



(d)



(e)



(f)

Fig. 2. Digestive enzyme **(a)** Amylase, **(b)** Trypsin, **(c)** Chymotrypsin, **(d)** Total protease, **(e)** Pepsin, and **(f)** Lipase activity of *A. testudineus* fed with varying levels of *I. aquatica* incorporated diet for 60 days. Different letters indicate statistically significant variations (n= 3, P< 0.05)

DISCUSSION

The results of this study show that the diet containing *I. aquatica* influenced the growth performance of *A. testudineus*, with the IA15 diet resulting in the best growth performance (Table 2). Similar observations were also reported in *L. rohita* when fermented *I. aquatica* was reported to successfully replace up to 25% for optimal growth (**Ali & Kaviraj, 2018**). The highest FW, BMG, and SGR were exhibited by fish fed with IA15 in this study. Our results also align with previous studies on other fish, where partially replacing with *I. aquatica* resulted in enhanced growth metrics **(Yousif *et al.*, 2019)**. Higher apparent protein digestibility of feed, WG, SGR, and lower FCR were also recorded in *H. fossilis* fed with 50% fermented *I. aquatica* leaf meal-supplemented feed (**Ali & Kaviraj, 2021).**

The significantly higher growth performance observed in the IA15 group can be attributed to the optimal nutrient provided by a 15% inclusion of *I. aquatica*. This inclusion level ensures a balanced supply of essential amino acids, vitamins, minerals, and bioactive compounds, which are crucial for the metabolic processes that support the growth and development of the fish. Several studies have indicated that *I. aquatica* contains high levels of essential nutrients such as protein, lipids, and minerals, which enhance growth performance **(Roy *et al.*, 2022a)**. The presence of bioactive compounds like flavonoids, saponins, and alkaloids in *I. aquatica* was found to improve immune function and overall health, leading to better growth rates **(Austin, 2007; Adedokun *et al.*, 2019)**. Lower values of FCR indicate efficient utilization of the feeds, which may minimize production costs and make feed production a sustainable process (**Martinez-Cordova *et al.*, 2016**). The lowest FCR observed in the IA15 group indicates better digestion and absorption of *I. aquatica* at this level of inclusion, suggesting better utilization of the diet for growth. Congruent to our observations, **Nandi *et al*. (2023)** also observed enhanced growth, reproductive parameters, and health status of *H. fossilis* when fed with a diet containing fermented water spinach (up to 50% replacement of fish meal). Similarly, a replacement of fishmeal up to 25% by *I. aquatica* in the Nile tilapia (*O. niloticus*) diet showed no negative impact on its growth performances and nutrient utilization **(Yousif *et al*., 2019)**. In addition, **Chepkirui *et al*. (2022)** reported the dietary incorporation of water spinach meal for improved growth performance in the Nile tilapia.

However, replacing fish meal with plant sources at higher levels can often lead to poor fish growth **(Abdel-Warith *et al*., 2013)**. This may be due to a deficiency of essential amino acids in most plants **(El-Saidy & Gaber, 2003; Furuya *et al.*, 2004)** or increased fibres or antinutrients present in them (**NRC, 2011)**. Moreover, lower dietary nutrient digestibility may also result due to microbial activities for fermentation of increased fibre in the lower gut of the fish, leading to reduced growth and a higher FCR **(Glencross, 2009)**. Similar observations were noted in our study, as the growth performance of *A. testudineus* declined at a higher inclusion level (IA20) of *I. aquatica*. This indicates the threshold level beyond which further inclusion may adversely affect the fish's growth and digestive enzyme activity. Higher inclusion levels of plant proteins can also interfere with nutrient absorption and metabolism in fish **(Francis *et al.*, 2001)**. Similar results were reported in *Clarias gariepinus* by **Odulate *et al.* (2013)** and in *O. niloticus* by **Yousif *et al.* (2019)** and **Manuel *et al.* (2020)** when fed with *I. aquatica* incorporated diet. Regression analysis is an effective technique for identifying the optimum dosage of feed supplements **(Yossa & Verdegem, 2015)**, which has been utilized in previous studies to determine the optimal inclusion of plant-based diets in fish **(Shekarabi *et al.*, 2022; Hossain *et al.*, 2023)**. The polynomial regression analysis in this study showed that the optimal inclusion level range is 16.64-17.50% for optimum growth performance of *A. testudineus*. The analysis of the carcass proximate composition showed no deterioration in the nutritional content of the fish among all the groups. This may be due to the efficient utilization of nutrients from the *I. aquatica*-incorporated diet, resulting in improved protein, lipid and ash contents in the plant-supplemented diet groups. Our results concur with the findings of **Ali and Kaviraj (2018)**, where *Labeo rohita* was fed *I. aquatica* in its diet.

One critical factor to consider when determining the efficiency of fish nutrient uptake is the digestive enzyme activity. Digestive enzyme activities are known to be significantly influenced by the ingested foods, diet composition, and feeding habits of the fish **(Almeida *et al.*, 2018; Goswami *et al*., 2020; Roy *et al*., 2022b**). Results of our study indicate a general trend of increase in all the digestive enzyme activity in *A. testudineus*, up to 15% incorporation of the *I. aquatica* and then a subsequent decline at higher (20%) inclusion. Compared to other diet groups, the IA15 diet showed a significant increase ($P < 0.05$) in amylase, trypsin, total protease, pepsin and lipase activities. Higher enzyme activity can enhance nutrient digestion and absorption, improving growth performance. The decline in enzyme activity at higher levels of plant inclusion (IA20) may be due to increased levels of fibers or antinutrients. Similar findings have been observed in earlier studies by **Manuel *et al.* (2020)**. The results of growth parameters are also in agreement with those of the digestive enzyme activities. Higher enzyme activities in IA15 might result in efficient FCR, leading to a better growth performance (SGR).

Amylase is a carbohydrase and is responsible for the conversion of complex polysaccharides into their simpler forms. The amylase activity increased with increasing % inclusion of *I. aquatica* in the diet by up to 15%, beyond which the activity declined. Among all the treatments, the IA15 diet-fed fish recorded the highest amylase activity ($P < 0.05$), probably in response to increasing plant incorporation in its diet, indicating the effective utilisation of carbohydrates in the diets. Higher amylase activity has been associated with a higher proportion of plant materials in its diet, such as in herbivorous and omnivorous fish (**Gioda *et al.*, 2017**). Better activity of amylase may result in efficient utilisation of carbohydrates for protein sparing effect, thereby making the proteins available for growth in the fish. Our results agree with those of **Ali and Kaviraj (2018**), where the enhanced activity of α-amylase was reported in *L. rohita* fed with a diet supplemented with 25% *I. aquatica* (fermented) leaf meal, beyond which the activity decreased at a higher level of inclusion (50 and 75%). However at higher (20%) incorporation of *I. aquatica*, the amylase activity showed a decreasing trend. This may be due to the increased inhibitory effects of certain phytochemicals present in *I. aquatica* on the activities of enzymes such as α-amylase and α-glucosidase **(Saikia *et al.*, 2023)** with increasing levels of incorporation of the plant in the diet. Higher lipase activity in the IA15 diet may be attributed to the essential fatty acids in *I. aquatica*, which result in the efficient utilisation of dietary fats for energy and fish growth **(Gisbert *et al.*, 2009)**. Similar findings were reported in *L. rohita* when fed with *I. aquatica*-incorporated diet (**Ali & Kaviraj, 2018)** and in *Heteropneustes fossilis* when fed with mulberry leaf by **Ali *et al*. (2019)** and soybean meal by **Khanom *et al*. (2022)**.

Activities of proteases, *viz.* total protease, serine proteases (trypsin and chymotrypsin), and acidic protease pepsin, showed similar trends in their activities in this study. *I. aquatica* supplemented diet resulted in the enhanced activities of these proteases in the fish. Enhanced protease activities may be indicative of the efficient digestibility of *I. aquatica* at this level of inclusion in the fish. Maximum activities of all these proteases were recorded in IA15, while activities declined at a higher (20%) inclusion, which suggests that the addition of the plant beyond 15% may not be beneficial for the fish. Enzyme activities at higher inclusion of plant proteins are often affected by factors such as the presence of anti-nutritional factors or increased dietary fiber **(NRC, 2011)**. Reduced digestibility of plants at higher levels may be due to an increased proportion of ash (mineral) and fiber in the diet **(Cruz-Velásquez *et al.*, 2014)** and the absence of cellulase enzymes in fish to hydrolyze cellulose, the main component of plant cell walls **(Ray *et al.*, 2012)**. The results of growth performance at the IA20 level are also consistent with that of enzyme activity. Improved protein digestion, as a result of enhanced trypsin and total protease levels, indicates their role in growth and muscle development **(Chamchuen *et al.*, 2014)**. Increased pepsin activity suggests an improved protein breakdown in the stomach, leading to better absorption of amino acids **(Buddington *et al.*, 1992)**. A similar rise in protease activity was also noted in *L. rohita* when fed with a diet containing fermented *I. aquatica* at 50% and 75% replacement levels **(Ali & Kaviraj, 2018)**. **Goswami *et al.* (2022)** reported enhanced protease (total protease, trypsin and chymotrypsin activity) in *Cyprinus carpio* fed with 15-20% inclusion of *Lemna minor* in its diet. Similar observations were also made by **Shrivastav *et al*. (2022)** in the juvenile common carp (*Cyprinus carpio*) when fed with *Spirodela polyrhiza* supplemented feed (up to 20%).

CONCLUSION

Overall, the findings of this study have demonstrated the potency of *I. aquatica* as an alternative for fish meal or animal-based protein in the diet of climbing perch, thereby reducing the cost of feed production. Further studies may be recommended to maximize its utilization by investigating ways to increase its level of supplementation through the application of biotechnological processes such as microbial fermentation, extrusion technology, thermal treatment, etc. The utilization of *I. aquatica* in the diet of *A. testudineus* at 15%, replacing a fish meal, improves the growth performance and digestive enzyme activity, resulting in better digestion and utilisation of the feed ingredients. The inclusion of the plant in the diet of climbing perch did not alter the carcass composition of the fish. This study has given useful insights into the application of *I. aquatica* in the feed of *A. testudineus* as a cost-effective alternative source of protein for the sustainable culture of the species.

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