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EFFECT OF DIETARY *Wolffia globosa* SUPPLEMENTATION ON GROWTH PERFORMANCE AND CAROTENOID CONTENT OF GOLDFISH (*Carassius auratus*)

Khushbu Kumari[1], Sagarika Swain[1*], Sarvendra Kumar[1], Ravi Shankar Kumar[2] and Abhiman[3]

[1]*Department of Fish Nutrition, Physiology and Biochemistry,* [2]*Department of Fisheries Economics, Extension and Statistics,* [3]*Department of Aquaculture, College of Fisheries, Kishanganj, Bihar Animal Sciences University, Patna, Bihar (India)*
*****e-mail: *swain_sagarika@ymail.com*

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ABSTRACT

In fish production, feed accounts for the highest cost, making it essential to explore natural, cost-effective, and locally available feed alternatives to ensure the growth and sustainability of ornamental aquaculture. In addition to its high protein content, *Wolffia* is a valuable source of carotenoids such as lutein, zeaxanthin, and β-carotene. The present study assessed the effect of *Wolffia globosa* supplemented diet on the growth and colouration of goldfish. The experiment was conducted in 15 tanks of 2000 L capacity each, containing 30 fish tank^{-1}. The study employed five treatments, each replicated three times, using a completely randomized design. The experimental duration was 90 days. The effects of four different doses (2.5, 5.0, 7.5, and 10%) of *W. globosa* supplemented diet were compared to a control basal diet (approximately 40% protein content). Results revealed that of the five test diets, the diet supplemented with 10% *W. globosa* powder showed significantly higher weight gain and specific growth rate (1.46 ± 0.01% day^{-1}). This supplemented diet revealed improved feed conversion ratio (1.74 ± 0.07) and protein efficiency ratio (1.44 ± 0.06) in fish compared to the control. In present study, the total carotenoid content of skin was higher than that of fish muscle and whole fish. The carotenoid content (µg g^{-1}) in skin (112.53 ± 0.01), muscle (32.73 ± 0.03), and whole fish (9.64 ± 0.06) was significantly highest in treatment T_4 (10% of *W. globosa* supplementation). Thus, it may be concluded that a diet containing 10% *Wolffia* powder is effective in enhancing growth and carotenoids in goldfish.

Keywords: Carotenoids, colouration, goldfish, growth performance, *Wolffia*

INTRODUCTION

Ornamental fish display a wide variety of colours and patterns, and their bright appearance plays a crucial role in their market value. Globally, colour is one of the most important factors influencing the price of aquarium fish (Saxena, 1994). With rising demand worldwide, the ornamental fish sector has expanded rapidly, with fishkeeping recognized as the second most popular hobby world-wide (Ghosh *et al.,* 2003). The European Union is the largest consumer of ornamental fish, and Indian species enjoy strong international demand. In India, the ornamental fish industry annually earns around Rs. 500 crore and is growing at an annual rate of 11.6%, driven largely by increasing urbanization (Shinoj, 2021). Ornamental fish farming offers meaningful livelihood support to rural youth and women, strengthening economic independence and encouraging social inclusion. Besides providing income, it also boosts the family happiness index by promoting gender equality, reducing labour migration, and contributing to the upliftment of marginalized groups (Bhattacharya *et al.,* 2025).

Among ornamental fish, goldfish *Carassius auratus* (family Cyprinidae) are a popular species that is easy to maintain, hardy, and readily available. It is also one of the earliest fish species domesticated for aquarium keeping. Its striking colour range, from bright orange to deep red, adds to its appeal among hobbyists (Mohanta *et al.*, 2008). The colour, pattern, and intensity of ornamental fish strongly shape customer preference and market value. Carotenoids serve as the primary pigments responsible for skin colouration in ornamental fish (Sinha and Asimi, 2007). However, fish that naturally display vibrant hues may experience colour fading when reared in crowded or intensive aquaculture systems (Pulcini *et al.*, 2020). Fish colouration is influenced by three major factors namely genetics, nutrition, and the nervous system. Carotenoid supplements can be natural or synthetic, but synthetic carotenoids may pose environmental risks (Kaur and Shah, 2017). Like other animals, fish cannot produce carotenoids on its own and must obtain them through their diet. A variety of natural carotenoid sources, including plant petals, flower extracts, bacteria, and microalgae, are commonly used to enhance colour, while also supporting growth (Maiti *et al.*, 2017). As synthetic additives are costly, recent research has focused on affordable and eco-friendly natural alternatives.

Wolffia sp. is considered a potential alternative protein source and a rich carotenoid source, as it possesses the desired characteristics such as wide adaptability to varied agro-climatic zones, easy of cultivation, low production cost, relatively small size, high biomass yield, high protein content (25-40%) with a favourable amino acid profile, relatively high lysine and methionine contents compared with other plant protein sources, and richness in β-carotene (Culley *et al.*, 1981; Appenroth *et al.*, 2018). The genus *Wolffia*, commonly known as watermeal/ duckweed, belongs to the family Lemnaceae. According to leaf size, duckweed is one of the smallest members of the Lemnaceae family measuring 0.4-1.3 mm and being oval-shaped and green with yellow patterns (Said *et al*., 2022). Numerous studies have reported the use of various duckweed species in aquaculture feed, particularly as dietary supplements to enhance growth in freshwater carps and tilapia (Yilmaz *et al.*, 2004). Additionally, it is more cost-effective than formulated feed and significantly reduces the risk of nutrient leaching. Hence, the present study aimed to assess the impact of a *Wolffia*-supplemented diet on growth and total carotenoid content in different tissue (skin, muscle, and whole fish) of goldfish.

MATERIALS AND METHODS

Preparation of Wolffia powder

Fresh culture of *Wolffia globosa* was collected from a clean pond, ensuring no contamination with unwanted plants or debris. It was rinsed thoroughly 2-3 times with clean water to remove dirt, and suspended particles. The washed *Wolffia* was spread on a clean mesh to completely drain the excess water and dried under mild sunlight for 1-2 days with a fine net cover to prevent contamination. Further drying was carried out using a hot air oven at 50-60°C for 4-6 h until fully dry. Dried *W. globosa* finely powdered using a grinder. The powdered *W. globosa* was stored in an airtight container and kept in a cool, dry place away from moisture and sunlight.

Experimental setup and feed formulation

This study was carried out at the wet laboratory of the College of Fisheries, Kishanganj, Bihar (India). The experiment was conducted to explore the inclusion level of *W. globosa* powder as an alternative protein source, along with its impact on the colour enhancement of goldfish (*Carassius auratus*). Goldfish, weighing 2.64 to 2.69 g, were procured from a local ornamental fish supplier. A 30 min $KMnO_4$ bath treatment @10 ppm was administered to disinfect the fish prior to stocking them into circular fibre-reinforced plastic (FRP) tanks of 2000 L capacity. Prior to the start of 90-day feeding experiment, fish were acclimatised to the laboratory conditions for 15 days. Thirty fish per tank were randomly distributed into five experimental groups, in triplicates, in a completely randomised design. Aeration was provided in each experimental unit. Experimental tanks were cleaned manually by

siphoning the water up to 15 to 20% along with faecal matter on a daily basis. Fish were fed with respective diets @ 5% body weight day^{-1}, divided into two equal feedings. Daily feeding rate was recalculated periodically based on the updated body weight of fish during the experimental period.

Five iso-nitrogenous (40% CP) and isolipidic diets, *viz*., diet 1 (T_0), diet 2 (T_1), diet 3 (T_2), diet 4 (T_3) and diet 5 (T_4), were formulated as under:

Ingredients (g 100 g^{-1} dry matter)	T_0	T_1	T_2	T_3	T_4
Fish meal	34	34	34	33	30
Soybean meal	24	23	23	22	22
Rice bran	13.98	14.98	16.98	15.98	16.98
Mustard oil cake	10	9	7	8	11
Wheat flour	5	6	6	8	7
Corn flour	10	7.5	5	2.5	0
CMC[a]	1	1	1	1	1
BHT[a]	0.02	0.02	0.02	0.02	0.02
VMM[a]	2	2	2	2	2.0
Wolffia powder	0	2.5	5.0	7.5	10.0

[a]CMC = Carboxymethyl cellulose; BHT = Butylated hydroxyl toluene; and VMM = Vitamin mineral mixture. The composition of VMM (kg^{-1}): Vitamin A - 55,00,000 IU; Vitamin D_3 - 11,00,000 IU; Vitamin B_2 - 2,000 mg; Vitamin E - 750 mg; Vitamin K - 1000 mg; Vitamin B_6 - 1000 mg; Vitamin B_{12} - 6 µg; Calcium pantothenate - 2,500 mg; nicotinamide - 10 g; choline chloride - 150 g; Mn - 27,000 mg; I - 1000 mg; Fe - 7500 mg; Zn - 5000 mg; Cu - 2000 mg; Co - 450 L-lysine - 10 g; DL-methionine - 10 g; Selenium - 50 ppm.

The basal diet was formulated using common ingredients like fish meal, soybean meal, mustard oil cake, rice bran, wheat flour, corn flour, CMC, BHT, vitamin-mineral mixture. All ingredients, in powdered form, were mixed thoroughly to form a homogenous blend, and an appropriate ratio of water was added to make a dough. The dough was cooked in an autoclave for 15 min. Then the dough was cooled down to room temperature by spreading it in a tray and vitamin-mineral mixture was added. *Wolffia* powder @ 2.5, 5.0, 7.5, and 10% were added separately, as per treatment, and mixed in basal feed. The control diet was maintained without *Wolffia* powder. The dough was extruded through a hand pelletizer.

Proximate analysis of ingredients and diets

Proximate analysis of ingredients and diets was done by standard methods (AOAC, 1995).

Moisture: Moisture content of experimental feed was estimated by placing pre-weighed sample in a petri-dish and drying it in a hot air oven at 100-105°C until it attained a constant weight. The weight difference was then calculated to record the percentage moisture content, using the following formula:

$$\text{Moisture (\%)} = \frac{\text{Initial weight of sample (g)} - \text{Dried weight of sample (g)}}{\text{Initial weight of sample (g)}} \times 100$$

Crude protein (CP): The nitrogen content of dried experimental diet was determined quantitatively by Kjeltec automated method (4800 Kjeltec Auto Distillation, Foss, Sweden) using titration to assess the nitrogen content. Crude protein content was estimated by multiplying the nitrogen content by 6.25.

Ether extract (EE): The ether extract (or crude lipid) of dried experimental diets was determined using Soxhlet apparatus (Borosil) with diethyl ether (boiling point 65±5°C) as solvent. The percentage of ether extract was calculated as follows:

$$\text{EE (\%)} = \frac{\text{Initial weight of sample(g)} - \text{Final weight of sample (g)}}{\text{Initial weight of sample (g)}} \times 100$$

Ash: The ash content in diet samples was determined by taking a known weight of dried samples in a silica crucible and heating them in a muffle furnace at 600°C for 5-6 h. The ash content was calculated as follows:

$$\text{Ash(\%)} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

Nitrogen-free extract (NFE): NFE was calculated by subtracting the all other nutrients (%) from 100.

Growth parameters

At the end of experiments, fish were starved overnight before taking the final weight using an electronic balance. The growth parameters *viz*., weight gain, specific growth rate, feed conversion ratio

and protein efficiency ratio were estimated as per the methods of Guillaumme *et al.* (2001).

Weight gain (WG, %): The WG % was determined using the following formula:

$$\text{WG \%} = \frac{\text{Final weight (g)} - \text{Initial weight (g)}}{\text{Initial weight (g)}} \times 100$$

Specific growth rate (SGR, % day^{-1}): Specific growth rate was determined using the following formula:

$$\text{SGR} = \frac{\text{Log}_e\text{Final weight} - \text{Log}_e\text{Initial weight}}{\text{Number of days}} \times 100$$

Feed conversion ratio (FCR): FCR was calculated at the end of feeding trial. Feed intake was determined based on the quantity of feed supplied to each tank during the experimental period, while weight gain was calculated as the difference between the final and initial body weight of fish. FCR was calculated using the following formula:

$$\text{FCR} = \frac{\text{Feed given (g)}}{\text{Increase body weight (g)}}$$

Protein efficiency ratio (PER): PER was calculated at the end of feeding trial. Protein intake was determined based on the quantity of protein supplied to each tank during the experimental period while weight gain was calculated as the difference between the final and initial body weight of fish.

$$\text{PER} = \frac{\text{Weight gain(g)}}{\text{Protein fed (g)}}$$

Feed intake was determined based on the quantity of feed supplied to each tank during the experimental period.

Dietary energy (DE): DE was calculated using following formula of Halver (1976)

$$\text{DE (kcal 100 g}^{-1}\text{)} = (\text{CL\%} \times 9) + (\text{\%CP} \times 4) + (\text{\%NFE} \times 4)$$

Collection of blood and serum

After 90 days of feeding trial, blood samples were collected from three randomly selected fish from each tank. Before sampling, fish were anesthetized using clove oil @ 50 µL L^{-1} water. Blood samples were drawn from the caudal vein of fish using a medical syringe pre-rinsed with 2.7% EDTA solution. The collected blood samples were immediately transferred to the test tubes containing 2.7% EDTA solution as an anticoagulant and gently mixed to prevent hemolysis. Besides blood serum protein, albumin, globulin and glucose assays, this blood was also used for nitroblue tetrazolium (NBT) assay (Stasiack and Bauman, 1996). Additionally, fresh blood was extracted from the caudal vein without using anticoagulants and transferred into a dried Eppendorf tube. It was left in a slanted position for 1 h at room temperature to allow clot formation. The clot was centrifuged at 10,000 rpm in a refrigerated centrifuge (REMI C-24 Plus, India) for 10 min. The resulting yellow straw-coloured serum was transferred precisely to a separate Eppendorf tube and kept at -20°C before analysis for serum protein, albumin, globulin and serum glucose.

Respiratory burst activity

The NBT assay was performed using the method of Stasiack and Baumann (1996). Blood samples (50 uL) were added to the wells of U-bottom microtiter plates and incubated at 37°C for 1 h to allow cell adhesion. The supernatant was then removed, and the wells were washed thrice with phosphate-buffered saline. Following the washes, 50 µL of 0.2% NBT solution was added to each well and kept for another 1 h. The cells were fixed with 100% methanol for 2-3 min and washed three times with 70% methanol. The plates were air-dried, and 60 µL of 2N KOH along with 70 µL of dimethyl sulfoxide (DMSO) were added to each well to dissolve the formazan blue precipitate. The absorbance of turquoise blue solution was recorded using an ELISA reader (Thermo Scientific Multiskan FC).

Serum protein

Serum protein content was estimated using biuret method with a total protein kit (Erba Diagnostic Mannheim, Transasia Bio-medicals Ltd., India). In this method, serum proteins react with copper ions

in alkaline biuret reagent, forming a purple-colored complex. The absorbance of this complex is directly proportional to the concentration of protein. Three test tubes labelled as blank (B), standard (S), and test (T) were prepared, and 1 mL biuret reagent added to each tube. Distilled water (20 µL), serum sample (20 µL), and protein standard (20 µL) were added to the blank, test, and standard tubes, respectively. The mixtures were thoroughly mixed and incubated at 37°C for 10 min. The absorbance of test sample and standard was measured against reagent blank using a microplate reader at 546 nm. Each sample was analyzed in triplicate, and protein concentration estimated by the following formula:

$$\text{Serum protein}(g/dL) = \frac{\text{Concentration of standard} * \text{absorbance of test (T)}}{\text{absorbance of standard (S)}}$$

Concentration of standard = 6 g dL^{-1}

Albumin and globulin

Albumin reacts with dye bromocresol green (BCG) in a buffered medium, forming a green-coloured complex. The intensity of colour depends on albumin concentration in the sample and is directly proportional to each other (Erba Diagnostic Mannheim, Transasia Bio-medicals Ltd., India). The albumin concentration was estimated by the following formula:

$$\text{Serum albumin (g } dL^{-1}) = \frac{\text{Concentration of standard} \times \text{Absorbance of test (T)}}{\text{Absorbance of standard (S)}}$$

Concentration of standard = 3.6 g dL^{-1}

Globulin (g dL^{-1}) was estimated by subtracting serum albumin content from total protein in serum.

Serum glucose

Serum glucose level was measured as per Trinder's method using a kit (Erba Diagnostic Mannheim, Transasia Ltd., India). In this method, glucose in serum sample is oxidized in presence of glucose oxidase, producing gluconic acid and hydrogen peroxide. The reaction forms a coloured complex, with absorbance proportional to the glucose concentration. For assay, 1 mL enzyme reagent was added to both test sample and standard tubes. The reaction mixtures were incubated at 37°C for 10 min, and absorbance of test sample and standard measured against a reagent blank using a spectrophotometer (ThermoFisher, GENESYS 1XX) at 505 nm. Each sample was analyzed in triplicate, and glucose concentration was determined by the formula:

$$\text{Glucose}(mg/dL) = \frac{\text{Absorbance of test sample}}{\text{absorbance of standard}} \text{ x N}$$

Where N is the concentration of standard (100 mg dL^{-1})

Total carotenoid content

The total carotenoid content was analysed using the method of Olson (1979). Red-coloured muscle tissue (excluding the head and alimentary canal), skin, and whole fish (1 g) was placed in a 10 mL screw-capped clear glass vial. To this, 2.5 g anhydrous sodium sulphate was added. The sample was then gently mashed with a glass rod against the side of vial. Then 5 mL chloroform was added into the vial and mixture left to incubate overnight at 0°C. When a clear 1-2 cm layer of chloroform formed above the caked residue, the optical density was measured at 500 nm spectrophotometerically. Aliquots of 0.3 mL chloroform were taken and diluted to 3 mL with absolute ethanol. A blank was prepared similarly for comparison. The wavelength at which the maximum absorption was recorded was used for the subsequent calculations. The total carotenoid content was calculated as µg per wet weight of tissue as follows:

$$\text{Total carotenoids content} = \frac{10 \text{ x absorption at maximum wavelength}}{0.25 \text{ x Extinction coefficient}}$$

Where, 10 is Dilution factor, 0.25 is factor for extinction coefficient

Statistical analysis

The data obtained was statistically analyzed by using a suitable statistical method (SPSS 22.0). Analysis of variance (one-way ANOVA) was performed to determine the significant differences

between the mean values of different treatments. Duncan's test was used for post hoc comparison of means ($p < 0.05$) between different groups.

RESULTS AND DISCUSSION

Proximate composition of Wolffia globasa and experimental diets

The proximate composition of *W. globosa* on dry basis revealed moisture content of 95.65 ± 0.12%, crude protein 26.71 ± 1.86%, ethyl extract 6.53 ± 1.58%, nitrogen free extract 36.42 ± 3.99%, ash 17.0 ± 1.52%, crude fibre 13.33 ± 1.66% and dietary energy of 311.33 ± 3.17 kcal 100 g^{-1}.

The proximate composition of experimental diets (Table 1) revealed that dry matter content in diets ranged from 90.07 ± 0.71 to 91.33 ± 0.88%, crude protein from 39.96 ± 0.32 to 40.36 ± 0.12%, and ether extract from 6.0 ± 0.38 to 6.88 ± 0.58%. Crude fibre ranged from 9.33 ± 0.73 to 10.73 ± 0.13%, ash content from 11.83 ± 1.16 to 13.33 ± 0.33%, and total nitrogen-free extract from 29.38 ± 1.06 to 31.09 ± 0.36%. The calculated digestible energy (DE) of diets was found in the range of 339.7±5.14 to 342.2±5.59 kcal 100 g^{-1}. The minimal variation in protein and energy contents among the diets confirmed that the experimental diets formulated were isonitrogenous and nearly isoenergetic, thereby ensuring that observed differences in fish performance could be attributed primarily to the dietary inclusion levels of *W. globosa* powder.

Table 1: Proximate composition of experimental diets (% DW basis)

Treatments	T_0	T_1	T_2	T_3	T_4
Dry matter	91.33 ± 0.88	90.27 ± 0.63	91.33 ± 0.33	90.43 ± 0.72	90.07 ± 0.71
Crude protein (CP)	40.04 ± 0.36	39.96 ± 0.32	40.36 ± 0.12	40.23 ± 0.26	40.00 ± 0.05
Crude lipid (CL)	6.44 ± 0.22	6.44 ± 0.58	6.00 ± 0.38	6.88 ± 0.44	6.88 ± 0.58
Crude fibre (CF)	10.00 ± 1.03	9.33 ± 0.73	10.73 ± 0.13	10.33 ± 0.46	10.20 ± 0.70
Ash	12.67 ± 0.16	13.17 ± 0.16	11.83 ± 1.16	13.17 ± 0.44	13.33 ± 0.33
NFE[a]	30.85 ± 1.08	31.09 ± 0.36	31.07 ± 1.40	29.38 ± 1.06	29.58 ± 0.67
Digestible energy[b]	341.60 ± 2.77	342.2 ± 5.59	339.7 ± 5.14	340.4 ± 3.06	340.30 ± 4.38

[a]NFE - Nitrogen free extract; [b]Digestible energy (kcal 100 g^{-1}) was calculated using the following formula: = (CL% × 9) + (%CP × 4) + (%NFE × 4)

Growth performance

The mean initial body weight and mean final body weight varied from 2.64 ± 0.01 to 2.69 ± 0.03 g, and 6.94 ± 0.07 to 9.84 ± 0.08 g, respectively. During 90-days of experimental period, the fish exhibited weight gain ranging from 162.87 to 272.75% (Fig. 1). The weight gains among the treatments differed significantly ($p < 0.05$) with highest weight gain in T_4 treatment, followed by T_3, T_2, and T_1, and lowest weight gain in control treatment (T_0) (Fig. 2). The average specific growth rate (SGR) of goldfish differed significantly ($p < 0.05$) among the treatments with highest SGR (1.46 ± 0.01% day^{-1}) in treatment T_4. The present findings align with Chareontesprasit and Jiwam (2001) who effectively used various high-protein aquatic plants such as *Azolla*, *Lemna*, and *Wolffia* as supplementary feeds. The outstanding growth observed in this study indicates that the presence of growth-promoting factors in *W. globosa* may have directly or indirectly

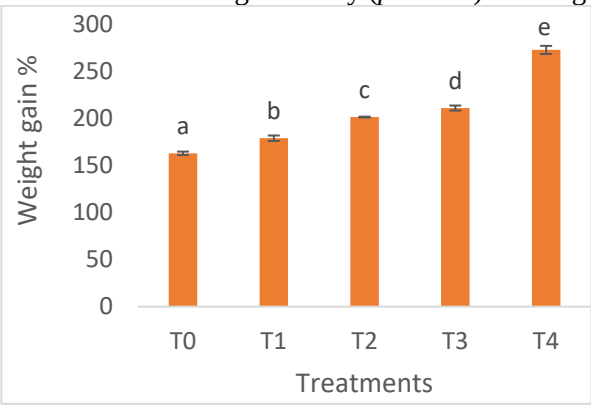

Fig. 1: Weight gain % of goldfish fed with different doses of *Wolffia* incorporated diets

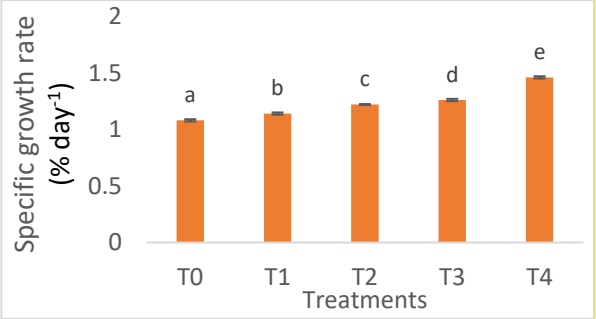

Fig. 2: Specific growth rate of goldfish fed with different doses of *Wolffia* incorporated diets

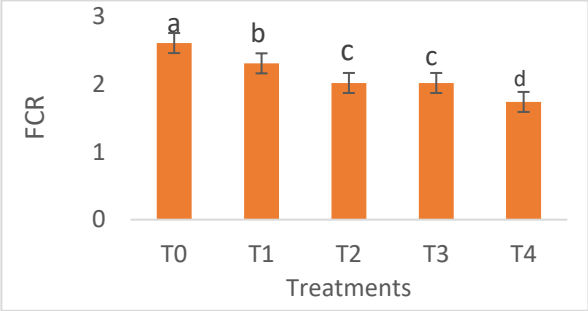

Fig. 3: Feed conversion ratio of goldfish fed with different doses of *Wolffia* incorporated diets

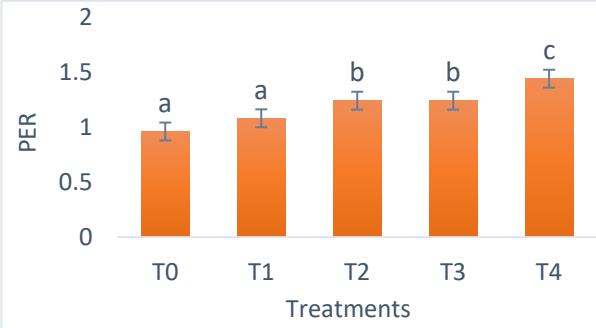

Fig. 4: Protein efficiency ratio of goldfish fed with different doses of *Wolffia* incorporated diets

enhanced the overall growth performance (Pradhan *et al*., 2019). Gogoi *et al*. (2018) observed similar results on dietary supplementation of *Wolffia arrhiza* on growth performance of *Botia dario*, while Jayakumar and Swaminathan (2023) reported growth improvement when goldfish (*Carassius auratus*) was supplemented with 9% *Wolffia* dried powder. Sein *et al*. (2020) observed the better growth of Indian major carp (*Labeo rohita*) fingerlings when fed *Wolffia* (10 g)-supplemented diet.

The feed conversion ratio (FCR), an essential metric in aquaculture, measures the efficiency of feed utilization for unit weight gain in the cultured species. A lower FCR not only indicates improved growth efficiency but also reflects reduced environmental impact, as it minimizes nutrient losses that could lead to eutrophication, biodiversity loss, and disruption of ecosystem services (Waite *et al*., 2014). FCR in present study differed significantly among the treatments, except between T_2 and T_3 treatments, with lowest FRC (1.74 ± 0.07) in T_4 treatment (Fig. 3). Similar findings have been also observed by Pradhan *et al*. (2019) when live *Wolffia*-supplemented diet was fed to *Labeo rohita*. Bandyopadhyay *et al*. (2005) reported similar growth improvements when formulated diet was fed to the goldfish, *Carassius auratus* against commercial feed.

The protein efficiency ratio (PER) was significantly highest (1.44 ± 0.06) in T_4 treatment, while no significant differences ($p > 0.05$) were observed between treatments T_0 and T_1, as well as between T_2 and T_3 (Fig. 4). These results are consistent with Bandyopadhyay *et al*. (2005) who reported a PER range of 1.21 ± 0.01 to 1.74 ± 0.02 for goldfish. The lower FCR and higher PER observed in this study underscore the potential of *W. globosa* as an economical and efficient feed ingredient, contributing to the sustainability and profitability of ornamental fish production. The remarkable growth performance of goldfish fed with *W. globose*-incorporated experimental diets, observed in present study, compared to the control diets may be attributed to its rich and unique nutritional profile.

Carotenoid content in goldfish

The carotenoid content in skin, muscle, and whole body of goldfish across the treatments is given in Fig. 5. Treatment T_4 yielded significantly highest ($p < 0.05$) carotenoid content of 112.53 ± 0.01 µg g^{-1} in skin, while lowest value was observed in control T_0 (52.17 ± 0.01 µg g^{-1}). The carotenoid content in muscle ranged from 5.03 ± 0.01 µg g^{-1} in T_0 to 32.73 ± 0.03 µg g^{-1} in T_4 treatment. Similarly, in whole fish the carotenoid content was highest in T_4 (9.64 ± 0.06 µg g^{-1}) and lowest in T_0 (2.85 ± 0.01 µg g^{-1}). The result demonstrated significant variation in carotenoid deposition across the treatments, and T_4 treatment showed superior carotenoid levels. In present study, the total carotenoids content of

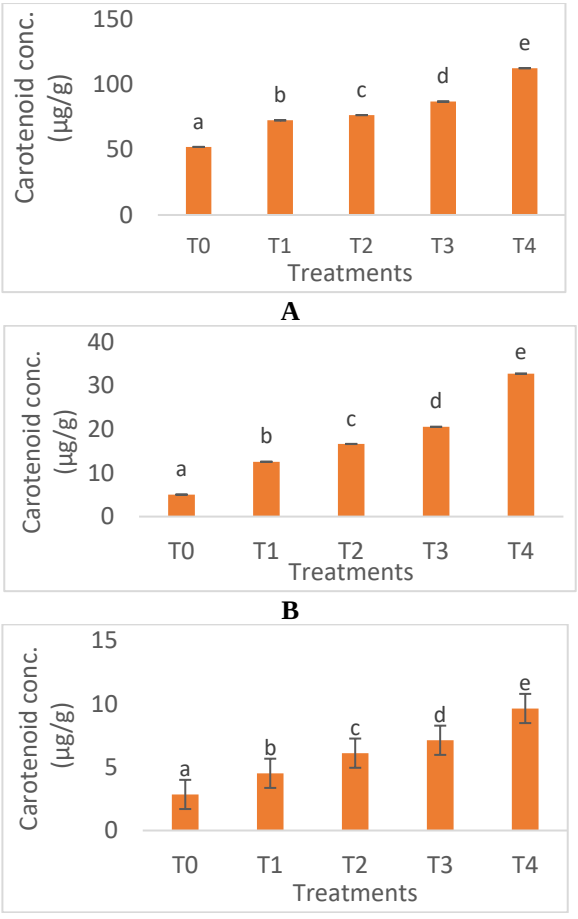

Fig. 5: Effect of different *Wolffia* concentration on total carotenoid content in A) skin; B) muscle, and C) whole body of goldfish

skin was highest in comparison to fish muscle and whole fish. These findings are in conformity with Gogoi *et al*. (2018) who reported similar observations in *Botia dario* by substituting *Spirulina* with *Wolffia*. Chantiratikul *et al*. (2010) found that skin pigmentation of broilers increased with increasing inclusion of dietary *Wolffia* meal. Tiewsoh *et al*. (2019) reported that carrot powder supplementation in goldfish diet increases total carotenoid content compared to control diet. The reason behind low carotenoid content in fish muscle compared to fish skin y may be attributed to the transport of assimilated carotene from muscle to skin and fins for necessary skin pigmentation (James *et al*., 2006). In salmon, Arctic char and trout, the pigmentation of integument and fins occurs only during sexual maturation. The reduced muscle carotenoids indicate that carotenoids might be assembled directly to the integument and fins from the muscle during breeding season. These phenomena may be due to reductive metabolic pathways from the muscle to the fins and skin.

Effect on serum biochemical parameters

Significant variations ($p < 0.05$) were observed in total serum protein among different dietary treatments (Table 2), with highest serum protein in T_3, which was statistically comparable to T_2 and T_1, but significantly higher than T_0 and T_4. The lowest serum protein was noticed in T_4. Serum globulin levels showed significant differences among the treatments. Globulin levels are commonly associated with immune competence, as globulin fractions include immunoglobulins and other immune-related proteins. The highest globulin content was noted in T_2 and T_0, which were statistically similar, and lowest in T_4 treatments. Serum albumin concentration was significantly higher in T_3 and T_4 compared to T_0. Overall, dietary treatment T_3 resulted in improved serum protein profile, particularly total protein and albumin concentration, indicating enhanced physiological and metabolic status of fish. Increased serum protein levels are generally associated with better nutrient utilization, efficient digestion, and improved health condition. Enhanced total protein may reflect improved liver function, as liver is the primary site of plasma protein synthesis. Serum glucose is widely considered a reliable indicator of stress and metabolic disturbance in fish. The serum glucose was significantly low in T_4 group compared to the rest treatments (Fig. 6) which suggests reduced physiological stress and better metabolic homeostasis at this inclusion level. Hyperglycemia in fish is commonly associated with stress responses mediated by cortisol and catecholamine release. Therefore, reduced glucose concentration in T_4 revealed better stress tolerance and efficient energy use. Possibly, the optimal inclusion of *W. globosa* improved carbohydrate metabolism or enhanced insulin-mediated glucose uptake. Pradhan *et al*. (2019) have reported that improved levels of plasma protein, blood glucose in *Wolffia*-fed group is an indicator of better health status of *Labeo rohita* fry.

Table 2: Serum protein albumin and globulin levels of gold fish fed with *Wolffia*-based diets

Treatments	Serum protein (g dL^{-1})	Globulin (g dL^{-1})	Albumin (g dL^{-1})
T0	$5.91^{a} \pm 0.04$	$2.56^{b} \pm 0.05$	$3.35^{a} \pm 0.08$
T1	$6.09^{ab} \pm 0.08$	$2.39^{ab} \pm 0.18$	$3.63^{ab} \pm 0.11$
T2	$6.25^{b} \pm 0.04$	$2.59^{b} \pm 0.19$	$3.66^{ab} \pm 0.22$
T3	$6.33^{b} \pm 0.11$	$2.32^{ab} \pm 0.08$	$4.01^{b} \pm 0.03$
T4	$5.85^{a} \pm 0.73$	$2.00^{a} \pm 0.02$	$3.90^{b} \pm 0.01$

Mean values with different superscripts in the same column differ significantly ($p < 0.05$). The data is given as mean ± S.E., n = 3.

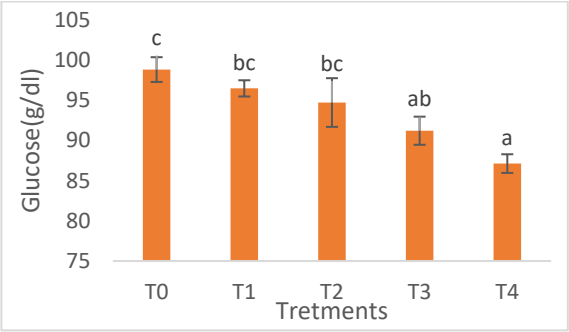

Fig. 7: Effect of different *Wolffia* on serum glucose level of goldfish

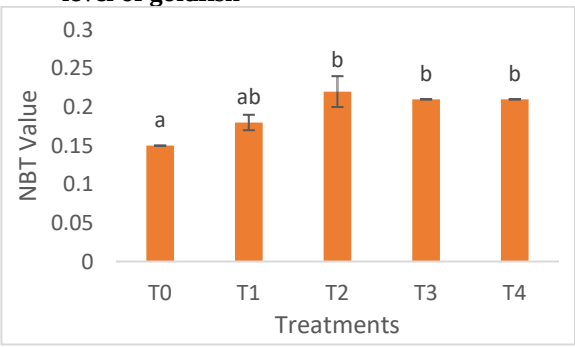

Fig. 6: Effect of different *Wolffia* on NBT activity of goldfish

Effect on immunological parameter

Dietary inclusion of *W. globosa* significantly influenced innate immune response and metabolic status of *Woffia* as evidenced by enhanced nitro blue tetrazolium (NBT) activity. All experimental *Wolffia* fed groups showed significantly elevated NBT activity compared to the control ($p < 0.05$), thereby indicating enhanced respiratory burst activity in gold fish (Fig. 9). NBT reduction reflects the production of reactive oxygen species (ROS) by neutrophils and macrophages during pathogen defense. Enhanced respiratory burst activity suggests improved non-specific immune response, which is a primary defense mechanism in fish. The bioactive compounds, antioxidants, essential amino acids, and micronutrients present in *Wolffia* may have contributed to the activation of immune cells. Plant-based functional feed ingredients are known to enhance macrophage activity and oxidative killing capacity, thereby improving disease resistance and overall health status. The immunostimulatory effect observed aligns with previous findings where phytogenic or plant-derived feed additives enhanced innate immunity in ornamental and cultured fish species. Improved NBT activity indicates that *W. globosa* supplementation may strengthen the fish's ability to combat infectious agents without inducing pathological stress. The NBT activity was significantly affected in *Labeo rohita*; fed with floating weed *W. globosa* as an inorganic extractive and Nath *et al*., 2021observed similar phenomena when bivalve *Lamellidens marginalis* fed with *W. globosa* as an organic extractive.

Conclusion: Incorporating *Wolffia globosa* into ornamental fish feed offers a promising, eco-friendly alternative to synthetic carotenoids. This aquatic plant is easy to culture due to its rapid doubling time, low production cost, and high adaptability to various water conditions. Its inclusion in fish diets not only reduces overall feed costs but also enhances the colouration of ornamental fish and meets their nutritional requirements. Therefore, *W. globosa* can serve as a sustainable, low-cost source of natural carotenoids and nutrients. This approach can empower farmers by enabling them to produce high-quality ornamental fish at competitive prices, helping them thrive in the market.

Author contributions: KK participated in sampling and data analysis, SS conceptualized the study, designed the experiments, and imparted overall guidance. SK was responsible for feed formulation and preparation, and contributed to manuscript editing. Abhiman contributed to the feeding trial and manuscript editing. RSK carried out the statistical analysis.

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