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Colour Enhancement in Rosy Barb (Pethia Conchonius) Through Dietary Natural Carotenoid Supplementation

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Abstract
Around the world, it is highly usual to keep ornamental fish in glass tanks because of their vibrant colors and ability to naturally reduce tension. Because ornamental fish are unable to produce their own color-producing carotenoid pigments, they must rely on natural or synthetic carotenoids in their food to produce their color pigmentations. Total Carotenoids in the tissues of rosy barb (both experimental and control) were analysed. Among the five experimental diet, the maximum amount of total carotenoid was found in Feed D5 (1.7330±0.0151 µg/g), D2 (1.6346±0.0201 µg/g) and D4 (1.3666±0.0293 µg/g) and the minimum value (0.2785±0.0139 µg/g) was recorded in D1. The rosy barb fed with D5 diet revealed maximum retention of carotenoids. Carotenoids were isolated from the control and experimental group fish tissues at the end of the 10th week using column chromatography with silica gel as the stationary phase and wet packing technique. In HPLC technique, the retention time and absorption spectral characteristics of carotenoid isomers were used in identifying the unknown peaks in the samples. The results shows, the carotenoid standards, including E-Violaxanthin, 9'-Z Neoxanthine, E-Lutein, E-Zeaxanthine, E-β-Cryptoxanthine, α-Carotene and β-Carotene. The carotenoid content was estimated from five different experimental group fish tissues. The samples were analysed using C30 column on HPLC. In D1, D3 and D5 group, E-Violaxanthin and E-Zeaxanthine as the principal carotene were present. There was no carotenoid content was observed in D2 group fishes. In D4 group, E-Violaxanthin (6.814 Rt) as the principal carotene were present. In addition to highlighting the potential of natural components like beet root and spirulina for integration and development of feeds for rosy barb color improvement, the study underscores the significance of carotenoids in the commercial rosy barb business.
Keywords: *Beet root, Carotenoids, HPLC, Ornamental fish, Spirulina*

INTRODUCTION

The most prevalent class of pigments found in nature are carotenoids, which are lipid-soluble pigments with conjugated double bonds. There are around 750 different types of carotenoids known to exist. Carotenoids are naturally occurring pigments that dissolve in fat. They are made from the backbone of a 40-carbon polyene chain, which has functional groups that contain oxygen and cyclic end groups. Conjugated double bonds in the hydrogen backbone of carotenoids give them their color (Bendich and Olson, 1989). They are found in every family of plants and animals. Animals must get them from their diet because only microbes, fungus, and plants can synthesis them from scratch. Because of their biological properties and lack of cytotoxicity or adverse effects, carotenoids have been employed extensively in the food, feed, pharmaceutical, and cosmetic sectors. They also demonstrate strong antioxidant activity (Khachik 2006; Maoka 2015; Lim et al. 2018; Britton et al. 2020). Mammals' carotenoids have been linked to a number of advantageous biological benefits through epidemiological research and clinical trials. Additionally, carotenoids have a number of physiological impacts, including those on the immune system, reproduction, lipid metabolism, skin photoprotection, cardiovascular disease, hypertension, atherosclerosis, cancer, and inflammation of the skin (Nakano et al. 1999; Nakano 2003; Maoka 2015; Zhang and Wnag 2015; Maoka 2020; Zheng et al. 2020). According to recent estimates, the global market for carotenoids is worth over $1.5 billion USD (Sathasivam 2018; Nakao and Sakata 2019).

It is well known that carotenoids are important for fish health. According to De Carvalho and Caramujo (2017), carotenoids influence fish development, performance, and general health in addition to providing consistent pigmentation. These factors have an impact on important fish production characteristics. Carotenoids are involved in cellular processes that raise an animal's metabolism. One of the strongest

antioxidants, carotenoids offer defense against a variety of stressors, such as UV rays, reactive oxygen species, and free radicals (Maoka et al. 1989; Bendich 1989). Additionally, carotenoids are essential for the immune system and act as precursors of transcription regulators (Hill and Johnson 2012; Anbazahan et al. 2014). Fish with higher levels of carotenoid have also been demonstrated to be more resilient to bacterial and fungal infections (Shahidi and Brown 1998; Chavarria and Flores 2013). As a result, carotenoids are frequently included in diets for both pigmentation and overall health.

The nutritional value of fish tissue comes from a variety of physiologically active compounds, such as carotenoids, which are vital to human health, in addition to protein and lipids. As a result, several writers have been doing in-depth research on the biological role and occurrence of carotenoids in people, animals, and plants. Therefore, understanding the carotenoid content of meat or certain fish species in fisheries appears to be crucial. It is possible to supplement feed with naturally occurring carotenoids, such as those found in plants high in carotenoid content, to improve color. Commercial fish producers usually add synthetic carotenoids to fish meals to preserve overall fish health and accelerate color intensification, whereas natural carotenoids are often safer (Gupta et al. 2007). Since natural carotenoids are commonly accessible, can be simply added to fishmeal, and are typically free, incorporating them into aquaculture is a more realistic technique.

A diet rich in carotenoids enhances the fish's skin color and market value. Many fishes' appealing red, orange, and yellow coloring is caused by carotenoid pigments. Based on this knowledge, we developed a food and supply to enhance the fish's natural pigmentation. A diet rich in carotinoid enhances the fish's skin color and market value.

MATERIALS AND METHODS

Animals and experimental condition

Pethia conchonius (Rosy barb) was purchased from a local commercial aquarium fish supplier who obtained them from Kurunthancode, Kanyakumari, Tamil Nadu, India and kept for 15 days for acclimation in the laboratory. The final experiment lasted 90 days in an aquarium (50 ml size).

Estimation of Carotenoid

One g of sample was collected from the fish's head, middle, and tail regions to determine the total carotenoid content of the fish. Then samples were transferred to a 10 mL pre-weighed glass tube. After the samples were ground in acetone containing anhydrous sodium sulphate, the extractions were made up to 10 mL with acetone. The samples were stored in a refrigerator for 3 days at 4°C and then extracted three to four times until no more colour could be obtained. The solutions were centrifuged at 5000 rpm for 5 min, and then absorptions were measured in a spectrophotometer. Before the trial, the carotenoid content of the fish body was estimated, and subsequently, carotenoid content was measured every week for the whole experiment. The following equation was used, and the total carotenoid in the fish body was calculated and expressed as mg/10 kg carotenoid.

$$\text{Total carotenoid content } \left(\frac{\text{mg}}{10\text{kg}}\right) : \text{Abs } \times 1000 \times 10 \text{ kg } 1900$$

Where,

V= 10, Abs = Pigment Absorption Rate

Isolation of carotenoid

Column chromatography

Column was packed using wet packing technique using silica gel (15 g) as the adsorbent. Slurry was prepared using hexane and the slurry was poured in to the column. 8 ml of crude extract was added over the top of the column. Gradient elution technique was followed for column chromatography. The column was eluted with acetone and fractions were collected. The fractions collected were concentrated and TLC was performed to identify the presence of compound. After that to collect fractions and stop the procedure when the fraction become colourless (Gujjeti and Mamidala, 2013).

Identification of carotenoid

HPLC

Carotenoid extracts were analysed via reverse phase analytical HPLC using solvents and conditions described previously. Samples were analysed using an isocratic method (90% CH_3CN/H_2O) on an Alltech Alltima HP C18 (250 × 4.6) 5 µm column at a flow rate of 1.0 mL/min. Analytical HPLC analyses were performed on a Dionex P680 solvent delivery system equipped with a PDA100 UV detector. The column temperature was 30°C and UV detection was monitored at 465 nm. The carotene contents was quantified with reference to samples of commercial standard of bcarotene (Sigma Chemical, St. Louis, USA) of

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known concentrations ranging from 10 to 400 µg/ml. The analyses were performed in triplicate for all the samples and the results were presented as fresh weight basis (FW).

RESULTS

Total carotenoid in fish tissue

After a 90-day feeding examination, diet made using natural carotenoid sources showed greater carotenoid content than control diets (Table 1; Fig. 1). In control fish exhibited low carotenoid levels, ranging from 0.0517±0.0012 to 0.8297±0.0350 µg/g at the end of the experiment. The initial carotenoid content of 0.0647±0.0036 µg/g in the tissue was raised to 0.2785±0.0139 µg/g in D1 fed group after 90 days. The initial carotenoid content of 0.1482±0.0134 µg/g in the tissue was raised to 1.6346±0.0201 µg/g in D2 fed group after 90 days. Carotenoid content in the tissue increased from 0.4450±0.0176 µg/g to 0.8670±0.0228 µg/g in D3 fed group after 90 days of experiment. The initial carotenoid content of 0.6414±0.0334 µg/g in the tissue was raised to 1.3666±0.0293 µg/g in D4 fed group after 90 days. Carotenoid content in the tissue increased from 0.3666±0.0293 µg/g to 1.7330±0.0151 µg/g in D5 fed group after 90 days of experiment.

Table 1. Carotenoid content in control and experimental fish treated with different experimental diets at the end of the experiment

Groups	Carotenoid content (µg/g) at two week interval				
	18th day	36th day	54th day	72th day	90th day
Control	0.0517±0.0012	0.0549±0.0029	0.0869±0.00704	0.1662±0.0144	0.8297±0.0350
D1	0.0647±0.0036	0.0152±0.0014	0.1334±0.0071	0.2426±0.0376	0.2785±0.0139
D2	0.1482±0.0134	0.2304±0.0121	0.3465±0.00879	0.4683±0.0098	1.6346±0.0201
D3	0.4450±0.0176	0.5617±0.0377	0.6237±0.0284	0.6576±0.0220	0.8670±0.0228
D4	0.6414±0.0334	0.9184±0.0140	1.0192±0.0052	1.2506±0.0109	1.3666±0.0293
D5	0.3666±0.0293	1.0281±0.0013	1.1746±0.0013	1.4376±0.0224	1.7330±0.0151

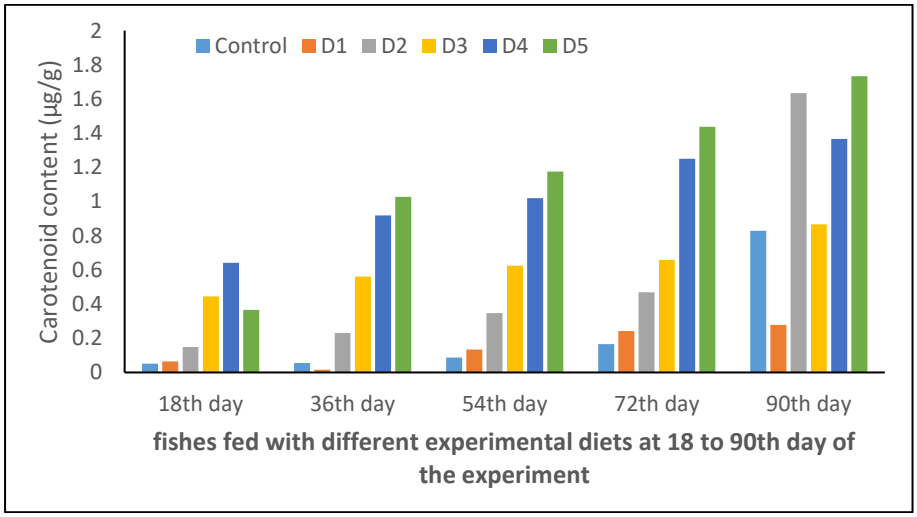


Fig. 1. Carotenoid content in control and experimental fish after fed with experimental diets

Fig. 2. Pethia conchonius fed with control diet and carotenoids diet (D1, D2, D3, D4 and D5)

Column chromatography

Carotenoids were isolated from the control and experimental group fish tissues at the end of the 10th week using column chromatography with silica gel as the stationary phase and wet packing technique (Fig 3). Elution was continued until the collected fractions were colourless, indicating complete elution of carotenoids and other coloured compounds. The collected carotenoid fractions were pooled, dried, and weighed to determine the total yield, providing a quantitative measure of the isolated carotenoids.

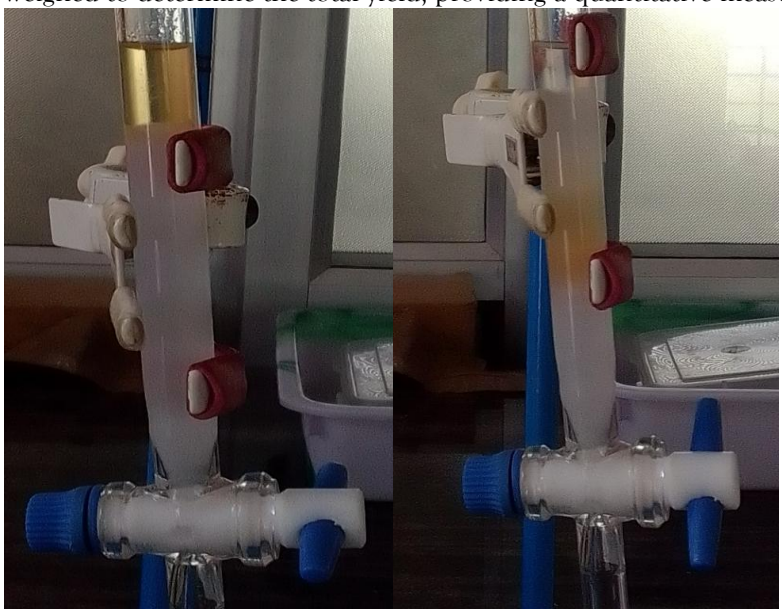
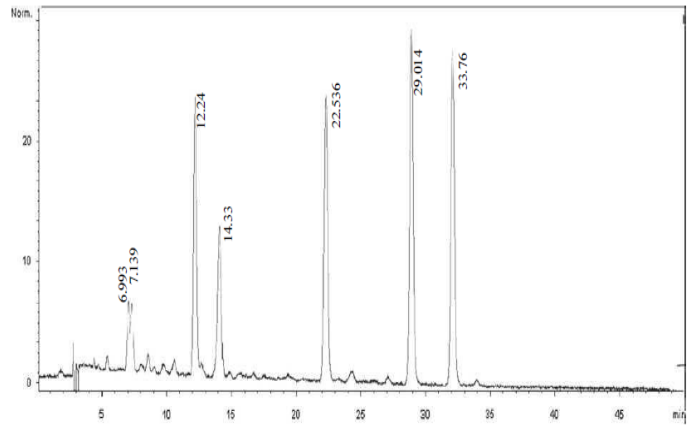


Fig. 3. A small section of the column showing the separation of carotenoid

HPLC

For identification of cis-carotenoids, the seven carotenoid standards were illuminated to accelerate cis isomers formation by using a procedure described in the methods section. The retention time and absorption spectral characteristics of carotenoid isomers were used in identifying the unknown peaks in the samples. Figure 4 and Table 2 shows the HPLC chromatograms of photoisomerized carotenoid standards, including E-Violaxanthin, 9'-Z Neoxanthine, E-Lutein, E-Zeaxanthine, E-β-Cryptoxanthine, α-Carotene and β-Carotene. The identification of the cis isomers was based on the cis peak, wavelength spectrum and Q ratio's (ratio of the height of the cispeak to the main absorption peak) with those in the literature. The retention time and absorption spectral characteristics of carotenoid isomers were used for identifying the unknown peaks in the samples.

The carotenoid content was estimated from five different experimental group fish tissues. The samples were analysed using C30 column on HPLC. In D1 group, E-Violaxanthin (6.957 Rt) and E-Zeaxanthine (14.352 Rt) as the principal carotene were present (Fig. 4; Table 3). There was no carotenoid content was observed in D2 group fishes. In D3 group, E-Violaxanthin (6.820 Rt) and E-Zeaxanthine (14.369 Rt) is the major carotenoid present (Fig. 4; Table 3). In D4 group, E-Violaxanthin (6.814 Rt) as the principal carotene were present (Fig. 4; Table 3). In D5 group, E-Violaxanthin (6.939 Rt) and E-Zeaxanthine (14.357 Rt) is the major carotenoid present (Fig. 4; Table 3).



HPLC profile of carotenoid standards

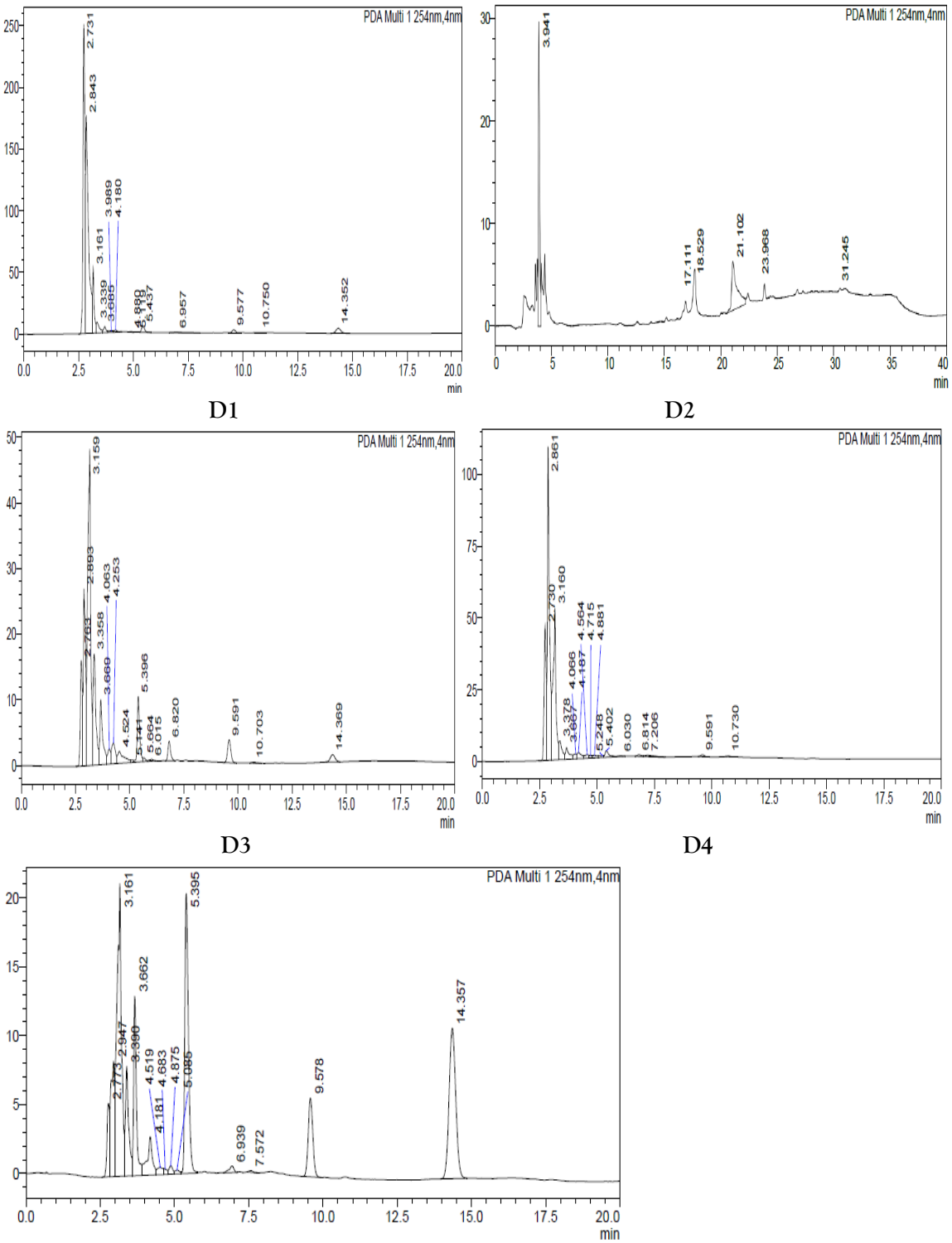


Fig. 4. HPLC profile of the major carotenoids in fish tissue of standard, D1, D2, D3, D4 and D4 group

Table 2. Identification and chromatographic data of carotenoid standards

Sl. No.	Retention time	Area	Name
1	6.993	129341	E-Violaxanthin
2	7.139	125636	9'-Z Neoxanthine
3	12.24	239658	E-Lutein
4	14.33	187423	E-Zeaxanthine
5	22.536	248632	E-β-Cryptoxanthine
6	29.014	266417	α-Carotene
7	33.76	25933	β-Carotene

Table 3. Identification and chromatographic data of carotenoid content in fish tissue of D1, D3, D4 and group

Sl. No.	D1	D3	D4	D5	Name of the carotenoid
	Retention time				
1	6.957	6.820	6.814	6.939	E-Violaxanthin
2	14.352	14.369	-	14.357	E-Zeaxanthine

DISCUSSION

Among the most prevalent natural pigments, carotenoids are in charge of many of the colors seen in nature and serve a number of purposes. Mostly found in plants, algae, fungus, mammals, photosynthetic bacteria, and certain non-photosynthetic bacteria, carotenoids constitute a family of 800 naturally occurring fat-soluble pigments. Carotenoids must be received from diet because mammals are unable to biosynthesize them; only plants, bacteria, fungus, and algae can synthesis them (Schiedt, 1998). After melanin, carotenoids are the most prevalent pigments in the animal kingdom. They provide a protective role against damage from light and oxygen and are essential to the photosynthetic process. Additionally, carotenoids serve as pro-vitamin A, antioxidants, immunoregulators, and are transported from muscle to the ovaries, all of which suggest a role in reproduction (Nakano et al., 1999; Bell et al., 2000). Additionally, fish with high carotenoids have been found to be more resilient to bacterial and fungal infections (Shahidi et al., 1998). A 40-carbon polyene chain, which may be thought of as the molecule's backbone, is the source of most carotenoids.

There are several types of carotenoids found in fish, with the main kind being specific to the species. Tunaxanthin (yellow), lutein (greenish-yellow), beta-carotene (orange), alpha, beta-doradexanthins (yellow), zeaxanthin (yellow-orange), canthaxanthin (orange-red), astaxanthin (red), eichinenone (red), and taraxanthin (yellow) are often found carotenoids that give fish their hues. Carotenoids are gathered by many fish in their gonads and integuments. On the other hand, fish belonging to the Salmonidae family oddly store astaxanthin in their muscles. Although many marine fishes also have lutein pigment, freshwater fishes are the most prevalent. Tunaxanthin is widely found in fish that are members of the Perciformes family. Tunaxanthin is the substance that gives marine fishes their vivid yellow skin and fins. Tunaxanthin was shown to be metabolized from astaxanthin via zeaxanthin in feeding tests in red sea bream and yellow tail (Matsuno et al., 2001). Certain carotenoids are unique to particular fish species. Fish often have lower levels of different carotenoids, and the percentage of each varies between samples, maybe as a result of their nutritional and/or physiological circumstances. Despite their inability to synthesis carotenoids from scratch, certain fishes can change one form of carotenoids into another.

Carotenoids are the main pigment source of ornamental fish, giving them their many colors, including red, yellow, and other similar shades. These are frequently produced from animals that have high concentrations of carotenoid in the aquatic food chain (Gouveia et al., 2003). Using a natural diet that included beetroot and spirulina as sources, this study investigated the effects of natural carotenoids on the pigmentation of rose barb fish (Pethia conchonius). The findings demonstrated that selected plant sources had a good effect on the fish's pigmentation.

The highest amount was discovered in the D5 diet, followed by D2 and D4, and the lowest in the D1 diet, according to the spectrophotometric study of the pigment content of rosy barb fish meals. The D5 diet-fed experimental fish sets had the highest concentration of carotenoid deposition in their bodies among the five experimental meal sets, whereas the D1 diet-fed fish sets had the lowest value. Fish development may be improved by carotenoid-mediated intermediate metabolism, which may be in charge of improving food metabolism (Segner et al., 1989; Amar et al., 2001). The quantity of natural carotenoid in the diet that may have a growth-promoting function may be the reason why the D5 diet had the considerably greatest growth percentage in the current investigation. Fish's carotenoid metabolism produces vitamin A and other nutrients that stimulate development (Maiti et al., 2017). Similar outcomes were observed in earlier research on red sword tail (Singh and Kumar, 2016), rainbow trout (De-la et al., 2006), goldfish (Sinha and Asimi, 2007), and guppy fish (Mirzaee et al., 2012). These findings suggest that the amount of carotenoid content in fish feed is linked to the enhancement of fish growth.

D5 diets raised the amount of carotenoid in the rosy barb fishes' tissue in the current investigation. This study demonstrated unequivocally that fish carotenoid deposition is directly related to dietary carotenoid concentration, which in turn enhances coloration. When given several natural carotenoid sources such as alfalfa (Yanar et al., 2008), red yeast (Xu et al., 2006), and spirulina (Kiriratnikom et al., 2005), goldfish had a similar pattern of findings. Fish tissue and skin have varying amounts of carotenoid deposition depending on the species (Ha et al., 1993). By consuming an optimal amount of carotenoid through feed,

the highest level of carotenoid content in fish tissue and skin may be achieved (Amla et al., 2013). Singh and Kumar's (2016) study, which found that dietary beet root powder at 15% had the maximum carotenoid deposition in the meat and enhanced the coloration in Red Sword Tail (Xiphophorus helleri), was comparable to the current study. In a similar vein, Somanath and Jasmin (2013) showed that a meal supplemented with spirulina promoted the formation of carotenoid pigments in the muscle and skin tissues of Carassius auratus.

Several carotenoid sources, such as spirulina and blue green algae, as well as plant sources, such as beet root (Singh and Kumar, 2016), tomato and carrot (Mirzaee et al., 2012), and paprika (Hancz et al., 2003), were discovered to have a significant impact on the color development of ornamental fishes. Several plant-based natural sources of carotenoid have been successfully tested in ornamental fishes up to this point. Non-traditional sustainable protein sources with a favorable amount of carotenoid were tried as fish feed in the current study because there is a dearth of research on ornamental fishes with natural carotenoid sources from animal origin. This was evident in the skin coloration of rosy barb fishes. The findings discussed in this study empirically support the idea that rosy barb fishes' skin pigmentation is greatly improved when they consume carotenoids. Interestingly, natural carotenoids from powdered beetroot and spirulina have positive results. Additionally, the comparative study of color enhancement efficacy between beetroot and spirulina carotenoid sources indicates that they have equal potential, establishing natural carotenoids as competitive substitutes for synthetic carotenoids, which are constrained by worries about safety, cost, and biological efficacy (Elbahnaswy and Elshopakey, 2023). These results are further supported by the isolation and characterisation of carotenoids from the experimental and control group using column chromatography. The experimental and control group diet's carotenoids were separated and examined.

Reverse phase analytical HPLC was used to analyze carotenoid extracts under previously mentioned conditions and solvents (Barba et al., 2006). It is commonly recognized that the characteristics of the stationary phase have a significant impact on the separation of carotenoids. Fraser et al. (2000) separated carotenes, xanthophylls, ubiquinones, tocopherols, and plastoquinones in a single run using an HPLC C30 column; nevertheless, their analytical duration exceeded 42 minutes.

The three main pigment groups that give higher plants their color are betalains, flavonoids, and carotenoids (Tanaka et al., 2008). Carotenoids, which are present in the chloroplasts of higher plants, are a mixture of α-and β-carotene, lycopene, xanthophyll, lutein, cryptoxanthin, zeaxanthin, violaxanthin, and neoxanthin. They are most widely distributed in both photosynthetic and non-photosynthetic organs (Delgado-Vargas et al., 2000). In photosynthetic organs, they are biosynthesised and stored in the chloroplast, where they perform two crucial functions: photosynthesis and photoprotection (Young 1991; Maoka, 2020). Fruits, flowers, seeds, roots, and leaves of higher plants are known to contain natural carotenoids (Priyadarshani and Jansz, 2014; Das, 2016), and several research have shown how interested people are in utilizing carotenoid colors derived from plants. In addition to improving color, studies have examined other aspects of carotenoids from higher plants in fish diets, including sensory perception, immunity, feed conservation ratio, growth performance, social behavior, and ornamental fish survival rate (Dananjaya et al., 2017). According to this study, the two main carotenoid found in fish tissue are violaxanthin and zeaxanthin. All four groups, with the exception of the D2 group, had carotenoid content, which I obtained via a diet high in beetroot and spirulina.

CONCLUSION

Research on the nutrition and color improvement of ornamental fish is scarce. The results show that the commercial ornamental fish industry depends heavily on carotenoids. Because synthetic carotenoids have detrimental impacts on aquatic ecosystems, natural plant sources can be used and included into specific meals for color retention or augmentation in limited situations. The ornamental fish business, the color-enhancing feed industry, and employment development will all benefit from it.

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