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Dietary Administration of Karonda (*Carissa carandas*) on the Growth, Digestive Enzymes, Skin Mucosal Immunity, and Pigmentation in Siamese Fighting Fish (*Betta splendens*)

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ABSTRACT

Karonda (*Carissa carandas*) fruit contains natural colorants; however, no research has investigated its effects on fish growth and coloration. Thus, this study examined the impacts of *C. carandas* fruit powder (CCFP) on growth, skin mucosal immunity, digestive enzymes, and pigmentation in Siamese fighting fish (*Betta splendens*). Flavonoids (15.97 ± 0.48 mg quercetin equivalent/g CCFP), phenolics (43.52 ± 1.73 mg gallic acid equivalent/g CCFP), terpenoids (350.00 ± 15.66 mg linalool equivalent/g CCFP), tannins (40.97 ± 0.15 mg tannic acid equivalent/g CCFP), carotenoids (5.53 ± 0.73 µg/g CCFP), and β-carotene (0.67 ± 0.05 µg/g CCFP) were estimated in CCFP. In the 2,2-diphenyl-1-picrylhydrazyl assay, CCFP showed antioxidant activity with an IC_{50} of 256.12 ± 7.68 µg/ml. Fish (0.42 ± 0.02 g weight and 3.20 ± 0.07 cm length) were fed diets containing CCFP at 0 (control), 3, 6, and 9 g/kg for 8 weeks. The results showed that CCFP administration significantly enhanced final weight, length, weight gain, specific growth rate, and average daily gain ($p < 0.05$). No changes in feed conversion ratio, survival, and condition factor were observed ($p > 0.05$). Dietary CCFP significantly increased skin mucus lysozyme, alkaline phosphatase, IgM, myeloperoxidase, total protein, antioxidant capacity, catalase, and superoxide dismutase. Fish treated with CCFP showed significant increases in intestinal protease, lipase, and amylase activity, as well as skin, muscle, and fin carotenoid levels. In conclusion, the administration of CCFP at 9 g/kg is optimal for improving growth, digestive enzymes, skin mucosal immunity, and pigmentation in *B. splendens*.

Keywords: Aquaculture, feed additive, feed utilization, natural colorants, ornamental fish, phytochemicals

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INTRODUCTION

The Siamese fighting fish (*Betta splendens*) is one of the most significant ornamental fish species in Thailand. The export value of this species in 2016 reached 529 million baht, the most valuable among ornamental

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fish (Krueahong et al., 2022). The primary sources of *B. splendens* in the ornamental fish market are generally obtained from cultivation processes and natural water sources. Recently, the natural habitat of *B. splendens* has been threatened by the expansion of modern agriculture, pesticide use, and increasing water pollution. Some contaminants can alter reproductive system function and decrease hatch rates, leading to a decline in the *B. splendens* population in nature (Paulos et al., 2010). Ornamental fish production systems have been developed for large-scale export. However, raising fish under intensive conditions can result in infectious diseases and significant mortality rates (Gruneck et al., 2022). Moreover, fish stress responses to long-term capture, starvation, water pollution, and low-quality feed may substantially decrease skin pigmentation (Nascimento et al., 2019; Pailan et al., 2012).

Fish skin color is a specific feature that needs to be controlled according to market demands (Pailan et al., 2012). The factors affecting skin color in fish include the carotenoid content in their diet. Like other aquatic animals, fish cannot synthesize carotenoids and must obtain them through digestion (Thongprajukaew et al., 2011). In the ornamental fish industry, various additives, such as sex hormones, synthetic carotenoids, and astaxanthin, have been incorporated into diets to improve coloration (Karslı, 2021; Keleştemur & Çoban, 2016; Wang et al., 2006). On the other hand, fish that receive continuous hormone administration may experience stress, liver enlargement, and abdominal edema. Furthermore, according to Paulos et al. (2010), using artificial colorants increases production costs and harms fish. This report suggests that natural colors from plants or animals can be a more affordable option for fish culture than chemicals. Interestingly, natural supplements may improve fish growth and health (Clotfelter et al., 2007; Wang et al., 2006).

Karonda (*Carissa carandas*) is a member of the flowering plants of the Apocynaceae family. It is a huge shrub with 3 to 5-cm long branches and stems covered in thick, sharp spines. It features clusters of white blooms and single leaves. The fruit has smooth, waxy skin and is rounded or slightly oval. When young, the fruit is white and gradually turns pink and dark red as it ripens. The analysis of the phytogenics in different parts of *C. carandas* indicated the existence of flavonoids, alcohol, cardiac glycosides, terpenoids, saponins, tannins, and phenolics (Itankar et al., 2011; Singh et al., 2020). Gas chromatography-mass spectrometry (GC-MS) identified various phytochemical substances in the extract of dried *C. carandas* fruit. These chemicals include myo-inositol, 4-c-methyl, 2*R*-acetoxymethyl-1,3,3-trimethyl-4t-(3-methyl-2-buten-1-yl)-1t-cyclohexanol, dichloroacetic acid, 2-ethylhexyl ester, 12-Oleanen-3-yl acetate, (3-alpha), 2*R*-acetoxymethyl-1,3,3-trimethyl-4t-(3-methyl-2-buten-1-yl)-1t-cyclohexanol, and 1-pentatriacontanol (Anupama et al., 2014). Previous research has shown that *C. carandas* has antioxidant, anti-inflammatory, analgesic, anticancer, antidiabetic, and hepatoprotective activities (Itankar et al., 2011; Neimkhum et al., 2021). Additionally. *C. carandas* has demonstrated efficacy in treating diabetes, diarrhea, fever, ulcers, pain, asthma, and malaria in the application of traditional

medicine (Singh et al., 2020; Verma et al., 2015). Importantly, data indicates that the fruit of *C. carandas* has the potential to be used as a natural pigment in the dietary supplement, fabric, and pharmaceutical industries (Manicketh et al., 2020). As a result, these findings support the use of *C. carandas* fruit as a supplement in ornamental fish production.

The specific growth rate and weight gain are commonly used as indicators of the growth-promoting properties of herbs or their derivatives in aquaculture studies (Hoseinifar et al., 2019; Jahazi et al., 2020). Digestive enzymes are crucial for the breakdown of nutrients in fish diets. Diets enriched with herbal plants increase the activities of digestive enzymes, leading to potential improvements in fish development, feed intake, and feed efficiency (Mohammady et al., 2022). Fish are protected from infectious disorders caused by different pathogens through the secretion of skin mucus by goblet cells. Fish skin mucus is a non-specific immunological defense mechanism and contains vital biological molecules such as lysozymes, myeloperoxidase, and antimicrobial peptides (Hoseinifar et al., 2015). Medicinal plants in fish diets enhance skin mucus components, indicating an improvement of innate immune function by certain phytogenic chemicals (Promprom et al., 2024). As stated above, lighter coloration in fish is directly related to the consumption of dietary carotenoids. Previous studies have suggested that incorporating carotenoids or β-carotene into the diet could increase the pigmentation levels in numerous fish species (Keleştemur & Çoban, 2016; Wang et al., 2006; Yanar et al., 2007). Therefore, it is postulated that incorporating natural products containing carotenoids and β-carotene into the diet of ornamental fish could be beneficial in enhancing their coloration (Dananjaya et al., 2015; Sathyaruban et al., 2021).

As mentioned above, different parts of *C. carandas* have been used to treat various ailments in folk medicine. However, no scientific research has documented the utilization of *C. carandas* in aquafeed or any other animal species. A recent study performed an acute toxicity test on male Wistar rats and observed that administering acetone extracts of *C. carandas* fruits at doses ranging from 500 to 5000 mg/kg body weight did not cause any deleterious effects or mortality (Saher et al., 2020). Moreover, *C. carandas* fruit contains natural pigments such as carotenoids and β-carotene. These findings suggest that *C. carandas* fruit could be utilized as a feed additive without toxicity. Thus, this work investigates the impacts of dietary administration of *C. carandas* fruit powder (CCFP) on the growth, digestive enzymes, skin mucosal immunology, and pigmentation in *B. splendens*.

MATERIALS AND METHODS

Chemicals and Reagents

Unless otherwise specified, the chemicals and reagents used throughout the present research were of analytical grade and obtained from Sigma-Aldrich (St. Louis, USA).

Plant Samples

Ripe fruits of *C. carandas* were collected from a local market in the Khueang Nai District, Ubon Ratchathani, Thailand. A plant specimen was collected and taxonomically classified to the species level by the plant taxonomist. The plant specimen (Munglue 0018) was kept for future reference in the Biology program of the Faculty of Science, Ubon Ratchathani Rajabhat University.

Preparation of *C. carandas* Fruit Powder

The sample of CCFP was made according to the method of Santos et al. (2019) with minor modifications. The fruits were rinsed with flowing tap water, and the seeds were separated. The seedless fruits were dried in a hot air oven at 60ºC for three days and blended using an electronic blender. The dried sample was mixed with distilled water at the ratio of 100 mg/ml and boiled at 100ºC for 15 min. The solution was then passed through a filter paper of Whatman No. 1. The filtrate was mixed with maltodextrin (1:1 w/w) and processed in a Büchi mini spray dryer (Büchi Labortechnik AG, B-290, Switzerland). The conditions of operation for spray drying were as follows: the inlet air temperature was 150ºC, and the outlet air temperature was 107ºC. The atomization pressure was 4 bars. The average feed rate was 0.5 L/h, and the average drying air flow rate was 75.63 m^3/h. The sample was kept at -20ºC for use in experiments.

Phytochemical Determination

The colorimetric method of Jankham et al. (2024) was used to measure total flavonoid concentration in mg quercetin equivalent per gram of CCFP (mg QE/g CCFP). The Folin-Ciocalteu reagent method evaluated total phenolic content (Verma et al., 2015), reported as mg gallic acid equivalent per gram of CCFP (mg GAE/g CCFP). The terpenoid level was assessed using the methodology developed by Łukowski et al. (2022), and the outcomes are quantified as milligrams of linalool equivalent per gram of CCFP (mg LNOLE/g CCFP). The tannin concentration was determined using the Folin-Ciocalteu method (Anh & Tan, 2023), and the findings are shown as milligrams of tannic acid equivalent per gram of CCFP (mg TAE/g CCFP). The carotenoid content was assessed using the Foss et al. (1984) method, and the findings are shown as micrograms of carotenoids per gram of CCFP (µg/g CCFP). The β-carotene content was evaluated using the Biswas et al. (2011) method, and the findings are reported as µg of β-carotene per gram of CCFP (µg/g CCFP). All phytochemical tests were triplicated using a microplate reader (SPECTRO Star Nano, BMG LabTech, Germany).

Antioxidant Activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) test was used to assess the antioxidant activity of CCFP (Verma et al., 2015). The CCFP was diluted to final 0–1000 µg/ml concentrations

in methanol. After mixing 2 ml of each concentration with 1 ml of 0.2 mM DPPH reagent, the mixture was incubated at room temperature for 30 min. The absorbance was measured at 517 nm using a UV-visible spectrophotometer (Lambda 12, PerkinElmer, Connecticut, USA). The standard reference used in this research was ascorbic acid. IC_{50} values and percentages of DPPH inhibition were computed.

Diet Preparation

The fish diet (Product name: Higade 9006T) used in this study was obtained from Charoen Pokphand Foods PCL, Bangkok, Thailand. The levels of CCFP used were 0 (control), 3, 6, and 9 g/kg diet, based on the reports of Doan et al. (2020) and Pailan et al. (2012) with some modifications. The diet samples were combined with distilled water (1:4 w/v), homogenized using an electric meat mincer to produce extruded string shapes, and dried at 40ºC for 24 h. The diets were then crushed into small pellets (1 mm) and kept at 4ºC for future use. The basal diet's moisture, crude protein, crude fat, and ash were measured using Association of Official Analytical Chemists methods (AOAC, 2019). The fiber content was quantified following the methodology outlined by AOAC (2010). The proximate analysis of the basal diet is provided in Table 1.

Table 1
Proximate analysis of the basal diet used in this research

Proximate analysis	Results (%)
Moisture	8.10
Crude protein	43.37
Crude lipid	6.26
Nitrogen-free extract	30.62
Ash	10.48
Fiber	1.16

Note. The crude protein, crude lipid, moisture, ash, and fiber were measured values. The nitrogen-free extract was calculated value

Animal Ethics and Regulation

The study was carried out at the Ubon Ratchathani Rajabhat University Farm with permission from the Institutional Animal Care and Use Committee (AN64003).

Fish Preparation

Male *B. splendens* with a solid red phenotypic was purchased from Ubon Ratchathani Fish Cooperatives and acclimatized in the laboratory for two weeks. Daily monitoring was conducted during the acclimatization period to observe the experimental fish's exterior appearance, general behavior, and health. Fish with an initial weight of 0.42 ± 0.02 g and an initial length of 3.20 ± 0.07 cm were raised individually in plastic beakers of 11.5 cm in height and 8 cm in diameter, filled with 250 ml of dechlorinated water and exposed to a 12-hour light/12-hour dark cycle. The basal diet was given to the fish twice a day, at 8:00 and 17:00. Water quality parameters, including temperature (29.51 ± 0.34ºC), pH (7.65 ± 0.17), alkalinity (110.02 ± 1.68 mg/L), nitrite (0.0029 ± 0.0003 mg/L), nitrate (0.042

± 0.005 mg/L), and dissolved oxygen (6.78 ± 0.20 mg/L), were checked and maintained under standard conditions for *B. splendens* cultivation (Thongprajukaew et al., 2011). The water in the containers was changed daily.

Experimental Design

The study used a completely randomized design (CRD). There were four treatments, each with three replications with 20 fish. The treatments included a control group (0 g CCFP/kg) and three experimental groups (3, 6, and 9 g CCFP/kg). The experiment was carried out over eight weeks.

Growth and Survival Rate

At the end of the experimental period, the fish were starved for 24 h. All the fish from each replicate were harvested for the evaluation of growth and survival using the following equations:

$$\text{Weight gain (WG, g)} = \text{Final weight (g)} - \text{Initial weight (g)} \quad [1]$$

$$\text{Specific growth rate (SGR, \%/day)} = \frac{\text{Ln final weight (g)} - \text{Ln initial weight (g)}}{\text{the experimental period}} \times 100 \quad [2]$$

$$\text{Average daily gain (ADG, g/day)} = \frac{(\text{final weight (g)} - \text{initial weight (g)})}{\text{the experimental period}} \quad [3]$$

$$\text{Feed conversion ratio} = \frac{\text{feed consumed by fish (g)}}{\text{final fish weight (g)}} \quad [4]$$

$$\text{Condition factor (g/cm}^3) = 100 \times [\text{fish weight (g)}]/[\text{fish length}^3\ \text{(cm)}] \quad [5]$$

$$\text{Survival rate (\%)} = \frac{\text{number of fish at the end of the experiment}}{\text{number of fish at the beginning of the experiment}} \quad [6]$$

Skin Mucus Collection

Four fish per replication were subjected to a 24-hour starvation period, after which skin mucus was collected using the method mentioned by Hoseinifar et al. (2015). The fish were anesthetized using clove oil (5 ml/L) in an appropriate container with an electric air pump. Each fish was placed in a plastic bag with 10 ml of 50 mM NaCl. To collect the skin mucus, the fish were moved gently by hand for 1 min. The skin sample was then transferred to a test tube and centrifuged at 3000 × *g* for 5 min at 4ºC. After being collected, the supernatant was kept at -20ºC for use in other tests.

Skin Mucus Assays

The gram-positive bacterium *Micrococcus lysodeikticus* was lysed using the turbidimetric method to evaluate lysozyme activity. Alkaline phosphatase (ALP) activity was quantified

using *p*-nitrophenyl phosphate as a substrate (Wangkahart et al., 2022). Hoseinifar et al. (2015) used the method to measure total immunoglobulin M (IgM). The activity of mucosal myeloperoxidase (MPO) was determined based on the protocol of Doan et al. (2020). Skin mucus protein levels were estimated using a Lowry assay (Lowry et al., 1951), with bovine serum albumin as the standard reference. The ferric ion-reducing antioxidant power assay, as reported by Fernández-Alacid et al. (2019), was used to determine the total antioxidant capacity (T-AOC). The activities of catalase (CAT) and superoxide dismutase (SOD) were assessed using the protocols described by Wangkahart et al. (2022).

Digestive Enzyme Collection

After a 24-hour starvation period, four fish from each replicate were harvested and anesthetized individually using a specific dose of clove oil. The abdominal wall was then opened, and the internal organs were collected, cleared of connective tissues, cleaned with 0.90% normal saline solution, and weighed. The digestive tract was extracted using 0.2 M Na_2HPO_4-NaH_2PO_4 buffer (pH 8) at a ratio of 1:10 (w/v). After that, the samples were centrifuged for 30 minutes at -4ºC at 15,000 × *g*. The supernatants were kept at -20ºC until analysis.

Digestive Enzyme Activities and Protein Content

Soluble starch served as the substrate to test the amylase activity. A standard curve of reducing sugar was used to compare the absorbance, which was determined at 540 nm (Willora et al., 2022). The lipase activity was evaluated using *p*-nitrophenyl palmitate (*p*-NPP) as a substrate, as Winkler and Stuckmann (1979) reported. The solutions were quantified at a wavelength of 410 nm and compared to a standard curve of *p*-nitrophenol. The assessment of protease activity was conducted using the method presented by Wangkahart et al. (2022), using azocasein as a substrate. The solutions were measured at 440 nm and compared with a standard curve of tyrosine. The specific digestive enzyme activities are given in units per milligram of total protein (U/mg total protein). Lowry et al. (1951) outlined the method to determine the total protein concentration in the digestive tract supernatant. The activities of digestive enzymes were calculated using the following equation (Willora et al., 2022):

$$\text{Specific enzyme activities (U/mg total protein)} = \frac{(\Delta\text{Abs} \times \text{V total})}{\varepsilon \times \text{V sample} \times \text{t}} \times \frac{\text{ml}}{\text{mg total protein}} \quad [7]$$

Where, ΔAbs represents the test samples' absorbance, V_{total} is the total volume of the reaction solution, ε is the molar extinction coefficient, V_{sample} is the volume of the supernatant used in the reaction, and *t* is the reaction time.

Carotenoid Contents

The carotenoid content in the fish was assayed according to the method of Thongprajukaew et al. (2012). Briefly, four fish from each replicate were starved for 24 hours. Each fish was then anesthetized with a specific concentration of clove oil in an appropriate container equipped with an electric air pump. Skin, muscle, pectoral fin, caudal fin, pelvic fin, dorsal fin, and anal fin samples were collected and stored separately in centrifuge tubes. Three milligrams of each sample were introduced into microcentrifuge tubes containing 1 ml of 90% acetone and stored in the dark at 4ºC for three days. The samples were shaken twice daily. To determine the carotenoid content, the samples were centrifuged at 5000 × *g* for 10 mins at a temperature of 4ºC. Collected supernatants were measured at 474 nm. The carotenoid content was calculated according to Thongprajukaew et al. (2012). The results are presented as μg per gram of sample (μg/g wet weight).

Data Analysis

Kolmogorov–Smirnov and Levene's tests were used to test data normality and variance homogeneity, respectively. If the data distribution was abnormal, square-root and arcsine transformations were used. The data are shown as mean ± SEM (standard error of the mean). Duncan's multiple range test and one-way analysis of variance (ANOVA) were used to assess significant variations among treatments. There was a statistically significant difference between the treatments when the *p*-value was less than 0.05.

RESULTS

Phytochemical Determination and Antioxidant Activity

The results of the phytochemical determination in CCFP are presented in Table 2. The phytochemical compounds found in CCFP were flavonoids, phenolics, terpenoids, tannins, carotenoids, and β-carotene. Moreover, the IC_{50} value of the antioxidant activity estimated by the DPPH assay in CCFP was 256.12 ± 7.68 μg/ml, lower than the IC_{50} value of ascorbic acid.

Growth Parameters

The final weight, length, WG, ADG, and SGR of the fish fed the CCFP diets were significantly greater than those of the control fish ($p < 0.05$; Table 3). However, the FCR, condition factor, and survival rate were not significantly different among the treatments after eight weeks of experimentation ($p > 0.05$).

Mucosal Skin Immune Activity

Figure 1 illustrates the effects of CCFP diets on skin mucus immune parameters. After an eight-week experimental period, dietary supplementation with CCFP led to a significant

Table 2
Qualitative determination and antioxidant activity of C. carandas *fruit powder*

Phytochemicals	Results
Total flavonoid content (mg QE/g CCFP)	15.97 ± 0.48
Total phenolic content (mg GAE/g CCFP)	43.52 ± 1.73
Terpenoids (mg LNOLE/g CCFP)	350.00 ± 15.66
Tannins (mg TAE/g CCFP)	40.97 ± 0.15
Carotenoids (µg/g CCFP)	5.53 ± 0.73
β-carotene (µg/g CCFP)	0.67 ± 0.05
DPPH assay (IC_{50})	
CCFP (µg/ml)	256.12 ± 7.68
Ascorbic acid (µg/ml)	60.97 ± 0.48

Note. Data are represented as mean ± SEM, $n = 3$ for each test, QE = quercetin equivalent, GAE = gallic acid equivalent, LNOLE = linalool equivalent, TAE = tannic acid equivalent, DPPH = 2,2-diphenyl-1-picrylhydrazyl

Table 3
Growth parameters of B. splendens *fed* C. carandas *fruit powder diets and control diet for eight weeks*

Parameters	CCFP (g/kg diet)			
	0 (Control)	3	6	9
IW (g)	0.42 ± 0.03	0.38 ± 0.02	0.44 ± 0.03	0.41 ± 0.02
FW (g)	0.92 ± 0.05^{b}	1.14 ± 0.06^{a}	1.25 ± 0.13^{a}	1.27 ± 0.07^{a}
IL (cm)	3.12 ± 0.09	3.13 ± 0.09	3.26 ± 0.06	3.30 ± 0.06
FL (cm)	4.37 ± 0.15^{b}	5.02 ± 0.14^{a}	5.00 ± 0.25^{a}	5.27 ± 0.16^{a}
WG (g)	0.49 ± 0.03^{b}	0.76 ± 0.06^{a}	0.81 ± 0.11^{a}	0.85 ± 0.07^{a}
ADG (mg/day)	8.92 ± 0.02^{b}	13.57 ± 0.12^{a}	14.46 ± 0.25^{a}	15.35 ± 0.14^{a}
SGR (%/day)	1.04 ± 0.07^{b}	1.33 ± 0.02^{a}	1.19 ± 0.08^{a}	1.36 ± 0.05^{a}
FCR	1.46 ± 0.08	1.41 ± 0.09	1.28 ± 0.17	1.41 ± 0.09
CF (g/cm^3)	1.07 ± 0.12	0.92 ± 0.08	1.03 ± 0.11	0.87 ± 0.04
SR (%)	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

Note. The different superscripts indicated in each row show a significant difference at $p < 0.05$. Data are represented as mean ± SEM. IW = initial weight (g), FW = final weight (g), IL = initial length (cm), FL = final length (cm), WG = weight gain (g), ADG = average daily gain (mg/day), SGR = specific growth rate (%/day), FCR = feed conversion ratio, CF = condition factor (g/cm^3), SR = survival rate (%)

increase in lysozyme, IgM, and myeloperoxidase levels compared to the basal diet. According to the results, fish fed a 3 g CCFP-containing diet showed a significant increase in ALP levels compared to other groups. The results also demonstrated that fish-fed diets containing 6 and 9 g of CCFP had substantially higher total protein concentrations than the control and 3 g CCFP-treated groups.

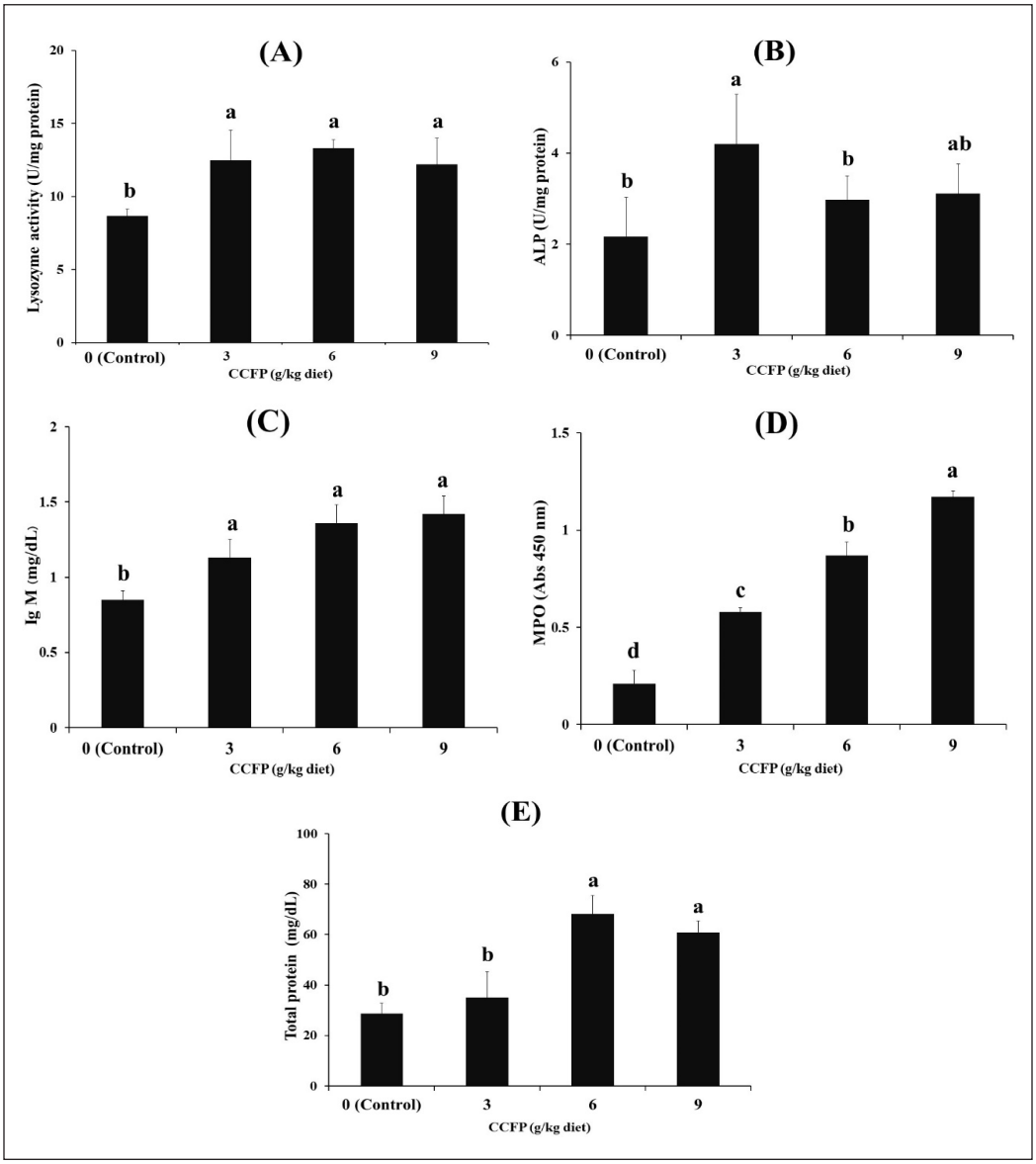

Figure 1. Non-specific skin mucus immune parameters of *B. splendens* fed *C. carandas* fruit powder diets and control diet for eight weeks. (A) Lysozyme activity, (B) Alkaline phosphatase (ALP), (C) Immunoglobulin M (IgM), (D) Myeloperoxidase (MPO), (E) Total protein. The different superscripts indicated a significant difference at $p < 0.05$. $n = 12$ for each group. Data are represented as mean ± SEM

Antioxidant Capacity

The results illustrate the effects of dietary CCFP on the antioxidant capacity of *B. splendens*, as depicted in Figure 2. The levels of T-AOC in the fish-fed diets containing 6 and 9 g CCFP/kg were notably greater than those in the control fish. Moreover, fish fed a diet

containing 6 g CCFP/kg showed the highest CAT activity compared to the other groups. Also, the SOD activities in the experimental groups were significantly greater compared to those in the control group.

Digestive Enzyme Activities and Protein Content

Figure 3 displays the levels of digestive enzyme activity, and protein content of fish fed different levels of CCFP. The results revealed that fish fed a 9 g CCFP/kg diet showed the highest protease activity compared to the other groups. Furthermore, the CCFP diets significantly increased fish lipase activity compared to the control diet. In addition, fish fed 6 and 9 g CCFP/kg diets had higher amylase activities than other groups. However, the total protein contents did not differ significantly among the groups.

Carotenoid Contents

Table 4 presents the effects of dietary supplementation with CCFP on the carotenoid content of *B. splendens* over 8 weeks. The results indicated that fish fed 3 and 9 g CCFP/kg diets had a significant increase in carotenoid content in the skin compared with the other groups. In addition, fish fed a 3 g CCFP/kg diet showed the highest muscle carotenoid content. Furthermore, fish fed with CCFP-containing diets had higher carotenoid levels in their caudal fins than control fish. Fish fed a 9 g CCFP/kg diet showed the highest levels of carotenoid in the pectoral, pelvic, dorsal, and anal fins.

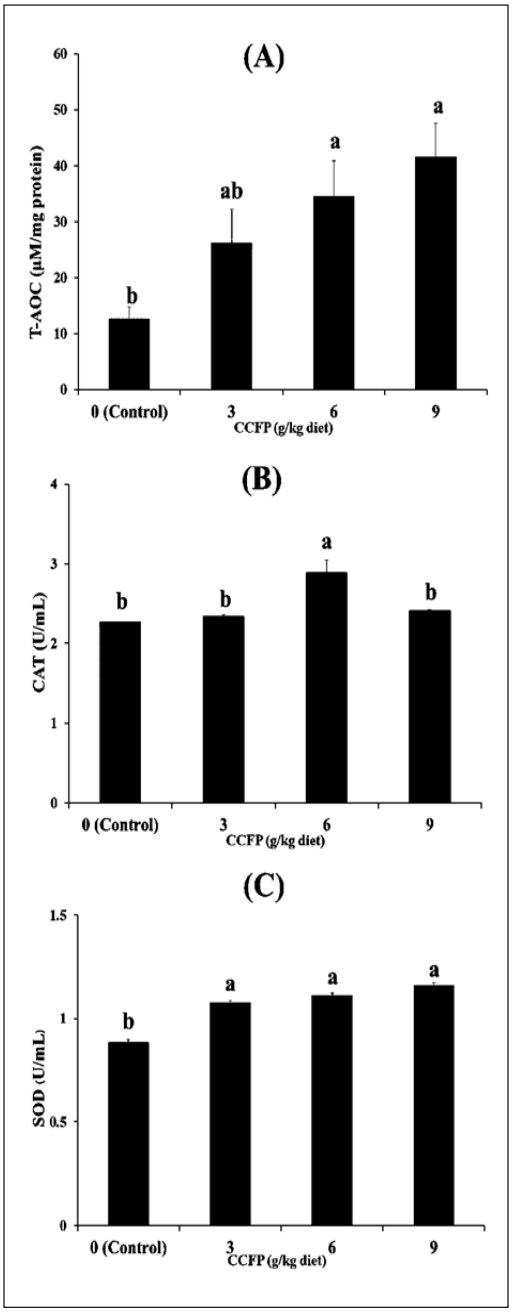

Figure 2. Antioxidative enzyme activity in *B. splendens* fed *C. carandas* fruit powder diets and control diet for eight weeks. (A) Total antioxidant capacity (T-AOC), (B) Catalase (CAT), (C) Superoxide dismutase (SOD). The different superscripts indicated a significant difference at $p < 0.05$. $n = 12$ for each group. Data are represented as mean ± SEM

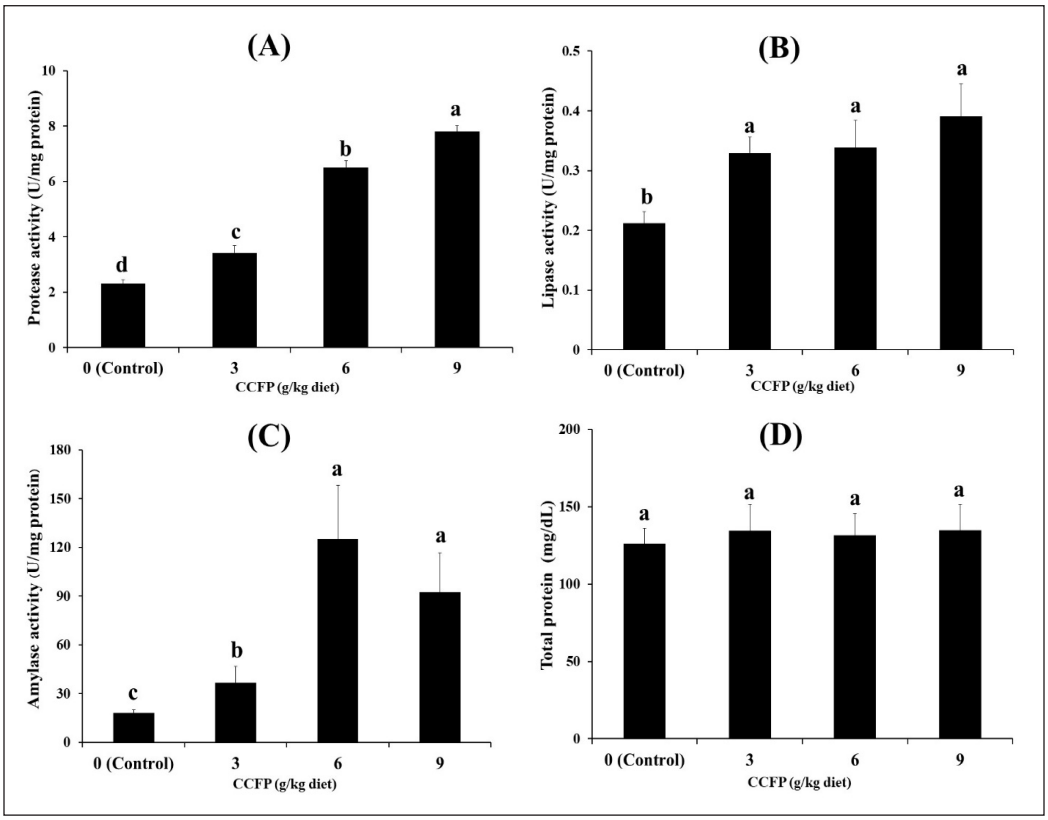

Figure 3. Digestive enzyme activities and protein contents in *B. splendens* fed *C. carandas* fruit powder diets and control diet for eight weeks. (A) Protease activity; (B) Lipase activity; (C) Amylase activity; (D) Total protein. The different superscripts indicated a significant difference at $p < 0.05$. $n = 12$ for each group. Data are represented as mean ± SEM

Table 4
Total carotenoid contents (µg/g wet weight) in skin, muscle, and fins of B. splendens fed C. carandas fruit powder diets and control diet for eight weeks

Parameters	CCFP (g/kg diet)			
	0 (Control)	3	6	9
Skin	21.34 ± 4.64^{c}	99.86 ± 2.39^{a}	80.25 ± 8.42^{b}	112.13 ± 5.84^{a}
Muscle	34.61 ± 9.85^{c}	86.24 ± 2.00^{a}	56.25 ± 1.38^{b}	60.33 ± 9.65^{b}
Pectoral fin	419.68 ± 25.18^{c}	473.80 ± 19.20^{c}	655.89 ± 37.74^{b}	1902.83 ± 78.52^{a}
Caudal fin	241.82 ± 19.63^{b}	717.79 ± 32.17^{a}	721.34 ± 12.67^{a}	724.53 ± 13.33^{a}
Pelvic fin	323.15 ± 19.00^{c}	775.07 ± 50.74^{b}	702.83 ± 34.07^{b}	1387.93 ± 57.88^{a}
Dorsal fin	319.64 ± 12.79^{d}	609.69 ± 16.88^{c}	787.15 ± 48.21^{b}	1847.33 ± 74.39^{a}
Anal fin	475.55 ± 17.90^{d}	696.55 ± 31.39^{c}	886.71 ± 88.32^{b}	1176.44 ± 81.74^{a}

Note. The different superscripts indicated in each row show a significant difference at $p < 0.05$. $n = 12$ for each group. Data are represented as mean ± SEM

DISCUSSION

Phytochemical Determination and Antioxidant Activity

The accumulation of reactive free radicals can damage cellular components. Natural products have been used to prevent oxidative processes in cells (Almarri et al., 2023). Plants contain a wide range of antioxidants, such as phenolics, flavonoids, and carotenoid pigments (Promprom et al., 2024). These compounds have been isolated and tested for their potential use in various applications (Dananjaya et al., 2015; Manicketh et al., 2020). The aquaculture industry has used medicinal herbs and their secondary metabolites as feed additives to enhance the growth and physiological features of several aquatic species (Hoseinifar et al., 2015; Jahazi et al., 2020; Jankham et al., 2024; Mohammady et al., 2022). This research indicated that CCFP consists of flavonoids, phenolics, terpenoids, tannins, carotenoids, and β-carotene. In addition, the CCFP exhibited antioxidant properties with an IC_{50} value of 256.12 ± 7.68 µg/ml as determined by the DPPH assay. As a result, this study supports using CCFP as a natural supplement to improve fish growth, health, and coloration.

Growth Performance

In this study, dietary supplementation with CCFP at concentrations of 3, 6, and 9 g/kg for eight weeks resulted in significant increases in growth parameters compared to those in the control group. Moreover, the application of CCFP to the diet had no noticeable effects on FCR, CF, or SR in *B. splendens*. Similar to the previous study, juvenile *B. splendens* fed a microwave-irradiated diet showed a significant increase in body weight, CF, SGR, net weight, and ADG when compared with the control, gamma-irradiated diet, probiotic-containing diet, and carbohydrase-supplemented diet (Thongprajukaew et al., 2011). Furthermore, Patria et al. (2024) indicated that dietary supplementation with Spirulina powder from *Arthrospira maxima* at 15% significantly increased the growth of *B. splendens*. The improvement in growth and survival rates is crucial for ensuring the productivity and profitability of the aquaculture industry. The addition of plants or their bioactive compounds to feeds can improve growth and nutrient utilization in various aquatic species. The growth-promoting properties of CCFP detected in this research may be due to phytochemicals like flavonoids and phenolic compounds, which have been shown to stimulate appetite, enhance the secretion of digestive enzymes in the gastrointestinal tract, boost immune functions, resist bacterial infections, and mitigate environmental stress, ultimately promoting overall fish health (Jankham et al., 2024; Patel & Rao, 2013). This present study suggests that CCFP could be a useful novel dietary supplement to improve fish growth (Almarri et al., 2023). However, further investigations should focus on elucidating the effects of CCFP on nutrient digestion and absorption in different aquatic animals.

Mucus Immunological Parameters

The findings of this study showed that fish fed CCFP diets had considerably higher levels of total protein, IgM, lysozyme, and ALP than fish fed a control diet. It is generally accepted that fish skin mucus acts as the first line of defense against pathogen invasion (Doan et al., 2020; Promprom et al., 2024). It contains various molecules, such as lysozyme, ALP, and IgM (Hoseinifar et al., 2015). Lysozyme plays a crucial role in disease prevention by lysing pathogenic bacteria. ALP is involved in the defense of fish against several pathogenic agents. IgM neutralizes invading antigens, and MPO protects by destroying foreign substances and pathogens through phagocytosis. The results of this study were in agreement with Motlagh et al. (2020), who noted that female guppy fish (*Poecilia reticulata*) fed diets supplemented with garlic extract showed a significant increase in lysozyme activity, IgM, and ALP when compared with the control. The improvement of immunological parameters in the skin mucus of *B. splendens* by CCFP is likely due to the effects of flavonoids, phenolics, and carotenoids, which regulate the production of active components in the immune system (Hoseinifar et al., 2015; Motlagh et al., 2020). Nevertheless, additional experiments are required to investigate the molecular mechanisms by which CCFP influences non-specific immunological responses in aquatic animals.

Antioxidant Capacity

The present study demonstrated that dietary CCFP increased the activity of T-AOC, CAT, and SOD in *B. splendens* skin mucus. T-AOC is commonly used to assess the antioxidant potential of phytochemicals in fish (Hendam et al., 2024). The primary function of CAT is to degrade H_2O_2 into water and oxygen, thereby preventing oxidative damage to cells and their components (Shekarabi et al., 2022). SOD plays a crucial role in reducing free superoxide anion radicals in the body (Hendam et al., 2024). It is possible that phenolics, flavonoids, tannins, terpenoids, and carotenoids found in CCFP may be responsible for a significant increase in the antioxidant capacity in skin mucus (Neimkhum et al., 2021). Therefore, the increase in antioxidant enzyme activity caused by dietary CCFP would reduce oxidative stress in fish (Singh et al., 2020; Verma et al., 2015). A possible reason for this is that CCFP may enhance the expression of fish antioxidant genes (Mohammady et al., 2022). Nevertheless, more research is necessary to verify this hypothesis.

Digestive Enzyme Activities and Protein Content

The present investigation showed that CCFP supplementation in fish diets significantly increased intestinal protease, lipase, and amylase activities in *B. splendens*. Similar research by Thongprajukaew et al. (2011) noted that *B. splendens* fed a microwave-irradiated diet revealed a significant increase in amylase activity when compared with the other modified

diets. Moreover, the application of curcumin to the diet at 5 g/kg produced a significant enhancement of trypsin and lipase activities in the intestine of crucian carp (*Carassius auratus*) (Jiang et al., 2016). It is well-established that the activity of digestive enzymes is a major indicator of digestive processes in animals (Jiang et al., 2016; Mohammady et al., 2022). The addition of medicinal plants to diets can increase digestive enzyme activity in the tract, resulting in growth improvements in cultured fish (Mohammady et al., 2022; Promprom et al., 2024). It has been reported that phytochemicals such as phenolics, flavonoids, tannins, and pigments have been reported to promote the secretion of digestive enzymes (Almarri et al., 2023; Jiang et al., 2016). Based on the findings of this study, the flavonoids, phenolics, and tannins present in CCFP may be responsible for the elevated digestive enzyme activities in *B. splendens* (Almarri et al., 2023). Therefore, the increased digestive enzyme activities could support the growth of fish.

Carotenoid Contents

This study revealed that dietary supplementation with CCFP significantly increased the total carotenoid contents in various parts of *B. splendens*. It was found by Patria et al. (2024) that a 15% spirulina power-supplemented diet significantly enhanced the color brightness in *B. splendens* when compared with the control diet. The coloration of fish is influenced by the accumulation of pigments, such as carotenoids, in chromatophores. Carotenoids are absorbed in the intestinal mucosa and distributed in various organs of fish, including the skin, muscle, reproductive organs, and liver, through the action of lipoproteins (Sathyaruban et al., 2021). Genetics, the life cycle, stress, diet compositions, and environmental conditions are among the factors influencing the distribution and accumulation patterns of carotenoids in fish (Nascimento et al., 2019; Thongprajukaew et al., 2011; Sathyaruban et al., 2021). Ornamental fish are unable to synthesize carotenoid pigments and must obtain coloring pigments from their diet. The application of synthetic carotenoids to fish feed can enhance the integument coloration (Keleştemur & Çoban, 2016; Wang et al., 2006). However, using synthetics increases the cost of fish production (Dananjaya et al., 2015). Therefore, there is a need to develop novel feed additives with skin color-promoting properties for cultivating ornamental fish (Sathyaruban et al., 2021). The present study showed that dietary supplementation of CCFP enhanced the carotenoid contents in the skin, muscles, and fins of *B. splendens*. The current study also revealed that CCFP contains significant amounts of carotenoids and β-carotene. Previous research has indicated that the skin pigmentation of fish can be enhanced by the application of plants that contain carotenoids, flavonoids, and betalains (Thongprajukaew et al., 2012; Sathyaruban et al., 2021; Yanar et al., 2007). According to the results of this investigation, CCFP may be used as a natural carotenoid source to improve skin color in ornamental fish.

CONCLUSION

This research indicates that *C. carandas* fruit powder (CCFP) is a promising novel feed additive for enhancing various aspects of *B. splendens* health and performance. Specifically, CCFP supplementation improved fish growth, the mucosal immune response, digestive enzyme activity, and skin pigmentation. The estimated optimal CCFP supplementation concentration was 9 g/kg of diet.

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