



EFFECT OF BLACK MULBERRY (*MORUS NIGRA*) POWDER ON GROWTH PERFORMANCE, BIOCHEMICAL PARAMETERS, BLOOD CAROTENOID CONCENTRATION, AND FILLET COLOR OF RAINBOW TROUT

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Abstract

The degree of flesh pigmentation is one of the most important quality criteria dictating the fish market value. It is well known that fish, like other animals, cannot synthesize their own coloring pigments *de novo*, and must obtain these pigments from their diet. In this study, four levels of black mulberry (*Morus nigra*) juice powder (BMP) as a natural pigment source were incorporated into a basal diet at concentrations of 0, 0.25, 0.5, and 0.75% and fed to rainbow trout weighing 100±5 g for 8 weeks in triplicate. At the end of the feeding trial, the effect of BMP on growth performance, blood biochemical parameters and fillet color was examined. Fish fed BMP showed significant enhancements in weight gain (WG), specific growth (SGR), food conversion ratio (FCR), and survival rates (SR) ($P<0.05$). SGR, WG and SR values were increased significantly following dietary supplementation with BMP in a dose dependent manner with the highest values in fish fed 0.75%, while the FCR was decreased ($P<0.05$). Body crude protein, lipid, and moisture contents were increased significantly in fish fed BMP ($P<0.05$). Dietary BMP has significantly decreased the levels of blood ALT, AST, and glucose ($P>0.05$). While the blood carotenoid concentration was increased in fish fed 0.5% BMP compared to other treated groups. Fish fed BMP showed increased fillet yellowness (b^*) and redness (a^*), while the fillet lightness (L^*) was decreased when compared to the control ($P<0.05$). In conclusion, diets supplemented with BMP increased the growth performance, muscle pigmentation, and health status of rainbow trout.

Key words: *Morus nigra*, growth performance, carotenoid, fillet color, rainbow trout

Rainbow trout (*Oncorhynchus mykiss*) is one of the most important farm-raised freshwater species and allocated the greatest part of aquaculture production in Iran

(Iran Fisheries Organization, 2017). Its global production reached 814 thousand tonnes and ranked fifteenth (FAO, 2017).

In aquaculture industry, organoleptic properties play a key role in the consumer acceptance (Safari and Atash, 2014). Salmonids cannot biosynthesize the carotenoids from *de novo* pathways, and they achieve their basal needs from artificial diets comprised entirely of natural and/or synthetic pigment sources (Storebakken and No, 1992). There are four main pigment groups that give color to the skin and tissues of animals and plants, namely melanins, purines, pteridines and carotenoids (Kop and Durmaz, 2008). Meanwhile, plant color is predominantly due to three organic pigments, namely betalains, carotenoids, and anthocyanins (Tanaka et al., 2008). The contents of carotenoids in fish diets are markedly responsible for red to pink color of the flesh, which is an important criterion for consumer choice (Choubert et al., 2009; Teimouri et al., 2013). Moreover, consumption of carotenoid pigments can decrease the risk of cancer cases and cardiovascular diseases in human beings (Gaziano and Hennekens, 1993; Gouveia and Rema, 2005; Krinsky, 1991; Mayne, 1996; Ziegler, 1989). Natural pigment resources rather than synthetic ones have been selected as alternative substrates due to their public health concern (Kim et al., 2019; Storebakken et al., 2004). Several studies reported that red pepper (Yilmaz and Ergün, 2011), sweet red paprika and hot pepper capsicum oleoresins (Yilmaz et al., 2013), marigold flower (Büyükċapar et al., 2007), *Haematococcus pluvialis* microalgae (Sommer et al., 1992), and annatto (Safari and Atash, 2014) could be used as dietary supplements to enhance farm-raised rainbow trout flesh pigmentation.

Mulberry (*Morus nigra*) is a widely distributed flowering plant in many temperate world regions (Erciřli and Orhan, 2007; Imran et al., 2010). Mulberry leaves, shoots, and fruits have been used in traditional Chinese medicine with a historical background (Zhishen et al., 1999). It is known to be a rich source of flavonoids, particularly anthocyanin (Katsube et al., 2006). Anthocyanin is the largest and the most important group of water-soluble phenolic pigments that donate protons to highly reactive free radicals and thereby can be used against various diseases (Chahar et al., 2011; He and Giusti, 2010; Ignat et al., 2011). Previous studies show that anthocyanin, anthocyanin rich blackberry syrup and blueberry powder improved fish immune parameters, antioxidant status, as well as survival rate against high ammonia stress, *Aeromonas veronii* and *Streptococcus iniae* (Kesbiç, 2019; Kim et al., 2019; Yilmaz, 2019 a, b). However, the effects of anthocyanin rich additives on fish fillet color have not yet been determined.

Therefore, the current study aimed to investigate the effect of dietary mulberry juice powder on some blood biochemical parameters, growth performance, and fillet color of juvenile rainbow trout.

Material and methods

Fish and experimental diets

The experiment was performed at Abzi Exir Kowsar trout farm (Tehran, Iran). Fish were acclimatized for 2 weeks in fiberglass tanks (1000 L). Fish health status

was verified by normal coloration, the absence of cysts, spots or patches over the body and gills and normal behavioral signs (swimming and feeding reflexes). During acclimatization period fish were fed the commercial basal diet (Chineh Company, Alborz Province, Iran) manually at three designated times of a day with a feeding ration of 3% body weight. The commercial basal diet contained 40% crude protein, 14% fat, 10% ash, 5% moisture, and 2% fiber.

Fresh black mulberry was purchased from a local market (Damavand, Iran) and transported to the Science and Research Branch Lab (Tehran, Iran). Black mulberry juice powder (BMP) was spray dried through a Buchi B-191 spray dryer (Buchi Laboratoriums-Technik AG, Switzerland) at 140–150°C of inlet and 80–85°C of outlet temperatures for 6–8 seconds (Gharsallaoui et al., 2007). BMP was then packed in three-layer waterproof nylon bags and maintained at –20°C until being used. Basal diet was supplemented with four levels of mulberry juice powder: 0 (control), 0.25, 0.5, and 0.75%. All diets were made into pellets, allowed to dry and stored in sterile plastic bags at 4°C until use.

Then, a total of 120 healthy rainbow trout (100±5 g) were randomly divided into 4 groups, in triplicates (10 fish per 100 L tank). The feeding rate was at 3% of body weight during the trial which lasted for 8 weeks. Fish were weighed every 2 weeks to readjust the weight of feed intake. Water quality during acclimation and actual experiment was maintained as follows: dissolved oxygen at 7.8 ± 0.3 mg/L, pH at 7.2 ± 0.1 and temperature at 16.6 ± 0.4 °C. The fish were subjected to a 16L:8D photoperiod regime.

Growth performance

All fish were deprived of food for 24 h before weighing and sampling and the following parameters were measured at the end of 8 weeks according to Mabrok and Wahdan (2018) using the following equations:

$$\text{Weight gain} = W2 \text{ (g)} - W1 \text{ (g)}$$

$$\text{Specific growth rate (SGR)} = 100 (\ln W2 - \ln W1)/T$$

$$\text{Feed conversion ratio (FCR)} = FI \text{ (g)} / WG \text{ (g)}$$

$$\text{Survival rate\%} = (\text{final amount of fish}/\text{initial amount of fish}) \times 100.$$

Where, W2 is final body weight (g), W1 is initial body weight (g), t is period of culture (day), FI is feed intake (g), and WG is weight gain (g).

Biochemical parameters

At the end of the trial, the fish were fasted for 24 h prior to sampling. Fish were anesthetized with clove oil (100 mg L⁻¹, Sigma Aldrich, Germany) before sample collection. Blood samples were drawn from the caudal vein and were immediately transferred to non-heparinized tubes for serum collection (30 fish per group). Serum was collected after centrifugation at 3000 g for 20 min, divided into several aliquots and stored at –20°C for further study. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and glucose levels were detected by commercial kits (Pars Azmoon Chemical Co., Tehran, Iran) using an auto analyzer (Hitachi 917, Japan).

Proximate chemical composition

At the end of study, five fish were collected from each tank (15 fish/treatment) at the termination of feeding trial for this intent and any fish were analyzed individually. Body proximate composition was conducted according to the standard methods described by the Association of Official Analytical Chemists (AOAC, 2005): moisture content was determined by drying the samples in an oven (Behr, Germany) at 105°C; crude lipid was determined by chloroform methanol extraction (2:1, v/v); crude protein was determined (Kjeldahl procedure: $N \times 6.25$) using an automatic Kjeldahl system; ash was measured by incineration in a muffle furnace at 500°C for 6 h.

Blood carotenoid concentration

The blood carotenoid concentration was measured according to the protocol described by Barbosa et al. (1999). Briefly, 200 μ l of serum sample was mixed with 400 μ l of ethanol (95%) and 1 ml of hexane. The mixture was vortexed for 1 min and hexane was collected following centrifugation at 4500 rpm for 10 min. The carotenoid absorption was read using Tean F-200 multiwell plate reader (Tecan Männdorf, Zurich, Switzerland) at 450 nm in n-hexane. Carotenoid concentration was calculated using a specific extinction coefficient ($E1\%$, 1 cm) 2500 in n-hexane (Weber, 1988).

Determination of fillet color

Twelve rainbow trout (3 fish per treatment) were slaughtered and two fillets from each fish with dimensions 10×5 cm and net weight of 50 g were packed in plastic vacuum plastic bags. The right side fillet was stored at 4±1°C until being used (for maximum 6 hours), while the left one was selected to evaluate the color parameters using a Colorflex Hunter Lab colorimeter (Hunter Lab Inc., Reston, USA) according to Teimouri et al. (2013). The color parameters were ranging from 0 for black to 100 for white and referred to as L^* (luminosity) for lightness, a^* (redness) for red/green, and b^* (yellowness) for yellow/blue. A standard white tile with reflectance values of $L^* = 95.91$, $a = +0.09$, and $b = +2.02$ was used as the reference (Choi et al., 2016).

Statistical analysis

The data were subjected to statistical analysis using the SPSS software version no. 21 (SPSS Inc., Chicago, IL, USA). The data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. P value of < 0.05 was considered statistically significant.

Results

Fish growth

Growth performance of rainbow trout fed diets enriched with different levels of mulberry juice powder for 8 weeks are summarized in Table 1. Based on the result, SGR, WG, and SR values were increased significantly following dietary supple-

mentation with mulberry juice powder in a dose dependent manner with the highest values in fish fed 0.75% ($P<0.05$). Additionally, the feed conversion ratio (FCR) was decreased when fish were fed mulberry juice powder ($P<0.05$).

Table 1. Growth performance of rainbow trout fed diets enriched with different levels of mulberry juice powder for 8 weeks

| Parameter | 0 | 0.25% | 0.5% | 0.75% |
|-----------|--------------|--------------|---------------|--------------|
| WG (g) | 25.60±3.00 c | 32.50±7.00 b | 42.50±17.50 a | 43.00±9.00 a |
| FCR | 1.50±0.02 a | 1.28±0.03 b | 1.16±0.02 b | 1.15±0.02 b |
| SGR | 1.90±0.21 b | 2.02±0.14 b | 2.06±0.18 ab | 2.13±0.24 a |
| SR% | 76.67±5 c | 83.3±5 b | 93.3±5 a | 95±5 a |

Data are presented as mean ± S.D. Values in each row with different letters show significant difference ($P<0.05$). WG – weight gain; FCR – feed conversion ratio; SGR – specific growth rate; SR – survival rate.

Proximate composition

Chemical composition of rainbow trout fed diets enriched with different levels of mulberry juice powder after 8 weeks are shown in Table 2. Body crude protein and moisture contents were increased significantly in fish fed mulberry juice powder at 0.5 and 0.75%, whereas fish showed improved lipid content by feeding BMP supplemented at 0.25, 0.5, and 0.75% when compared to the control group ($P<0.05$). On the other hand, body ash content was increased in fish fed BMP enriched diet ($P<0.05$).

Table 2. Chemical composition (%) of rainbow trout fed diets enriched with different levels of mulberry juice powder for 8 weeks

| Group | Protein | Lipid | Ash | Moisture |
|-------|---------------|-------------|-------------|---------------|
| 0 | 17.26±0.18 c | 3.37±0.02 d | 1.26±0.01 b | 62.35±0.88 c |
| 0.25% | 17.83±0.06 bc | 3.81±0.00 c | 1.83±0.07 a | 67.23±1.42 bc |
| 0.5% | 18.30±0.25 b | 3.87±0.01 b | 1.78±0.08 a | 72.18±2.08 a |
| 0.75% | 18.86±0.0 a | 3.98±0.01 a | 1.81±0.01 a | 68.25±0.70 b |

Data are presented as mean ± S.D. Values in each column with different letters show significant difference ($P<0.05$).

Biochemical parameters

Liver enzymes activity (ALT and AST), blood glucose levels among different treated groups are summarized in Table 3. The dietary supplementation of mulberry juice powder has significantly decreased the levels of ALT and AST in fish fed BMP at 0.5 and 0.75%, while the blood glucose level was decreased in fish fed BMP at 0.25% when compared to the control ($P>0.05$).

Statistically, a significant increase in blood carotenoid concentration was also observed in fish fed 0.5% mulberry juice powder enriched diet, compared to other treated groups (Table 3).

Table 3. Biochemical indices and blood carotenoid concentration of rainbow trout fish fed diets enriched with different levels of mulberry juice powder for 8 weeks

| Group | ALT (U/I) | AST (U/I) | Glucose (mg/dl) | Carotenoid (mg/L) |
|-------|--------------|----------------|-----------------|-------------------|
| 0 | 16.83±1.08 a | 307.50±35.93 a | 20.02±9.50 a | 0.8±0.02 a |
| 0.25% | 20.97±6.65 a | 363.50±24.00 a | 13.37±2.26 b | 0.8±0.04 a |
| 0.5% | 15.85±4.03 b | 302.75±16.70 b | 20.39±10.83 a | 1.7±0.03 b |
| 0.75% | 10.21±1.76 b | 263.00±45.86 b | 22.55±6.17 a | 0.8±0.02 a |

Data are presented as mean ± S.D. Values in each column with different letters show significant difference ($P<0.05$).

Fillet color parameters

Fillet color parameters (L^* : lightness, a^* : redness, and b^* : yellowness) of rainbow trout fish fed diets enriched with different levels of mulberry juice powder after 8 weeks are represented in Figure 1 and Table 4. Fillet lightness (L^*) was significantly decreased by increasing the level of BMP supplementation ($P<0.05$). Fish fed BMP at 0.75% showed increased fillet yellowness (b^*) when compared to the other groups ($P<0.05$). However, fillet redness (a^*) of fish fed BMP showed increased values when compared to the other groups ($P<0.05$).

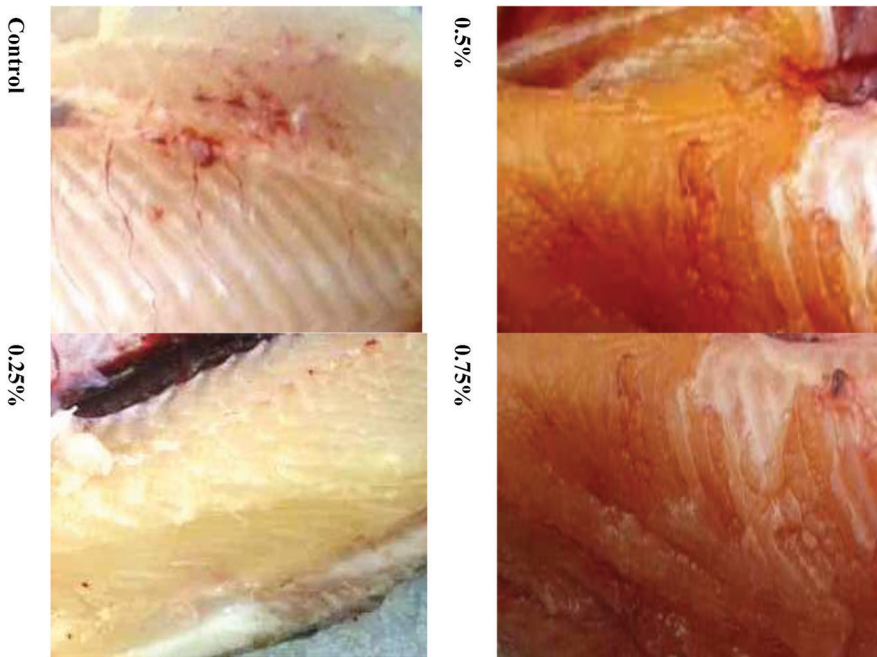


Figure 1. Fillet color of juvenile rainbow trout fed the experimental diets for 8 weeks

Table 4. Fillet color parameters (L^* : luminosity, a^* : redness, and b^* : yellowness) of rainbow trout fish fed diets enriched with different levels of mulberry juice powder after 8 weeks

| Group | L^* | a^* | b^* |
|-------|--------------|-------------|--------------|
| 0 | 48.42±1.83 a | 0.15±0.04 d | 8.95±0.02 b |
| 0.25% | 39.17±2.69 b | 1.04±0.06 c | 8.38±0.72 b |
| 0.5% | 38.22±1.18 b | 1.55±0.05 b | 9.16±0.57 ab |
| 0.75% | 39.77±1.82 b | 2.28±0.73 a | 9.63±0.33 a |

Data are presented as mean ± S.D. Values in each column with different letters show significant difference ($P < 0.05$).

Discussion

For the first time, the present study was conducted to evaluate the possible effects of dietary supplementation of black mulberry powder (BMP) on the growth performance, blood parameters, and fillet color of rainbow trout. The efficacy of PMB has been attributed to the flavonoids compounds which may help in improving the health status and performance of fish (Katsube et al., 2006). Although PMB is widely used as a medicinal herb globally, there were no brief studies explaining its application as feed additives in aquaculture.

Growth performance and feed conversion ratio are essential parameters to judge the potential of using feed additives in aquaculture. In the present study, we found that dietary PMB played an important role in improving the WG, SGR, and FCR of rainbow trout. Fish fed a diet supplemented with PMB showed a significant WG increase and concurrent FCR decrease. Our observation is consistent with previous findings that reported that using of medicinal herbs addition increased the growth-related parameters while decreasing feed conversion ratio (Acar et al., 2018; Parrino et al., 2019; Zemheri-Navruz et al., 2019; Zhou et al., 2015). Li et al. (2012 a, b) and Weisburger and Chung (2002) reported that the improved WG and FCR by dietary herbs is related to improved utilization of nutrients and the activation of the functionality of the intestinal flora. The results are consistent with Zhou et al. (2015) and Cho et al. (2007) who showed that the addition of herbs at high levels decreased the WG and feed utilization in channel catfish, olive flounder, and black rockfish, respectively. Results of the current study demonstrated that dietary incorporation of BMP significantly affected growth and feed efficiency in rainbow trout through the improvement of FCR. Berry plants are reported to contain a variety of bioactive compounds such as anthocyanins, flavonols, or ellagitannins (Kaume et al., 2011; Yilmaz, 2019 b). A stimulation of digestion and enhanced protein assimilation in the intestinal tract of fish take place via various active compounds of herbs (Kesbiç, 2019; Kim et al., 2019; Yilmaz, 2019 a, b). Further, Van Doan et al. (2019 a, b) reported that herbs, such as Thai ginseng (*Boesenbergia rotunda*) powder and elephant's foot (*Elephantopus scaber*) extract can stimulate digestion in Nile tilapia through a possible increase in digestive enzyme activity. Interestingly, BMP supplementation increased the body protein and lipid contents in the current study. This

may be attributed to its role of BMP in enhancing the protein and lipid metabolism that related to improved feed utilization.

Blood biochemical and physiological markers, for example, serum compounds, could be utilized to recognize probable beneficial effects of using natural feed additives on the health status of aquatic animals (Dossou et al., 2018; El Basuini et al., 2017; Zaineldin et al., 2018). The outcomes for the enzymatic and metabolic activities in the current study showed decreased ALT and AST levels following supplementation with different ratios of BMP. ALT and AST have been accounted for the examinations of fish wellbeing, liver enrichment with proteins, irresistible sicknesses, and contamination with toxics (Alexander et al., 2011). Thus, the low levels of ALT and AST in the current study suggest an improvement of health status and wellbeing of rainbow trout fed BMP.

Blood cortisol and glucose contents are among the important signs against environmental stressors in fish (Dawood et al., 2017). High blood glucose levels are a result of increased cortisol levels, which would promote gluconeogenesis in the liver (Leach and Taylor, 1982). In this study, glucose level decreased in fish fed BMP at 0.25% when compared with the control group. The significantly lower levels of glucose in blood suggested that BMP had an antistress effect and inhibited the amplitude of elevated cortisol, thereby reducing the magnitude of elevated glucose levels in blood.

It has been reported that the carotenoid absorption and deposition in rainbow trout is strongly affected by several factors, some of which being the nature of carotenoids used, the dietary carotenoid concentration, the fish size or physiological state and stage of sexual maturation (Storebakken and No, 1992; Torrissen et al., 1989). The results of the current study revealed that supplementation of BMP at 0.5% increased the blood carotenoid level, whereas the higher inclusion level (0.75%) did not show any effect when compared to the control. The obtained results indicated that the carotenoid concentration in the body of rainbow trout does not increase when the dietary BMP concentration is increased. The lack of response to higher dietary doses has been attributed to the reduction of carotenoid digestibility at higher inclusion levels (Rahman et al., 2016; Storebakken and No, 1992; Torrissen et al., 1990).

Fish fillet color directly correlates with the presence of carotenoids within hypodermal tissues (Barbosa et al., 1999). Color has successfully been quantified in fish using colorimeter, especially the a^* (redness) color parameters can explain the color differences from the visual inspection of the fish fillet by dietary supplemented PMB. By visual inspection of the muscle of fish in the current study, fish fed BMP showed increased fillet and yellowness (b^*) when compared to the other groups, while the fillet lightness (L^*) decreased. However, fillet redness (a^*) of fish fed BMP showed increased values when compared to the other groups. The increment in the average values of a^* and b^* and a reduction of L^* in the muscle of salmonids with increasing total dietary carotenoid concentration and feeding period is well documented in previous literature (Rahman et al., 2016; Schubring, 2009). These results also confirmed earlier observations on fish flesh pigmentation and supported the notion that the L^* values of fillets were inversely correlated with their carotenoid contents, whereas their a^* and b^* values were directly correlated with carotenoid contents. It

has been demonstrated that increasing carotenoid concentrations in the fish fillets led to increased a^* and b^* , and decreased L^* (Metusalach and Shahidi, 1997). Similarly, supplementation of astaxanthin showed increased redness and yellowness in Atlantic salmon (Christiansen et al., 1995; Skrede and Storebakken, 1986) and coho salmon (Smith et al., 1992). Combining with current and previous studies, it may be inferred that dietary BMP can be digested effectively in gastrointestinal tract to be deposited in tissues as pigmentation.

To conclude, the present study revealed that BMP supplementation enhanced the growth performance and feed utilization. Further improvements in the fillet color and blood chemical parameters were observed.

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