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Original Research Article

Effect of Supplementation with Lemon (*Citrus lemon*) Pomace Powder on the Growth Performance and Antioxidant Responses in Common Carp (*Cyprinus carpio*)

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ABSTRACT

This study was aimed to investigate beneficial effects of lemon pomace powder (peel and pulp), on growth performance and antioxidant responses in common carp. Fish were divided randomly into four groups of 30 each. Group 1 fish were fed with basic diet, serving as the control. Fish in group 2, 3 and 4 were fed the basic diet supplemented with 1.5%, 3% and 5% lemon pomace powder, respectively. Results showed that the values of glutathione peroxidase (GPX), superoxide dismutase (SOD) and glutathione peroxidase (GSH) in erythrocyte hemolysate were significantly increased in all of the treatment groups compared with the control (P<0.05). Significantly lower level of plasma malondialdehyde (MDA) concentrations were observed in fish receiving 5% lemon powder. Ferric reducing antioxidant power (FRAP) values increased significantly as compared to control and the most increase was observed in group 4 which received 5% lemon pomace. No significant differences were found in plasma values of total protein, albumin, bilirubin, urea, lactate dehydrogenase, alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase among the experimental groups. Fish that were fed diet supplemented with 3% and 5% of lemon pomace powder displayed improved growth performance including final weight, weight gain, feed conversion ratio and specific growth rate. These data suggest that supplementation of lemon pomace powder can be effective in improving the antioxidant capacity and growth performance in common carp.

Keywords: Antioxidant, Common Carp, Diet, Lemon Pomace

INTRODUCTION

Common carp is considered a potential species for commercial aquaculture in Asia due to its excellent ability to adapt to environmental conditions and food changes and has a high survival rate (Rahman *et al.* 2006; Rajabiesterabadi *et al.* 2020). Carp production represents approximately 8.3% of world aquaculture production (Anton-Pardo *et al.* 2014). Due to the increase in human consumption and the demand for fish, the aquaculture industry is forced to strengthen the breeding system, which may increase the risk of infectious diseases and inhibit growth performance (El-Haroun *et al.* 2006; Bowyer *et al*. 2019). These challenges have prompted farmers to test new ingredients to improve fish health and improve growth performance (Hoseini *et al*. 2018; Yousef *et al.* 2019). Therefore, it may be an interesting research area to study the evaluation of new components such as bioactive compounds and lemon pomace in diets of common carp. The addition of natural antioxidants, antibacterial, and biologically active compounds from different fruits and vegetables to the fish diet plays a vital role in enhancing the immune system and disease resistance of fish (Qadiri *et al*. 2019; Dash *et al*. 2015).

Citrus is a genus of Rutaceae, including 3 genera and 18 different species, which are widely distributed all over the world. Lemon (*Citrus limon*) is the third most important species of citrus in the world, behind orange and mandarin (González-Molina *et al.* 2010). It has a variety of biological properties, including anti-inflammatory, antibacterial, anti-obesity, anti-cancer, immunomodulatory, antioxidant, and hepatoprotective activities (Lee *et al.* 2011; Abirami *et al.* 2015). Its main biological components include carotenoids, citric acid, flavonoids, potassium, magnesium, phosphorus, vitamin C, flavanones, ascorbic acid, hesperetin, naringenin, limonin, minerals, flavone glycosides, hesperidin (Bouzenna *et al*. 2016). Citrus by-products have many biological properties; lemon peel and palm has antioxidant effects on fish (Fukada *et al.* 2014). Previous studies on the possible effects of bioactive compounds in citrus peels on different fish species revealed that pectin derived from citrus peels or pomace is considered one of the most promising immunomodulation agents, antiviral and antibacterial effects due to its high content of bioactive compounds (Sharma *et el.* 2006; Debbarma *et al.* 2013). Therefore, citrus peel and pomace provide beneficial physiological and nutritional effects against the larvae (Campolo et al. 2016), bacteria

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(Ramadan-Hassanien 2015), and fungus (Hernawan et al. 2015) when tested by *in vitro* assays (Ho *et al.* 2015). Biochemical methods and evaluation of blood parameters is considered as an important biomarker which can be used for evaluation of health status of fish. Alterations in enzymatic activities directly reflect metabolic disturbances and cell damage in specific organs (Wood *et al.* 2012). Thus, this study aimed to explore the possible effects of lemon pomace powder (peel and pulp) on growth, antioxidant defense and serum immune response of common carp.

MATERIAL AND METHODS

Preparation of diets

Lemon pomace were obtain from a local lemon juice factory in Mashhad, Iran. Pomace was dried under shade at room temperature. Dried pomace were powdered and passed through a 40-mesh screen (Tumane *et al.* 2014). A commercial pellet diet (Skretting, Spain) was crushed and mixed with tap water before adding the correct amount of crushed lemon pomace powder (LPP) and pelleting to obtain diets supplemented with 0% (control), 1.5%, 3% and, 5% LPP. All the experimental diets were allowed to dry and were stored at 4 °C. Diet formulation and proximate composition of control group are shown in Table 1.

Table 1. The feed ingredients and proximate composition.

Ingredients	**Amount (g/kg)**
Fishmeal	300
Soybean meal	160
Corn meal	240
Wheat flour	180
Rice bran	80
Fish oil	20
Soybean oil	20
Proximate composition of LPP (% dry matter)	
Crude protein	8.4
Moisture	5.9
Ash	6.7
Dry matter	94.1
Calcium	0.61
Phosphorus	0.33

Chemical analysis

Analyzed proximate composition of lemon pomace powder were determined according to the Method of (AOAC, 2002). Crude protein content was determined by Kjeldahl method using an Auto Kjeldahl System (Kjeltec™ 2300, Foss, Sweden). Moisture content by a dry measurement of protein percentage, ash percentage, calcium, phosphorus and dry matter content were measured by AOAC (2002) method.

Fish preparation and experimental design

One hundred and twenty common carp (*Cyprinus carpio*), weighing 63.16 ± 0.72 g, were obtained from a local farm (Mazandaran, Iran). They were divided randomly into 4 equal groups and held in four glass aquaria, each containing 250 L fresh water. Fish were acclimatized for 7 days before commencement of the experiment and were fed with a pellet diet at a rate of 2% body weight day^{-1}. Physicochemical conditions of the water during the experimental period were dissolved oxygen 5.5–6 ppm, temperature 25 ± 1 ºC, pH 7 ± 0.5. Photoperiod was a 12:12 light– dark cycle the water in the aquaria was renewed every 48 h. Group 1 fish were fed with basic diet, serving as the control. Fish in groups 2, 3 and 4 were fed the basic diet supplemented with 1.5, 3 and 5% lemon pomace powder, respectively. The fish in each group were fed three times daily at 8:00, 13:00 and 19:00 throughout the experiment period (5 weeks).

At the end of the experiment, 10 fish were selected randomly from each aquarium and anesthetized in diluted MS-222. Blood samples were taken by cardiac puncture using heparinized syringes and tubes. After plasma separation by centrifugation at 1000×g for 20 min, erythrocyte pellet was washed three times with normal saline solution. The washed centrifuged erythrocytes were hemolyzed by the addition of an equal volume of ice-cold redistilled water and prepared plasma and hemolysate aliquots were stored at -70°C until analysis.

Blood biochemical analysis

Activity of glutathione peroxidase (GPX) in erythrocyte hemolysate was measured using RANDOX-Ransel enzyme kit (RANDOX, Crumlin, UK). Activity of superoxide dismutase (SOD) in erythrocyte hemolysate was assayed by a modified method of iodophenyl nitrophenol phenyltetrazolium chloride applying the RANDOX-Ransod enzyme kit (RANDOX, Crumlin, UK) (Boanca *et al.* 2014).

Malondialdehyde (MDA) measurement in plasma and erythrocyte hemolysate was based on spectrophotometric analysis of the pink colored product of thiobarbituric acid reactive substances (TBARS), as described by Latha and Pari (2003) using UV-VIS spectrophotometer (OPTIZEN, South Korea). Concentration of MDA was determined using a molar absorptivity value of 156,000 M^{-1} cm^{-1}.

The FRAP value was determined as previously described by Benzie and Strain (1996). This method is based on the reduction of a ferric tripyridyltriazine reagent to the ferrous form by antioxidants in the sample. This reaction produces an intense blue color that can be determined spectrophotometrically at 593 nm. Using a calibration curve of Fe^{2+}, FRAP values were computed and expressed in μmol Fe^{2+} formed per L of plasma.

Plasma biochemical analysis including total protein, albumin, bilirubin, urea, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) were done using commercial colorimetric kits (Pars Azmoon, Iran).

Growth performance

All fish in the different experimental groups were weighed at the end of 5-week feeding trial for estimation of growth. Growth performance parameters were calculated according to the following formulae (Zhai *et al*. 2014):

Weight Gain (WG) = (final weight-initial weight) × 100 / (initial weight) (1)
Specific Growth Rate (SGR) = (final weight - initial weight) × 100 / days (2)
Feed Conversion Ratio (FCR) = feed given (dry weight) / total wet weight gain (3)
Survival= 100 × (final fish number / initial fish number) (4)

Statistical analysis

The data (means ± SEM) were analyzed by using one way analysis of variance (ANOVA) followed by Duncan's post hoc test to compare the means between treatments and differences were considered significant when ($P<0.05$). The statistical analyses were carried out with SPSS software version 20 (SPSSInc, Chicago,IL,USA).

RESULTS

Growth and feeding parameters

The growth performance of common carp fed diets supplemented with varying levels of lemon pomace powder is presented in Table 2. At the end of experimental periods the survival was 100% in all groups. The final fish weight, weight gain (WG) and specific growth rate (SGR) were significantly higher in fish fed with diets containing lemon pomace powder compared with the control diet ($P< 0.05$) and increased with increasing levels of LPP in the diet up to 5%. The FCR significantly reduced with increasing dietary inclusion levels of dietary lemon powder up to 5%.

Table 2: Growth performance of common carp fed various levels of LPP.

Group	Initial weight (gr)	Final weight (gr)	WG (%)	SGR (%)	FCR	Survival (%)
Control	61.12±0.68	65.57±0.62	7.33±1.07[a]	7.41±2.1[a]	5.72±2.7[a]	100
1.5% LPP	60.21±1.12	65.80±1.04	9.41±1.55[a]	9.31±2.89[a]	4.46±1.4[a,b]	100
3% LPP	68.13±1.38	81.11±1.24	19.23±1.64[b]	21.63±3.08[b]	2.18± 0.15[c]	100
5% LPP	63.18±1.15	85.27±0.91	35.22±1.9[c]	36.81±2.95[c]	1.19± 0.88[c]	100

Values are mean ± SEM of each experimental group. Mean values with different superscripts in each column are significantly different (P < 0.05).

Blood biochemical and oxidative parameters

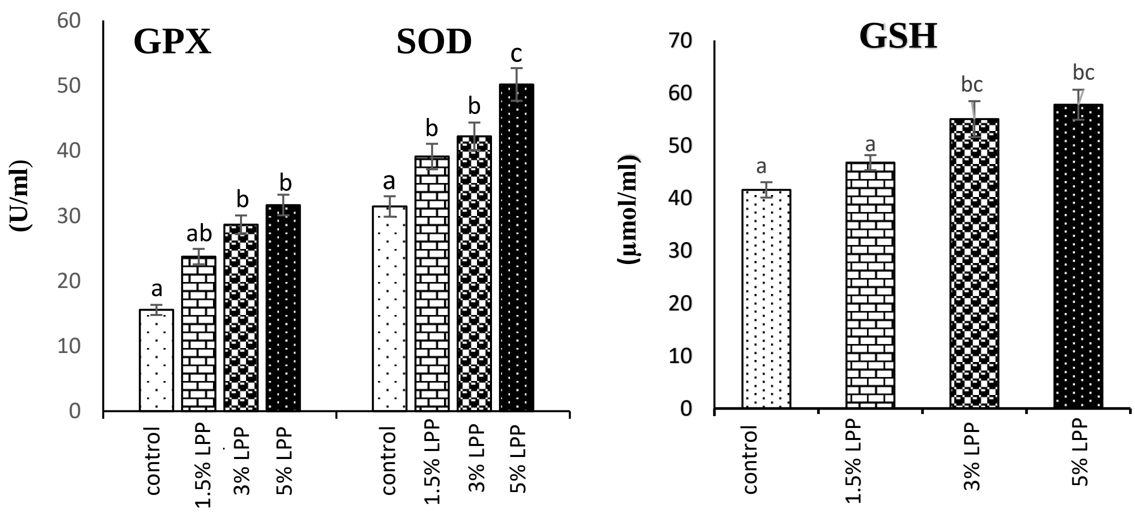
Measured plasma biochemical parameters are shown in Table 3. No significant differences were found in plasma ALP, AST, ALT, LDH, albumin, bilirubin, total protein and urea values among the experimental groups.

Table 3: Effects of varying doses of dietary LPP supplementation on plasma biochemical parameters of common carp.

Biochemical parameters	Control	Treatment (1.5%)	Treatment (3%)	Treatment (5%)
ALT(U/L)	81.25±12.97	78.67±13.37	74.76±14.65	60.01±15.89
AST(U/L)	68.15±13.04	63.65±5.67	63.78±8.47	58.16±4.67
ALP(U/L)	128.38±4.73	113.68±10	110.55±19.29	101.18±31.11
LDH(U/L)	1098.58±545.83	1036.07±521.29	966.07±314.05	939.35±391.17
Total protein (g/dl)	4.05±1.15	4.24±0.3	3.4±0.21	3.5±0.28
Albumin (g/dl)	2.91±0.18	2.54±0.17	2.17±0.17	2.21±0.2
Bilirubin (mg/dl)	1.62±0.4	1.27±0.31	1.53±0.61	1.03±0.2
Urea (mg/dl)	10.47±1.15	8.44±2	8.4±2.12	5.73±1.21

Values are mean ± SEM of each experimental group. Three is no significant difference in all treatment groups as compared to control.

The effects of lemon pomace powder supplementation on the levels of some antioxidant in erythrocyte hemolysate of common carp are presented in Figure 1. Glutathione (GSH) values increased significantly in group 3 and 4 as compared to control group and the most increase was observed in group 4 which received 5% LPP. As shown in Figure 1, glutathione peroxidase (GPX) and superoxide dismutase (SOD) activities were increased significantly as compared to control in all treatment groups. MDA values decreased significantly as compared to control in all treatment groups and the most decreased was observed in group 4 which received 5% lemon pomace powder. As shown in Figure 1, the increasing effect of lemon pomace powder on FRAP concentration in plasma of common carp was only significant in group 4, when compared with the control group.



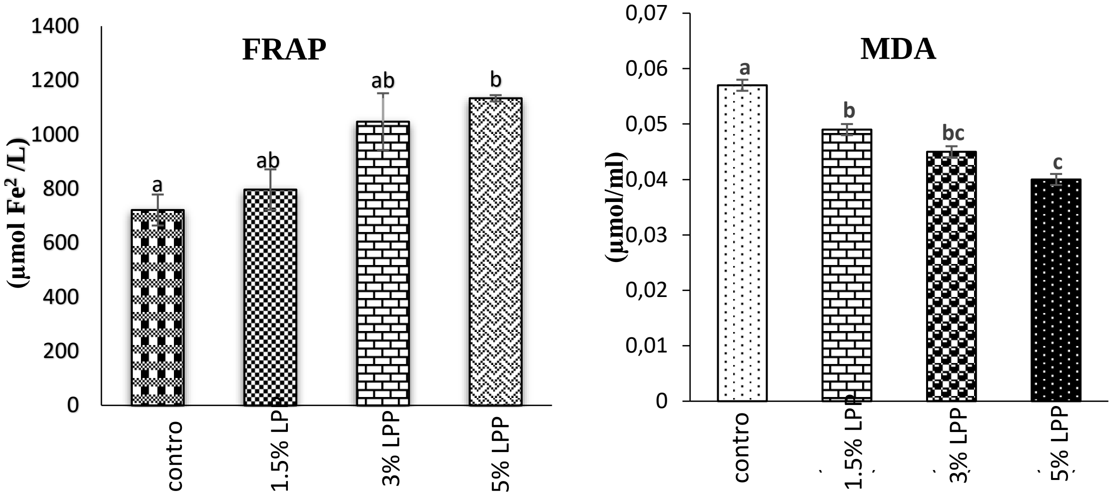


Figure 1: Effect of dietary lemon pomace powder supplementation on erythrocyte glutathione (GSH), glutathione peroxidase (GPX), and superoxide dismutase (SOD) as well as plasma MDA and FRAP values. Data are mean ± SEM (n = 10 in each group). Different letters indicate significant difference (p<0.05).

DISCUSSION

Modern aquaculture has made a significant contribution to human food security through a large number of high-quality and pleasant products. Therefore, research activities must be directed toward the use of functional and safe additives to refine aqua feeds and improve the growth and health of fish (Hoseinifar *et al.* 2021). Among the plants analyzed, those considered as medicinal plants can stimulate growth promotion, weight gain, appetite and early maturity of cultivated species (Bulfon *et al.* 2015).The antioxidant properties and high content of flavonoids in the peels and pomace can make this waste material a good source of nutraceutical and healthy compounds, especially to be used as anti-ageing products, due to the high content of polyphenols (Harikrishnan *et al.* 2011). Previous studies have demonstrated that plant extracts enhance the immune system of fish by enhancing the innate and adaptive immune response against pathogens (Van Hai 2015; Reverter *et al.* 2014). The antimicrobial and antioxidant activity of lemon being associated with flavonoids and essential oil (Adham 2015).

The present findings showed that fish fed diets supplemented with LPP significantly increased WG, and SGR, as well as improved FCR compared to the control diet. However, fish fed the 5% LPP diets significantly increased WG, and SGR, compared to other experimental groups (p<0.05). The present results are in agreement with very recent researches work in L. victorianus, Lates calcarifer, S. aurata, O. mossambicus, and O. niloticus treated with different levels of orange and lemon peel supplementation diets against pathogens (Baba *et al.* 2016; Shiu *et al.* 2016; García Beltran *et al.* 2017; Doan *et al.* 2018; Van Doan *et al*. 2019). Also, this observation is in agreement with the results obtained in *Oreochromis mossambicus* fed with *citrus sinensis* peel essential oil (Acar *et al*. 2015) and in *Labeo victorianus* fed with *citrus limon* peel essential oil (Ngugi *et al.* 2017). A 100% survival was observed in the all treatment groups that fed with LPP. This is in line with the earlier studies in *L. calcarifer* and *O. niloticus* (Shiu *et al.* 2016; Vicente *et al.* 2019). Plants contain a variety of active compounds that can enhance the growth response of fish and animals. For example, the biological compounds of aloe-emodin, emodin, kaolin, anthracenedione, and cinnamic acid actively enhance the growth performance of various fish (Harikrishnan *et al.* 2019; Harikrishnan *et al.* 2020). The exact mechanism of action to enhance the growth response of fish remains to be elucidated.

Endogenous antioxidant activity plays a precise role in cellular pathways that resist oxidative damage (Schieber *et al.* 2014). CAT and SOD are essentially the first line of defense against ROS. Its activation invalidates the destructive effects of ROS (Halliwell 2012). In addition, SOD, GPx and CAT are the first antioxidant defense and biomarkers for fish to resist stress or pathogens. The antioxidant activity such as SOD, GPx, and GSH were significantly influenced in both groups fed with 3% and 5% LPP than control group (p<0.05). Lemon and citrus leaf supplemented diet significantly modulate SOD activity in *L. calcarifer* (Shiu *et al*. 2016). In *Sparus aurata* the lemon peel enriched diet significantly modulated glutathione reductase (GR), SOD, and CAT enzyme activity (García Beltran *et al.* 2017). In O. niloticus the orange peel enriched diet significantly influence SOD, CAT, and GPx were reported by Vicente et al. (2019) and Abdel Rahman et al. (2019). In addition, significantly lower level of MDA were observed in fish receiving 5% of lemon pomace powder. These data suggest that supplementation of 5% seems to be more effective than lower levels of lemon pomace powder in strengthening the antioxidant system against oxidative stress.

Biochemical analysis is a fundamental tool used to diagnose and predict the outcome of diseases and to monitor the effects of therapeutic, nutritional, and environmental management in human and veterinary medicine. Alterations in enzymatic activities directly reflect metabolic disturbances and cell damage in specific organs. AST and ALT are known as indicators of liver injury (Harikrishnan *et al.* 2011). Furthermore, AST and ALT activities might be altered by a variety of chemical, biological, and physiological factors or by a disturbance of the Krebs cycle. Increased serum ALP activity may be observed in the case of extra hepatic obstruction, intrahepatic cholestasis, infiltrative liver disease, and hepatitis (Latha and Pari 20003). Result showed no significant differences in plasma ALP, AST, ALT, LDH, albumin, bilirubin, total protein and urea values among the experimental groups. It shows that lemon pomace powder at doses applied in the work have no damaging effects on tissues of common carp. Present results indicated that 5% LPP supplemented diet was the best option to improve growth of common carp.

CONCLUSIONS

The results of the present study indicate that dietary lemon pomace powder supplementation has potential to decrease oxidative stress to some extent by improvement of antioxidant system and reducing lipid oxidation in blood of common carp. Additionally, based on biochemical analysis results, it could be suggested that the lemon doses applied in this study might have no deleterious influence on organs of common carp. However, a detailed investigation is needed in other fish and animals before the incorporation of citrus and lemon by-products as a feed supplement. Moreover, the clarification of the molecular basis of lemon pomace powder beneficial effects on health and oxidative status indices requires further work.

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