Sains Malaysiana 52(12)(2023): 3357-3370
http://doi.org/10.17576/jsm-2023-5212-02

Gene Expression, Growth Performance and Body Composition of *Labeo rohita* Fingerlings Fed on Polyphenols Supplement

(Pengekspresan Gen, Prestasi Tumbesaran dan Komposisi Badan Anak *Labeo rohita* Diberi Makan Suplemen Polifenol)

Syed Makhdoom Hussain[1,*], Muniba Jamil[1], Shafaqat Ali[2,3], Muhammad Mudassar Shahzad[4], Majid Hussain[5], Nisar Ahmad[6] & Danish Riaz[4]

[1]*Fish Nutrition Lab, Department of Zoology, Government College University, Faisalabad, Punjab 38000, Pakistan*
[2]*Department of Environmental Science, College of Agriculture and Environmental Sciences, Government College University, Faisalabad, Punjab 38000, Pakistan*
[3]*Department of Biological Sciences and Technology, China Medical University, Taichung 40402, Taiwan*
[4]*Department of Zoology, Division Science and Technology, University of Education, Lahore, Pakistan*
[5]*Department of Fisheries and Aquaculture, University of Okara, Pakistan*
[6]*Department of Zoology, University of Jhang, Pakistan*

Received: 19 December 2022/Accepted: 30 November 2023

ABSTRACT

The aim of this research was to investigate the impacts of polyphenols-supplemented diet on growth, body composition and gene expressions of insulin-like growth factor (IGF) and growth hormone (GH) in *Labeo rohita* fingerlings fed canola meal-based diets. Two plant extracts; *Salvadora persica* and *Salvadora oleoides* were used to assess the efficacy of *L. rohita*. Twelve test diets, including control (no supplementation) and supplemented with *S. persica*, *S. oleoides* and their mixture at the levels of 200, 400 and 800 mg/kg, were formulated. The gene expression of IGFs and GH was observed to be regulated significantly ($p< 0.05$) in the liver tissue of fingerlings when fed with supplemented diet. The highest expression of GH was observed at 200 mg/kg mixture diet. The results of growth performance showed that maximum weight gain% (215%) and SGR (1.63) was recorded at 200 mg/kg mixture diet. The results of carcass analysis showed that they were significantly ($p< 0.05$) influenced by supplemented diet when compared with control diet. As growth performance is under genetic control, the synthesis and release of IGF are stimulated by binding of GH to receptors present in the liver. Conclusively, the results showed that rohu fingerlings shown the best results of growth performance, body composition and gene expression of IGF and GH, when fed on mixture of *S. persica* and *S. oleoides* at 200 mg/kg.

Keywords: Gene expression; growth hormone; growth performance; insulin-like growth factors; polyphenols

ABSTRAK

Matlamat penyelidikan ini adalah untuk mengkaji kesan diet tambahan polifenol ke atas tumbesaran, komposisi badan dan pengekspresan gen faktor tumbesaran seperti insulin (IGF) dan hormon tumbesaran (GH) pada anak *Labeo rohita* yang diberi diet berasaskan canola. Dua ekstrak tumbuhan; *Salvadora persica* dan *Salvadora oleoides* digunakan untuk menilai keberkesanan *L. rohita*. Dua belas ujian diet, termasuk kawalan (tiada suplemen) dan tambahan suplemen *S. persica, S. oleoides* serta gabungannya pada tahap 200, 400 dan 800 mg/kg telah diformulasi. Pengekspresan gen IGF dan GH diperhatikan telah dikawal dengan ketara ($p<0.05$) dalam tisu hati anak apabila diberi makanan tambahan. Pengekspresan tertinggi GH diperhatikan pada diet gabungan 200 mg/kg. Keputusan prestasi tumbesaran menunjukkan peningkatan% berat badan maksimum (215%) dan SGR (1.63) direkodkan pada diet gabungan 200 mg/kg. Keputusan analisis karkas menunjukkan bahawa mereka secara signifikan ($p< 0.05$) dipengaruhi oleh diet tambahan jika dibandingkan dengan diet kawalan. Oleh kerana prestasi tumbesaran berada di bawah kawalan genetik, sintesis dan pembebasan IGF dirangsang oleh pengikatan GH kepada reseptor yang terdapat dalam hati. Secara konklusif, keputusan menunjukkan bahawa anak rohu menunjukkan hasil terbaik dalam prestasi tumbesaran, komposisi badan dan pengekspresan gen IGF dan GH apabila diberi makan gabungan *S. persica* dan *S. oleoides* pada 200 mg/kg.

Kata kunci: Faktor tumbesaran seperti insulin; hormon tumbesaran; pengekspresan gen; polifenol; prestasi tumbesaran

Introduction

The paucity of proteinaceous foods even in highly developed regions can be overcome with suitable aquaculture development. Since the last few decades, the aquaculture industry has been popularized and served us with highly rich source of nutrients in the form of fish (Béné et al. 2016). A large proportion of essential micronutrients and proteins are present in fish and fish items that are of absolute importance for good health and achieving nutritional requirements (Reverter et al. 2017). Over few decades, the use of fish meal has become intensive, rendering it a highly expensive ingredient to be used for feed formulation (Salin et al. 2018). The most important factors of aquaculture to gain highest output are feed and feeding. The use of fish meal is one of the principle expenses that are faced by fish cultivators; as they spend a large part of their farming income in gaining high quality feed. So, it has become the major interest of most of the aquaculturists to supply cost-effective and good quality fish feed to fish farmers (Alhazzaa, Nichols & Carter 2019).

Canola meal is a high-quality protein source being rich in all essential amino acids and minerals (Yigit & Olmez 2009). Second to soybean meal, it is highly nutritional plant protein source (Glencross 2016). Because of its favorable amino acid content, canola oil is used in aquaculture industry to replace fish oil. It has 12% crude fiber, 35% crude protein, 3.5% residual oil and 6% ash (Burel & Kaushik 2008). However, due to several anti-nutritional factors (ANFs) like saponins, tannins and glucosinolates; canola meal use in fish feed is limited. These ANFs are the reason of low feed intake, poor nutrient digestibility, and disturbed digestive system in monogastric fishes (Landero et al. 2011).

Polyphenols or phenolic compounds belongs to one of the amplest categories of phytochemicals found in plant kingdom and are largely available (Maqsood et al. 2014). These plant polyphenols are very important part of the fish feed and have been utilized largely to prevent oxidative stress (Nijveldt et al. 2001). To fulfill the protein demands in short interval of time, the growth boosting substances are continuously being manipulated in aquaculture. The use of synthetic chemicals in the feed improves growth and reproductive performance, however, produces drug resistance and other problems which affect the sustainable establishment of aquaculture, food security and human health (Ming et al. 2012). Inclusion of herbal-enriched diets in aquaculture provided us positive outcomes in terms of immunity, growth performance and stress resistance (Amin et al. 2019; Moustafa et al. 2020). Therefore, to enhance fish immunity and to improve anti-stress activity via nutritional ways is the main concern for researchers. *Salvadora persica* and *Salvadora oleoides* from genus Salvadora and family Salvadoraceaeare are the two common species. *S. persica* is locally called miswak or toothbrush tree (Salehi & Sh 2006). Its extract has tannins, terpenes, alkaloids, carbohydrates, sterols and flavonoids. In addition, the phytochemicals analysis showed that extract of *S. persica* has low content of saponins, anthraquinones and coumarins (Verma et al. 2009). *S. oleoides* is grown on large scale in Saudi Arabia, India, and Pakistan. Its common names are badapilu, meethajal, and virdhpilu (Mathur 2015). Phytochemicals screening also showed that stem, leaves and seeds of *S. oleoides* have highest concentrations of hydrocarbons (41.3%), phenolic compounds (25.7%), and flavonoids, respectively. It is observed that the methanolic extract of aerial parts of plant contain fatty acid, tannins, vitamin C, urosolic acid, chlorides, resins, and stearic acid (Geetha, Manavalan & Venkappayya 2010).

The metabolic and physiological processes of fish need to be studied in response to dietary alterations, in order to have better knowledge of fishmeal replacers and plant by-products on fish body performance. Nutrigenomics is the analysis of gene expression pattern to check the effects of feed on genome of fish (Mutch, Wahli & Williamson 2005). The insulin-like growth factors (IGF-1) and growth hormone (GH) in fish control the growth performance (Tatar, Bartke & Antebi 2003). Genes that encode the expressions of IGF-1 and GH can be influenced by the dietary protein intake source. The nutrient metabolism, growth and development are affected by the fish nutritional status that regulate the actions of both IGF-I and IGF-II (Kumar et al. 2017). *L. rohita* is well known for its adaptability to grow fast in confined water areas. It is herbivorous being highly selective in its natural food (Singh, Gaur & Chari 2006). *L. rohita* was selected in the present research study due to its fast growth rate, good taste, high nutritional status, adaptability to climate, high market demand and economic value in Pakistan (Wahab, Rahman & Milstein 2002). The main objective of the current research study was to evaluate the impacts of polyphenols, derived from *S. persica* and *S. oleoides*, on gene expression of IGF-1 and GH, body composition and growth performance of fingerlings of *L. rohita*.

Materials and Methods

Formulation of test diets

First, basal diet was made by mixing dietary feed ingredients. All the feed ingredients were purchased from local market and analyzed chemically according to AOAC (AOAC 2005). Chemical composition of feed ingredients is shown in Table 1. Polyphenols enriched *S. persica* and *S. oleoides* extract was used in the study, which was obtained directly from the Department of Chemistry, Government College University, Faisalabad. Feed ingredients were crushed to powder form in grinding machine and sieved by a mesh of size 0.5 mm in diameter. Ingredients composition (%) of polyphenols-supplemented diets is shown in Table 2. Then, water (15%) and fish oil were added gradually into the mixture to make dough. Then, the dough was passed via feed pelleting machine to form pellets of size 0.2-0.3 mm diameter; not bigger than fish mouth size. Twelve test diets including control (no supplementation) and supplemented with *S. persica*, *S. oleoides* and their mixture (1:1) at the levels of 200, 400 and 800 mg/kg were formulated. The supplements were dissolved in water and sprayed over the pellets which were dried in oven at 55 °C. At last, pellets were stored at room temperature in separate plastic boxes.

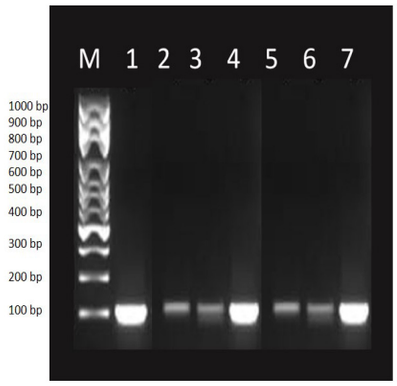

FIGURE 1. Pictorial representation of agarose gel. M is marker ladder of 100 bp

TABLE 1. Chemical composition (%) of feed ingredients

Ingredients	Dry matter (%)	Crude Protein (%)	Crude Fat (%)	Crude Fiber (%)	Ash (%)	Gross Energy (kcal/g)	Carbohydrates
Fish meal	90.4	47.4	6.60	1.70	23.5	2.03	16.0
Wheat flour	91.5	09.1	2.52	2.03	2.60	2.67	79.3
Corn-gluten 60%	91.3	58.8	4.65	1.20	1.93	4.23	28.6
Rice polish	93.9	10.2	12.4	11.2	09.8	3.27	48.3
Canola meal	90.7	37.9	4.91	3.73	12.3	3.43	34.9

TABLE 2. Ingredients composition (%) of basal diet

Ingredients	Ratio (%)
Canola meal	50
Fish meal	15
Wheat flour	11
Rice polish	13
Fish oil	7
Ascorbic acid	1
Vitamin Premix*	1
Mineral premix**	1
Chromic Oxide	1

Note: *Vitamin (Vit.) premix kg^{-1}: Vit. A: 15,000,000 IU, Vit. C: 15,000 mg, Vit. E: 30000 IU, Vit. B_2: 7000 mg, Vit. B_6: 4000 mg, Vit. B_{12}: 40 mg, Vit. D_3: 3,000,000 IU, Vit. K_3: 8000 mg, Ca pantothenate: 12,000 mg, Nicotinic acid: 60,000 mg, Folic acid: 1500 mg
**Mineral premix (kg^{-1}): Se: 3 mg, I: 40 mg, Co: 40 mg, Cu: 600 mg, Fe: 1000 mg, Mn: 2000 mg, Zn: 3000mg, Na: 45 g, Mg: 55 g, P: 135 g, Ca: 155 g

FISH AND EXPERIMENTAL CONDITIONS

The experiment was conducted at the Fish Nutrition Laboratory, Department of Zoology, Government College University, Faisalabad, Pakistan. The *L. rohita* fingerlings (6.57 ± 0.02 g) were bought from Government Fish Seed Hatchery, Faisalabad. The fingerlings were transferred to lab in oxygen containing plastic bags. Fish were given basal fish diet for 14 days. Each tank was supplemented with separate air pump for proper aeration during the whole experimental period. All tanks were covered with thin net to prevent accidental eescape of fish. Dissolved oxygen (6.0 ± 0.9 mg/L) and temperature (25.0 ± 0.8 °C) were sustained throughout the period. A total of 150 fingerlings were distributed equally into 12 tanks with triplicates (50 L water carrying capacity). Feeding trial was conducted following completely randomized design (CRD) in triplicates. The total duration of the experimental period was 70 days.

FEEDING TRIAL

Fish was fed twice a day at 5% of live wet weight of fish. Tanks were washed and water was changed after every feeding interval to clean any uneaten feed particles. Feces were collected after 2 h via fecal collecting tube twice a day. Proper care was taken to prevent breaking of fecal strings that can cause leaching of nutrients. Then, feces were weighed (5 g) and stored for further analysis.

GENE EXPRESSION

ISOLATION OF RNA

Experimental fish was dissected very carefully to extract liver tissue of *L. rohita* fingerlings and stored instantly in TRIzol reagent for further process. After RNA extraction, the purity of total RNA was checked by NanoDrop micro-volume spectrophotometer.

FIRST STRAND cDNA SYNTHESIS

The complete total RNA isolated from liver tissue of *L. rohita* fingerlings was reverse transcribed into complementary DNA with 20 µL total volume of using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, #k1622) with manufacturer's instructions. The samples of cDNA were then stored at -80 °C.

REVERSE TRANSCRIPTASE-QUANTITATIVE POLYMERASE CHAIN REACTION (RT-qPCR)

Ten µL reaction mixture containing 5 µL SYBR® Green, 1 µL forward primer, 1 µL reverse primer and 3 µL cDNA was prepared. β-actin was used as a reference gene and a house keeping gene as in Irm et al. (2020). It is commonly used as an internal reference for gene expression studies. The process was divided into three steps. First step was at 90 °C for 3 min. Second step comprised of 50 cycles, each cycle with three sub steps: (a) at 95 °C for 15 s, (b) at 60 °C for 30 s, (c) at 72 °C for 30 s. Third step comprised of 71 cycles, began at 60 °C and increased every 10 s up to 95 °C. Variations in gene expression (fold changes) were calculated by ΔΔCt method (Livak & Schmittgen 2001; Zhong et al. 2012). The primers were designed by following the protocols of Ye et al. (2012). Furthermore, following Kayama et al. (2021), cross amplification, hairpin loops, and dimer probability were investigated. The sequences of forward and reverse primers are given in Table 3. The annealing temperature and efficiency of primers were 45-50 ℃ and 98-99%, respectively. The agarose gel image of PCR is presented in Figure 1.

TABLE 3. Primers used for RT-qPCR analysis followed (Mun et al. 2019)

Gene	Primer sequence (5'–3')[a]	Amplicon Size (bp)	Accession Number
Gh	F: GAA CTG ATG CCA GCC ATG A R: AGC TAC AGA GTG CAG TTT G	87	XM_051106058.1
ghr-1	F: CCA TCA GAT GAG CAA CTT CTG AAA AGT R: ACT TCC TGG TGA ATC AGC CTT A	122	XM_051106058.1
ghr-2	F: CAC AGA CTT CTA CGC TCA GGT CA R: TGA GTT GCT GTC CAG GAG ACA	97	XM_051106058.1
igf-1	F: GTC TGT GGA GAG CGA GGC TTT R: AAC CTT GGG TGC TCT TGG CAT G	115	XM_051107790.1
igf-1 r_a	F: CTAAGGGCGTGGTTAAGCAC R: TTGTTGGCGTTGAGGTATGC	121	XM_051134507.1
igf-1 r_b	F: AGG GAC GAG CCA GAG ACG R:TTC AGA GGA GGG AGG TTG	126	XM_051114311.1
act-β	F: GTG ATG TGA CGC TGG ACC AAT C R: CCA TGT CAT CCC AGT TGG TCA CAA T	114	XM_051096690.1

Abbreviations; *gh*=Growth hormone, *ghr-1*=Growth hormone receptor-1, *ghr-2*=Growth hormone receptor-2, *igf-1*= Insulin-like growth factor-1, *igf-1* r_a= Insulin like growth factor-1 receptor a, *igf-1* r_b= Insulin like growth factor-1 receptor b, *act-β*=β-Actin

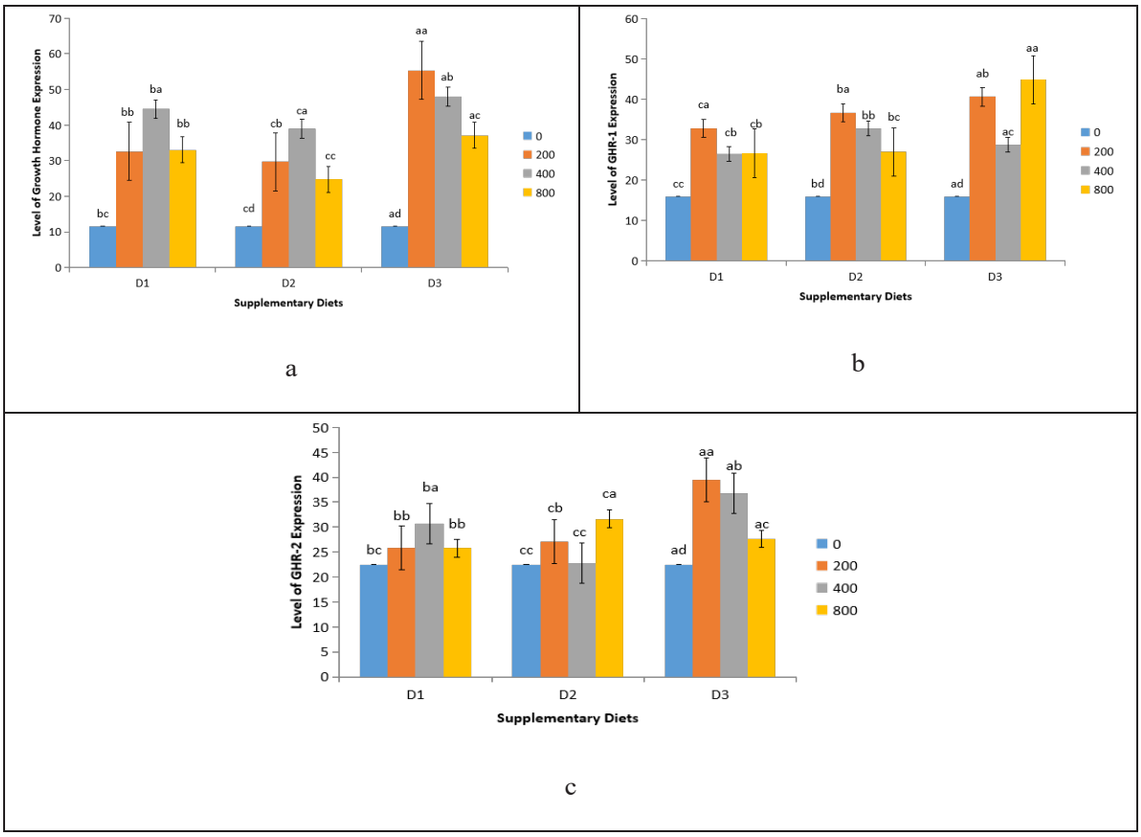

FIGURE 2. Relative mRNA expression of (a) GH (b) GHR-1 (c) GHR-2 in liver of *L. rohita* fingerlings fed on polyphenols-supplemented diets

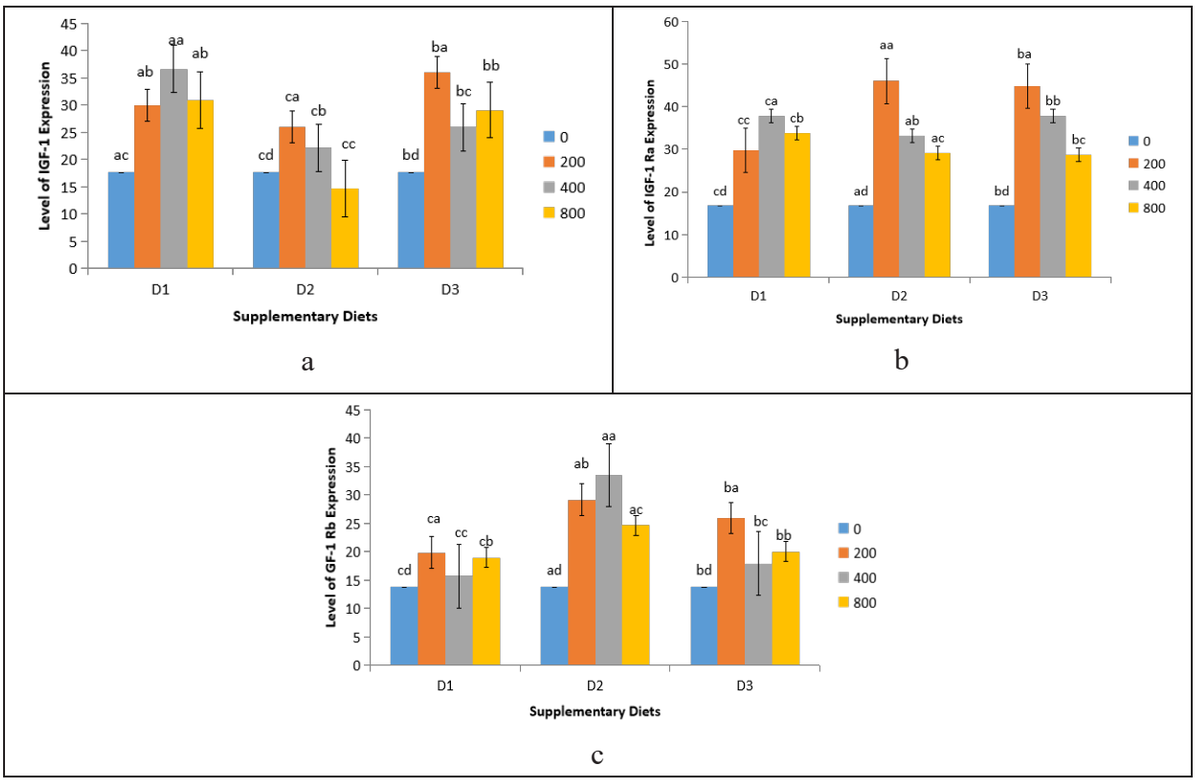

FIGURE 3. Relative mRNA expression of (a) IGF (b) IGF-1 R_a (c) IGF-1 R_b in liver of *L. rohita* fingerlings fed on polyphenols-supplemented diets

GROWTH PERFORMANCE

To check the feed and growth performance, fingerlings of each tank were bulk weighed at the start and completion of trial and calculated by using following standard formulae (Hussain et al. 2015).

$$\text{Weight gain \%} = \frac{\text{Final weight (g)-Initial weight(g)}}{\text{Initial weight}} \times 100 \quad (1)$$

$$\text{FCR} = \frac{\text{Total dry feed intake (g)}}{\text{Wet weight gain (g)}} \quad (2)$$

$$\text{SGR} = \frac{[\ln(\text{final weight}) - \ln(\text{initial weight})]}{\text{Duration of trial in day}} \times 100 \quad (3)$$

CHEMICAL ANALYSIS OF PROXIMATE BODY COMPOSITION

Fish body was analyzed by the standard methods of AOAC (2005) to find out their proximate composition. The crude protein and fat were determined with the micro-Kjeldahl apparatus and with the Soxtec HT2 1045 system, respectively. The moisture content was checked by placing the fish in oven at 105 °C for 12 h and ash content by burning the fish body in electric furnace at 650 °C for 12 h.

STATISTICAL ANALYSIS

Data of gene expression, growth performance and body composition of fingerlings was evaluated statistically by two-way analysis of variance (Steel, Torrie & Dickey 1996). The differences among means were compared by Tukey's Honesty Significant Difference Test and considered significant at $p<0.05$ (Snedecor & Cochran 1991). The CoStat-computer package (Version 6.303, PMB 320, Monterey, CA, 93940 USA) was utilized for statistical analysis.

RESULTS

EXPRESSION OF INSULIN-LIKE GROWTH FACTORS AND GROWTH HORMONE

After the 70 days of feeding period, the results showed that expressions of IGF and GH in the liver were noticed to be regulated significantly ($p< 0.05$) when fed with test diets having different levels of polyphenols. The IGF and GH gene expression were increased significantly in supplemented test diets when compared to control diet. The highest GH expression (55.32) and GHR-2 (39.52) were observed at 200 mg/kg mixture diet (Figure 2(a), 2(c)) which was significantly improved than other diets. Improved GHR-1 gene expression (44.78) was present in fish treated with 400 mg/kg of mixture diet while lowest GHR-2 expression (22.87) when fed with *S. oleoides* at 400 mg/kg (Figure 2(c)). At 800 mg/kg mixture diet, maximum (44.78) GHR-1 gene expression was noted (Figure 2(b)). The highest IGF-1 gene expression (36.67) at 400 mg/kg *S. persica* based diet was obtained when compared to control diet (Figure 3(a)). IGF-1 R_a and lowest IGF-1 R_b expression was observed at 400 mg/kg *S. oleoides* and mixture diet (Figure 3(b), 3(c)).

GROWTH PERFORMANCE

Growth performance was affected significantly in fingerlings fed with polyphenol supplemented diet as compared to control diet. The maximum specific growth rate (SGR) (1.63) and weight gain (WG) % (215%) was noticed in fingerlings fed with diet having 200 mg/kg polyphenols followed by 400 mg/kg (WG%: 192%, SGR: 1.53) (Table 4). In *S. oleoides* based diet, highest value of WG% and SGR was observed at 400 mg/kg followed by 200 and 800 mg/kg diet. The highest level (800 mg/kg) of *S. oleoides* in diet decreased the weight gain (141%) of fingerlings. WG of fingerlings at 800 mg/kg in *S. oleoides* based diet was lower than the other two levels but still higher than control level. All the experimental groups showed the significant increase in the WG and SGR when compared to control group. The lowest value of FCR (1.04) was observed at 200 mg/kg diet in mixture diet followed by *S. persica* based diet.

BODY COMPOSITION

Proximate analysis of body composition showed that fingerlings fed on mixture diet at 200 mg/kg had highest (63%) crude protein. The protein content in mixture diet increased with the increase in polyphenols levels. In *S. oleoides* based diet, highest protein content was observed at 200 mg/kg (59%) followed by control in diet (88%) (Table 5). In *S. persica* based diet, at 200 mg/kg significant increase in protein content was noticed while

TABLE 4. Growth performance of *L. rohita* fingerlings fed polyphenols-supplemented canola meal-based diet

Test Diets	Polyphenols	Levels (mg/kg)	Initial weight	Final weight	Weight Gain (%)	FCR	SGR
T_1		0	7.30±0.33	17.4±0.11^{bd}	138.9±12.4^{bd}	1.53±0.05^{aa}	1.24±0.07^{bc}
T_2		200	7.6±0.12	21.4±0.27^{ba}	181.3±1.40^{ba}	1.29±0.04^{ad}	1.47±0.007^{ba}
	S. persica						
T_3		400	7.30±0.17	19.4±0.06^{bb}	165.6±5.74^{bb}	1.37±0.01^{ac}	1.39±0.03^{ba}
T_4		800	7.32±0.20	18.6±0.07^{bc}	154±7.36^{bc}	1.49±0.03^{ab}	1.33±0.04^{bb}
T_5		0	7.30±0.33	17.4±0.11^{cd}	138.6±12.4^{cd}	1.52±0.05^{ba}	1.23±0.07^{cb}
T_6		200	7.43±0.03	18.9±0.04^{ca}	154.9±1.61^{ca}	1.36±0.02^{bc}	1.33±0.009^{ca}
	S. oleoides						
T_7		400	7.30±0.17	19.9±0.05^{cb}	173.0±7.10^{cb}	1.28±0.04^{bd}	1.43±0.03^{ca}
T_8		800	7.22±0.06	17.4±0.03^{cc}	141.5±1.84^{cc}	1.41±0.01^{bb}	1.25±0.01^{cb}
T_9		0	7.30±0.33	17.4±0.11^{ad}	138.2±12.4^{ad}	1.51±0.05^{ca}	1.22±0.07^{ad}
T_{10}		200	7.37±0.32	23.2±0.09^{aa}	215.3±12.6^{aa}	1.04±0.02^{cc}	1.63±0.05^{aa}
	Mixture						
T_{11}		400	7.43±0.26	21.7±0.17^{ab}	192.4±12.7^{ab}	1.11±0.04^{cb}	1.53±0.06^{ab}
T_{12}		800	7.45±0.04	20.5±0.10^{ac}	174.7±3.12^{ac}	1.08±0.01^{cc}	1.44±0.01^{ac}

Means in columns with distinct superscripts are significantly different at $p< 0.05$. Data are means of three replicates

at 400 mg/kg and control, no significant effect was seen between these two levels. The lowest value of protein content in all three diet groups was observed when fed with 800 mg/kg supplementation of polyphenols. Maximum fat content (15%) was observed in mixture diet when supplemented at 800 mg/kg. In all supplemented diets, ash and moisture content of the fish body were found to be minimum at 200 mg/kg in diet.

DISCUSSION

Plant extracts having active biomolecules or phytochemicals are being administered orally to augment the growth of farmed species and is considered a strategy of considerable attention nowadays (Abdel-Tawwab & Monier 2018; Adeshina et al. 2019). These possess anti-inflammatory, anti-microbial, immunostimulatory, antioxidant, growth promoting and microbiota regulation abilities (Reverter et al. 2017). Fish growth performance is under genetic control and it is mainly affected by various physiological and environmental factors such as temperature and photoperiod (Ranjan et al. 2018). The synthesis and release of IGF is stimulated by binding of GH to receptors

TABLE 5. Body composition (%) of *L. rohita* fingerlings fed on polyphenols-supplemented diets

Test diets	Polyphenols	Levels (mg/kg)	Protein	Fat	Ash	Moisture
T_1	*S. persica*	0	58.8±0.39^{bd}	13.5±0.33^{bc}	10.2±0.37^{ba}	17.5±0.32^{bc}
T_2		200	62.2±0.28^{ba}	13.4±0.28^{bd}	8.1±0.43^{bd}	16.3±0.34^{bd}
T_3		400	58.3±0.33^{bb}	14.3±0.14^{bb}	8.9±0.07^{bc}	18.5±0.24^{bb}
T_4		800	54.6±0.14^{bc}	15.2±0.39^{ba}	9.6±0.25^{bb}	20.6±0.34^{ba}
T_5	*S. oleoides*	0	58.4±0.39^{cb}	13.7±0.33^{ac}	10.4±0.37^{aa}	17.5±0.32^{ad}
T_6		200	59.8±0.15^{ca}	12.8±0.07^{ad}	9.6±0.02^{ad}	17.8±0.22^{ac}
T_7		400	55.9±0.28^{cc}	15.4±0.14^{ab}	9.9±0.03^{ab}	18.8±0.37^{ab}
T_8		800	51.7±0.03^{cd}	16.2±0.05^{aa}	10.4±0.04^{aa}	21.7±0.22^{aa}
T_9	Mixture	0	58.3±0.39^{ac}	13.8±0.33^{cc}	10.2±0.37^{ca}	17.7±0.32^{cb}
T_{10}		200	63.4±0.27^{aa}	13.1±0.28^{cd}	7.8±0.04^{cd}	15.7±0.03^{cd}
T_{11}		400	60.6±0.37^{ab}	14.2±0.15^{cb}	8.5±0.05^{cc}	16.7±0.03^{cc}
T_{12}		800	56.4±0.14^{ad}	15.6±0.30^{ca}	9.6±0.25^{cb}	18.4±0.03^{ca}

Means in columns with distinct superscripts are significantly different at $p< 0.05$. Data are means of three replicates

present in the target organ specifically in the liver (Miandare et al. 2016). Many studies have documented previously that GH and IGF expression were affected by adding supplements into fish feed (Tan et al. 2017). Kumar et al. (2017) reported that nutritional status of fish feed affected the IGF-1 expression in many fish species. In this study, GH and IGF-1 expression was significantly affected by diet supplemented with polyphenols. The results of our study are in accordance with results of Kumar et al. (2017) who stated that addition of de-oiled rice bran in the feed of *L. rohita* significantly affected IGF-I expression. In our study, the growth of fingerlings showed a significant increase when given 200 mg/kg polyphenols in diet (Table 4). The growth results showed a positive correlation with IGF-I and IGF-II expression. In line with our studies, Hoseinifar et al. (2017) recorded an upregulation of GH, IGF-1 and IGF-2 gene expression in liver and brain of common carp, by using polyphenol-enriched extract of date palm for the period of 60 days. Safari et al. (2020) found the improvement in GH

gene expression and in turn, the release of IGF while feeding the Beluga sturgeon on diet supplemented with dietary tannins (polyphenols). This effect increased in dose-dependent manner. The basic mode of action of polyphenols is to improve the gastrointestinal function and morphology and ultimately the enhanced fish growth and nutritional results (El-Bakary & El-Gammal 2010). Similarly in juvenile puffer fish, expression of IGF-1 gene showed significant results when added vitamin C in the diet (Cheng et al. 2018). Gómez Requeni et al. (2004) findings were in line with our results in that the addition of plant protein into feed of Gilthead sea bream (*Sparus aurata*) affected the GH expression. Midhun et al. (2016) stated the upregulation of GH and IGF-1 in brain and skeletal muscles of Nile tilapia when treated with polyphenolic curcumin.

In the present study, the *L. rohita* fingerlings fed with 200 mg/kg polyphenols showed the best growth performance. The highest weight gain of fingerlings was observed in mixture diet at 200 mg/kg. The growth determining phenomenon of polyphenols in fish feed is quite variable such as some polyphenols have been found to increase the growth rate in Parrotfish (Wang et al. 2003), African catfish (Turan & Akyurt 2005) and Yellow perch (Lee & Dabrowski 2004). Satisfying results of WG% (125%), SGR (1.80) and FCR (2.13) were obtained by Jahazi et al. (2020), while feeding the polyphenols extracted from chestnut (0.2%) supplemented diet to the common carp. The experiment conducted by Zhang and Wen (2012) supported our results that addition of chlorogenic acid into fish feed at 200 mg/kg improved the growth performance. Although in Angelfish juveniles, no effect on growth was recorded (Blom, Dabrowski & Ebeling 2000). It was observed that the growth performance of *L. rohita* fingerlings was significantly improved at lowest level, indicating that lower dose of polyphenols is suitable for enhancing the growth of fish. The maximum feed intake was recorded at 200 mg/kg diet. The lowest feed intake was observed at higher level 800 mg/kg, which means that highest concentration affects the palatability of feed and reduced the feed intake ultimately leading to low weight gain. Omnes et al. (2017) reported that addition of dietary tannin into feed of European seabass (*Dicentrarchus labrax*) reduced the feed intake of *Cyprinus carpio* when added above 10 g/kg diet. Such disparity can be due to difference in supplementation levels of the polyphenols than ours. Zhong et al. (2020) also found significant effect of tea polyphenols at 50 mg/kg on the growth performance of Black carp. Furthermore, Xu et al. (2019) described the growth promoting effects, WG% (861%), FCR (1.33) of quercetin (source of polyphenols) at 0.4mg/kg in Grass carp.

From our study, it is obvious that the increase in polyphenol level and its positive outcomes showed inverse relation after an optimal limit. It is also supported by Halliwell (2007), who said that phenolic compounds at high concentrations can lead to toxicity of host animal. The highest crude protein and crude fat content were present at the lowest level of combination diet, which supports the fact that at lower dose, polyphenols give better results. According to Deng et al. (2011), the increased fat and protein content in the fish body explained that flavonoid content in the plant extracts increased the ingestion of food, absorption and metabolism of nutrients. Similarly, with the increase of polyphenols level, improved fat and protein contents in the body were observed in Grass carp (Liang et al. 2012).

Conclusion

It was concluded that polyphenols extracted from *S. persica* and *S. oleoides* supplemented in canola meal are useful to achieve upregulated gene expressions of insulin-like growth factors and growth hormone, maximum growth performance and proximate body composition of *L. rohita*. Mixture of both polyphenols at 200 mg/kg worked well because of their synergistic action. Overall, these polyphenols can serve as functional feed ingredients so that successful fish farming is possible.

ACKNOWLEDGMENTS

The authors are thankful to Higher Education Commission (HEC), Islamabad, Pakistan for its support by providing Project#5649/Punjab/NRPU/R&D/HEC/2016 and Project#20-4892/NRPU/R&D/HEC/14/1145 to conduct this research work. The authors declare that there is no conflict of interest regarding publication of this paper. The data presented in this study are available on request from the corresponding author.

REFERENCES

Abdel-Tawwab, M. & Monier, M.N. 2018. Stimulatory effect of dietary taurine on growth performance, digestive enzymes activity, antioxidant capacity, and tolerance of common carp, *Cyprinus carpio* L., fry to salinity stress. *Fish Physiology and Biochemistry* 44(2): 639-649. https://doi.org/10.1007/s10695-017-0459-8

Adeshina, I., Jenyo-Oni, A., Emikpe, B.O., Ajani, E.K. & Abdel-Tawwab, M. 2019. Stimulatory effect of dietary clove, *Eugenia caryophyllata*, bud extract on growth performance, nutrient utilization, antioxidant capacity, and tolerance of African catfish, *Clarias gariepinus* (B.), to *Aeromonas hydrophila* infection. *Journal of the World Aquaculture Society* 50(2): 390-405. https://doi.org/10.1111/jwas.12565

Alhazzaa, R., Nichols, P.D. & Carter, C.G. 2019. Sustainable alternatives to dietary fish oil in tropical fish aquaculture. *Reviews in Aquaculture* 11(4): 1195-1218. https://doi.org/10.1111/raq.12287

Amin, A., El Asely, A., Abd El-Naby, A.S., Samir, F., El-Ashram, A., Sudhakaran, R. & Dawood, M.A. 2019. Growth performance, intestinal histomorphology and growth-related gene expression in response to dietary *Ziziphus mauritiana* in Nile tilapia (*Oreochromis niloticus*). *Aquaculture* 512: 734301. https://doi.org/10.1016/j.aquaculture.2019.734301

Association of Official Analytical Chemists (AOAC). 2005. *Official Methods of Analysis*. 15th ed. Washington: Association of Official Analytical Chemists. p. 1094.

Béné, C., Arthur, R., Norbury, H., Allison, E.H., Beveridge, M., Bush, S., Campling, L., Leschen, W., Little, D., Squires, D. & Thilsted, S.H. 2016. Contribution of fisheries and aquaculture to food security and poverty reduction: assessing the current evidence. *World Development* 79: 177-196. https://doi.org/10.1016/j.worlddev.2015.11.007

Blom, J.H., Dabrowski, K. & Ebeling, J. 2000. Vitamin C requirements of the Angelfish *Pterophylum scalare*. *Journal of the World Aquaculture Society* 31(1): 115-118. https://doi.org/2F10.1111/2Fj.1749-7345.2000.tb00705.x

Burel, C. & Kaushik, S.J. 2008. Use of rapeseed/canola in diets of aquaculture species. In *Alternative Protein Sources in Aquaculture Diets*, edited by Lim, C., Webster, C.D. & Lee, C-S. New York and London: Haworth Press. pp. 343-408. doi: 10.3923/jfas.2011.22.36

Cheng, C.H., Guo, Z.X., Ye, C.X. & Wang, A.L. 2018. Effect of dietary astaxanthin on the growth performance, non-specific immunity, and antioxidant capacity of pufferfish (*Takifugu obscurus*) under high temperature stress. *Fish Physiology and Biochemistry* 44(1): 209-218. https://doi.org/10.1016/j.aquaculture.2019.734341

Deng, J., An, Q., Bi, B., Wang, Q., Kong, L., Tao, L. & Zhang, X. 2011. Effect of ethanolic extract of propolis on growth performance and plasma biochemical parameters of rainbow trout (*Oncorhynchus mykiss*). *Fish Physiology and Biochemitry* 37(4): 959-967. https://doi.org/10.1007/s10695-011-9493-0

El-Bakary, N.E.R. & El-Gammal, H.L. 2010. Comparative histological, histochemical and ultrastructural studies on the liver of flathead grey mullet (*Mugil cephalus*) and sea bream (*Sparus auratus*). *Global Veterinaria* 4(6): 548-553.

Geetha, K., Manavalan, R. & Venkappayya, D. 2010. Control of urinary risk factors of stone formation by *Salvadora persica* in experimental hyperoxaluria. *Methods and Findings in Experimental and Clinical Pharmacology* 32(9): 623-629. https://doi.org/10.1358/mf.2010.32.9.1549114

Glencross, B. 2016. Understanding the nutritional and biological constraints of ingredients to optimize their application in aquaculture feeds. In *Aquafeed Formulation*, edited by Nates, S.F. Academic Press: New York. pp. 32-73. https://doi.org/10.1016/b978-0-12-800873-7.00003-8

Gómez-Requeni, P., Mingarro, M., Calduch-Giner, J.A., Médale, F., Martin, S.A.M., Houlihan, D.F., Kaushik, S. & Pérez-Sánchez, J. 2004. Protein growth performance, amino acid utilization and somatotropic axis responsiveness to fish meal replacement by plant protein sources in gilthead sea bream (*Sparus aurata*). *Aquaculture* 232(1-4): 493-510. https://doi.org/10.1016/S0044-8486(03)00532-5

Halliwell, B. 2007. Dietary polyphenols: good, bad, or indifferent for your health? *Cardiovascular Research* 73(2): 341-347. https://doi.org/10.1016/j.cardiores.2006.10.004

Hoseinifar, S.H., Dadar, M., Khalili, M., Cerezuela, R. & Esteban, M.Á. 2017. Effect of dietary supplementation of palm fruit extracts on the transcriptomes of growth, antioxidant enzyme and immune-related genes in common carp (*Cyprinus carpio*) fingerlings. *Aquaculture Research* 48(7): 3684-3692. doi:10.1111/are.13192

Hussain, S.M., Nisar, A., Farhat, J., Arshad, J., Nosheen, A., Majid, H., Shahtaj, A., Arsalan, M.Z.H., Danish, R. & Shahzad, M.M. 2015. Effects of citric acid and phytase supplementation on nutrient digestibility and growth performance of *Cirrhinus mrigala* fingerlings fed on corn gluten (30%) meal based diets. *International Journal of Biosciences* 6(7): 82-91. http://www.innspub.net/wp-content/upl

Irm, M., Taj, S., Jin, M., Luo, J., Andriamialinirina, H.J.T. & Zhou, Q. 2020. Effects of replacement of fish meal by poultry by-product meal on growth performance and gene expression involved in protein metabolism for juvenile black sea bream (*Acanthoparus schlegelii*). *Aquaculture* 528: 735544. https://doi.org/10.1016/j.aquaculture.2020.735544

Jahazi, M.A., Hoseinifar, S.H., Jafari, V., Hajimoradloo, A., Van Doan, H. & Paolucci, M. 2020. Dietary supplementation of polyphenols positively affects the innate immune response, oxidative status, and growth performance of common carp, *Cyprinus carpio* L. *Aquaculture* 517: 734709. doi: https://doi.org/10.1016/j.aquaculture.2019.734709

Kayama, K., Kanno, M., Chisaki, N., Tanaka, M., Yao, R., Hanazono, K. & Endoh, D. 2021. Prediction of PCR amplification from primer and template sequences using recurrent neural network. *Scientific Reports* 11(1): 7493. https://doi.org/10.1038/s41598-021-86357-1

Kumar, S., Sándor, Z.J., Nagy, Z., Fazekas, G., Havasi, M., Sinha, A.K., De Boeck, G. & Gál, D. 2017. Potential of processed animal protein versus soybean meal to replace fish meal in practical diets for European catfish (*Silurus glanis*): Growth response and liver gene expression. *Aquaculture Nutrition* 23(5): 1179-1189. https://doi.org/10.1111/ANU.12487

Landero, J.L., Beltranena, E. & Zijlstra, R.T. 2012. The effect of feeding lentil on growth performance and diet nutrient digestibility in starter pigs. *Animal Feed Science and Technology* 174(1): 108-112.

Lee, K.J. & Dabrowski, K. 2004. Long-term effects and interactions of dietary vitamins C and E on growth and reproduction of yellow perch, *Perca flavescens*. *Aquaculture* 230(1-4): 377-389. doi: 10.1016/S0044-8486(03)00421-6

Liang, J.J., Tian, L.X., Liu, Y.J., Yang, H.J. & Liang, G.Y. 2012. Dietary magnesium requirement and effects on growth and tissue magnesium content of juvenile grass carp (*Ctenopharyngodon idella*). *Aquaculture Nutrition* 18(1): 56-64. https://doi.org/10.1111/j.1365-2095.2011.00876.x

Livak, K.J. & Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *Methods* 25(4): 402-408. https://doi.org/10.1006/meth.2001.1262

Maqsood, S., Benjakul, S., Abushelaibi, A. & Alam, A. 2014. Phenolic compounds and plant phenolic extracts as natural antioxidants in prevention of lipid oxidation in seafood: A detailed review. *Comprehensive Reviews in Food Science and Food Safety* 13(6): 1125-1140. https://doi.org/10.1111/1541-4337.12106

Mathur, S. 2015. Medicinally potent and highly salt tolerant plant of arid zone *Salvadora persica* (Meswak): A review. *Journal of Plant Sciences* 3: 45-49. https://doi.org/10.11648/J.JPS.S.2015030101.17s

Miandare, H.K., Farvardin, S., Shabani, A., Hoseinifar, S.H. & Ramezanpour, S.S. 2016. The effects of galactooligosaccharide on systemic and mucosal immune response, growth performance and appetite related gene transcript in goldfish (*Carassius auratus gibelio*). *Fish and Shellfish Immunology* 55: 479-483. https://doi.org/10.1016/j.fsi.2016.06.020

Midhun, S.J., Arun, D., Edatt, L., Sruthi, M.V., Thushara, V.V., Oommen, O.V., Sameer, Kumar, V.B. & Divya, L. 2016. Modulation of digestive enzymes, GH, IGF-1 and IGF-2 genes in the teleost, Tilapia (*Oreochromis mossambicus*) by dietary curcumin. *Aquaculture International* 24(5): 1277-1286. https://doi.org/10.1007/s10499-016-9984-1

Ming, J., Xie, J., Xu, P., Ge, X., Liu, W. & Ye, J. 2012. Effects of emodin and vitamin C on growth performance, biochemical parameters and two HSP70s mRNA expression of Wuchang bream (*Megalobrama amblycephala* Yih) under high temperature stress. *Fish and Shellfish Immunology* 32(5): 651-661. https://doi.org/10.1016/j.fsi.2012.01.008

Moustafa, E.M., Dawood, M.A., Assar, D.H., Omar, A.A., Elbialy, Z.I., Farrag, F.A., Shukry, M. & Zayed, M.M. 2020. Modulatory effects of fenugreek seeds powder on the histopathology, oxidative status, and immune related gene expression in Nile tilapia (*Oreochromis niloticus*) infected with *Aeromonas hydrophila*. *Aquaculture* 515: 734589. https://doi.org/10.1016/j.aquaculture.2019.734589

Mun, S.H., You, J.H., Oh, H.J., Lee, C.H., Baek, H.J., Lee, Y.D. & Kwon, J.Y. 2019. Expression patterns of growth related genes in juvenile red spotted grouper (*Epinephelus akaara*) with different growth performance after size grading. *Development and Reproduction* 23(1): 35. https://doi. 10.12717/DR.2019.23.1.035

Mutch, D.M., Wahli, W. & Williamson, G. 2005. Nutrigenomics and nutrigenetics: the emerging faces of nutrition. *The FASEB Journal* 19(12): 1602-1616. https://doi.org/10.1096/fj.05-3911rev

Nijveldt, R.J., van Nood, E.L.S., van Hoorn, D.E., Boelens, P.G., van Norren, K. & van Leeuwen, P.A. 2001. Flavonoids: A review of probable mechanisms of action and potential applications. *The American Journal of Clinical Nutrition* 74(4): 418-425. https://doi.org/10.1093/AJCN%2F74.4.418

Omnes, M.H., Le Goasduff, J., Le Delliou, H., Le Bayon, N., Quazuguel, P. & Robin, J.H. 2017. Effects of dietary tannin on growth, feed utilization and digestibility, and carcass composition in juvenile European seabass (*Dicentrarchus labrax* L.). *Aquaculture Reports* 6: 21-27. https://doi.org/10.1016/j.aqrep.2017.01.004

Ranjan, A., Jain, K.K., Srivastava, P.P. & Muralidhar, P.A. 2018. Dietary energy requirement of *Pangasianodon hypophthalmus* (Sauvage, 1878) juveniles reared at two temperatures. *Turkish Journal of Fisheries and Aquatic Sciences* 18(1): 101-108. https:// doi.org/2F10.4194/2F1303-2712-v18_1_11

Reverter, M., Tapissier-Bontemps, N., Sasal, P. & Saulnier, D. 2017. Use of medicinal plants in aquaculture. In *Diagnosis and Control of Diseases of Fish and Shellfish*, 1st ed., edited by Austin, B. & Newaj-Fyzul, A. John Wiley & Sons Ltd. pp. 223-261. doi: 10.1002/9781119152125.ch9

Safari, R., Hoseinifar, S.H., Imanpour, M.R., Mazandarani, M., Sanchouli, H. & Paolucci, M. 2020. Effects of dietary polyphenols on mucosal and humoral immune responses, antioxidant defense and growth gene expression in beluga sturgeon (*Huso huso*). *Aquaculture* 528: 735494. https://doi.org/10.1016/j.aquaculture.2020.735494

Salehi, P. & Sh, M.D. 2006. Comparison of the antibacterial effects of persica mouthwash with chlorhexidine on streptococcus mutans in orthodontic patients. *DARU Journal of Pharmaceutical Sciences* 14(4): 178-182.

Salin, K.R., Arun, V.V., Mohanakumaran Nair, C. & Tidwell, J.H. 2018. Sustainable aquafeed. In *Sustainable Aquaculture*, edited by Hai, F., Visvanathan, C. & Boopathy, R. Springer, Cham. pp. 123-151. https://doi.org/10.1007/978-3-319-73257-2_4

Singh, P.K., Gaur, S.R. & Chari, M.S. 2006. Effect of varying protein levels on the growth of Indian Major Carp Rohu, *Labeo rohita* (Hamilton). *International Journal of Zoological Research* 2: 186-191. https://dx.doi.org/10.3923/ijzr.2006.186.191

Snedecor, G.W. & Cochran, W.G. 1991. *Statistical Methods.* 8th ed. Iowa State University Press. p. 503.

Steel, R.G.D., Torrie, J.H. & Dickey, D.A. 1996. *Principles and Procedures of Statistics.* 3rd ed. New York: McGraw Hill International Book Co. pp. 336-352.

Tan, X., Sun, Z., Huang, Z., Zhou, C., Lin, H., Tan, L., Xun, P. & Huang, Q. 2017. Effects of dietary hawthorn extract on growth performance, immune responses, growth-and immune-related genes expression of juvenile golden pompano (*Trachinotus ovatus*) and its susceptibility to *Vibrio harveyi* infection. *Fish and Shellfish Immunology* 70: 656-664. https://doi.org/10.1016/j.fsi.2017.09.041

Tatar, M., Bartke, A. & Antebi, A. 2003. The endocrine regulation of aging by insulin-like signals. *Science* 299(5611): 1346-1351. https://doi.org/10.1126/science.1081447

Turan, F. & Akyurt, I. 2005. Effects of red clover extract on growth performance and body composition of African catfish *Clarias gariepinus*. *Fisheries Science* 71(3): 618-620. https://doi.org/2F10.1111/2Fj.1444-2906.2005.01006.x

Verma, R., Purohit, S., Bhandari, A., Kumar, B. & Priyanka, P. 2009. *Salvadora persica* L (tooth brush tree): A review. *Journal of Pharmacy Research* 2(12): 1809-1812.

Wahab, M.A., Rahman, M.M. & Milstein, A. 2002. The effect of common carp, *Cyprinus carpio* (L.) and mrigal, *Cirrhinus mrigala* (Hamilton) as bottom feeders in major Indian carp polycultures. *Aquaculture Research* 33(8): 547-556. https://doi.org/10.1046/j.1365-2109.2002.00654.x

Wang, X., Kim, K.W., Bai, S.C., Huh, M.D. & Cho, BY. 2003. Effects of the different levels of dietary vitamin C on growth and tissue ascorbic acid changes in parrot fish (*Oplegnathus fasciatus*). *Aquaculture* 215(1-4): 203-211. http://dx.doi.org/10.5713/ajas.14.0532

Xu, Z., Li, X., Yang, H., Liang, G., Gao, B. & Leng, X. 2019. Dietary quercetin improved the growth, antioxidation, and flesh quality of grass carp (*Ctenopharyngodon idella*). *Journal of the World Aquaculture Society* 50(6): 1182-1195. https://doi.org/10.1111/jwas.12663

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S. & Madden, T. L. 2012. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 13: 1-11. https://doi.org/10.1186/1471-2105-13-134

Yigit, N.O. & Olmez, M. 2009. Canola meal as an alternative protein source in diets for fry of tilapia (*Oreochromis niloticus*). *Israeli Journal of Aquaculture – Bamidgeh* 61: 35-41. https://doi.org/10.46989/001c.20535

Zhang, C. & Wen, A. 2012. Different of chlorogenic acid doses on growth performance, non-specific immunity and antioxidant functions of *Cyprinus carpio* var. *Journal of Sichuan Agricultural University* 30: 92-97. https://doi.org/10.1111/ANU.12500

Zhong, H., Zhou, Y., Liu, S., Tao, M., Long, Y., Liu, Z. & Liu, Y. 2012. Elevated expressions of GH/IGF axis genes in triploid Crucian carp. *General and Comparative Endocrinology* 178(2): 291-300. https://doi.org/10.1016/j.ygcen.2012.06.006

Zhong, L., Hu, Y., Hu, Y., Li, J., Tian, Y., Chen, J., Ai, Q, & Xiao, T. 2020. Effects of dietary tea polyphenols on growth, immunity and lipid metabolism of juvenile black carp *Mylopharyngodon piceus*. *Aquaculture Research* 51(2): 569-576. https://doi.org/10.1111/are.14403

*Corresponding author; email: drmakhdoomhussain@gcuf.edu.pk

SUPPLEMENTARY FILES

Details of how the RNA was extracted, the concentration and purity of the samples and how it was stored.

1. Dissection of the experimental fish was done very carefully and extracted liver tissue of *L. rohita* fingerlings and collected in eppendorf for the gene expression study and stored instantly in TRIzol reagent for further process.

2. The cell or tissue samples were homogenized so that all fractions of the sample could be identical in composition. Tissue was homogenized through tissuelyser. Beads were added into eppendorf and set it into tissuelyser for 30-50 sec.

3. TRIzol reagent method is used to isolate the RNA of *L. rohita* fingerlings from liver and brain (including pituitary).

a. Homogenized sample was taken in a fresh eppendorf tube
b. 1 mL TRIzol reagent (Invitrogen, Germany) per 50 mg samples was added in the homogenized sample
c. 200 µL chloroform was added in the homogenate sample
d. Vortex the sample for 30 s and place it immediately in ice box
e. Centrifuge the sample at 12000 rpm for 15 min at 4 °C to get separation phase
f. After centrifugation separation phase was formed and transfer the upper phase in fresh nuclease free eppendorf tube
g. 0.5 mL isopropanol was added and centrifuged it at 12000 rpm for 10 min at 4 °C to get a pellet
h. Washed the pellet with 1 mL 70% ethanol, centrifuged it at 7500 rpm for 10 min at 4 °C and removed the supernatant
i. The pellet was then air dried in eppendorf tube stand
j. 20 µL RNase free H_2O was added to dissolve RNA pallet in eppendorf tube

Concentration of chemicals for RNA extraction by TRIzol reagent method

Sr. No	Chemicals	Concentration
1	TRIzol reagent	1 ml
2	Chloroform	200 µl
3	Ethanol 70%	1 ml
4	Isopropanol	O.5 ml
5	RNase free H_2O	20 l

4. The purity of total RNA was checked by NanoDrop micro-volume spectrophotometer. First placed a drop of double distilled water at the lens of spectrophotometer and then loaded 1 µl RNA sample at the less. Click the 'measure' button and record the readings.
5. RNA was stored at -20 ℃ until it was use.