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Research Article

Effect of phytogenic feed additive (Digestarom PEP) on growth performance, hematological parameters, immunity system, and carcass quality of giant gourami (*Osphronemus goramy*)

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Abstract

There is a growing interest among researchers to use herbal supplementations in aquafeed to enhance growth performance as well as the immunity systems of aquatic animals. Therefore, this study was conducted to investigate the effect of phytogenic feed additive (Digestarom PEP) on growth performance, chemical composition, hematological and immune responses of giant gourami (*Osphronemus goramy*). For this purpose, giant gourami with the average weight 3.1±0.2 g with selective experimental treatments included, control (0 g Digestarom PEP/kg), T1 PEP 0.5% (5 g Digestarom PEP/kg), T2 PEP 1% (10 g Digestarom PEP/kg), and T3 PEP 1.5% (15 g Digestarom PEP/kg) were feed for 60 days. Based on the growth performance results, a significant difference was observed in the specific growth rate (3.19 %/day), weight gain (18.47 g), and feed conversion factor (0.78) of PEP 1.5% treatment compared to other treatments (p<0.05). Based on the hematological results, there was a significant difference in the red and white blood cells numbers, neutrophil and monocyte percentages, and hematocrit level in PEP 1.5% treatment compared to other treatments (p <0.05). The levels of immune indices including lysozyme activity, IgM, complement proteins (C3 and C4) were significantly increased (p<0.05) in PEP 1.5% treatment compared to others, especially the control one. Also, there was a significant difference in the crude protein (15.37%) and lipid (3.87%) contents among PEP 1% and PEP 1.5% compared to control and PEP 0.5% (p<0.05). In conclusion, the results of this study showed that dietary administration of Digestrom™ PEP, especially at 1.5% concentration, could improve growth performance and innate immune responses of giant gourami.

Keywords: Digestrom PEP, Growth promoter, Immunostimulant., *Osphronemus goramy*

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Introduction

High stocking density, especially in the intensive aquaculture systems has generated potential environmental stress, leading to high susceptibility to various diseases including bacteria, fungi, viruses, and parasites. Recently, the application of antibiotics has been banned due to increasing the resistance to the most infectious bacteria as well as serious environmental hazards (Lazado and Caipang, 2014; Wang, 2014). For this reason, using natural feed additives as a growth promoter and immunostimulants were recommended (Syahidah *et al*., 2015).

Phytogenic additives as an alternative of antibiotics were divided into several types, including sensory additives, technological additives (antioxidants and toxin binder), zootechnical additives (immunomodulators, digestive stimulants, growth promoters, etc.) and nutritional additives (vitamins, minerals, etc.) (Karaskova *et al.*, 2015). Improving the health status, growth performance, and immunity system of several fish species were reported in several literatures by phytogenic compounds containing carvacrol, thymol, cineole, linalool, anethole, 1,8-cineol, fennel, allicin, capsaicin, allylisothiocynate, and piperine (Grashorn, 2010; Giannenas *et al.*, 2012; Gultepe *et al.*, 2014; Sutili *e al.*, 2017; Mohammadi *et* al., 2018; Samavat *et al*., 2019; Shekarabi *et al*., 2019).

Digestarom PEP is a phytogenic feed additive that contains essential oils included carvacrol, thymol, anethol, and limonene and also a prebiotic (fructooligosaccharide). Carvacrol in this product extracted from *Mentha pulegium* has strong antimicrobial and antioxidant activities (Kamimura *et al*., 2014). Anethol is used in Digestarom PEP. extracted from *Pimpinella anisum* has antifungal activity and appetite properties. The limonene in this product has strong antimicrobial properties and improved the taste of the food (Reisinger *et al.*, 2011). Fructooligosaccharide (FOS) is a nondigestible oligosaccharides that has positive effects on growth performance, metabolism, gastrointestinal microbiota composition and non-specific immunity of Atlantic salmon *Salmo salar* (Grisdale-Helland *et al.*, 2009) *Acipenser baerii*, beluga sturgeon (*Huso huso*) (Hoseinifar *et al.*, 2011). In previous studies, the positive effects of Digestarom PEP. As a feed supplement on health, immunity and chemical composition of beluga sturgeon (Defaee *et al.*, 2016), Nile tilapia (*Oreochromis niloticus*) fingerlings (Gaber *et al.*, 2014), bester juvenile fish (*H. huso*×*Acipenser ruthenus*) (Taghavi *et al*., 2015), and channel catfish (*Ictalurus punctatus*) (Peterson and Bosworth, 2014) were reported.

Giant gourami (*Osphronemus goramy*) is an important fish species in the aquarium trade of Iran, but it is commercially farmed for food in Southeast Asia and other locations due to introductions (Roberts, 1992). The fish also has been used for controlling invasive aquatic plants like *Salvinia molesta*. The growth rate of giant gourami is very fast and also it is easily

adapted to formulated feed (Budi *et al.*, 2015). Therefore, the main objective of this study was to evaluate the effects of Digestarom PEP. on growth performance, chemical composition, haematological and immune response of giant gourami.

Materials and methods

Experimental fish

One hundred and eighty healthy *O. goramy* with an average weight of 3.1±0.2 g were purchased from a private sector farm and transferred to Science and Research Laboratory (Tehran province, Iran). In this study, 12 aquaria (35×50×100 cm) with air pumps for each aquarium were designed. After 14 days of acclimation, fish were distributed into 4 treatments aquaria; each contains 3 aquaria as replication (each with 15 fish). The water was maintained at 25.0±1.1°C, dissolved oxygen at 7.1±0.2 mg L^{-1}, pH 7.4±0.1.

Preparations of experimental diets

Digestarom PEP is a mixture of herbal feed additives prepared by Biomin GmbH Company (Herzogenburg, Austria). In this study, Digestarom PEP treatments included: control (0 g Digestarom PEP/kg), T1 PEP 0.5% (5 g Digestarom PEP/kg), T2 PEP 1% (10 g Digestarom PEP/kg), and T3 PEP 1.5% (15 g Digestarom PEP/kg) were sprayed on basal feed (21 Beyza Feed Mill Company, Iran), then dried and kept at 4°C until used. The basal diet included 58±0.5% crude protein, 15±0.3% crude lipid, 1.6±0.1% ash, 11.5±0.4% moisture and 0.5±0.1% fibre. Fish were fed on experimental diets at 4-5% of body weight and 4 times per day for 60 days. Fish were fed three times of the day.

Growth performance

All fish were deprived of food for 24 hrs before weighing and sampling, and the following parameters were measured at the end of the feeding trial after 2 months:

Weight gain (WG; g) = W_2 (g) - W_1 (g)

Where W1 is the initial weight, W_2 is the final weight (Tacon, 1990)

Specific growth rate (SGR; % d^{-1}) = 100 (ln W_2 - ln W_1)/ T

Where ln = Natural logarithm, W_2 = Final weight at time t_2, W_1 = Initial weight at time t_1, and T is the number of days in the feeding period.

Feed conversion ratio (FCR) = feed intake (g) /weight gain (g)

Survival rate (SR; %) = 100 (N_t/N_0)

Where N_t and N_0 are final and initial numberof fish, respectively.

Blood sampling

After 60 days, the fish were fasted for 24 h prior to sampling and anesthetized with clove oil (125 mg L^{-1}) before sample collection (Hushangi and Hosseini Shekarabi, 2018). Blood samples (0.5 ml) were drawn from the caudal vein and were immediately

transferred to non-heparinized tubes for plasma collection. Blood samples of each replicate (9 fish per replicate, 27 per treatment) were immediately divided into two half parts. One half was transferred to a tube containing anti-coagulant (heparin) for studying the hematological analysis, while the other half was transferred to non-heparinized tubes for immunological studies. Sera samples were obtained by coagulated blood samples after centrifugation for 5 min at 3000×*g* at 4°C and stored at -80°C until use (Beheshtipour, 2019).

Hematological assay

The total red blood cells (RBC, 10^6 mm^{-3}) and white blood cells (WBC, 10^3 mm^{-3}) were enumerated in an improved Neubauer hemocytometer using Hayem and Turck diluting fluids (Blaxhall and Daisley, 1973). Haematocrit (Ht, %) was determined by the standard microhematocrit method and expressed as percentage. The haemoglobin (Hb, g dL^{-1}) level was determined according to the cyanomethemoglobin procedure. Furthermore, differential leukocyte cells were measured by preparing Giemsa stained smears. Blood smears were studied by light microscopy in order to make blood cell counts (Hushangi and Hosseini Shekarabi, 2018).

Immunological parameters

Lysozyme activity was measured according to the method described by Ellis (1990). Briefly, 50 µL sera of was added to 950 µL of a suspension of *Micrococcus lysodeikticus* (0.2 mg mL^{-1} in a 0.05 M sodium phosphate buffer (Sigma, USA) (pH 6.2) and the absorbance was measured at 520-560 nm and 22°C after 30 s and 180 s by spectrophotometer (2100-VIS model Unico, USA).

Serum immunoglobulin M (IgM) content was measured according to the method recommended by Sun *et al.* (2010) by using a microprotein determination method (C-690; Sigma), prior and after precipitating down the immunoglobulin molecules by means of a 12% solution of polyethylene glycol (Sigma). Serum levels of complements (C3 and C4) were measured using commercial kits (Pars Azmoon, Alborz, Iran) based on the immunoturbidimetry method .

Chemical composition

At the end of the feeding trial, nine fish from each tank (27 fish per group) were sampled randomly. The captured fish were humanly sacrificed and stored at -20°C prior to further analysis. The proximate composition of the fish fillets was conducted according to the standard methods described by the Association of Official Analytical Chemists (AOAC, 2005). Moisture was determined by drying the samples to a constant weight in a hot air oven (Behr, Germany) at 105°C. Crude lipid, protein and ash were determined by chloroform methanol extraction (2:1, v/v), Kjeldahl method (N×6.25) and incineration in a muffle furnace at 500 °C for 6 h respectively.

Statistical analysis

All data are shown as mean± SD. The data were subjected to statistical analysis

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

Results

Growth performance

The results of growth performance and survival rate of giant gourami fed diets enriched with different levels of Digestrom™ PEP were presented in Table 1. Based on results, weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) in PEP 1.5% were significantly different than other treatments (p<0.05). According to the results, by increasing the Digestrom™ PEP concentration from 0.5% to 1.5%, the growth parameters (except for FCR) were increased (p<0.05). Also, the highest level of survival was observed in PEP 1.5% treatment, which showed a significant difference with other treatments (p<0.05).

Table 1: Growth performance and survival rate of giant gourami fed diets enriched with different levels of Digestrom™ PEP after 60 days (Mean ±SD).

Parameter	Control	PEP 0.5%	PEP 1%	PEP 1.5%
WG (g)	7.00±0.45 ^{d}	10.17±0.57^{c}	12.33±0.25^{b}	18.47±0.85^{a}
SGR (%)	1.99±0.18^{d}	2.42±0.09^{c}	2.67±0.13^{b}	3.19±0.19^{a}
FCR	1.07±0.04^{a}	0.94±0.05^{b}	0.90±0.04^{b}	0.78±0.01^{c}
SR%	91.11±3.85^{b}	93.33±6.67^{b}	93.33±6.67^{b}	97.78±3.85^{a}

WG, weight gain; SGR, specific growth rate; FCR, fed conversion ratio; SR%, survival rate. Means in the same rows with different superscript are significantly different

Hematological profile

The number of red and white blood cells significantly increased (p<0.05) in Digestrom™ PEP treatments compared to control (Table 2). The highest number of white blood cells was observed in PEP 1.5% treatment which showed statistically significant differences with other treatments, especially control (p<0.05). Based on the results, there was a significant difference in neutrophil and monocyte percentages, and hematocrit value in PEP 1.5% treatment compared to other treatments (p<0.05). Also, the haemoglobin concentration did not significantly change by increasing dietary levels of PEP up to 1% (p>0.05), while the highest level was obtained in PEP 1.5% treatment (p<0.05). No significant difference was observed in lymphocyte percentage in studied treatments (p>0.05).

Table 2: Hematological changes of giant gourami fed diets enriched with different levels of Digestrom™ PEP after 60 days (Mean ±SD).

Parameter	Control	PEP 0.5%	PEP 1%	PEP 1.5%
RBC (10^6 mm^{-3})	5.08±0.06^b	5.43±0.05^a	5.44±0.02^a	5.42±0.01 ^{a}
WBC (10^3 mm^{-3})	6.44±0.03^d	9.31±0. 04^c	13.1±0.10^b	13.41±0.2^a
Hct (%)	35.7±0.52^b	35.47±0.50^b	35.40±0.36^b	36.20±0.72^a
Hb (g dL^{-1})	5.39±0.03^b	5.83±0.03^a	5.38±0.02^b	5.35±0.06^b
Monocyte (%)	2.43±0.12^d	3.17±0.31^c	4.0±0.20 ^{b}	5.23±0.06^a
Lymphocyte (%)	77.67±2.20^a	75.67±1.53^a	76.0±1.73^a	74.67±1.15^a
Neutrophil (%)	11.56±0.12^d	12.30±0.10^c	13.33±0.21^b	15.43±0.31^a

Note: RBC, red blood cells; WBC, white blood cells; Hct, hematocrit; Hb, hemoglobin concentration. Data are presented as mean ± S.D (n =9 fish from each group). Means in the same rows with different superscript are significantly different (p<0.05).

Immunity responses

As shown in Figures 1-4, the majority of studied immune indices were significantly increased (p<0.05) in all treatments that received dietary Digestrom™ PEP (0.5%, 1%, and 1.5%), compared to the control one (p<0.05). However, the highest levels of lysozyme activity, IgM, and C3 were observed in 1.5% PEP treatment (p<0.05). The level of C4 was not significantly different among Digestrom™ PEP treatments while, it was significantly higher than the control group (p<0.05).

Carcass chemical composition

The chemical composition of giant gourami fed diets enriched with different levels of Digestrom™ PEP is shown in Table 2. Based on the results, there was a significant difference in the crude protein percentage and crude lipid content among PEP 1% and PEP 1.5% compared to control and PEP 0.5% (p<0.05). Although, no significant difference was observed in moisture content and ash percentage in all studied treatments (p>0.05) (Table 3).

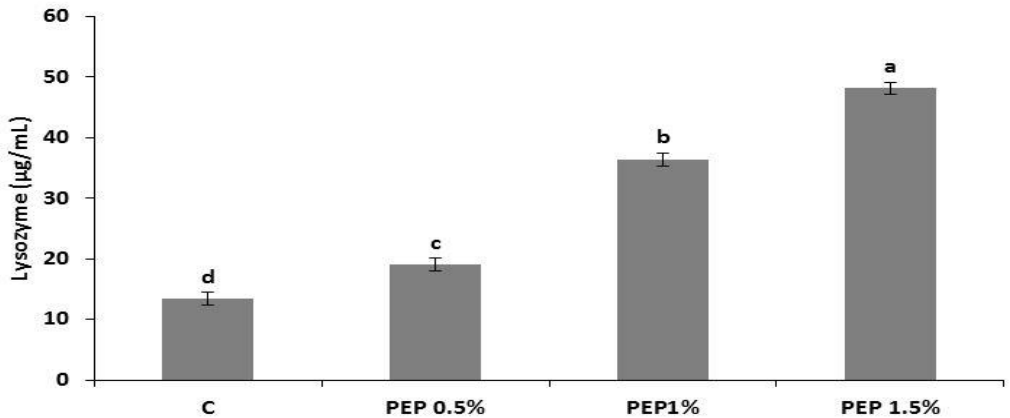

Figure 1: The effect of different concentrations of Digestrom™ PEP on lysozyme activity of giant gourami. C: control (0 g Digestarom PEP/kg), PEP 0.5% (5 g Digestarom PEP/kg), PEP 1% (10 g Digestarom PEP/kg), and PEP 1.5% (15 g Digestarom PEP/kg). Error bars indicate the standard error of the mean (n=3). Different superscript letters indicate statistically significant differences (p<0.05).

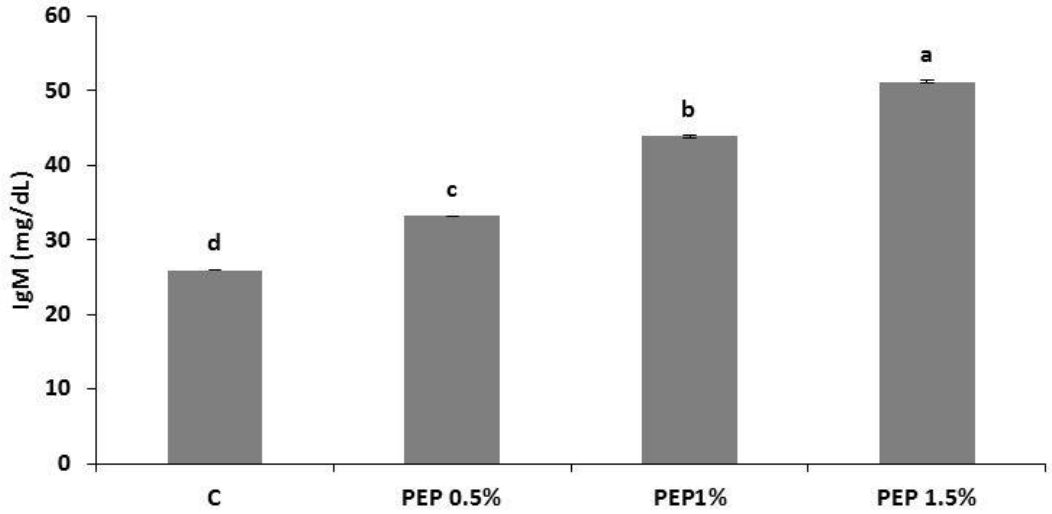

Figure 2: The effect of different concentrations of Digestrom™ PEP on IgM level of giant gourami. C: control (0 g Digestarom PEP/kg), PEP 0.5% (5 g Digestarom PEP/kg), PEP 1% (10 g Digestarom PEP/kg), and PEP 1.5% (15 g Digestarom PEP/kg). Error bars indicate the standard error of the mean (n=3). Different superscript letters indicate statistically significant differences (p<0.05).

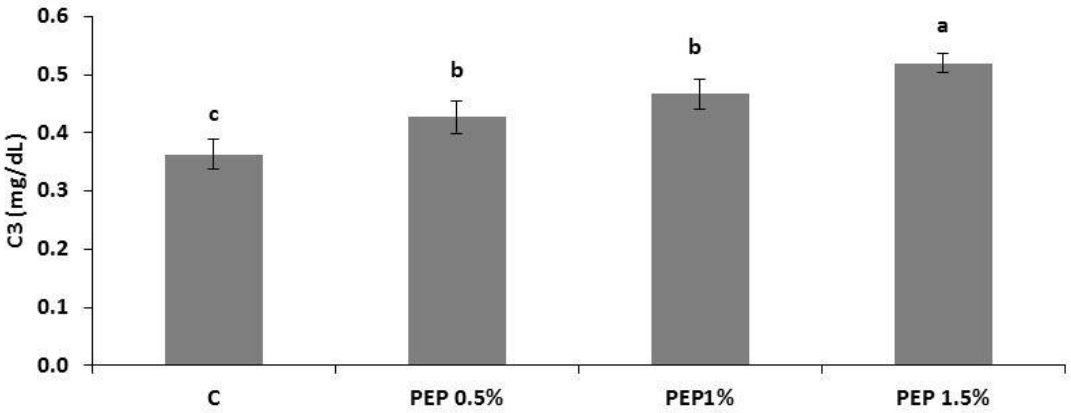

Figure 3: The effect of different concentrations of Digestrom™ PEP on C3 level of giant gourami. Figure 2: The effect of different concentrations of Digestrom™ PEP on IgM level of giant gourami. C: control (0 g Digestarom PEP/kg), PEP 0.5% (5 g Digestarom PEP/kg), PEP 1% (10 g Digestarom PEP/kg), and PEP 1.5% (15 g Digestarom PEP/kg). Error bars indicate the standard error of the mean (n=3). Different superscript letters indicate statistically significant differences (p<0.05).

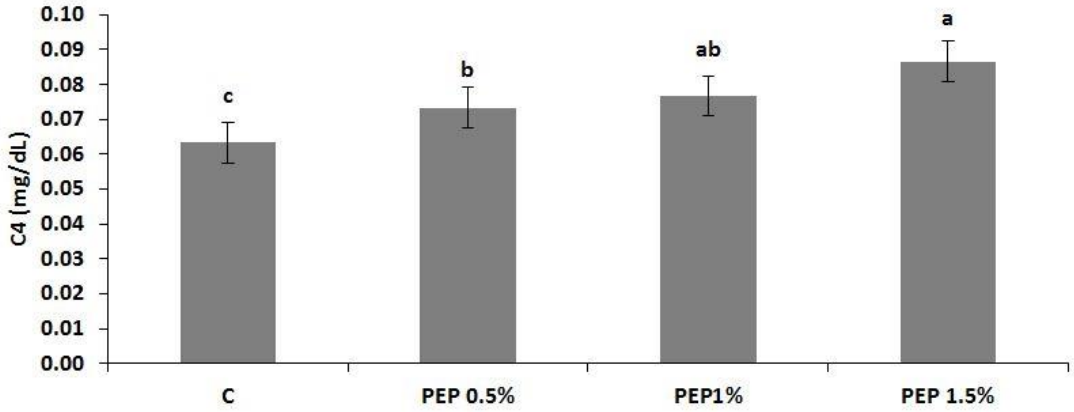

Figure 4: The effect of different concentrations of Digestrom™ PEP on C4 level of giant gourami. Figure 2: The effect of different concentrations of Digestrom™ PEP on IgM level of giant gourami. C: control (0 g Digestarom PEP/kg), PEP 0.5% (5 g Digestarom PEP/kg), PEP 1% (10 g Digestarom PEP/kg), and PEP 1.5% (15 g Digestarom PEP/kg). Error bars indicate the standard error of the mean (n=3). Different superscript letters indicate statistically significant differences (p<0.05).

Table 3: Chemical composition (% in wet basis) of giant gourami fed diets enriched with different levels of Digestrom™ PEP after 60 days (Mean ±SD).

Parameter	Control	PEP 0.5%	PEP 1%	PEP 1.5%
Crude protein	13.6±0.45^{c}	14.27±0.12^{bc}	15.1±0.20^{a}	15.37±0.74^{a}
Crude lipid	4.7±0.23^{a}	4.27±0.12^{ab}	4.03±0.21^{b}	3.87±0.21^{b}
Ash	4.87±0.12^{a}	4.73±0.06^{a}	4.77±0.15^{a}	4.77±0.12^{a}
Moisture	76.3±2.0^{a}	76.7±1.53^{a}	76.1±1.73^{a}	76.0±1.0^{a}

Means in the same rows with different superscript are significantly different (p<0.05).

Discussion

Phytogenics can act as natural growth promoters that improve diet palatability, stimulate appetite and increase feed consumption and growth performance (Karaskova *et al.,* 2015). According to the results, the growth indices were increased by increasing the Digestrom™ PEP concentration from 0.5% to 1.5%, while FCR were decreased by increasing the Digestrom™ PEP concentration.. At the end of the study, 1.5% PEP treatment has highest levels of weight gain, specific growth factor and survival rate. These results may be related to stimulation of digestive secretions, increasing the population of positive bacteria such as LAB, improvement of feed digestibility, especially for proteins and amino acid (Peterson *et al.,* 2015; Ran *et al.,* 2016; Sutili *et al.,* 2017). In a same study, El-Hawarry *et al.* (2018) showed that phytogenic compounds including oregano oil in Nile tilapia diet significantly improved the growth performance. Similar to our results, Gaber *et al.* (2014) showed that final weight and SGR of Nile tilapia were significantly affected in treatment containing 30% level wet date and 0.03% Digestrom™ PEP. Also, in Peterson and Bosworth (2016) study, channel catfish fed diets enriched with Digestarom™ significantly increased the weight gain and decreased the FCR. Also, Defaee *et al.* (2016) reported that

the Digestrom™ PEP at 2% of juvenile beluga diet could enhance growth parameters (average daily growth, specific growth rate and protein efficiency) and survival of the juvenile fish. Results of the present study were similar to Zheng *et al.* (2009) and Giannenas *et al.* (2012) studies that added carvacrol and thymol in catfish (Zheng *et al*., 2009) and rainbow trout (*Oncorhynchus mykiss*) feed, respectively (Giannenas *et al.*, 2012). Present results showed that Digestrom™ PEP increased the survival rate of giant gourami. The highest level of survival rate was observed in the PEP 1.5% treatment, which had a significant difference from the control. It was in agreement with Peterson *et al.* (2015) and Giannenas *et al.* (2012) studies that added Digestrom™ PEP in channel catfish and Biomin™ (commercial products containing carvacrol and thymol) in rainbow trout feed, respectively.

Hematology indicators are known as valuable biological indicators that reflect the body's general health (Haghighi *et al*., 2017). Based on the results, there was a significant difference in neutrophil and monocyte percentage, haematocrit and haemoglobin values in PEP 1.5% treatment compared to other treatments (p<0.05). Increasing the number of white blood cells and neutrophils in this study may be related to the presence of fructooligosaccharide, carvacrol and thymol in the PEP supplement that has stimulation effects on the immune system. In Hoseinifar *et al.* (2011) study, Hct, Hb and WBC were significantly higher in fish fed 2% oligofructose than the control group (p<0.05). Defaee *et al.* (2016) reported that WBC and neutrophils of juvenile beluga in the PEP 1% and 2% treatment was significantly higher than other treatments. Also, Gultepe *et al*. (2014) found higher counts of white and red blood cells and hematocrit levels in the tilapia (*Oreochromis mossambicus*) groups that received the 1% extract of thyme (*Thymus vulgaris*), rosemary (*Rosmarinus officinalis*), and fenugreek (*Trigonella foenum graecum*) (Beheshtipour *et al*., 2018). In a similar study, the erythrocyte number, hemoglobin content, hematocrit and leukocyte numbers were increased significantly after adding garlic to fish diets (Martins *et al.*, 2002) which could be due to the presence of limonene in both garlic and Digestrom™ PEP (Peterson and Bosworth, 2016;)

Lysozyme is responsible for activating other important defense molecules,, including the complement system and phagocytic cells (Ellis, 1990). At the end of the study, lysozyme activity, IgM, C3 and C4 levels were increased in all treatments that received Digestrom™ PEP (0.5%, 1% and 1.5%), compared to control group (p<0.05). These results are mainly attributed to carvacrol and thymol in Digestrom™ PEP (Volpatti *et al.*, 2013). In the same study, lysozyme activity and complement levels were higher in phytogenic supplemented groups containing carvacrol and thymol compared to the control (Giannenas *et al.*, 2012). Also, a significant increase in

the phagocytic and lysozyme activities were observed in rainbow trout that received allicin (Nya et al., 2010), Persian shallot powder (*Allium stipitatum*;)and dandelion flower extract (*Taraxacum officinale*;) compared to the control. Baba *et al.* (2016) showed that limon peel essential oil supplementation increased the Nitro blue tetrazolium (NBT) positive cell count and lysozyme activity of tilapia compared to control. Haghighi *et al*. (2018) reported that dietary inclusion of *O. vulgare* extract 1% improved the nonspecific immune parameters (respiratory burst activity, phagocytic activity and serum lysozyme activity) of juvenile rainbow trout. Also, Peterson *et al*. (2018) demonstrated that different herbal essential oils in Digestrom™ PEP could enhance survival of channel catfish challenged with *Edwardsiella ictaluri* through upregulation of RBL1a and RBL3b in the gill.

Based on the results, there was a significant difference in the crude protein percentage and crude lipid content among PEP 1% and PEP 1.5% compared to control and PEP 0.5% (p<0.05). In Peterson *et al.* (2015) survey, chemical analysis of channel catfish showed that fillet lipid was lower and protein content was higher than the control group. In the same study, a combination of 30% level wet date and 0.03% Digestrom™ PEP in Nile tilapia feed significantly increased the protein content, the protein efficiency ratio (PER) and protein productive value (PPV%) compared to the control group (Gaber *et al.*, 2014). Also, the beluga chemical analysis revealed a significantly higher protein content in the PEP 2% treatment than control group (Defaee *et al.*, 2016). It was confirmed that phytogenic feed additive induce transcription, increasing RNA, leading to an increase in total amino acids and enhancing the protein content (Karaskova *et al.*, 2015).

In conclusion, the results suggested that Digestrom™ PEP supplement, especially in the 1.5% concentration, has positive effects on growth performance, chemical composition and immune response of giant gourami (*O. goramy*). However, further studies are needed to determine the non-specific mechanism of Digestrom™ PEP on giant gourami and survey the effects of PEP on fish physiology.

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