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Effects of Pomegranate (*Punica granatum*) Peel Methanolic Extract Dietary Supplementation on *Oreochromis Niloticus* Performance, Blood Health, Intestine Morphometry and Immunity

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THIS study evaluated the dietary pomegranate peel extract (PPE) effects on performance, blood health, intestine morphometry and *Aeromonas hydrophila* resistance of monosex Nile tilapia (*Oreochromis niloticus)*. Fish (n=120, 9.5±0.5 g) were randomly distributed into four equal triplicate groups. A basal diet supplemented with PPE at a rate of P0, 0.05, 0.1 and 0.3 % representing P0 (control group), P0.05%, P0.1% and P0.3% groups respectively. Fish were fed diets 2 times daily for 70 days at a rate of 4% of fish body weight. The P0 and P0.05% showed significantly (P<0.05) improved growth performance (final weight, weight gain and feed conversion ratio) in respect to P0.1% and P0.3%. Fish that received PPE showed significantly (P<0.05) higher haematological assay (RBCs, WBCs, lymphocyte, and monocyte) compared to P0. A significant (P<0.05) increase in total protein, globulin, albumin and decrease in aspartate aminotransferase, alanine aminotransferase, urea, uric acid, creatinine, triglycerides, and cholesterol was reported in fish received PPE compared to P0. Immune response (higher lysozyme activity, phagocytic activity, phagocytic index, and immunoglobulin M), oxidative/antioxidant status (higher superoxide dismutase activity and lower malondialdehyde level) and intestinal morphometry (higher goblet cell number and villi length) were significantly (P<0.05) higher in fish received PPE in respect to P0 in a dose dependent manner. Fish received PPE showed low mortality rate through achieving the highest protection against *Aeromonas hydrophila* infection compared to P0. In conclusion, PPE dietary supplementation improved blood health, intestine morphometry and immunity of Nile tilapia while inducing an improvement in fish performance in P0.05 group.

Keywords: *Oreochromis niloticus*, Pomegranate peel extract, Growth performance, Oxidative status, Intestinal morphometry, *Aeromonas hydrophila*.

Introduction

Aquaculture, especially tilapia could play an important role in fighting against food diffidence, and malnutrition [1]. After carp, Tilapia is the second main species of cultivated fin fish worldwide [2]. From aquaculture production in Egypt, Nile tilapia represents about 65% [3]. Food and Agriculture Organization[4], recorded that Egypt is considered the main source of cultivated tilapia in Africa. Nile tilapia is a proper fish to culture as it characterized by high growth rate, high nutritional values, low feed conversion ratio, and resistance against diseases[5]. However, in an intensive aquaculture system; higher stocking density combined with common management actions like fish handling, harvesting, and transportation, may be sources of stress to fish [6]. These may subsequently lead to several conditions which include low quality of meat [7], poor metabolism capacity, increased diseases susceptibility and in critical cases ended by death [8].

Hence, fish diseases and mortalities induced by infection of bacteria; accordingly, it has an effect on both consumption demand and general health. Aeromonas species is a facultative pathogen, Gram-negative bacterium and one of the *Aeromonadaceae* family. Aeromonas spp. causes high mortality in infected cultured fish, resulting in a massive economic losses [9]. *Aeromonas hydrophila* was stated as the most pathogenic bacterial species infected tilapia which responsible for septicemic infection and

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high mortality rates in fish farms resulting in a global economic loss in a commercial aquaculture industry [9, 10,11].

Usage of chemotherapeutics and antibiotics for treatment the diseases can still play an important role in managing fish health if used properly however under inappropriate use it has resulted in severe problems like drug-resistance development for bacterial pathogens, aquatic animals immunity suppression, a high risk to human health, besides many environmental hazard [12]. So, antibiotics with other synthetic drugs must be substituted with natural plant extracts which can offer an alternative as they are naturally biodegradable when compared to synthetic drugs besides they are cheap and readily available, and in addition, some of them has immunostimulants, growth promoters, antioxidant, antimicrobial, digestive enhancing, appetite stimulating and hepatoprotective effects [13].

Many studies have shown that using herbal extracts as additives increased the fish growth and protect fish from diseases, antioxidant enhancing, immunostimulant and had appetite stimulating effect [13-16]. Pomegranate (*Punica granatum*) is a Mediterranean native fruit that has been broadly consumed by many countries in traditional medicine[17]. Pomegranate production is projected to be around 1.5 million tons globally, as reported by FAO[14]. This fruit in its entirety (seed, peel, flower, leaf, root, juice, and bark) have antioxidant, hypoglycemic, anti-inflammatory, and anti- bacterial properties[18], Pomegranate peel (PP) constitutes about 26–30% from the total weight of the fruit, the discarding of such huge amount of PP waste causes an environmental problem and food industry waste as they contain bioactive compounds such as phenolic compounds, including flavonoids (ellagitannins, gallotannins, anthocyanins, gallegly esters, hydroxycinnamic acids, hydroxybenzoic acids, and dihydroflavonol) and hydrolyzable tannins (gallic acid, ellagic acid, punicalin, and punicalagin)[19]. The PP has higher antioxidant levels than pomegranate seed and pulp. Thus, PP is a natural rich supplement of antioxidants for animal feed [20]. The pomegranate peel extract (PPE) has widely been studied for its strong antimicrobial, anti-inflammatory, and antioxidant properties [20, 21]. The PPE also improved liver, kidney, and intestine functions in Nile tilapia [14]. Antimicrobial activity of PPE phenolics is based on membrane proteins precipitation which result in lysis of microbial cell[19]. As well, PPE has hypoglycemic effect [22], hepatoprotective effect and hypolipidemic effect [23]. Accordingly, Punicalagin could be a prospective candidate for the therapeutics of many immune pathologies, and immune system strengthening[24], liver fibrosis prevention, heart disease prevention, wound healing promotion and connective tissue strengthening which has the ability to prevent spreading of cancer cells [14] . There is no known drug interactions and no side effects from PPE [24].

However, studies of pomegranate use on fish growth, blood health, and disease resistance are scanty and unclear as some studies show a negative effect of PPE on fish growth [20, 25]while other studies show no effect of PPE on growth[1]. Hence, it was important to throw some additional light on PPE's effects on fish performance, feed utilisation efficiency, and immunity. Therefore, the present study evaluated PPE effects as a natural feed additive on growth performances, haemato-biochemical profile, oxidative status, immune response, intestinal histomorphology, and resistance against *A. hydrophila* in Nile tilapia (*O. niloticus*).

Material and Methods

Ethical approval

The protocol and management of the present experiment was approved by "the Institutional Aquatic Animal Care and Use in Research Committee, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University, Egypt" (approval number: IAACUC-KSU-62-2018).

Preparation of pomegranate peel extract

Pomegranate were gathered from the local markets in Gharbia, Egypt (2019), and then washed with purified water before being used. After that the peels were removed then they dried for 48 h in hot air oven (40 ˚C). By a mixing grinder, dried peels were grounded to get fine powder (60- mesh size). The powder (100 g) was mixed with 400 ml methanol 70% as a solvent and extracted in a rotary shaker (55 xg) for 6 h. Whatman No. 41 filter paper was used for filtration the extract to remove the peel particles in Buchner funnel. The extract was collected and evaporated in a flash evaporator at reduced pressure (40 ˚C) (Büchi, Flawil, Switzerland, Rotavapor® R-100). The methanol free extract was dried by using lyophilizer (Bench top freeze dryer Model LY-10N standard manifold- Taisite lab, USA). The dried extract was frozen until used.

High- performance liquid chromatography (HPLC)

Pomegranate peel extract was analyzed by HPLC using an Agilent 1260 series according to manufacture instruction. The separation was carried out using Kromasil C18 column (4.6 mm x 250 mm i.d., 5 µm). The mobile phase consisted of water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) at a flow rate 1 ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (82% A); 0–5 min (80% A); 5-8 min (60% A); 8-12 min (60% A); 12-15 min (85% A) and 15-16 min (82% A). The multi-wavelength detector

was monitored at 280 nm. The injection volume was 10 µl for each of the sample solutions. The column temperature was maintained at 35 °C.

Experimental fish

Apparently healthy monosex Nile tilapia (*Oreochromus niloticus*) (n= 120 and average body weight= 9.5±0.5 g) were obtained from a local farm of tilapia in Kafr El-Sheikh, Egypt. Fish was kept in twelve glass aquaria (80 x 45 x 35 cm) filled with 90 L de-chlorinated aerated fresh water, and at 26 °C± 2°C, dissolved oxygen 7.63 ± 0.68 mg L^{-1} and pH 7.6 ± 0.6. For three weeks, the fish were adapted to experimental condition before the start of the experiment. Water was changed daily at a rate of 20 % of the whole volume. Everyday wastes of the fish were syphoned from the aquaria.

Experimental design, fish management and diets

Fish were randomly divided into four groups in triplicates (10 fish / replicate). A basal commercial diet with 0 % of PPE was prepared to achieve the fish nutrients requirement contained 2940 kcal/kg digestible energy and 30.80% crude protein representing the control group (P0%). Other three experimental groups, diets were supplemented with PPE at rate of 0.05, 0.1 and 0.3 % representing P0.05%, P0.1% and P0.3% group, respectively [20]. To attain the previous concentrations of PPE in the experimental diets, different concentrations of PPE were dissolved in 100 ml of absolute ethanol and then sprayed uniformly on 1 kg of feed. The control diet was given the same amount of ethanol to maintain the same conditions with PPE diets. Before using the diets to feed fish, they were dried for one day at room temperature to ensure evaporation of ethanol. The fish were received 2 meals per day (8 am and 2 pm) and fed by hand at rate of 4% of body weight for 70 days. The photoperiod was adjusted to 12 h light and 12 h dark.

Fish growth performance parameters and feed utilisation efficiency

At the start and end of the experiment, each fish was weighed separately in order to determine the initial and final weight. Feed intake was determined on a daily base by collecting, drying and weighing of uneaten feed and subtracting it from the total delivered feed. The fish were caught with a fitting net and clove oil (Merck, Germany) at the rate of 50µl/L of water was used to anaesthetize them. The length (L) of the fish was measured by measuring board. The other performance parameters were determined as follow:

- Body weight gain (BWG) = final body weight (W1) – initial body weight (W0).
- Feed conversion ratio (FCR) = feed intake (g)/BWG (g).
- Condition factor (K)= 100 × ($W1/L^3$)[26].

Blood sampling, hematological and biochemical analysis

After 70 days of the experiment, the blood (6 fish/ treatment) was obtained from caudal vein into plastic syringe (3 ml size with 23Gx1.1/4 needle size) and transferred to heparinized tubes smeared with sodium heparin for hematological parameters. Another blood sample was collected into Eppendorf tubes without anti-coagulant then centrifuged (3,000 rpm for 15 min) then the serum samples were gathered and stored immediately in -20 °C until use for further analysis.

Hematological assay

The erythrocytes (RBCs) and leukocytes (WBCs) were counted using a hemocytometer using Natt-Herrik solution, as described by [27]. The cyanomet haemoglobin method using Drabkin's solution was used to determine haemoglobin concentration[27]. According to [28] the micro hematocrit method was used for estimation of the packed cell volume (PCV %). The method of [29] was used to calculate mean corpuscular volume (MCV), mean corpuscular Hb (MCH), and mean corpuscular Hb concentrations (MCHCs). For determination of differential leukocytic count (DLC), a thin blood films were prepared, left to dry in air, then fixed with methanol for 3-5 minutes, stained with Giemsa stain for 8-10 minutes, and rinsed with distilled water then allowed to dry. The white blood cells were counted among one hundred of blood smear according to [27]. The absolute DLC was calculated according to [30] .

Biochemical assay

The determination of total protein, albumin, and globulin (was calculated mathematically by subtraction of albumin from total protein), triglyceride, and cholesterol, blood urea nitrogen, uric acids, creatinine, alanine-aminotransferase (ALT), and aspartate aminotransferase (AST) were assessed by commercially diagnostic kit “Bioanalytic Diagnostic Industry, Turkey”.

Immunological assay

Phagocytic activity and phagocytic index assay

Smears of the whole blood were prepared for assessing of phagocytic activity and index as the method of [31]. The phagocytic activity and index were measured by the following calculations: Phagocytic activity = number of the phagocytic cell containing yeast /total number of phagocytic cell ×100. While, Phagocytic index = number of phagocytized cells/number of phagocytic cells.

Lysozyme activity

The activity of serum lysozyme was evaluated by ELISA; using the micro plate ELISA reader at the wave length 450 nm, as method mentioned by [32] .

Immunoglobulin M (IgM) assay

Immunoglobulin M (IgM) was determined using ELISA Kit (Cusabio Biotech Co., Ltd., Wuhan, China [33].

Antioxidant enzyme assay

According to the method described by [34], ELISA kits (Inova Biotechnology, China) were used to measure superoxide dismutase enzyme (SOD) activity and malondialdehyde (MDA) level in serum using the micro plate ELISA at the wavelength 450 nm.

Intestine histomorphology

After being anaesthetized with clove oil (Merck, Germany) at a rate of 50 l/L of water, one fish from each tank was sacrificed them, and the intestine was dissected out immediately and fixed in buffered formalin 10% for=24 hours. After the tissues fixed; the samples were dehydrated and flushed in absolute alcohol several times before being inserted in paraffin. On a Leica Rotary Microtome "RM 2145, Leica Microsystems, Wetzlar, Germany", serial 5-m longitudinal sections were sliced and placed on glass slides. Then, slides stained regularly with hematoxylin and eosin (H&E). Image J analysis software "National Institutes of Health, MD, USA." was used to perform the histomorphometric analysis, including the whole mucosal length (from the villus tip to the layer of muscle), villus height (from the villus tip to the villus- crypt junction), and villus width (measured from the villus's midpoint)**.** The goblet cells number per unit of surface area (mm^2) was used to calculate Density of goblet cells[35].

Challenge with *A. hydrophila*

After 70 days of trial, the challenge test was performed. The challenge test was done using *Aeromonas hydrophila (A. hydrophila)*, isolated and identified previously in the Faculty of Veterinary medicine, Kafrelsheikh University, Egypt [36]. Five apparently healthy fish from each replicate in the different treatments and control were challenged with a sub- lethal dose of *A. hydrophila* as informed by [30] where a 0.1 mL of 24-h broth of virulent *A. hydrophila* bacteria (5×10^5 CFU mL^{-1}) was injected in fish intraperitoneal (IP) [1]. The control group was injected with a 0.1 mL of phosphate-buffered saline (PBS). After that, fish were observed for 10 day [34] and the daily fish mortality was determined.

Statistical analysis

Data were tested for distribution normality and the residuals analysis confirmed the normality. Before processing percentage data, arcsine transformation was used. Data were analysed in Graph Pad Prism 6 "Graph Pad Prism v6.0, San Diego, CA, USA" and all results were stated as means with SEM. for comparison among different treatments, One-way ANOVA was used. Tukey's multiple comparison was used as a *post hoc* test where appropriate. Significance level was set at $P<0.05$. Log-rank (Mantel Cox) test was used for representation of cumulative survival of tilapia fingerlings injected at 5×10^5 CFU mL^{-1} of *A. hydrophila*.

Results

HPLC analysis

As shown in Table 1, PPE contains high levels of polyphenolic acids especially gallic acid, catechin, and ellagic acid were reported to be 933.37, 581.57 and 111.80 µg/ml respectively. In addition, high amounts of cholinergic acid, naringenin, and vanillin (supplementary file: 1).

TABLE 1. The analysis of the active ingredient components of the pomegranatepeel methanolic extract (PPE).

Polyphenolic components	RT (Retention Time) (min)	Concentration (µg/ml) standard	Concentration (µg/ml) PPE sample
Gallic acid	3.257	16.8	933.37
Catechin	4.451	67.5	581.57
Ellagic acid	8.101	34.3	111.80
Chlorogenic acid	4.104	28	84.50
Naringenin	10.205	15	70.89
Vanillin	9.867	12.9	23.51
Pyro catechol	7.160	29.2	2.41
Rutin	7.437	61	5.32
Methyl gallate	5.637	10.2	2.04
Coumaric acid	9.112	13.2	1.46
Syringic acid	6.504	17.2	1.54
Ferulic acid	10.083	12.4	0.00
Coffeic acid	5.952	18	9.29
Taxifolin	12.412	13.2	0.25
Cinnamic acid	14.317	5.8	0.16
Kaempferol	14.683	12	3.22

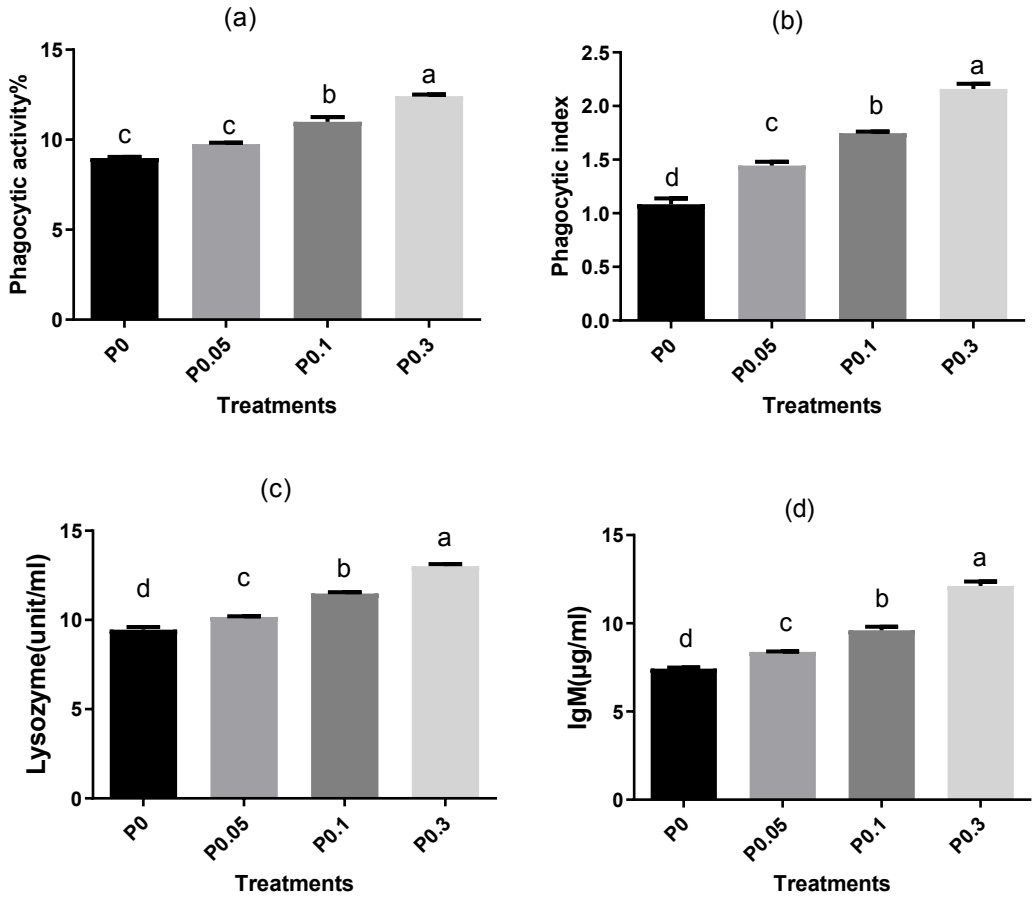

Figure 1.Effect of different dietary levels of PPE on the immune response parameters (a) phagocytic activity, (b) phagocytic index, (c) lysozyme activity and (d) IgM of Nile tilapia. The columns (mean± SEM) with different superscripts letters (a, b, c….) are significantly different (One-way ANOVA, *P*<0.05).

Fish growth performance and feed utilization efficiency

As shown in Table 2, the growth performance and feed utilization of Nile tilapia fed different concentrations of PPE showed significant (P<0.05) differences except for condition factor. Fish received PPE at 0.05% concentration showed insignificant (P>0.05) difference with control group (P0%) in fish growth performance (final body weight, body weight gain, and FCR) while, P0% and P0.05% showed significant (P<0.05) difference in respect to P0.1% and P0.3%. The highest final body weight and body weight gain were reported in P0.05% followed by P0%, P0.1% and P0.3% however the lowest FCR was recorded in P0.05% followed by P0%, P0.1% and P0.3%.

TABLE 2. Effects of dietary levels of the pomegranate peel methanolic extract on growth performance, and feed utilization of Nile tilapia

	P0%	P0.05%	P0.01%	P0.3%	*P*-value
Initial body weight (g)	9.246±0.090	9.202±0.054	9.250±0.067	9.420±0.092	0.241
Final body weight (g)	47.30±0.905^{a}	47.83±0.737^{a}	44.37±0.573^{b}	41.52±0.632^{b}	0.001
Final body weight gain (g)	38.05±0.842^{a}	38.62±0.787^{a}	35.12±0.535^{b}	32.10±0.610^{c}	0.001
Feed conversion ratio	1.211±0.027^{c}	1.141±0.023^{c}	1.298±0.014^{b}	1.360±0.026^{a}	0.001
Condition factor (K)	1.721±0.017	1.684±0.031	1.829±0.025	1.833±0.089	0.115

Means within the same row with different superscript letters (a, b, c….) are significantly different (P<0.05).

Hematological profile

Results in Table 3 demonstrated that RBCs, WBCs, heterophils, lymphocytes and monocytes of Nile tilapia fed different dietary concentrations of PPE showed significant (P<0.05) difference when compared to control group (P0%). Higher significant values of RBCs, WBCs, lymphocytes and monocytes were found in fish fed 0.3% PPE supplemented diet compared with other groups with significant decrease in heterophils of fish fed PPE diet. However, PCV, HB, MCV, MCH, MCHC, esinophils and basophils showed non-significant (P>0.05) difference in respect to control group.

TABLE 3. Effects of dietary levels of the pomegranatepeel methanolic extract on hematological profile of Nile tilapia

	P0%	P0.05%	P0.1%	P0.3%	*P*-value
RBCs (x10^{6}/mm^{3})	2.053±0.231^{a}	2.110±0.120^{ab}	2.100±0.311^{ab}	2.177±0.120^{b}	0.0313
PCV%	20.00±2.577	19.50±2.500	19.67±1.333	20.67±3.333	0.3422
Hb (g/100ml)	6.127±0.364	6.285±0.595	6.363±0.542	6.523±0.218	0.0560
MCV(fl)	97.36±3.311	93.66±2.925	93.64±4.176	97.78±3.054	0.1040
MCH (pg)	29.84±2.208	29.17±2.780	30.30±2.291	30.31±1.123	0.1687
MCHC (g/dl)	30.67±3.587	30.67±1.285	31.41±2.498	32.38±2.722	0.2211
WBCs (x10^{3}/mm^{3})	9.55±0.205^{c}	9.81±0.160^{c}	11.06±0.223^{b}	12.49±0.248^{a}	0.0001
Heterophil (x10^{3}/mm^{3})	1.337±0.060^{a}	1.125±0.035^{ab}	1.117±0.127^{b}	1.013±0.034^{b}	0.0053
Lymphocyte (x10^{3}/mm^{3})	7.453±0.023^{a}	7.765±0.015^{b}	8.413±0.057^{c}	9.567±0.061^{d}	0.0001
Monocyte (x10^{3}/mm^{3})	0.603±0.028^{a}	0.645±0.015^{a}	0.696±0.006^{a}	0.906±0.026^{b}	0.0001
Esinophil (x10^{3}/mm^{3})	0.096±0.003	0.100±0.000	0.066±0.033	0.036±0.036	0.3976
Basophil (x10^{3}/mm^{3})	0.066±0.033	0.100±0.000	0.100±0.000	0.036±0.036	0.3703

Means within the same row with different superscript letters (a, b, c….) are significantly different (P<0.05).

Biochemical profile

Table 4 revealed that AST, ALT, total protein, globulin, albumin, urea, uric acid, creatinine, triglyceride and cholesterol of Nile tilapia fed varying dietary concentrations of PPE were significantly (P<0.05) different when compared to control group. The levels of ALT, AST, urea, uric acid, creatinine, triglyceride and cholesterol were significantly (P<0.05) lower in the fish that received PPE than those of the control fish with better findings being observed in fish fed diet supplemented with 0.3% PPE. Higher significant (P<0.05) levels of total protein, globulin, and albumin were found in fish fed PPE supplemented diet compared to control group with the high values being detected in fish fed diet supplemented with 0.3% PPE.

TABLE 4. Effects of dietary levels of the pomegranate peel methanolic extract on biochemical profile of Nile tilapia

	P0%	P0.05%	P0.1%	P0.3%	*P*-value
ALT (U/l)	4.373±0.012[a]	4.065±0.055[b]	3.600±0.051[c]	2.510±0.075[d]	0.0001
AST (U/l)	73.02±0.470[a]	70.98±0.165[b]	69.68±0.245[b]	67.64±0.266[c]	0.0001
Total protein (g/dl)	3.070±0.036[a]	3.265±0.025[a]	3.680±0.090[b]	4.130±0.065[c]	0.0001
Albumin (g/dl)	1.417±0.008[a]	1.405±0.025[a]	1.477±0.003[b]	1.510±0.0100[b]	0.0009
Globulin (g/dl)	1.653±0.036[a]	1.860±0.00[a]	2.203±0.093[b]	2.620±0.055[c]	0.0001
Urea (mg/dl)	4.530±0.023[a]	4.080±0.050[b]	3.480±0.005[c]	2.210±0.026[d]	0.0001
Creatinine (mg/dl)	0.323±0.008[a]	0.285±0.005[b]	0.256±0.006[b]	0.210±0.005[c]	0.0001
Uric acid (mg/dl)	0.620±0.032[a]	0.485±0.005[b]	0.453±0.012[b]	0.393±0.003[b]	0.0003
Triglyceride (mg/dl)	100.6±0.872[a]	91.58±0.935[b]	82.63±1.378[c]	73.52±1.364[d]	0.0001
Cholesterol (mg/dl)	93.98±1.909[a]	84.60±0.940[b]	75.88±1.738[c]	68.96±0.571[d]	0.0001

Means within the same row with different superscripts letters (a, b, c….) are significantly different (P<0.05).

Immune responses

Nile tilapia Immune responses fed different concentrations of PPE diets are presented in Figure 1. Dietary PPE significantly (P<0.05) increased lysozyme activity, IgM, phagocytic activity and index, and compared with control group in a dose dependent effect. The highest significant (P<0.05) increase was detected in P0.3% followed by P0.1%, P0.05% and P0%.

Oxidant/antioxidant parameters

Activity of SOD and level of MDA in serum of fish fed with different concentrations of PPE are presented in Figure 2. In comparison to P0%, dietary PPE significantly (P<0.05) increased SOD activity in a dose dependent effect and the fish fed 0.3% PPE recorded the highest significant value (P<0.05). However, dietary PPE significantly (P<0.05) decreased MDA level in a dose dependent effect compared with P0%. The lowest MDA level was obtained by feeding the fish on diet containing 0.3% PPE.

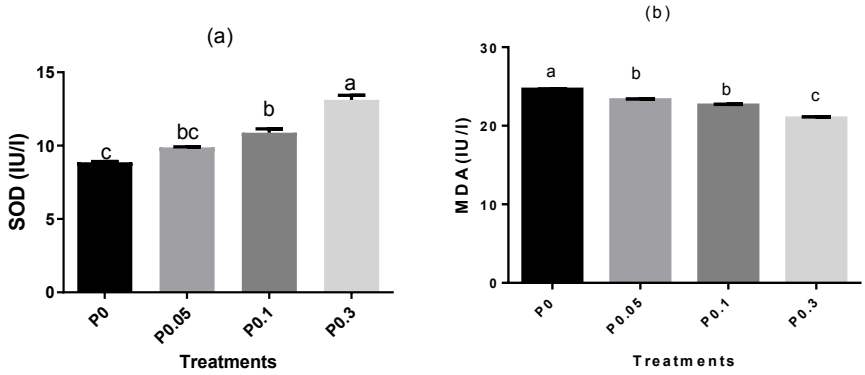

Figure 2. Effect of different dietary levels of PPE on the antioxidant parameters (a) SOD and (b) MDA of Nile tilapia. The columns (mean± SEM) with different superscripts letters (a, b, c….) are significantly different (One-way ANOVA, *P*<0.05).

Intestinal morphometry

Dietary supplementations of PPE made a significant improvement in the intestinal histomorphology (goblet cells number and villi length) of the fish fed different PPE concentrations compared with P0% (P<0.05) (Table 5, Figure 3). In fish fed with PPE diets, morphometry of different parts of intestine showed an increase in goblet cells numbers and villi length (P<0.05). The best findings were recorded in fish fed with 0.3% PPE. Fish received diet supplemented with PPE demonstrated reduction in inter villi space compared with control group in terminal part (P<0.05).

TABLE 5. Effects of dietary levels of the pomegranatepeel methanolic extract on intestinal morphometry of Nile tilapia.

Intestine parts	P0%	P0.05%	P0.1%	P0.3%	*P*-value
Anterior part					
Villi length (mm^2)	180.0±10.75^{a}	257.5±21.34^{ab}	299.1±22.74^{b}	323.8±13.04^{b}	0.0021
Villi width (mm^2)	73.34±13.70	84.61±4.556	98.87±4.784	110.7±10.94	0.0924
Inter villi Space (mm^2)	55.25±4.622	44.66±4.563	42.65±7.403	42.48±5.971	0.3993
Goblet cells (no/mm^2)	8.66±0.881^{a}	11.67±0.881^{ab}	12.33±0.666^{b}	13.33±0.333^{b}	0.0098
Middle part					
Villi length (mm^2)	255.1±17.48^{a}	338.6±35.74^{ab}	420.9±26.84^{bc}	504.5±27.71^{c}	0.0012
Villi width (mm^2)	57.26±2.243	57.58±7.269	62.65±7.212	65.14±5.104	0.7295
Inter villi space (mm^2)	45.97±2.475	41.99±3.723	35.28±5.00	32.55±3.559	0.1234
Goblet cells (no/mm^2)	20.00±1.155	20.67±2.186	24.67±1.453	28.33±2.333	0.0419
Terminal part					
Villi length (mm^2)	79.85±6.485^{a}	83.77±4.278^{a}	109.5±4.392^{a}	185.5± 14.33^{b}	0.0001
Villi width (mm^2)	70.88±6.572	60.95±7.715	62.29±2.343	81.87±6.355	0.1331
Inter villi Space (mm^2)	84.89±9.329	92.64±14.26	53.42±10.65	44.99±7.195	0.0343
Goblet cells (no/mm^2)	5.000±1.00^{a}	7.000±0.577^{ab}	8.000±0.577^{ab}	9.667±0.881^{b}	0.0173

Means within the same row with different superscript letters (a, b, c….) are significantly different (P<0.05).

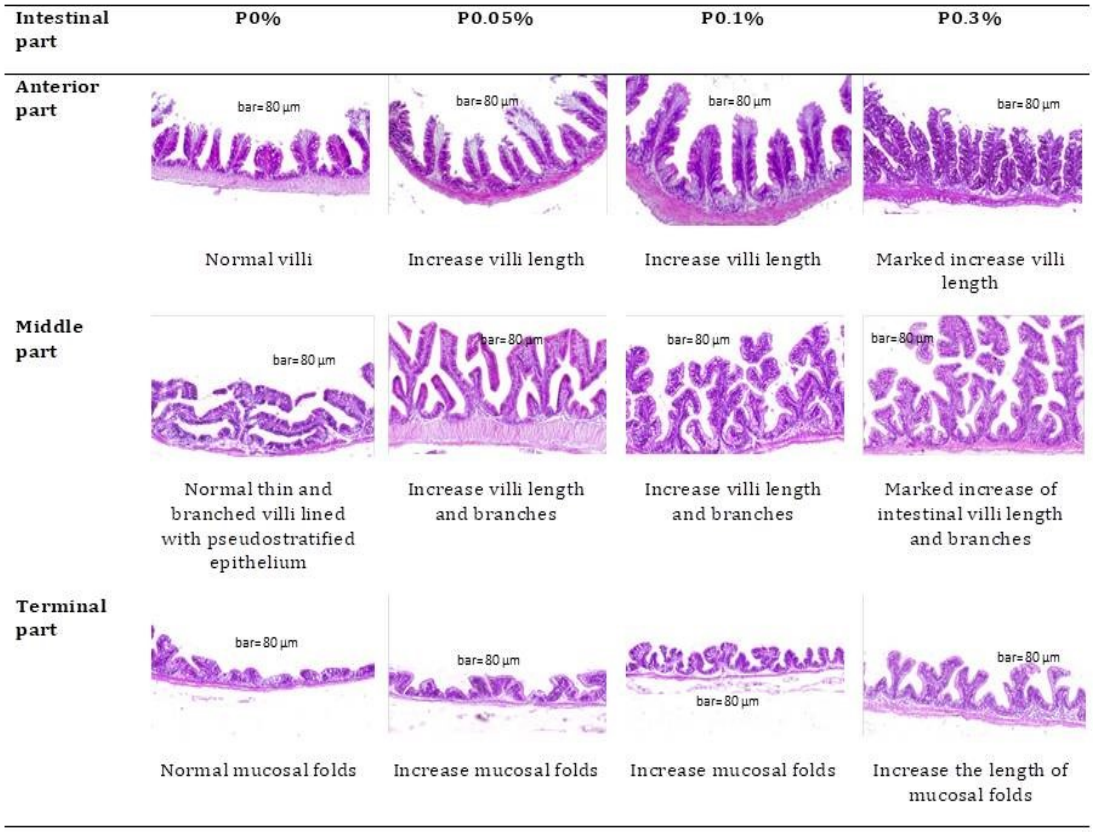

Figure3. Haematoxylin–eosin-stained (H&E, X100, bar= 80 µm) photomicrograph of the anterior, middle and terminal parts of the intestine of Nile tilapia fed with different dietary levels of PPE.

Bacterial challenge with Aeromonas hydrophila

The relative percentage of survival (RPS) and morality rate of groups challenged with *A. hydrophila* are presented in figure 4. The groups fed diets with different concentrations of PPE showed reduced mortality compared to P0% (P<0.05). The highest protection was recorded in fish fed with 0.3% PPE followed by the 0.1% PPE group. The main clinical sigs in the infected fish were cloudy eyes, haemorrhage on the skin, haemorrhage on pectoral fin and ulcers in the skin (fig. 5).

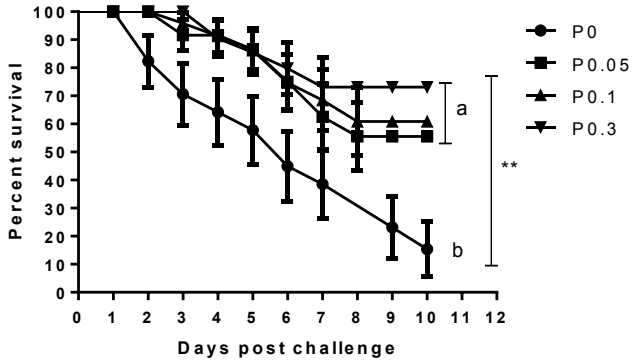

Figure 4. Log-rank (Mantel Cox) representation of cumulative survival of tilapia fingerlings injected at 5 × 10^5 CFU mL^{-1} of *Aeromonas hydrophila*. Each curve represents the average ±standard error results of three parallel tanks holding 5 fish/tank. Groups that do not share letters are significantly different (*P*<0.05). Stars (**) denotes statistical significance (*P*<0.0037) as determined by Log-rank (Mantel Cox) test.

Figure 5. Signs of *Aeromonas hydrophila* infection in experimentally challenged Nile tilapia. (a) cloudy eye (circle); (b) haemorrhages on the skin (black arrow); (c) ulcers in different areas of body (red arrow) and (d) haemorrhages on the base of pectoral fin (blue arrowhead).

Discussion

Herbal extracts contain a lot of active compounds which have a beneficial effect on Nile tilapia performance[37]. Therefore, the present study was aimed to evaluate using different concentrations of dietary PPE as a feed additive on growth performance, haemato-biochemical profile, oxidative status, immune response, intestinal histomorphology, and resistance of *O. niloticus* against *A. hydrophila.*

The PPE is characterized by high polyphenolic compounds which are natural sources of antioxidants. Polyphenolic compounds include phenolic acids, flavonoids and tannins. Flavonoids (Catichin and rutin) and hydrolysable tannins (Ellagic acid and gallaic acid) are mainly components of PPE. These polyphenolic compounds posse high antioxidant, antimicrobial activities ,and immunostimulant effects[38, 39, 40,41, 42]. According to HPLC analysis of PPE that collected from local markets in Gharbia, Egypt in this study; it contained high levels of polyphenolic compounds (gallic acids, catichin, ellagic acids, cholinergic and vanillin).

The study results showed that Nile tilapia fed diets contain lower concentration of PPE (P0.05%) and diet free from PPE showed higher final body weight and weight gain compared with fish received higher concentration of PPE (P0.1% and P0.3%). Nile tilapia fed diets contain lower concentration of PPE (P0.05%) and diet free from PPE showed lower FCR in respect to fish received higher concentration of PPE (P0.1% and P0.3%). These findings were similar to the results reported by Monir et al.; Badawi & Gomaa [1, 20] who used PPE with different concentrations as feed supplement in Nile tilapia diet as well as the results obtained by Toutou et al.[14] who studied the dietary effects of pomegranate peel with different levels (1, 2, 3, 5, 10,

15, 20 %) on Nile tilapia. The reduced body weight and weight gain could be due to PPE contains high content of polyphenols. Polyphenols may inhibit adipose tissue growth via their antiangiogenic activity and by modifying metabolism of adipocyte or reduce fat digestion and absorption[43,44,45]. Polyphenols are one of the phytochemicals' family which has health benefits [46]. Dietary phytochemicals can act as anti-obesity agents, suppress preadipocytes differentiation , induce apoptosis of existing adipocytes, and stimulate lipolysis, so they suppress and/or reduce adipose tissue growth[47].

Hematological parameters act as an important indices for general health, stress, and nutritional status of fish[48, 49] The present study showed a significant increase in RBCs, WBCs, lymphocytes and monocytes of Nile tilapia fed different concentrations of PPE diets in a dose dependent effect, especially with 0.3% PPE when compared to P0%. However, PCV, HB, MCV, MCH, MCHC, esinophils and basophils showed no significant difference from control group. The hematological values were within the tilapia normal range in this study and similar to the outcomes reported by Mohamed et al. [50]. Similar results were stated by Reda et al. [51] who reported that dietary pomegranate peel enhanced haematological parameters for *Clarias gariepinus* challenged by *A. hydrophila*. Furthermore, Harikrishnan et al. [52] reported that diet enriched with pomegranate enhanced the hematology in olive flounder against *Philasterides dicentrarchi.* Acar et al. [18] found in rainbow trout, a positive effect on some blood parameters of pomegranate seed oil against *Yersinia ruckeri*. Torell et al. [53] showed that flavonoids prevent polyunsaturated fatty acids peroxidation in cell membranes. Also, flavonoids have been stated to prevent superoxide ions and hydroxy radicals formation, which are two strong peroxidation agents. This antioxidant activity can protect both the formed blood cells as well as the hematopoietic committed stem from the reactive free radicals attack in the body [51]. However, the current study results differ from the results reported by Harikrishnan et al. [52] who stated a slight reduction in RBCs, Hb and PCV in the blood of monosex *O. niloticus* fed diets containing different concentrations of pomegranate peel compared to the blood of control fish. This might be due to using of pomegranate peels from different sources with different chemical composition. The chemical composition of the crude extract is mainly affected by locality and soil type.

Blood biochemical profile is useful for determining fish health status after different feeding trials [54]. In this study total protein, globulin, and albumin increased significantly in fish fed different concentrations of PPE in a dose dependent effect. Moreover, the liver enzymes levels (AST and ALT) were significantly reduced in serum of fish that received PPE than those of the control fish, the best findings being observed in fish fed diet supplemented with 0.3% PPE. These data indicated that the PPE preserved the structural integrity of the hepatocellular membrane and design of liver cell as stated by a previous histopathological study conducted by[55]. Many studies have revealed that antioxidants capacity to scavenge reactive oxygen species is an essential mechanism of hepato-protective effects [56, 57]. The current study results revealed lower serum urea, creatinine and uric acid in fish fed PPE in a dose dependent effect. Lower levels of urea, uric acid, and creatinine in fish that fed different concentrations of PPE may indicate kidney and gill health[58], requirements of amino acid (arginine)[59] and feed utilization[60] in fish. Effect of PEE on urea, creatinine, and uric acid are still not clearly understood in fish. Fish received diet supplemented with PPE in this study showed a significant decrease in triglycerides and cholesterol levels. The reduction in cholesterol may be elucidated by the polyunsaturated fatty acids and other components in pomegranate peel. The PPE inhibits the activity of pancreatic lipase which inhibits absorption of fat from the intestine[61]. In contrast, high levels of blood cholesterol and triglyceride are indicators of liver dysfunction since lipid homeostasis is one of the major functions of the liver[62]. These findings of blood biochemical profile in this study are similar to the findings obtained by Monir et al.; Badawi & Gomaa. [1 ، 20] who fed Nile tilapia with different concentrations of PP and PPE respectively. Similar results of biochemical profile for this study were revealed in the blood of monosex tilapia supplemented with PP for only 45 days but, after 90 days of feeding, PP supplementation in diet increased serum urea and creatinine levels in fish, indicating that the levels of urea and creatinine increased with increasing feeding trial duration[25]. Furthermore, Acar et al. [18] revealed similar results of biochemical profile for using pomegranate seed oil in rainbow trout.

Lysozyme activity, phagocytic activity and index, IgM activity, WBCs count, and differential leukocytes count are parameters and indicators that used for evaluation of fish immune response. Phagocytosis in fish has a necessary mechanism of cellular immunity. It decreases the pathogen outbreaks as it detects the pathogen and decreases its spread [63]. Activity of lysozyme has an important defense mechanism in the innate immune response. In both invertebrates and vertebrate's lysozyme is a significant parameter in the immune defense. As though, lysozyme has bactericidal activity; makes lysis of pathogenic bacteria[64], as a result of its role on peptidoglycan of cell wall, it has an important action in producing defense against bacterial invasion[65]. Lysozyme activity, IgM, phagocytic activity and index levels increased significantly in a

dose dependent effect in fish that fed diet containing PPE. Similar results revealed by Badawi & Gomaa [20] who stated a significant high levels of IgM and lysozyme activity in blood of Nile tilapia fed PPE diets in respect to fish received the control diet. In addition, Monir et al.[1] stated that serum lysozyme activity in Nile tilapia fed PPE-enriched diets was higher than the control. Furthermore, Badrey et al. [25] revealed that Nile tilapia fed diets containing PP had significantly higher IgM levels and lysozyme activity than control fish. Olive flounder that fed diet enriched with pomegranate had improved innate immune response against *Philasterides dicentrarchi* [52] and *Clarias gariepinus* groups challenged with *A. hydrophila* showed significant increase in phagocytic and lysozyme activity after feeding diets contain different concentrations of PPE [51]. These findings are attributed to high content of polyohenols in PPE which act as immunostimulants[66]. Dissimilar to Acar et al. [18] who reported that lysozyme activity showed no significant difference in the rainbow trout fed with pomegranate seed oil supplemented diet in respect to control. This dissimilarity may be due to species difference and herbal extract components and environmental conditions. In addition, PPE induced a significant increase in WBCs count with observed significant differences in monocytes and lymphocytes numbers among the experimental groups. These results agreed with Harikrishnan et al. [52] who reported that the monocytes and lymphocytes increased significantly in *Olive flounder* fed diet enriched with pomegranate and injected with or without parasite (*Philasterides dicentrarchi)* as compared to control.

Superoxide dismutase (SOD) and malondialdehyde (MDA) are important enzymes as indicators for antioxidant mechanism[67,68]. MDA is an end product of lipid peroxidation in serum espcially polyunsaturated fatty acid (PUFA), while enzymatic antioxidants interact with each other to limit reactive oxygen species (ROS) production mainly hydrogen perioxide and superoxide radicals so prevent ROS-induced oxidative damage to membrane lipid (lipid peroxidation). The SOD is a major cellular antioxidant enzyme responsible for the detoxification of ROS and controlling anti-oxidant mechanism and cell-mediated immunity in fish [4, 12]. The present study revealed a significant increase in SOD activity and a significant decrease in MDA in fish fed PPE diets in a dose dependent effect. Similar results of SOD were detected by Monir *et al.* [1] on Nile tilapia. Likewise, Abdel Moneim; Chidambara Murthy *et al.* [67,69] have reported similar results of PPE effect on SOD and MDA activity on rats. The present results were also coincided with that reported by Zeweil *et al.* [70] who revealed that PP addition to diets of rabbits had significantly decrease MDA activity and increase SOD activity. These findings might be due to the polyphenolic compounds in PPE as gallic acid, ellagic acid, cholinergic acid, catechin, naringeen, and vanillin that posse antioxidant activities through oxidative stress reduction by decreasing hydrogen peroxide and superoxide anion levels and lipid peroxidation ,and act as a potent free radical scavenger [67, 68, 71, 72]

Intestinal villi length and goblet cells number affect directly on fish digestion and nutrient absorption [73]and also affecting the absorption area capacity so they considered a good healthy intestine indicators[74]. Goblet cells produce mucous in the different parts of intestine to make a protection of the mucosal layer from injury, dehydration, and invasion of pathogenic agents [75]. The present study results revealed a significant increase in villi length and goblet cells numbers of some intestinal parts and non-significant decrease in the inter villi space in fish received PPE diets in a dose dependent effect. The increasing in villi length is related to an increase in the surface area available for nutrients absorption [76]. The longer villi found in intestine indicated that the absorption process is more effective in fish that fed PPE [77, 78]. Similar results were reported by Toutou et al.[14] who stated an enhancement in the gut histology of mono sex Nile tilapia fed PP. This effect could be attributed to polyphenolic compounds in PPE which modulate gut microbiota function and composition and interfere with membrane permeability of bacteria. Besides, polyphenols effect on immunity and metabolism of digestive tract and have anti-inflammatory properties [39, 79]

The current study demonstrated that fish fed with PPE supplemented diet induced mortality rate reduction in Nile tilapia challenged with pathogenic *A. hydrophila* and a significant higher survival rate was reported in fish fed 0.3% PPE. These results supported that PPE possess high antibacterial activity against *A. hydrophila*. Similar results were informed by Monir *et al*.[1] who concluded that PPE in diets has decreased mortality rates in Nile tilapia challenged with pathogenic *A. hydrophila*. Reda et al. [51] stated that mortalities after challenge with virulent *A. hydrophila* was low in *Clarias gariepinus* fed diet enriched with PP. In addition, Harikrishnan *et al.* [52] recorded mortalities decreased in *Olive flounder* challenged with *Philasterides dicentrarchi* and fed PPE supplemented diet. Low mortality rates of fish fed PPE may be due to polyphenolic compounds especially flavonoids and tannins which had high antibacterial activity, high immune-response and high antioxidant activity as shown in this study. Antibacterial activity related for degrading the cell wall, disrupting the cytoplasmic membrane, interact with membrane integrated enzymes, and damaging membrane proteins, which lead to cell death at the end [80,81].

Conclusion

Under the circumstances of this study, the crude extract of PPE contains polyphenolic compounds and flavonoids. Dietary PPE enhanced hematological and biochemical parameters, improved immune response, antioxidant activity, intestine histomorphology and increased survival of Nile tilapia challenged with *Aeromonas hydrophila* due to high polyphenolic content in PPE. Moreover, it induced an improvement in fish performance in P0.05 group. Hereafter, further studies are required to approve the recommended dose of PPE that could be recommended as natural feed additive in Nile tilapia diets in order to improve performance, health status and disease resistance.

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Data Availability Statement

All relevant data are available from the authors upon request.

Author contributions

All authors contributed equally in this work (conception, acquisition, samples analysis, statistical analysis, data interpretation, manuscript drafting, and manuscript revision).

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Conflict of interest

The authors declare that they have no conflict of interest.

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تأثير مستخلص قشر الرمان كمكمل غذائي على أداء البلطي النيلي، صحة الدم، شكل الأمعاء والمناعة

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قيمت الدراسة الحالية تأثير مستخلص قشر الرمان (PPE) على اداء النمو، صحة الدم، شكل الأمعاء ومقاومة بكتريا الايروموناس هيدروفيلا بالنسبة للبلطي النيلي أحادي الجنس حيث تم توزيع الأسماك (ن = 120، 9.5 ± 0.5 جم) بشكل عشوائي إلى أربع مجموعات ثلاثية متساوية. وتم امدادهم بنظام غذائي أساسي مكمل بمستخلص قشر الرمان بمعدل 0 و0.05 و 0.1و 0.3 ٪، حيث تمثل P0 (مجموعة التحكم) و ٪P0.05 و ٪P0.1 و ٪P0.3 على التوالي. تم تغذية الأسماك مرتين يومياً لمدة 70 يوماً بنسبة 4٪ من وزن جسم السمكة. أظهر P0 و P0.05% تحسنًا في أداء النمو (الوزن النهائي، وزيادة الوزن ونسبة تحويل العلف) فيما يتعلق بـ ٪P0.1 و ٪P0.3 (P<0.05)حيث أظهرت الأسماك التي تلقت مستخلص قشر الرمان أعلى مقايسة دموية (كرات الدم الحمراء، كرات الدم البيضاء، الخلايا الليمفاوية والوحيدات) مقارنة ب P0 (P<0.05) تم الإبلاغ عن زيادة في البروتين الكلي، الجلوبيولين، الألبومين وانخفاض في الأسبارتات أمينوترانسفيراز، ألانين أمينوترانسفيراز، اليوريا، حمض اليوريك، الكرياتينين، الدهون الثلاثية والكوليسترول في الأسماك التي تم الحصول عليها من معدات الوقاية الشخصية مقارنة بـ p0 (p<0.05).تم الإبلاغ عن الاستجابة المناعية (نشاط الليزوزيم العالي، النشاط البلعمي، مؤشر البلعمة، والغلوبولين المناعي الحالة المؤكسدة (ديسموتاز أعلى من أكسيد الفائق وانخفاض (malondialdehyde)وقياس التشكل المعوي (عدد خلايا الكأس وطول الزغابات) في الأسماك التي حصلت على معدات الوقاية الشخصية فيما يتعلق بـ (P <0.05) P0في تأثير يعتمد على الجرعة. أظهرت الأسماك التي تلقت معدات الوقاية الشخصية معدل وفيات منخفضًا من خلال تحقيق أعلى حماية ضد عدوى بكتريا الايروموناس هيدروفيلا مقارنة بـ .P0في الختام، أدت المكملات الغذائية الخاصة بمعدات الوقاية الشخصية إلى تحسين صحة الدم وقياس شكل الأمعاء ومناعة البلطي النيلي بينما أدت إلى تحسين أداء الأسماك في مجموعة P0.05.

الكلمات المفتاحية: البلطي النيلي، مستخلص قشر الرمان، أداء النمو، قياس التشكل المعوي، الحالة المؤكسدة، الايروموناس هيدروفيلا.