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Effect of dietary barberry fruit (*Berberis vulgaris*) extract on immune function, antioxidant capacity, antibacterial activity, and stress-related gene expression of Siberian sturgeon (*Acipenser baerii*)

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ABSTRACT

Barberry fruit (BF) has a long history as a folk remedy due to its antimicrobial, anti-inflammatory, antioxidant, and cholagogic properties. This study was performed to determine the dietary effect of BF extract on serum and skin mucus immune parameters, antioxidant status, and stress-selected gene expression in Siberian sturgeon. One hundred and fifty fish (30 ± 1 g initial weight) were fed with different levels of BF extract including 0 (control), 150, 300, 600, and 750 mg kg^{-1} for 8-week. After the feeding trial, the counts of white blood cells and lymphocytes were increased in BF-added groups compared to the control group ($P < 0.05$). The highest levels of serum complement component 4, lysozyme activity, and alternative complement were obtained in 750 mg kg^{-1} BF extract treatment ($P < 0.05$). The highest activities of protease, alkaline phosphatase, and esterase were obtained in the skin mucus samples of the fish fed with 750 mg kg^{-1} BF extract ($P < 0.05$). The group fed diets supplemented with 600 and 750 mg kg^{-1} BF extract showed the highest mucus lysozyme activity ($P < 0.05$). The activities of glutathione peroxidase and superoxide dismutase were increased in fish treated with different levels of BF extract ($P < 0.05$), while malondialdehyde content was decreased in BF-added groups compared to the control group ($P < 0.05$). Heat shock protein and cytochrome *P450* mRNA expressions were lowest in the 750 mg kg^{-1} BF extract treatment group, while the highest levels of both genes were found in the control group ($P < 0.05$). The results showed marked improved antibacterial capacity of Siberian sturgeon fed dietary BF against *Streptococcus iniae*, *Yersinia ruckeri*, *Escherichia coli*, *Listeria monocytogenes*, *Aeromonas hydrophila*, and *Lactococcus garvieae*. This study unveiled the promising results of dietary BF extract, especially at 750 mg kg^{-1}, in the regulation of immune and antioxidant defense systems along with the stress responses in Siberian sturgeon.

1. Introduction

During the last decade, sturgeon farming has received considerable attention due to its high meat quality, huge income from the production of caviar, and environmental issues (Fontagné et al., 2006). Siberian sturgeon (*Acipenser baerii* Nikolskii, 1896) is an appropriate candidate for freshwater pisciculture to produce meat and caviar due to its easy adaptation to rearing conditions, short sexual maturity, and relatively

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resistance to culture conditions (Dettlaff et al., 2012; Kolman and Kapusta, 2018). Thus, successful farming of *A. baerii*, especially under intensive systems, can support the global supply of caviar and meat, and reduce fishing pressure on the wild stocks. In intensive systems, this species like other commercial fish species are constantly exposed to opportunistic pathogens due to the presence of various stressors and suppressing the immune system (Hoseinifar et al., 2016; Shen et al., 2014). Therefore, developing effective strategies to boost the immune system and antioxidant defense capacity in the intensive and super-intensive productions of sturgeons is vital (Dawood et al., 2020; Reverter et al., 2014). In the last two decades, the overuse of antibiotics by farmers has led to weak the host immune system, notable development of strains resistant to synthetic compounds, disruption of the microbial ecosystem of the intestinal and the environment, and residual antibiotics in the meat (Dawood et al., 2018). In this regard, the application of feed additives such as medicinal plants is considered a promising solution for improving immunological or nutritional factors in the aquaculture industry (Bairwa et al., 2012; Rashmeei et al., 2020; Reverter et al., 2014). Herbal products have several advantages including eco-friendly, low side effects, easy to access, and cost-effectiveness (Reverter et al., 2014). Indeed, they are multifunctional supplements that exert beneficial effects on the physiological activities of many fish species (Awad and Awaad, 2017; Hoseinifar et al., 2020; Reverter et al., 2014; Yousefi et al., 2021). Furthermore, the enhancement of growth rate, antioxidant status, immune system trigger, decrement of susceptibility to environmental stress, and infectious agents have been found for several species of sturgeons treated by herbal additives such as rosemary oil in *Huso huso* (Ebrahimi et al., 2020), green tea extract in *H. huso* ♂ × *Acipenser ruthenus* ♀ (Ebrahimi et al., 2017), lemon verbena in *Acipenser baerii* (Adel et al., 2021a), proanthocyanidins in *A. baeri* ♀ × *A. schrenckii* ♂ (Xu et al., 2021), and *Sargassum ilicifolium* in *H. huso* (Yeganeh and Adel, 2019).

Barberry is a well-known medicinal plant that is used as a feed additive all around the world (Imenshahidi and Hosseinzadeh, 2019). Furthermore, a wide range of its therapeutic and medicinal activities has been explored in humans or animals models (Feng et al., 2019; Ji et al., 2012). The high antioxidant, antimicrobial, and anti-inflammatory activities of barberry are justified due to the presence of berberine as the main bioactive compound as well as other substances including acanthine, bargustanine, beriambine, flavonoids, ascorbic acid, triterpenes, and palmatine (Imenshahidi and Hosseinzadeh, 2019; Kremer et al., 2008; Rahimi-Madiseh et al., 2017). The dietary administration of barberry or berberine in the aquaculture industry has been considered and its beneficial effects on improving antioxidant capacity in *Megalobrama amblycephala* (Chen et al., 2016), innate immune responses and disease resistance in *O. niloticus* (Van Doan et al., 2020), and growth rate in *O. mykiss* (Ramezanzadeh et al., 2020) have been addressed. Moreover, the positive effect of barberry extract on the growth and feed utilization attributes of Siberian sturgeon was reported in our recent paper. However, no information is available on the effect of barberry fruit extract on immune-physiological responses and antioxidant capacity of sturgeons, which indicates the importance of conducting this research on these valuable fish. Therefore, the present study was the first attempt to investigate the dietary effect of barberry fruit extract on immune parameters (serum and skin mucus variables), antioxidant status, some stress-related gene expression, and skin mucus bactericidal activity in Siberian sturgeon.

2. Materials and methods

2.1. Barberry (*B. vulgaris*) fruit extraction and chemical complements analysis

Barberry fruit (BF) was purchased from a local market (Qaen, South Khorasan, Iran). The fresh ripe BF was dried in a conventional oven at 60 °C for 48 h and finely powdered. Afterward, ethanol (99% purity) was added at 1:10 ratio (w/v) and stirred for 72 h at room temperature. Then, the mixture was vacuum filtered through a Whatman #1 filter paper and ethanol was evaporated by a Laborota 4003 rotary evaporator (Heidolph Instruments GmbH Co., Schwabach, Germany). BF extract was kept at − 20 °C until further analysis (less than two weeks).

Total phenolic contents (mg gallic acid mL^{-1}) were measured based on the Folin-Ciocalteu method at 765 nm using gallic acid (Sigma-Aldrich) to plot the standard curve (Du et al., 2009). Total anthocyanins (mg cyanidin-3 glucoside L^{-1}) of BF extract were evaluated based on Lee et al. (2002) method at 700 nm. To determine the antioxidant activity of BF extract, 1 mL of the extract was added to 2 mL of 0.004% 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical solution. The mixture was stirred and kept at room temperature in a dark place for 30 min. Then, the absorbance of the samples was recorded at 517 nm and the percentage of DPPH scavenging was computed according to the method described by Kukić et al. (2008). Afterwards, the radical inhibitory activity was expressed based on the amount of the extract required for initial reduction of DPPH at 50% (half-maximal inhibitory concentration; IC_{50}). Finally, the IC_{50} value (mg mL^{-1}) was determined by plotting the percentage of DPPH radical inhibition against different BF extract. Berberine (μg mL^{-1}) was determined by spectrofluorometeric method as described by Yusupov et al. (1990). Briefly, 1 mL of the extract was added to 5 mL of sodium perchlorate solution and 10 mL of borate phosphate buffer and kept at room temperature. After 2 h, the mixture was transferred to the decanter and separate the organic phase by adding 10 mL of 1, 2-dichloroethane (Merck). Finally, the fluorescence value of the organic phase was measured by a Hitachi fluorescence spectrophotometer (Hitachi F 2500, Hitachi Co., Tokyo, Japan) at 518 nm.

2.2. Experimental diets preparation and fish culture conditions

A commercial sturgeon feed free from any medicinal plant additives was purchased from 21-Beyza® Company, Shiraz, Iran (Ex-APG1™ 48.6% crude protein, 14.2% lipid, 1.7% fiber, 0.9% phosphorus, 8.3% moisture, and 20.0 kJ/g diet energy) and considered as a basal diet. The basal diet was powdered and sieved on a 30-mesh screen (600 μm). In the next step, different levels of BF extract including 0 (control), 150, 300, 600, and 750 mg kg^{-1} were dissolved in a lukewarm water and added to the powdered basal diet to obtain a stiff dough. The paste was minced using an MG-1400R grinder (Pars Khazar Co., Tehran, Iran) equipped with 3 mm diameter die mesh and the spaghetti-like strands were air-dried at room temperature. The pellets were packed in zipper bags with silica gel and kept in a freezer (−20 °C) throughout the trial. The experimental diets were made semimonthly.

Siberian sturgeon (*A. baerii*) was purchased from International Sturgeon Research Institute (Rasht, Gilan, Iran). The fish were relocated to Khojir Agricultural Research Center (Tehran, Iran) and habituated with the new conditions for 14 days. The fish was fed with the control diet (re-pelleted basal diet) during the adaptation period. Afterward, 150-acclimated fish with an initial weight of 30 ± 1 g (mean ± SE) were randomly dispersed into 15 square concrete tanks (1 m × 1 m×1 m, 10 fish in each tank) with 0.2 L min^{-1} water flow. The fish were fed three times daily by hand to apparent satiation for 8 weeks. Some water quality variables and environmental conditions were controlled and recorded (17 ± 1°C water temperature, 6.2 ± 0.3 mg L^{-1} dissolved oxygen, 2.1 ± 0.2 mg L^{-1} nitrate, 0.02 ± 0.001 mg L^{-1} nitrite, and 7.2 ± 0.2 pH) during the experiment period. No mortality was also recorded in either BF-supplemented groups or the control group during the study period.

In this study, all the experimental studies related to the fish were undertaken following the international guidelines for the care and use of animals for scientific purposes (Jenkins et al., 2014), which were approved by the committee of Science and Research Branch University, Tehran, Iran (local approval number: 1127, 02/18/2018).

2.3. Sampling

After 56 days of the feeding trial, fish were starved for 24 h and three fish from each tank were randomly sampled. Then, the blood samples were withdrawn from the caudal vein of the anesthetized fish (150 mg L^{-1} clove flower powder). A partial part of the collected blood samples was poured into Na-heparin (as an anticoagulant) coated microtubes for white blood cell (WBC) and differential leukocyte counts. The remaining blood samples were transferred to non-heparin tubes and were allowed to clot at refrigerator temperature (4 °C) for 2 h. Afterward, the samples were centrifuged at 3000 ×*g* for 15 min at 4 °C. Then, the supernatants were collected and stored at − 80 °C for future analysis.

To collect the fish skin mucus samples, three starved fish from each tank were randomly sampled and anesthetized with the clove powder concentration. The anesthetized fish were rinsed with clean water to prevent any side effect from the anesthesia substance and transferred individually into zipper bags containing 50 mL of 50 mM NaCl (Ross et al., 2000). The collected skin mucus samples were transferred into sterile 15 mL tubes and centrifuged at 8000 ×*g* for 10 min at 4 °C and the supernatants were stored at − 80 °C until use.

2.4. Blood immune parameters

The total number of WBC was counted using a Neubauer hemocytometer under a Nikon TS100 inverted microscope (Nikon Corporation, Tokyo, Japan). After fixing (96% ethanol for 30 min) and staining (Giemsa solution) of the blood smear samples, the differential count of leukocytes was also determined under the microscope (Nikon TS100).

The total protein (TP) content of the serum samples was measured according to the Biuret method using a commercial kit (Catalog number: 3142803; Bionik®, Tehran, Iran) at 540 nm. Additionally, serum albumin content was determined spectrophotometry based on the bromocresol green method by a commercial kit (Catalog number: 3142103; Bionik®, Tehran, Iran). The total serum globulin was obtained by subtracting the albumin content from the total serum protein content (Nayak et al., 2007).

Serum lysozyme enzyme activity was measured by turbidimetric assay according to Ellis (1990) method with some modifications.

The concentrations of complement components (C3 and C4) and immunoglobulin M (IgM) were calculated using ELISA assay (ELX800, BioTek, Vermont, USA) according to the instructions of Hangzhou Eastbiopharm® (Hangzhou, China) diagnostic kits (Catalog numbers: CK-E90918, CK-E90919, and CT-E9091 for C3, C4, and IgM, respectively), which were used for fish (Shekarabi et al., 2021).

Serum alternative complement hemolytic 50% activity (ACH50) was performed based on the hemolysis of rabbit red blood cells according to the method described by Yano (1992).

2.5. Serum antioxidant enzymes activity and malondialdehyde level

The glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) activities, and malondialdehyde (MDA) level were measured in the serum samples using the relevant ZellBio® commercial kits, ZellBio GmbH, Ulm, Germany (Catalog numbers: ZB-GPX-A48, ZX-44108-96/192, ZX-44102-96, and ZX-44116-96 for GPx, SOD, CAT, and MDA, respectively) according to the manufacturers' protocols by spectrophotometry at 412, 420, 405 and 535 nm, respectively.

2.6. Skin mucosal immunity

2.6.1. Mucus non-specific immune parameters

The lysozyme activity and TP content of the mucus samples were performed similarly to the methods described for the serum samples.

Alkaline phosphatase (ALP) activity of the mucus samples was assayed using Pars-Azmoon commercial kit (Catalog number: 1400002, Pars-Azmoon Co., Alborz, Iran) by a biochemical Prestige 24i auto-analyzer (Boeki Medical Systems, Tokyo, Japan).

The activity of protease (PRT) in the mucus samples was quantified based on azocasein hydrolysis assay (Ross et al., 2000). Briefly, the mucus sample was added to 100 mM ammonium bicarbonate buffer (pH 7.8) containing 0.7% azocasein (at the of 1:1) and incubated for 19 h at 30 °C in a rotary thermo-shaker (THL 500, Gerhardt, Königswinter, Germany). After stopping the reaction by adding trichloroacetic acid (4.6% final concentration), the mixture was centrifuged at 15,000 ×*g* for 5 min. Then, 100 μl of the supernatants were added to 100 μl of 0.5 M NaOH in the 96-well microplate ELISA reader (BioTek ELX800). Trypsin and the assay buffer were used instead of the sample as positive and negative controls, respectively. The protease activity was measured by increasing the optical density (OD) absorbance at 450 nm.

Esterase (EST) activity was determined according to the method of Ross et al. (2000). In summary, the equal volume of mucus sample and 0.4 mM para-nitrophenyl myristate was added to 100 mM ammonium bicarbonate buffer containing 0.5% Triton X-100, pH 7.8, and incubated at 30 °C. The OD absorbance was read unceasingly for 2 h at 405 nm by the ELISA reader (BioTek ELX800).

2.6.2. In-vitro antibacterial activity of the skin mucus

The disc diffusion assay and minimum inhibitory concentration (MIC) measurements were undertaken to determine the bactericidal properties of the fish skin mucus against *Streptococcus iniae* (PTCC 1887), *Yersinia ruckeri* (PTCC 1888), *Escherichia coli* (PTCC 1860), *Listeria monocytogenes* (PTCC 1163), *Aeromonas hydrophila* (strain AH04; KP689330), and *Lactococcus garvieae* (strain LC2; KJ997915). Accordingly, 200 μl of the bacterial suspension (10^7 CFU/mL; 0.5 McFarland) was spread individually over a blood agar medium plate, except for *L. monocytogenes* that was cultured on tryptic soy agar. Next, a sterile paper disc was impregnated to 100 μl of the mucus and the discs were implanted on the bacterial culture medium and then incubated at 25 °C for 24 h under aerobic conditions. Afterward, the diameter of the inhibition zone (mm) was measured via a digital caliper (3 replications were considered for each bacterial culture).

The macro-dilution method was used to determine the MIC of skin mucus against the tested microorganisms. For this purpose, sequential dilution of mucus was prepared in sterile tubes containing 5 mL of tryptone soya broth medium. The prepared mucus dilutions were dissolved with sterile saline solution to achieve the following final concentration: 12.5, 25, 50, 75, 100, 125, 150, 200, 250, and 300 μl mL^{-1}. The tubes were inoculated with 10 μl of the bacterial suspension (McFarland 0.5) and incubated at 25 °C for 24 h. Afterward, the lowest concentration of the mucus in which the turbidity was not observed was considered MIC.

2.7. Gene expression

2.7.1. Tissue sampling, RNA extraction, and cDNA synthesis

At end of the trial, three fish from each tank were randomly selected and euthanized with an overdose of clove powder. The fish were dissected on ice and the liver samples collected from each tank were pooled to have one specimen for each tank (three samples per treatment). The tissue samples were immediately immersed in liquid nitrogen (−196 °C) and stored at − 80 °C until the RNA extraction assay.

Total RNA was isolated according to the manufacturer's instructions of Sinaclon® RNX-plus™ extraction kit (Catalog number: EX6101, Sinaclon Co., Tehran, Iran) that was described in detail by Paknejad et al. (2020a). The quantity and quality of the extracted RNA were assessed by a WPA Biowave II spectrophotometer (Biochrom Ltd., Cambridge, UK) at 260/280 nm absorbance ratio and agarose gel electrophoresis (1% agarose and stained with ethidium bromide), respectively.

The WizScript™ cDNA synthesis kit (Catalog number: W2201, Wizbio Solutions, Seongnam, South Korea) was used to synthesize complementary DNA (cDNA) according to the manufacturer's guidelines.

Reverse transcription-polymerase chain reaction (RT-PCR) was run in triplicate using a standard protocol (initial denaturation at 95 °C for 5 min, 40 cycles of denaturation, annealing at specific temperatures, and extension at 72 °C for 15 s).

2.7.2. Primer design and quantitative PCR

Primers for heat shock protein (*HSP70*), cytochrome P450 (*P450*), and ribosomal protein L6 (*RPL6*) genes were designed based on NCBI Gene Bank information using Oligo 7 program (Table 1). Ribosomal protein L6 (*RPL6*) gene was also used as an internal reference gene to normalize the expression levels of the candidate genes based on its expression stability in *A. baerii* (Abdolahnejad et al., 2015; Aidos et al., 2020).

Quantifying the mRNA transcription levels of *HSP70* and *P450* genes was performed by a real-time PCR detection system (Model: CFX96, Bio-Rad Laboratories, California, USA) using an SYBR Green qPCR master mix kit (WizPure™, Catalog number: W1711, Wizbio Solutions, Seongnam, South Korea) based on the guidelines provided by the kit. The efficiency of the qPCR amplification for each primer was evaluated according to the following equation: $E (\%) = (10^{1/slope} - 1) \times 100$. Moreover, the relative expression levels of the target genes were performed based on the mathematical model provided by Pfaffl (2001).

2.8. Statistical analysis

In this study, each treatment contained three replicates and three fish were randomly sampled from each replicate (nine fish per treatment) for the blood sampling, skin mucus collection, and gene expression assay. All results were reported as the mean ± standard error (SE). First, all data were checked for the assumption of parametric tests including normality of the distribution (Kolmogorov-Smirnov test) and homogeneity of variance (Levene test). Then, the data were analyzed by one-way analysis of variance (ANOVA), and the differences between the means were tested by Duncan's multiple comparisons post-hoc tests and the differences were considered significant when the *P*-value was less than 0.05 ($P < 0.05$). Correlations between some immune data were examined using Pearson correlation test. All statistical analyses were performed using SPSS 23.0 (Chicago, IL, USA).

3. Results

3.1. Chemical components of BF extract

The content of total phenolic compounds (mg gallic acid mL^{-1}), total anthocyanins (mg cyanidin-3 glucoside L^{-1}), IC50 (μg mL^{-1}), and berberine (μg mL^{-1}) in BF extract are shown in Table 2. The highest value of BF extract compounds was recorded for total phenolic content (51.3 ± 4.3 mg mL^{-1}).

3.2. Humoral immunity components

The highest count of WBC was observed in the fish fed with 750 mg kg^{-1} BF extract, while it was not significantly higher than 600 mg kg^{-1} BF extract treatment (Table 3; $P > 0.05$). The estimation of differential leukocyte counts showed that the addition of BF extract in the diets significantly enhanced lymphocyte and reduced neutrophil percentages compared to the control fish (Table 3; $P < 0.05$). Dietary BF extract had no significant effect on the counts of monocyte and eosinophil among different groups (Table 3; $P > 0.05$). Basophil was not observed in the blood samples.

Serum immune parameters of Siberian sturgeon fed with different levels of BF extract are illustrated in Table 3. Statistical analysis of data showed that different levels of BF extract in the diets had no significant effect on C3 level in serum ($P > 0.05$). The inclusion of BF extract

Table 2
Chemical composition of barberry (*Berberis vulgaris*) fruit extract.

Parameters	Test result	Unit
Total phenolic contents	51.3 ± 2.5	mg gallic acid mL^{-1}
Anthocyanins	39.6 ± 1.2	mg cyanidin-3 glucoside L^{-1}
IC50	88.0 ± 0.5	μg mL^{-1}
Berberine	443.9 ± 17.5	μg mL^{-1}

Notes. Values are presented as the mean ± SE (n = 3). IC50: The half maximum inhibitory concentration of 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity.

Table 3
Hematological and serum immunological parameters of Siberian sturgeon (*Acipenser baerii*) fed with dietary inclusion levels of barberry fruit (BF) extract for 8 weeks.

Parameters	Dietary BF extract levels (mg kg^{-1})				
	0 (Control)	150	300	600	750
WBC (10^3 mm^{-3})	4.83 ± 0.09^c	5.16 ± 0.14^b	5.12 ± 0.04^b	5.35 ± 0.08^{ab}	5.46 ± 0.06^a
Lym (%)	73.67 ± 0.29^c	78.67 ± 0.29^b	80.67 ± 0.50^b	86.00 ± 1.15^a	87.33 ± 0.76^a
Neu (%)	23.67 ± 0.33^a	19.33 ± 0.33^b	17.33 ± 0.88^b	12.00 ± 1.00^c	11.00 ± 1.00^c
Mon (%)	1.67 ± 0.55^a	1.33 ± 0.47^a	1.67 ± 0.55^a	1.33 ± 0.67^a	1.33 ± 0.47^a
Eos (%)	1.00 ± 0.55^a	0.67 ± 0.58^a	0.33 ± 0.29^a	0.67 ± 0.33^a	0.33 ± 0.47^a
C3 (mg d L^{-1})	55.61 ± 0.30^a	56.20 ± 0.81^a	59.53 ± 1.11^a	60.55 ± 1.46^a	61.05 ± 1.87^a
C4 (mg d L^{-1})	14.50 ± 0.41^c	17.50 ± 1.22^{bc}	20.50 ± 0.06^{ab}	21.00 ± 0.83^a	23.50 ± 0.41^a
IgM (mg d L^{-1})	71.48 ± 1.63^b	74.14 ± 0.81^b	76.21 ± 1.63^{ab}	82.20 ± 0.81^a	82.80 ± 1.19^a
Lyz (mL^{-1} min^{-1})	25.65 ± 0.53^d	28.59 ± 0.29^c	29.77 ± 1.31^{bc}	32.06 ± 0.76^{ab}	35.68 ± 1.22^a
ACH50 (U%)	126.92 ± 1.39^c	130.68 ± 0.51^b	131.69 ± 0.42^{ab}	133.27 ± 0.92^{ab}	135.22 ± 0.87^a
TP (g dL^{-1})	4.11 ± 0.11^c	5.08 ± 0.16^b	5.24 ± 0.27^b	5.67 ± 0.14^{ab}	6.21 ± 0.35^a
Globulin (g dl^{-1})	3.10 ± 0.10^c	3.55 ± 0.12^{bc}	3.79 ± 0.15^{ab}	4.16 ± 0.09^{ab}	4.45 ± 0.27^a
Albumin (g dl^{-1})	1.01 ± 0.06^b	1.53 ± 0.10^a	1.45 ± 0.16^a	1.51 ± 0.11^a	1.76 ± 0.08^a

Notes. Values are presented as the mean ± SE. Different letters denote significant differences between the treatments (n = 9, $P < 0.05$). WBC: white blood cell; Lym: lymphocyte; Neu: neutrophil; Mon: monocyte; Eos: eosinophils; C3 and C4: complement components 3 and 4; IgM: immunoglobulin M; Lyz: lysozyme; ACH50: alternative complement activity; TP: total protein.

Table 1
Oligonucleotide sequences for the study of heat shock protein (*HSP70*) and cytochrome P450 (*P450*) gene expression in Siberian sturgeon (*Acipenser baerii*).

Gene name	Primer name	Sequences of qPCR primers (5′−3′)	Amplicon (bp)	Tm (°C)	Efficiency (%)	Accession Number
HSP70	HSP70-F	ACGGACTCAGAGAGGCTGAT	110	59	97.2	AF228040.1
	HSP70-R	GAGTCGTCGAATCTCCGTCC				
P450	P450-F	TTCTTCCAGCCATTCGGGTC	124	60	96.8	KM258120.1
	P450-R	AGCATCCTGTCTGAAGCTGG				
RPL6	RPL6-F	TGCCTCGGTATTACCCTACC	115	59	98.0	MH702441.1
	RPL6-R	AGGACAGTGCCAGGAGTGAT				

Notes. RPL6: ribosomal protein L6, F: forward primer, R: reverse primer, Tm: melting temperature.

upregulated plasma C4 and IgM in the fish. The alteration of both C4 and IgM was started significantly takes effect at 600 mg kg^{-1} inclusion level ($P < 0.05$). However, there was no significant difference in C4 and IgM levels between treatments fed with 600 and 750 mg kg^{-1} BF extract ($P > 0.05$). The highest level of lysozyme activity was observed in 750 mg kg^{-1} BF extract, but it was not statistically different from 600 mg kg^{-1} BF extract treatment ($P > 0.05$). The measured levels of ACH50 in the fish fed with a diet containing 750 mg kg^{-1} BF extract showed a significant increase compared to those fed with 150 mg kg^{-1} BF extract and the control group ($P < 0.05$). The contents of serum TP, globulin, and albumin enhanced significantly in the fish fed with BF extract at 750 mg kg^{-1} compared to those treated with dietary BF extract at 0 mg kg^{-1} (control diet) ($P < 0.05$).

Moreover, the Pearson correlation analysis indicated that there was a significant positive correlation between the serum ACH50 level and C3, C4, and lysozyme (Table 4). There was also a tendency for a positive correlation between the serum IgM content and lymphocyte count and serum TP level (Table 4).

3.3. Antioxidant enzymes activity and MDA level

The activities of GPx, SOD, CAT as well as MDA content in the serum samples of Siberian sturgeon fed with different levels of BF extract are depicted in Fig. 1. GPx activity was increased significantly by the addition of 300, 600, and 750 mg kg^{-1} BF extract to the diet compared to the control group ($P < 0.05$). However, there was no considerable difference in the GPx activity between the control and 150 mg kg^{-1} BF extract groups ($P > 0.05$). SOD activity in the fish fed with diets containing BF extract was significantly higher than the control one ($P < 0.05$). The results showed that feeding Siberian sturgeon with different levels of BF extract had no remarkable effect on the serum CAT activity ($P > 0.05$). Besides, MDA level was significantly decreased by administration of the fish diet with BF extract at different concentrations compared to the control fish ($P < 0.05$).

3.4. Skin mucosal immune responses

The effect of different dietary levels of BF extract on the skin mucosal immunity of Siberian sturgeon is summarized in Table 5. The highest activities of PRT and ALP were obtained in the group fed with 750 mg kg^{-1} BF extract ($P < 0.05$). However, PRT and ALP activities in the mucus samples were not significantly affected by dietary BF extract at lower concentrations (150, 300, and 600 mg kg^{-1}) compared to the control fish ($P > 0.05$). The inclusion of BF extract promotes mucosal lysozyme activity. The activity of mucosal lysozyme was started significantly takes effect at 600 mg kg^{-1} inclusion level ($P < 0.05$). However, there was no significant difference in lysozyme activity between treatments fed with 600 and 750 mg kg^{-1} BF extract ($P > 0.05$). The lysozyme activity of the mucus samples significantly ($P < 0.05$) increased in the fish fed supplemented diet with 750 mg kg^{-1} BF extract compared to others except for 600 mg kg^{-1} BF extract treatment. The level of skin mucus EST activity was increased numerically with higher levels of BF extract and the highest value was observed in 750 mg kg^{-1} BF extract ($P < 0.05$). The highest concentration of mucosal TP was found in fish fed a diet containing 750 mg kg^{-1} BF extract, while the lowest concentration was found in control fish ($P < 0.05$).

Table 4
Pearson correlation coefficients (*R*) for relation between some of the humoral immune variables of Siberian sturgeon (*Acipenser baerii*) fed enriched diets with barberry fruit (BF) extract for 8 weeks.

Correlations	R
ACH50 – C3	0.81*
ACH50 – C4	0.84*
ACH50 – Lysozyme	0.89*
IgM – Lymphocyte	0.94*
IgM – TP	0.82*

*Correlation is significant at the 0.01 level(2-tailed, n = 45). IgM: immunoglobulin M, ACH50: alternative complement activity, TP: total protein, C3: complement components 3, C4:complement components 4.

3.5. Skin mucus antibacterial capacity

The antibacterial capacity of skin mucus of fish fed BF for 56 days is shown in Table 6 and Fig. 2. The minimum inhibitory concentration test (MIC) showed that the infection with *Streptococcus iniae*, *Yersinia ruckeri*, *Escherichia coli*, *Listeria monocytogenes*, *Aeromonas hydrophila*, and *Lactococcus garvieae* resulted in lower MIC in Siberian sturgeon fed BF than fish BF-free diet (Table 6). The inhibition zone diameter of fish fed BF and infected with *A. hydrophila* is increased by 150, 300, 600, and 750 mg kg^{-1} doses and by 300, 600, and 750 mg kg^{-1} in case of *S. iniae* and *Y. ruckeri* (Fig. 2). The inhibition zone in case of *L. garvieae* is increased by 150, 300, 600, and 750 mg kg^{-1} doses than the control and 300, 600, and 750 mg kg^{-1} doses than 150 mg kg^{-1} (Fig. 2). The inhibition zone in case of *L. monocytogenes* is increased by 600 and 750 mg kg^{-1} doses than the control and 150 and 300 mg kg^{-1} doses than 150 mg kg^{-1} (Fig. 2). In the case of *E. coli*, the inhibition is increased by 150, 300, and 750 mg kg^{-1} than the control (Fig. 2).

3.6. Stress-related genes expression

The relative transcription of *HSP70* and cytochrome *P450* mRNA levels in Siberian sturgeon fed with BF extract are presented in Fig. 3. The *P450* mRNA level was significantly down-regulated in fish fed with 750 mg kg^{-1} BF extract compared to the control group ($P < 0.05$), while the intermediate concentrations of dietary BF extract (150, 300, and 600 mg kg^{-1}) did not show a significant difference in the gene expression levels compared to the control and 750 mg kg^{-1} BF extract groups ($P > 0.05$). Moreover, the transcription of *HSP70* gene was significantly reduced in 750 mg kg^{-1} BF extract treatment compared to the control and 150 mg kg^{-1} BF extract groups ($P < 0.05$).

4. Discussion

Immunomodulation of the immune system in aquatic animals can be achieved using functional feed additives (Ali et al., 2022; Dawood, 2021). In this regard, dietary medicinal plants can attenuate the harmful effects caused by aquaculture operations on the fish due to their valuable bioactive compounds (Paknejad et al., 2020b; Rashmeei et al., 2020; Samavat et al., 2019). In this study, BF as a herbal supplement had a positive effect on the immune responses and antioxidant defense system of Siberian sturgeon according to its unique antibacterial, anti-oxidant, and anti-inflammatory properties (Chen et al., 2016; Van Doan et al., 2020).

Leukocytes are an important component of the defense system that plays a fundamental role in the immune responses in fish (Ellis, 1999). Our findings exhibited that the counts of leukocytes and lymphocytes were significantly increased by dietary levels of BF extract, especially at 750 mg kg^{-1}. However, the neutrophils and monocytes were decreased in the BF-added groups. Similarly, Ramezanzadeh et al. (2020) revealed an enhancement in the leukocytes count and lymphocyte percentage of rainbow trout fed diets supplemented with barberry root extract. In unfavorable conditions, physiological responses are characterized by a decrease in the lymphocytes count and an increase in the number of neutrophils (Ellis, 1999). This means that the fish receiving BF extract are able to elicit stronger responses to invasive pathogens. Earlier studies also have reported a remarkable enhancement in the leukocyte count of *A. baeri* after feeding with *Aloysia citrodora* (Adel et al., 2021a). Therefore, it seems that the presence of bioactive molecules such as alkaloids and phenols in barberry could stimulate the spleen and thymus

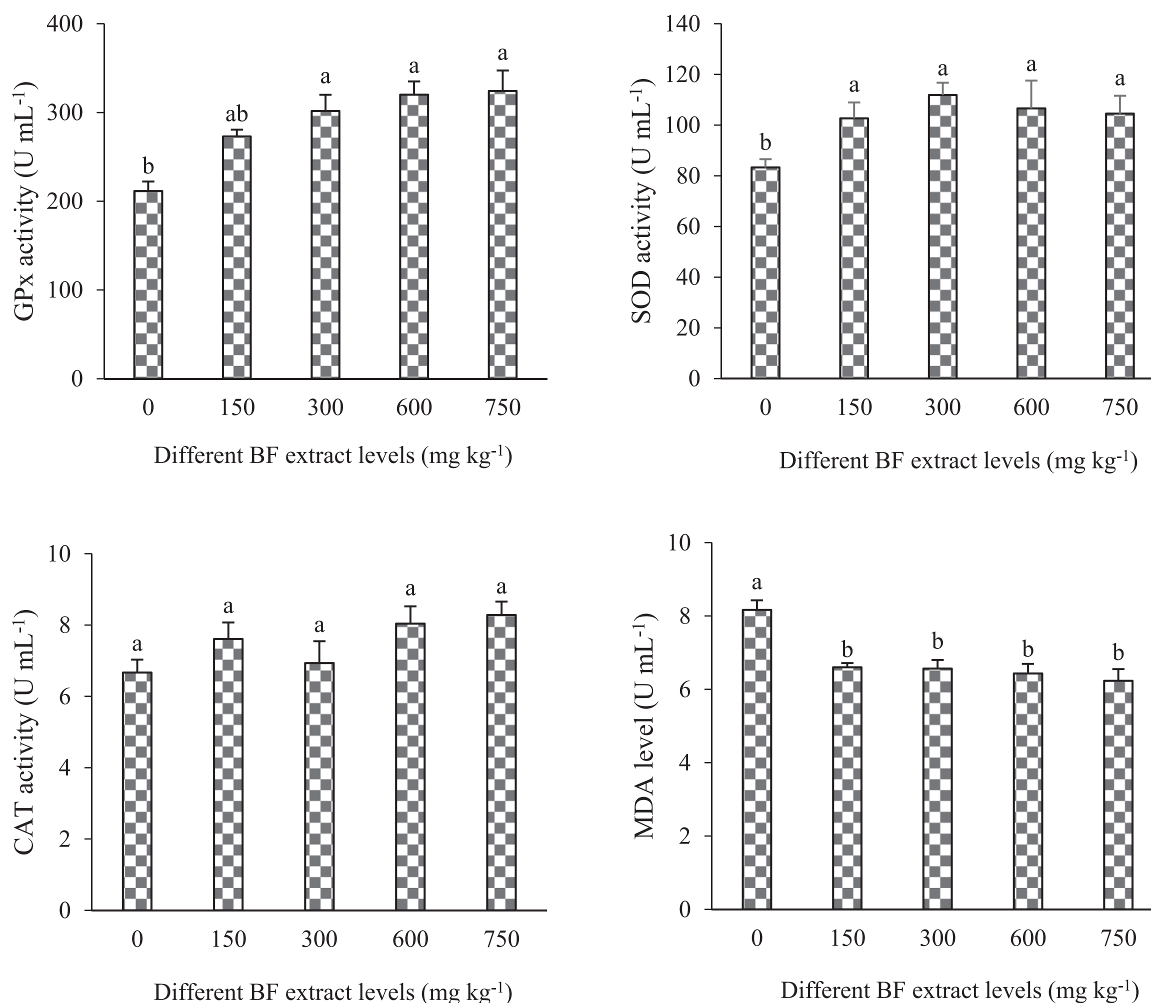

Fig. 1. Glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) levels in the serum samples of Siberian sturgeon (*Acipenser baerii*) fed with different levels of barberry fruit (BF) extract for 8 weeks. Different letters over the bars show significant differences (n = 9, $P < 0.05$). Error bars indicate the standard error of the mean.

Table 5
Skin mucus non-specific immune parameters of Siberian sturgeon (*Acipenser baerii*) fed with dietary inclusion levels of barberry fruit (BF) extract.

Parameters	Dietary BF extract levels (mg kg^{-1})				
	0 (Control)	150	300	600	750
PRT (U L^{-1})	72.20 ± 0.94^{b}	74.34 ± 0.61^{b}	73.91 ± 1.21^{b}	76.86 ± 1.36^{b}	87.49 ± 0.87^{a}
ALP (U L^{-1})	42.67 ± 1.45^{b}	44.00 ± 0.58^{b}	45.00 ± 1.15^{b}	48.33 ± 1.45^{b}	56.00 ± 1.73^{a}
Lyz (mL min^{-1})	19.31 ± 1.13^{b}	21.65 ± 0.85^{b}	23.57 ± 0.84^{b}	27.00 ± 0.60^{a}	29.83 ± 1.38^{a}
EST (U L^{-1})	0.30 ± 0.01^{c}	0.31 ± 0.01^{bc}	0.35 ± 0.01^{bc}	0.39 ± 0.00^{b}	0.56 ± 0.01^{a}
TP (g dL^{-1})	5.90 ± 0.27^{c}	7.30 ± 0.24^{b}	7.45 ± 0.39^{b}	8.15 ± 0.20^{b}	9.91 ± 0.39^{a}

Notes. Values are presented as the mean ± SE. Different letters denote significant differences between the treatments (n = 9, $P < 0.05$). PRT: Protease; ALP: Alkaline phosphatase; Lyz: lysozyme; EST: Esterase; TP: Total protein.

Table 6
The Minimum inhibitory concentration test (μg mL^{-1}) of Siberian sturgeon (*Acipenser baerii*) skin mucus fed with dietary levels of barberry fruit (BF) extract for 56 days.

Treatments (mg kg^{-1})	*Aeromonas hydrophila*	*Streptococcus iniae*	*Lactococcus garvieae*	*Yersinia ruckeri*	*Listeria monocytogenes*	*Escherichia coli*
0 (Control)	> 250	> 200	250	200	150	100
BF 150	200	150	> 150	150	100	75
BF 300	100	100	100	100	50	50
BF 600	100	100	100	50	50	50
BF 750	100	100	100	50	50	50

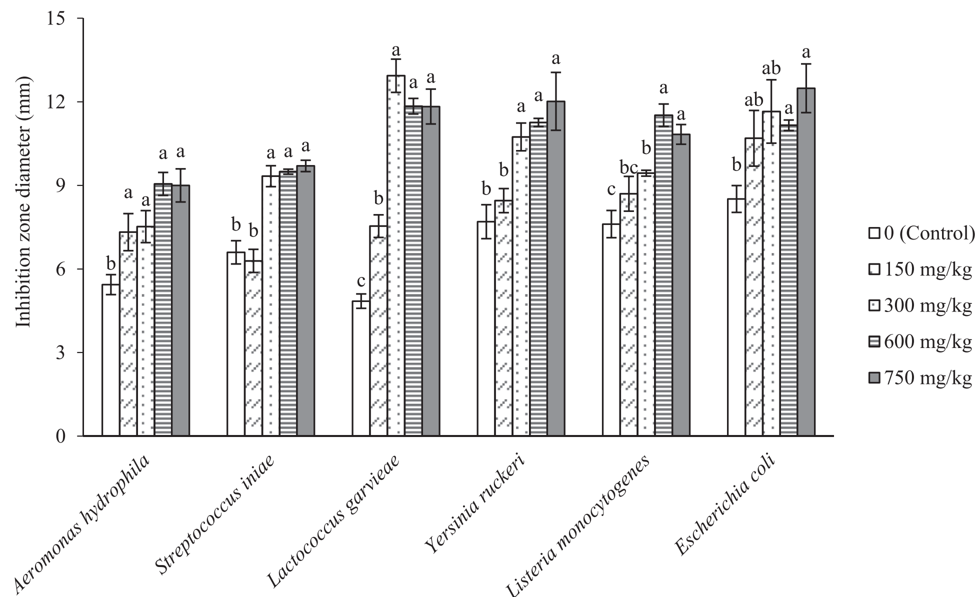

Fig. 2. The bacterial growth inhibition zone diameter (mm) of Siberian sturgeon (*Acipenser baerii*) skin mucus fed with dietary barberry fruit extract for 56 days. Different letters over the bars show significant differences (n = 9, $P < 0.05$). Error bars indicate the standard error of the mean.

to synthesize more leukocytes.

Herbal supplements are known as immune regulators that are involved in activating the innate immune responses of fish (Reverter et al., 2014). In this study, lysozyme activity as a bacterial wall-destroying agent was increased in fish receiving barberry diet (Choi et al., 2008; Secombes, 1996) which is similar to the results obtained in *Acanthopagrus schlegeliiang* (Wang et al., 2020), and *Oreochromis niloticus* (Van Doan et al., 2020) treated with berberine. High lysozyme activity in this work may be attributed to an increase in the number of leukocytes by the immune supplement. Besides, lysozyme participates in the activation of the complement system through the opsonization of foreign particles (Secombes, 1996).

In this study, C3 and C4 as main components factors in the classical complement pathway and ACH50 (alternative complement pathway activity) were significantly increased in the fish receiving supplemented diets. Improving the ACH50 and C3 and C4 by herbal medicine such as *Aloe vera* and *Polygonum minus* extracts has been well documented in Siberian sturgeon and rainbow trout, respectively (Adel et al., 2020; Bazari Moghaddam et al., 2017). It seems that increasing the activity of the complement system may be related to the improvement of liver functions by dietary BF extract levels or berberine as previously reported by Van Doan et al. (2020), and Xu et al. (2017).

Albumin and globulin are two major protein components of serum that are good indicators for assessing the effect of nutrients on the immune defense of fish (Wiegertjes et al., 1996). In this study, a significant increase was obtained in the levels of serum TP, albumin, and globulin in the fish fed with dietary BF extract, especially at 750 mg kg^{-1}, indicating a remarkable improvement in the immune response and function of organs involved in protein synthesis such as the liver. Similarly, several studies have also pointed out that the immune functions were simultaneously improved with an increase in serum TP, albumin, and globulin levels of fish after feeding with botanical substances (Ebrahimi et al., 2020; Safari et al., 2020). In fish systematic immunity, IgM is one of the main antibody isotypes with a variety of defense activities against pathogens such as antibacterial, anti-adhesion, antitoxin, anti-invasions, and activation of the complement classical pathway (Ellis, 1999). In this study, serum IgM value was enhanced with increasing levels of BF extract in Siberian sturgeon diet. The beneficial effects of several herbs such as *Coriandrum sativum* (Farsani et al., 2019) in rainbow trout, *Viscum album* (Yousefi et al., 2021) and *Zingiber officinale* in common carp (Mohammadi et al., 2020) on improving the IgM level have been previously reported. This improvement may be related to the increased expression of head kidney receptors such as the cluster of differentiation (*CD4*) along with increasing lymphocyte frequency after feeding with herbal bioactive compounds (Hølvold, 2007; Torrecillas et al., 2018). Therefore, the presence of valuable biomolecules such as cyanidin, peonidin, petunidin, malvidin, delphinidin, and especially berberine in BF (Yildiz et al., 2014) can be an influential factor in increasing serum immunoglobulins (Wang et al., 2020).

Fish epidermal mucus contains some vital immune elements to trap and slough off a variety of pathogens which each has its own biological functions (Guardiola et al., 2014). So far, a wide range of immune parameters such as lysozyme, esterase, proteases, alkaline phosphatase, and immunoglobulins have been discovered in fish skin mucus (Esteban and Cerezuela, 2015). In the current study, PRT, ALP, lysozyme, and esterase were increased in the skin mucus samples by the addition of BF extract at 750 mg kg^{-1} to Siberian sturgeon diet. In agreement with our results, Van Doan et al. (2020) reported that berberine as an isoquinoline alkaloid in barberry can effectively promote the immune functions in *O. nioticous*. Other studies have shown an improvement in mucosal immune responses in Persian sturgeon, *Acipenser persicus* (Adel et al., 2021b) and beluga sturgeon, *H. huso* (Safari et al., 2020) that were fed with different levels of *Gracilaria persica* powder and chestnut wood extract, respectively. It is generally accepted that phytomedicine activates the mucosal immune responses of fish by affecting the lymph tissues associated with the skin, gills, and intestines (Shekarabi et al., 2021). However, in the case of barberry, more evidence is needed to confirm this probable hypothesis.

One of the prominent benefits of herbal products in the fish diet is increasing the antioxidant capacity of the host and supporting the cellular structure by reactive oxygen species (ROS) scavenging and stimulating the secretion of endogenous antioxidant enzymes such as GPx, SOD, and CAT (Bilen et al., 2020). Several studies documented that using herbal extracts as a supplement in aquafeed can equilibrate the production of ROS and the antioxidant system of fish due to the high content of bioactive compounds (Bilen et al., 2020; Hasanpour et al., 2017; Karataş et al., 2020; Rufchaei et al., 2020; Sahin et al., 2014). The GPx enzyme decomposes peroxide radicals to alcohols and oxygen, while SOD and CAT enzymes play an important role in the detoxification of ROS (Fridovich, 1995; Yin et al., 2014). On the other hand, the

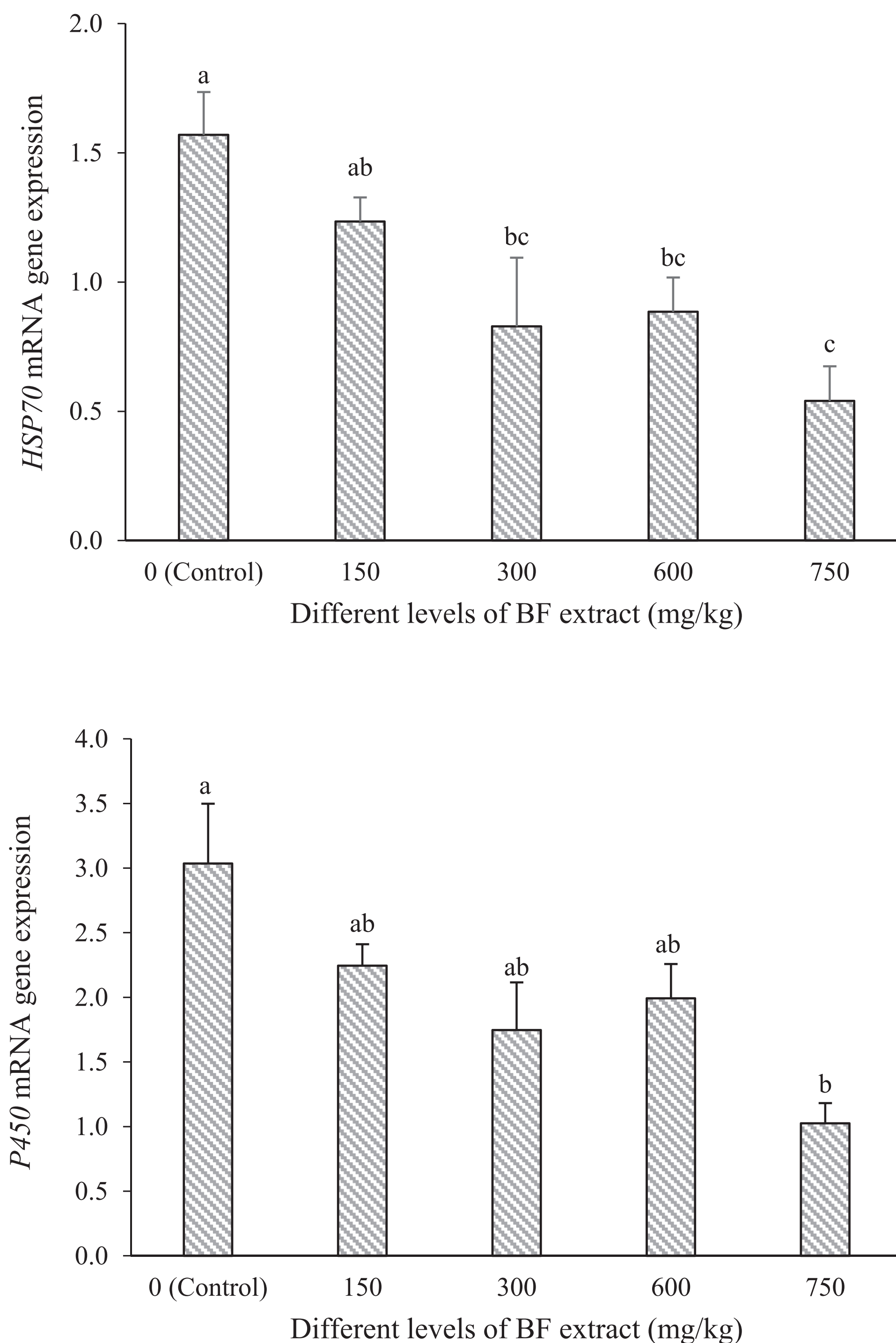

Fig. 3. Relative gene expression levels of heat shock protein (*HSP70*) and cytochrome P450 (*P450*) in the liver of Siberian sturgeon (*Acipenser baerii*) fed with different levels of barberry fruit (BF) extract for 8 weeks. Different letters over the bars show significant differences (n = 3, $P < 0.05$). Error bars indicate the standard error of the mean.

content of MDA in the serum samples as a bio-indicator of lipid damages by ROS was lessened by dietary administration of BF extract compared to the control fish. A higher level of MDA leads to higher cell toxicity as well as accelerates the cells and tissue damages due to the crosslink with the nucleophilic groups of proteins, nucleic acids, and amino phospholipids (Sahin et al., 2014). It can be assumed that the scavenging ability of ROS was enhanced in the fish fed with BF extract due to the high contents of phenolic compounds in BF (Hasanpour et al., 2017). Besides, Wang et al. (2020) found an increase in the activity of SOD in black sea bream, *Acanthopagrus schlegelii*, fed a diet supplemented with 50 mg kg^{-1} berberine. In another similar study, Yang et al. (2019) reported that a diet containing 5 μM berberine can decrease MDA level in the presence of sodium palmitate in grass carp (*Ctenopharyngodon idella*). Therefore, there is a close relationship between a diet high in antioxidants and the host health (Sarraf et al., 2019), and supplementing Siberian sturgeon diet with antioxidant substances can improve general health.

Heat shock proteins are well-known molecular chaperones that synthesize in response to environmental stress factors and infections to protect organisms from cellular damages (Ming et al., 2010; Mu et al.,

2013). Therefore, down-regulating of *HSP70* gene expression by adding BF extract to Siberian sturgeon diet can be indicated that dietary BF extract act as a cell protector against harmful conditions by offsetting the effects of environmental stressors (Su et al., 2010). Similarly, HSP70 gene expression was decreased by dietary administration of immunostimulants in fish previously (Ahmadi et al., 2014; Avella et al., 2010; Rollo et al., 2006). The cytochromes P450 is multigene of enzymes related to the detoxification process and the transformation of many endogenous and xenobiotics substrates (Miandare et al., 2016; Stegeman, 1993). In this study, decreasing in *P450* gene expression might be due to a higher tolerance of treated Siberian sturgeon with dietary BF extract against various potential stressors in fish husbandry conditions. In agreement with our results, Yang et al. (2019) reported that berberine significantly reduced cytochrome C levels in *C. idella*. Moreover, high antioxidant potential, reduce lipid peroxidation, and production of toxic compounds such as MDA were possible reasons for the decreased expression of these two genes in the groups treated with dietary BF extract compared to the control group.

The incorporation of herbal plants and their extracts is known for their antibacterial capacity against pathogenic infection (Dawood et al., 2021; Gaba et al., 2021). The skin mucus is the borderline involved in protecting against infection (Tiralongo et al., 2020). Hence, it is crucial to detect its antibacterial activity to evaluate the immunity activated by medicinal herbs. The results showed marked improved antibacterial capacity of Siberian sturgeon fed dietary BF against *Streptococcus iniae*, *Yersinia ruckeri*, *Escherichia coli*, *Listeria monocytogenes*, *Aeromonas hydrophila*, and *Lactococcus garvieae*. The results are in line with Ramezanzadeh et al. (2020), who indicated that rainbow trout (*Oncorhynchus mykiss*) fed BF had increased antibacterial capacity against *A. hydrophila*. The antibacterial capacity of BF is attributed to its high content of flavonoids and polyphenols that can actively enhance immunity and counteract bacterial infection (Ahmadifar et al., 2021; Jamshaid et al., 2020; Kong et al., 2021).

5. Conclusion

Based on our investigation, the supplementation of Siberian sturgeon diet with 750 mg kg^{-1} barberry fruit extract improved the serum and skin immune parameters as well as antioxidant status. In addition, stress-related gene expression was significantly down-regulated by dietary administration of BF extract in Siberian sturgeon. Obtained results confirmed a hypothesis that BF extract as a feed supplement act as an immune booster and this may be a great interest to those who are involved in the sturgeon aquaculture.

CRediT authorship contribution statement

Seyed Pezhman Hosseini Shekarabi, Mehdi Shamsaie Mehrgan, Farshad Ramezani, Mahmoud A.O. Dawood: Conceptualization. **Seyed Pezhman Hosseini Shekarabi, Mehdi Shamsaie Mehrgan, Farshad Ramezani**: Formal analysis. **Seyed Pezhman Hosseini Shekarabi, Mehdi Shamsaie Mehrgan, Farshad Ramezani, Hien Van Doan, Tossapol Moonmanee, Noor Khalidah Abdul Hamid, Zulhisyam Abdul Kari**: Funding acquisition. **Seyed Pezhman Hosseini Shekarabi, Mehdi Shamsaie Mehrgan, Farshad Ramezani, Mahmoud A.O. Dawood**: Investigation. **Seyed Pezhman Hosseini Shekarabi, Mehdi Shamsaie Mehrgan, Farshad Ramezani**: Project administration. **Seyed Pezhman Hosseini Shekarabi, Mehdi Shamsaie Mehrgan, Farshad Ramezani, Mahmoud A.O. Dawood, Hien Van Doan, Tossapol Moonmanee, Noor Khalidah Abdul Hamid, Zulhisyam Abdul Kari**: Writing – original draft.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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