



EFFECT OF DIETARY MOLDAVIAN BALM (*DRACOCEPHALUM MOLDAVICA* L.) ON GROWTH PERFORMANCE, ANTIOXIDANT STATUS, IMMUNE RESPONSE, AND GENE EXPRESSION OF COMMON CARP (*CYPRINUS CARPIO*)

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Abstract

Chemical compounds used to prevent and control fish disease often cause environmental hazards; thus, alternative approaches as new and effective strategies are needed. The current investigation was performed with the aim of exploring the effects of dietary Moldavian balm (MB, *Dracocephalum moldavica* L.) on the growth, immune parameters, and antioxidant status of common carp (*Cyprinus carpio*). Fish (n=300, w=3.80±0.02 g) in four groups in triplicates were supplemented with 0%, 0.5%, 1%, and 2% MB. After 42 days, it was found that feed supplements increased final weight (FW), weight gain (WG), and specific growth rate (SGR) and decreased the food conversion ratio (FCR) (P<0.05). In addition, fish supplemented with 2% MB significantly showed higher serum total protein (TP), alternative complement pathway (ACH50), and glutathione peroxidase (GPx) (P<0.05). The 0.5% MB-supplemented fish represented higher levels of LYZ, alkaline phosphatase (ALP), total Ig, and ACH50 in their skin mucus in comparison with the unsupplemented fish (P<0.05). The results also indicated that 2% MB resulted in a significantly higher expression level of intestinal tumor necrosis factor (TNF- α) (P<0.05); however, the level of LYZ, interleukin-1 β (IL-1 β), and TLRs decreased in supplemented fish. CAT and SOD expressions were increased in 0.5% MB supplement. In conclusion, MB could be recommended as an efficient feed additive to boost common carp's growth, immunity, and health status.

Key words: fish immunity, herbal immunostimulants, immune-related genes

In recent decades, aquaculture, with an annual growth rate of 8.8%, has dramatically played a critical role in food production (FAO, 2020). Today, global aquaculture vitally produces 15–20% of human animal-consumed protein (Ghafarifarani et al., 2021). Aquaculture has suffered numerous infectious diseases, causing an average of 15% economic losses, and is presently considered the most critical factor affecting the sustainable development of this industry (Faruk et al., 2004; Lebel et al., 2010; Bilen et al., 2019).

Unfortunately, traditional antimicrobials and chemical disinfectants as preventive measures are being applied as the first choice (Van Hai, 2015; Dawood and Koshio, 2016; Song et al., 2016; Hoseini et al., 2018). Approximate global antimicrobial consumption in aquaculture was evaluated by more than 10,000 tons in

2017, and it is predicted to rise by 33% by 2030 (Schar et al., 2020). The indiscriminate use of antibiotics leads to antibiotic-resistant bacteria and transferring of specific genes to other species (Rasul and Majumdar, 2017). Another consequence is the possibility of antibiotic tissue residues in cultured organisms reaching humans through the food chain (Ansari et al., 2014; Griboff et al., 2020). The growing demand for safe food has increased suitable alternatives for antibiotics and chemical compounds (Genc et al., 2007). Several studies have suggested natural alternatives to enhance health and production, including essential oils, alkaloid polysaccharides, and herbal medicines. (Punitha et al., 2008; Vallejos-Vidal et al., 2016; Awad and Awaad, 2017). Phytobiotic agents and their derivatives as biodegradable and safe compounds with fewer adverse effects have been frequently reported

as efficient chemotherapeutics in aquaculture, which promote growth, improve the immune system and increase the survival rate of fish (Reverter et al., 2014).

Moldavian balm (MB) (*Dracocephalum moldavica*) is a perennial medicinal plant belonging to the Lamiaceae family. The branched lateral root system of Moldavian balm makes it adaptable to environmental changes (Dastmalchi et al., 2007 a). Due to the extreme adaptation mentioned before, the Moldavian balm is found in many places; however, it is native to the Mediterranean, Central Asia, Europe, and Iran (Koch-Heitzmann and Schultze, 1988; Schultze et al., 1993; Mrlanova et al., 2001; Moradkhani et al., 2010). The medical use of Moldavian balm dates back to 2000 years ago by Greeks and Romans (Kennedy et al., 2002; Moradkhani et al., 2010). The Moldavian balm contains hydroxycinnamic acids, flavonoids, and quercetin (Dastmalchi et al., 2007 b). Flavonoids are potent antioxidants and work as scavengers of free radicals (Rice-Evans et al., 1996). Several studies have indicated Moldavian balm and its derivatives' antiviral and antibacterial effects (Allahverdiyev et al., 2004; Adinee et al., 2008; Bulfon et al., 2014; Mehrabi et al., 2018). Moldavian balm has also been recommended to cure neurological and functional diseases and cancer (Hohmann et al., 1999; Le Marchand, 2002; Mazzanti et al., 2008; Oniga et al., 2010; Amooaghaie, 2011). In numerous studies, scientists aimed to illustrate the benefits of the medical use of Moldavian balm in aquaculture. It is well documented that the application of the Moldavian balm increases the growth rate (Farahi et al., 2012; Bilen et al., 2019; Abdel-Latif et al., 2020 a); enhances antioxidative responses (Middleton, 1998; Koksai et al., 2011; Farahi et al., 2012; Bilen et al., 2019; Abdel-Latif et al., 2020 b); boosts and stimulates the immune system by increasing WBC levels – including T cells – and enzymes (Middleton and Kandaswami, 1992; Kelley and Bendich, 1996; Meydani and Beharka, 1998; Patora and Klimek, 2002; Koksai et al., 2011; Farahi et al., 2012; Bilen et al., 2019; Abdel-Latif et al., 2020 b).

Unsatisfactorily, available data on Moldavian balm effects on common carp are insufficient. This study, therefore, was designed to assess the impact of dietary Moldavian balm on common carp's growth, antioxidant defense, and immunity.

Material and methods

Moldavian balm analysis

Chemical composition of essential oils

MB was obtained from the local market in Gorgan, Iran. Following this, authentication of the plant was carried out at Gorgan University of Agricultural Sciences and Natural Resources. The dried aerial parts of the plants were hydrodistilled by a Clevenger-type apparatus and stored in the dark at 4°C. The chemical composition analysis of MB essential oils (EOs) was carried out by a

gas chromatograph (Hewlett-Packard, Santa Clara, CA; 6890N) equipped with a mass spectrometer (Hewlett-Packard 5973N).

Antibacterial activity

Seven bacterial species, including four gram-negative (*Escherichia coli* PTCC 1399, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Shigella dysenteriae*) and three gram-positive (*Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus cereus*) were used to study the antimicrobial properties of the EOs. The antibacterial effects were investigated through three methods as follows:

Agar disk diffusion

After inoculation of 0.1 mL suspension of microorganisms, paper disks were filled with each EO's nutritional agar media (Merck, Darmstadt, Germany), and they were then left to incubate. Negative control was impregnated with dimethyl sulfoxide (DMSO), and measurements for growth inhibition diameter were recorded (Moradi et al., 2014).

Agar well diffusion

Bacterial cultures that consisted of broth-like properties (1.5×10^6 cfu/mL) were placed in nutrient-molten agar (Merck, Darmstadt, Germany) and remained to solidify. As for negative control, DMSO was put to use, and the plates were incubated. The growth inhibition diameters were considered antimicrobial activity (Boyanova et al., 2005).

Broth microdilution assay

The EOs were dissolved in 10% DMSO, and the media that contained 10^6 CFU/mL of the microorganisms were added. Each bacterium was allocated three rows of the microplate, with the positive controls containing 125 microliters of growth medium and 15 microliters of the working culture bacteria. The negative controls (two rows of the microplate) contained 15 microliters of saline solution in place of the cultured bacteria. (Turgis et al., 2012). Minimum inhibitory concentration (MIC) is the lowest concentration at which an antimicrobial compound entirely inhibits bacterial growth without exhibiting any outward signs of development. Minimum bactericidal concentration (MBC) refers to the lowest concentration of antimicrobials necessary to eradicate at least 99.9% of the initial inoculum, as determined by subculturing 10 microliters from each well in nutrient agar plates in the absence of any discernible growth for a period of 72 hours at 37°C.

Antioxidant activity

Methanolic 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich Chemical Co. St. Louis, USA) solution (24 µg/mL) was dissolved in 50 µL of the EOs. Subsequently, the absorbance was measured and recorded post-incubation (Pharmacia LKB Novaspec, Sweden). The effectiveness of the EOs at scavenging DPPH radicals

was assessed using the method developed by Ehsani et al. (2016).

Based on the Folin-Ciocalteu method which was mentioned by Aminzare et al. (2017), a total phenols assay was conducted. Briefly, the EOs were mixed with Folin-Ciocalteu's phenol reagent (Merck, Darmstadt, Germany). After adding sodium carbonate solution, the absorbance was measured, and the outcome was given in grams per kilogram of gallic acid equivalents (GAEs). The β -carotene bleaching (BCB) (Sigma-Aldrich Chemical Co. St. Louis, USA) was added to linoleic acid (Sigma-Aldrich Chemical Co. St. Louis, USA) and Tween 40 (Merck, Darmstadt, Germany), all dissolved in chloroform (Merck, Darmstadt, Germany). After evaporation, distilled water was added, and the same process was carried out using the reference antioxidants butylated hydroxyanisole (BHA). Calculations were used to determine how well EOs protected β -carotene from oxidation (Taherkhani et al., 2016).

For ferric reducing antioxidant power, EOs, phosphate buffer, and $K_3Fe(CN)_6$ were mixed together. Trichloroacetic acid was added after incubation, and the mixture was centrifuged. A solution containing the upper layer, distilled water, and 0.1% aqueous $FeCl_3$ was made, and the absorbance was measured (Taherkhani, 2016). The concentration of EOs that provided 50% inhibition was used to calculate the reducing power of the EOs. Various quantities of MB, methanol and 2mM $FeCl_2$ were used to create a solution for the chelating capacity experiment. Following the addition of ferrozine, the mixture's absorbance was measured. It was determined how quickly ferrozine- Fe^{2+} complex formation was inhibited (Guleria et al., 2013).

Preparation of diets and feeding trial

The ingredients of the basal diet are shown in Table 1, which is used as a control. Four experimental diets (in triplicate) with the dose of 0 (control), 0.5, 1, and 2% Moldavian balm (MB) were maintained as the T1, T2, T3, and T4, respectively. The basal ingredients were mixed with 100 mL of water per 1 kg diet until obtaining a smooth dough. Subsequently, pellets were constructed using a commercial grinder and dried overnight at 40°C. All ingredients were sieved through a 2 mm mesh and stored in covered plastic containers at 4°C until use.

Table 1. Analysis of the commercial feed for common carp (Fardaneh Co. Shahrekord, Iran)

Analyses (dry matter)	Composition (%)
Protein	37
Lipid	6
Fiber	6
Digestible phosphorus	1.25
Moisture	7

Fish and culture system

Three hundred fingerlings of common carp who were seemingly healthy were transferred from a local farm in

Isfahan, Iran, to Azad University's Fisheries Research Center in Shahrekord. The fish were acclimated to the experimental conditions for two weeks and were fed twice daily with commercial feed during the acclimatization process. Thereafter, fish (3.80 ± 0.02 g) were placed in 12 glass aquariums (150 L) in a random manner at a rate of 25 fish/tank and fed on experimental diets for 42 days two times a day (3% body weight) (Yousefi et al., 2021). The water parameters like temperature, dissolved oxygen, pH, and hardness were maintained at $25.2 \pm 1.1^\circ\text{C}$, 6.6–6.9 mg L^{-1} , 6.9–7.1, and 182.43 ± 9.25 . They were kept in a natural photoperiod (12L:12D). Continuous aeration was provided, and aquariums were siphoned on a daily basis with 50% dechlorinated freshwater.

Sampling procedure

Sampling was carried out after 42 days. To analyze blood parameters, three randomly chosen specimens were sedated with 100 mg L^{-1} eugenol anesthesia. Using a 2 mL syringe, the required amount of blood was extracted from the caudal vein. Following centrifugation (1600 g for 10 min, Heraeus Labofuge 400), the sample went under serum extraction and was controlled at -20°C until further analysis.

Using Ross et al.'s (2000) indirect method, skin mucus samples were extracted. Six anesthetized (100 mg L^{-1} eugenol) fish were individually transferred to a zip-keep bag containing NaCl (50 mM; 5 mg L^{-1} fish, Merck, Germany) for nearly one minute. Then mildly rubbed and moved back to their tanks. Collected mucus was centrifuged at 1600 g for 10 min at 4°C and kept at -80°C .

Three fish per tank were humanely killed by a sharp blow to the skull after deep anesthesia to take intestinal samples. The fish intestine, after evisceration, was washed with sterile physiological cold saline solution 0.9% (10 volumes (w/v)), then homogenized. After centrifuge for 20 minutes at 4°C , 6000 g, the supernatants were collected and kept at -80°C for further experiments.

Growth performance

To evaluate the effect of different diets on the fish performance, the fish were individually weighed by a digital scale (Sartorius TE1502S digital gram scale) with an accuracy of 0.1 g. The growth parameters, including weight gain, specific growth rate, feed conversion ratio, and survival rate were calculated using the below equations:

$$\begin{aligned} \text{Weight gain (WG) (g)} &= \text{initial weight} - \text{final weight} \\ \text{Specific growth rate (SGR) (\% d}^{-1}\text{)} &= \{(\ln \text{ final weight (g)} - \ln \text{ initial weight (g)}) / \text{days}\} \times 100 \\ \text{Feed conversion rate (FCR)} &= \text{total feed given (g)} / \text{weight gain (g)} \\ \text{Survival rate (SR) (\%)} &= (\text{final numbers} / \text{initial numbers}) \times 100 \end{aligned}$$

To calculate the survival rate in different groups, the number of mortality in tanks was recorded.

Table 2. The sequences of the primers used for the real-time PCR

Primer name	Sequence (5'-3')	Product (bp)	Accession number
TLRs-I-F	TCAAATCTCAATTTCTTCCCAAGCT	179	LC150761.1
TLRs-R	TAGTGGCACCATTTCGCTCTG		
TNF α -F	GAACAATCAGGAAGGCGGAAA	128	JX181982
TNF α -R	GGGTTTCTGTGGACACTTCAG		
IL1 β -F	CATTGCTTGTACCCAGTCTGG	121	AJ401031
IL1 β -R	TCTGAAGAAGAGGAGGCTGTC		
Lyz-F	CAGGTGGAAAGAACAAGTGCA	150	AB027305
Lyz-R	ACATCTTACGCCCTTACAGT		
Catalase-F	TCCTGTGGGACGCCTTGTGTTG	115	GQ376154.1
Catalase-R	CTTGTCTGGGCCGGGCTCAATG		
SOD-F	AGGTCCGCACTTCAACCCT	179	JF342355.1
SOD-R	GGATCACCATGGTCCTCCC		
Beta actin-F	TCTGCTATGTGGCTCTTGACT	118	XM_042721308
Beta actin-R	AACCTCTCATTGCCAATGGTG		

Immunological parameters

Serum and mucus immunological components were determined by routine methods. Lysozyme (LYZ) activity was assayed following the methods described by Demers and Bayne (1997). In summary, a suspension of *Micrococcus luteus* at 0.2 mg/ml was made using sodium phosphate buffer (0.05 M, pH 6.2). After mixing 60 μ L of the sample with 2 mL of the bacterial solution and incubating for 3 minutes, the absorbance was measured. A unit of LYZ caused a decrease in absorbance 0.001 min^{-1} at 450 nm. Alternative complement pathway (ACH50), in samples of serum and mucus, was assayed consistent with Ortuno et al. (2000) studies using sheep red blood cells (SRBC). Total immunoglobulin (total Ig) was assessed with a polyethylene glycol solution (12.5%, Sigma) by measuring protein levels before and after sedimentation (Siwicki and Anderson, 1993). By utilizing a commercial kit (Pars Azmun, Co., Tehran, Iran), the total protein (TP) content was determined at 540 nm and the optical density (OD) was recorded at 540 nm (Bradford et al., 1976). Commercial kits from Xu et al. (2019 a) and their study (Pars Azmun, Co., Tehran, Iran), were used to measure the mucus alkaline phosphatase (ALP) activity.

Antioxidant status

The function of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) activity were detected by commercial equipment (Kiazist, Hamadan, Iran). CAT was utilized to decrease hydrogen peroxide to H_2O with a commercial kit following manufacturer's instructions (Kiazist, Iran). CAT was determined by decreasing H_2O_2 into a molecule of oxygen and water (Boveris and Chance, 1973). SOD activity was assayed using xanthine and xanthine oxidase to produce superoxide radicals. GPx activity was assayed by the NADPH

consumption with an ELISA reader (Flohé and Günzler, 1984).

Gene expression

Relative mRNA expression of immune-antioxidant genes (*TLRs*, *TNF- α* , *IL-1 β* , *LYZ*, *CAT*, and *SOD*) was analyzed by using quantitative real-time PCR. The primers for target genes were designed in Geneious IR 9.1.8 based on the mRNA sequences reported for common carp (Table 2). Total RNA was extracted using RNX-Plus (Sinaclon, Iran) as described by the manufacturer's instructions. The RNA quality and quantity were confirmed with 1% agarose gel electrophoresis and nanodrop spectrophotometry (Thermofisher 2000c, USA), respectively. 1 μ g of extracted RNA was used for cDNA synthesis by using 1 μ L reverse transcriptase (Sinaclon, Iran), 4 μ L RT buffer, 1 μ L oligodT primer, 2 μ L dNTPs and 1 μ L RNase inhibitor. Real-time PCR reactions with 10 μ L final volume contained 5 μ L 2X SYBR Green PCR Master Mix (Ampliqon, Denmark), 0.2 μ M from each primer, and 1 μ L cDNA. Thermal cycling was initial holding at 95°C for 5 minutes, 40 cycles at 94°C for 15 seconds, 58°C for 40 seconds, and 72°C for 30 seconds, followed by melt curve analysis. Three replicates for each sample were performed, and all reactions were normalized relative to control samples and the β -actin gene as an endogenous control. The change in relative mRNA fold was calculated according to $2^{-\Delta\Delta\text{Ct}}$ formula (Livak and Schmittgen, 2008).

Statistical analysis

The normality test was checked using the Kolmogorov-Smirnov test, the data were subjected to the analysis of variance (one-way ANOVA), and Duncan's post-hoc test was performed at a 95% confidence level. SPSS 20 software was employed for statistical analysis and data is expressed as means $\pm$ standard error (SE).

Table 3. Chemical composition of Moldavian balm essential oils using GC/MS analysis

Number	Component	Retention Time (Min)	Total (%)
1	6-Methyl-5-Hepten-2-one	12.24	1.31
2	(p) Cymene	14.36	0.12
3	Linalool oxide	15.12	0.23
4	(trans) Linalool oxide	17.31	0.51
4	Linalool	18.14	0.72
5	Nerol oxide	20.13	0.60
6	Nerol	22.16	0.20
7	(cis) Carveol	24.13	0.28
8	Neral	25.20	0.17
9	Geraniol	25.83	31.64
10	Geranial	26.10	27.73
11	(dihydro) Linalol acetate	27.25	1.13
12	Methyl geranate	28.30	0.26
13	Neryl acetate	29.40	0.80
14	Geranyl acetate	30.82	24.63
15	trans-Caryophyllene	31.28	0.40
16	(neo) Methyl lactate	32.60	0.65
17	Germacrene-D	33.50	0.15
18	Spathulenol	37.80	1.13
19	Caryophyllene oxide	38.45	0.83
20	Carotol	40.12	0.10
21	Cedrol	40.80	0.29
22	(1,10-Di-epi) Cubenol	41.62	0.30
23	(10-epi- γ) Eudesmol	42.71	0.23
24	(α) Acorenol	43.44	0.87
25	(β) Acorenol	44.42	0.71
26	Cedroxyde	46.10	1.70
27	Cryptomeridiol	49.14	0.12
28	Phytol	52.13	0.86
29	Eicosene	56.13	0.27
Total Number			98.94%

Table 4. Antibacterial effect of Moldavian balm by agar disk diffusion method (mean $\pm$ SD)

Diameter of inhibition zone (mm)	
<i>E. coli</i>	12.12 $\pm$ 0.10 Aa
<i>S. typhimurium</i>	8.32 $\pm$ 0.12 Ab
<i>P. aeruginosa</i>	9.23 $\pm$ 0.20 Ab
<i>S. dysenteriae</i>	14.76 $\pm$ 0.10 Ac
<i>S. aureus</i>	13.93 $\pm$ 0.15 Ac
<i>L. monocytogenes</i>	11.70 $\pm$ 0.20 Aa
<i>B. cereus</i>	14.60 $\pm$ 0.15 Ac

Different letters (a–b) in the same column indicate significant differences among the treatments ($P < 0.05$).

Results

Moldavian balm analysis

A GC/MS analysis was performed to identify the essential bioactive compounds, which resulted in the

quantification of twenty-nine components. The compounds are listed in Table 3 in order of their retention times. Geraniol (31.64%), geranial (27.73%), and geranyl acetate (24.63%) were the most abundant components.

Table 5. Antibacterial effect of Moldavian balm by agar well diffusion method (mean $\pm$ SD)

Diameter of inhibition zone (mm)	
<i>E. coli</i>	7.23 $\pm$ 0.10 Aa
<i>S. typhimurium</i>	6.94 $\pm$ 0.15 Aa
<i>P. aeruginosa</i>	8.57 $\pm$ 0.10 Ab
<i>S. dysenteriae</i>	9.59 $\pm$ 0.15 Ac
<i>S. aureus</i>	9.78 $\pm$ 0.05 Ac
<i>L. monocytogenes</i>	8.36 $\pm$ 0.10 Ab
<i>B. cereus</i>	10.48 $\pm$ 0.15 Ad

Different letters (a–b) in the same column indicate significant differences among the treatments ($P < 0.05$).

Table 6. Antibacterial effect of Moldavian balm by micro-dilution method

	MIC	MBC
<i>E. coli</i>	5000	5000
<i>S. typhimurium</i>	2500	5000
<i>P. aeruginosa</i>	5000	5000
<i>S. dysenteriae</i>	2500	5000
<i>S. aureus</i>	2500	2500
<i>L. monocytogenes</i>	5000	5000
<i>B. cereus</i>	2500	5000

MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration.

The antibacterial potency of MB EOs on the selected bacterial species was examined using the agar disk diffusion method as presented in Table 4. According to the results, all bacteria tested showed some degree of sensitivity to the essential oils. *S. dysenteriae* and *B. cereus* were sensitive to all EOs tested based on the growth inhibition zone.

Table 5 represents the antibacterial effects of EOs obtained from the agar well diffusion method. The highest antibacterial activity of MB EOs was observed in the gram-positive species *S. dysenteriae*, *S. aureus*, and *B. cereus*. In addition, the MICs and MBCs of the tested bacteria are presented in Table 6. In most cases, the MIC values were lower than MBC.

The antioxidant activity of the MB EOs was expressed in Table 7. The presented data shows no significant difference in the antioxidant values of MB.

Growth performance

The differences in growth rate and survival of the studied fish are shown in Table 8. While the fish's initial weight showed no statistical difference, the final weight and weight gain experienced an increase in the MB treatments ($P < 0.05$); however, T3 exhibited the most FW and WG levels among all the treatments significantly ($P < 0.05$). According to the findings, T3 and T4 treatments showed significantly augmented SGR compared to those that did not receive MB ($P < 0.05$). Also, T3 and T4 fish had lower FCR values compared to T1 and T2 ($P < 0.05$); however, no significant differences were found between T1 and T2 ($P > 0.05$). Also, there were no appreciable differences in the SR of experimental treatments compared to the control group ($P > 0.05$).

Fish immunity

Serum immune parameters of common carp supplemented with dietary Moldavian balm are shown in Table 9. LYZ was unaffected by plant supplementation, representing no statistical change in groups ($P > 0.05$). ACH50 and serum TP were found to be highest in T4 ($P < 0.05$); however, no significant difference was observed in other treatments in comparison to the control ($P > 0.05$). The highest value of serum total Ig level was measured in T2 ($P < 0.05$), with no meaningful difference between other treatments ($P > 0.05$).

Antioxidant parameters

The antioxidant status of the fish that received dietary Moldavian balm is presented in Table 10. The CAT and SOD levels of the MB-supplemented carps did not represent any statistical difference compared to those that received only basal diet ($P > 0.05$). GPx was found to be highest in T4 fish ($P < 0.05$); however, the differences between GPx in other groups were not statistically significant ($P > 0.05$).

Table 7. Antioxidant potential of Moldavian balm using different antioxidant assays

	2,2-diphenyl-1-picrylhydrazyl Ic ₅₀ * (mg/ml)	Total phenolic contents	β -carotene/ linoleic acid bleaching assay (%)	Reducing power Ic ₅₀ (mg/ml)	Chelating power at 0.5 (mg/ml)
Moldavian balm	5.52 $\pm$ 0.20 a	156 $\pm$ 4.21 a	79.24 a	10.34 $\pm$ 0.40 a	66.13 $\pm$ 1.20 a
Butylated hydroxyanisole	0.76 $\pm$ 0.005 c	**	82.33 c	2.14 $\pm$ 0.005 c	**
Quercetin	**	**	**	**	72.13 $\pm$ 1.10 c

* IC₅₀, defined as the concentration of the test material required to cause a 50% decrease in initial DPPH concentration.

** Not examined.

Different letters (a–b) in the same row indicate significant differences among the treatments ($P < 0.05$).

Table 8. Growth performance of common carp fed different levels of dietary Moldavian balm

Parameters	T1	T2	T3	T4
IW (g)	3.76±0.04	3.79±0.03	3.83±0.03	3.84±0.03
FW (g)	8.23±0.05 d	8.46±0.03 c	9.76±0.11 a	8.70±0.03 b
WG (g)	4.47±0.01 d	4.66±0.03 c	5.92±0.08 a	4.86±0.01 b
SGR (% d ⁻¹)	1.86±0.01 c	1.93±0.02 bc	2.00±0.02 a	1.94±0.02 b
FCR	1.43±0.007 a	1.43±0.022 a	1.08±0.016 c	1.30±0.023 b
SR (%)	95.55±2.22	100.0±0.00	97.77±2.22	95.55±2.22

Data are expressed as the mean ± SE (n= 3). Different letters (a–d) in the same row indicate significant differences among the treatments (P<0.05).

IW: initial weight; FW: final weight; WG: weight gain; SGR: specific growth rate; FCR: feed conversion ratio; SR: survival rate. T1 (0), T2 (0.5%), T3 (1%) and T4 (2%).

Table 9. Serum immunological indices of common carp fed different levels of dietary Moldavian balm

Parameters	T1	T2	T3	T4
LYZ (U/L)	32.66±1.76	31.00±0.57	24.33±3.28	30.00±3.60
ACH50 (U/ml)	20.56±0.82 b	22.06±0.53 b	20.16±2.46 b	28.30±0.40 a
Total Ig (g/L)	13.53±0.99 b	21.60±3.59 a	17.13±0.46 ab	19.30±0.81 ab
TP (g/L)	36.53±3.27 b	35.00±2.80 b	31.60±1.96 b	48.30±1.13 a

Data are expressed as the mean ± SE (n= 9). Different letters (a–b) in the same row indicate significant differences among the treatments (P<0.05).

LYZ: Lysozyme; ACH50: Alternative complement pathway; Total Ig: Total immunoglobulin; TP: Total protein. T1 (0), T2 (0.5%), T3 (1%) and T4 (2%).

Table 10. Serum antioxidant status of common carp fed different levels of dietary Moldavian balm

Parameters	T1	T2	T3	T4
CAT (mu/ml)	87.18±3.59 ab	80.11±2.57 b	85.91±3.15 ab	92.05±2.64 a
SOD (IU/ml)	72.10±1.73	64.81±4.25	69.99±3.27	68.91±0.60
GPx (mu/ml)	51.05±0.61 b	43.03±2.50 b	47.49±2.71 b	72.82±3.25 a

Data are expressed as the mean ± SE (n= 9). Different letters (a–b) in the same row indicate significant differences among the treatments (P<0.05).

CAT: Catalase; SOD: Superoxide dismutase; GPx: Glutathione peroxidase; T1 (0), T2 (0.5%), T3 (1%) and T4 (2%).

As shown in Figure 1, a significant elevation in mucus ACH50 levels was observed in T2, in comparison to other fish (P<0.05). In addition, ALP was augmented in T2 and T3 compared to T1; whereas no significant differences were found between T4 and T1 (P>0.05). Total Ig content in T2 fish was significantly higher than that of the control (P<0.05). However, no significant differences were found between other fish supplemented with MB and the control (P>0.05). Furthermore, MB was found not to significantly affect mucus LYZ and TP activity (P>0.05).

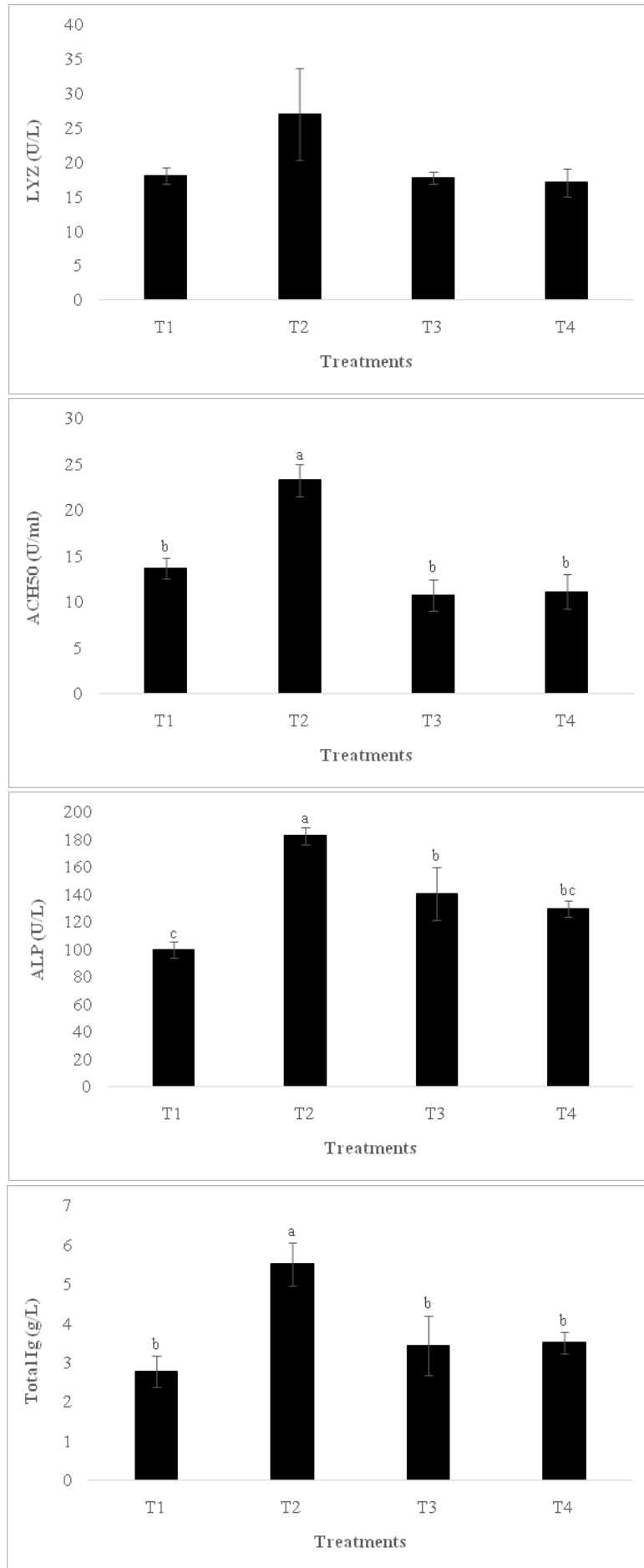
Gene expression

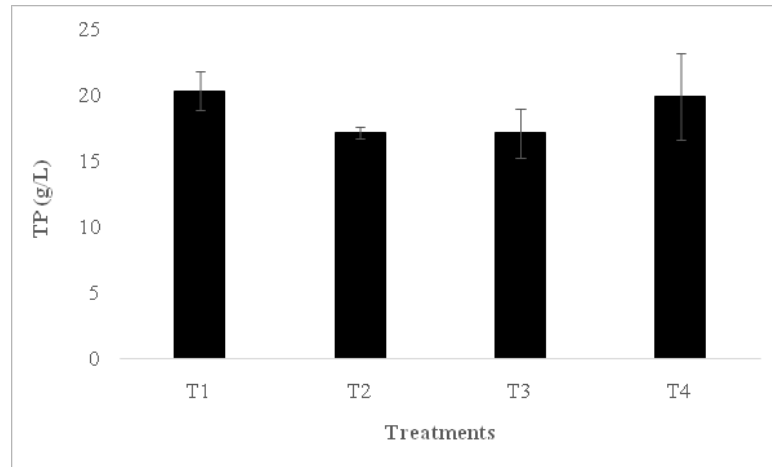
The degrees of expression of the studied genes relating to immune and antioxidant activities are presented in Figure 2. According to the findings, a significant decrease was observed in *IL-1β*, *LYZ* and *TLRs* expression in experimental groups concerning the control group. However, supplementation with 2% Moldavian balm re-

sulted in an elevation in *TNF-α* expression compared to the control (P<0.05). CAT and SOD genes experienced a significant reduction in fish that were fed high concentrations of Moldavian balm (T3 and T4) (P<0.05), while these parameters witnessed a statistical elevation in those that received T2 diet.

Discussion

Several reports indicate the positive role of medicinal herbs in fish aquaculture (Kong et al., 2021; Yousefi et al., 2021). Herbs, due to their various active ingredients, are proven to efficiently enhance health status by promoting innate immunity mechanisms. Therefore, in this study, the effects of Moldavian balm (MB) on growth performance, antioxidant status, serum and mucus immune parameters, and immune gene expression of common carp were assessed.

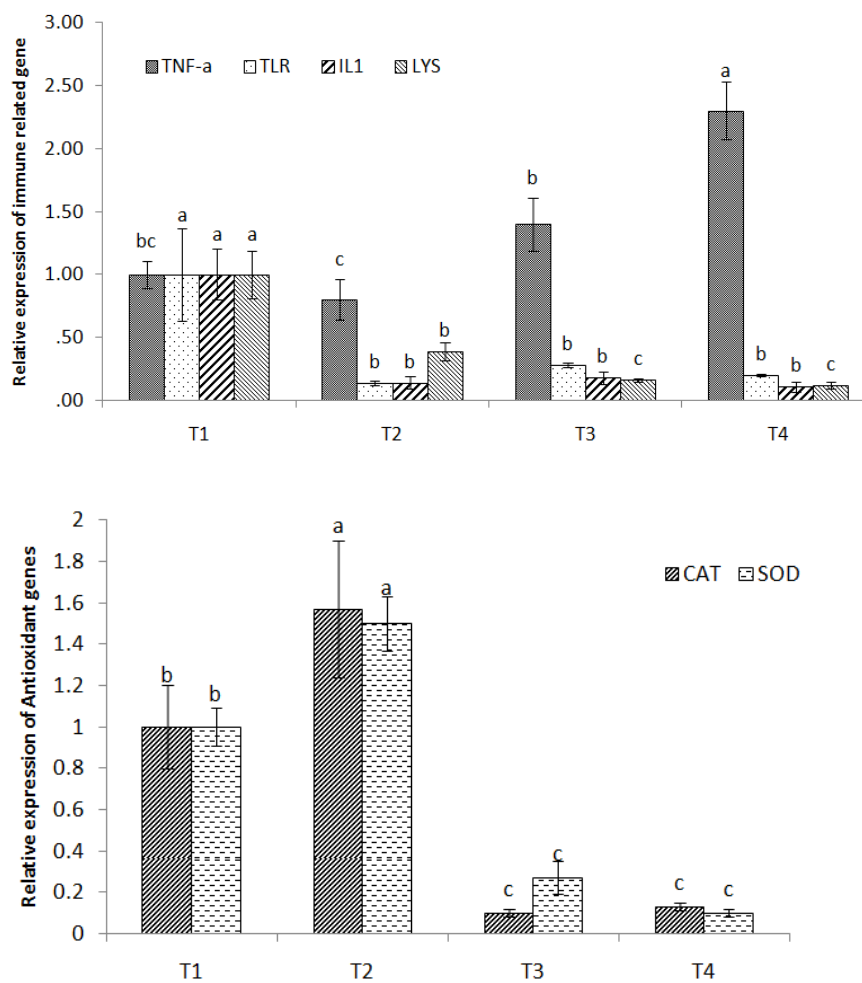




ALP: Alkaline phosphatase; Total Ig: Total immunoglobulin; LYZ: Lysozyme; ACH50: Alternative complement pathway; TP: Total protein; T1 (0), T2 (0.5%), T3 (1%) and T4 (2%).

Values are presented as the mean $\pm$ SE (n=6). Different letters (a-c) in each row indicate statistically significant differences ($P < 0.05$).

Figure 1. Skin mucus parameters of common carp different levels of dietary Moldavian balm



Values are presented as the mean $\pm$ SE. Different letters (a-b) in each row indicate significant differences ($P < 0.05$).

TNF- α (Tumor Necrosis Factor- α); TLRs: Toll-like receptors; IL-1 β (Interleukin 1); LYZ: Lysozyme; CAT: Catalase; SOD: Superoxide dismutase; T1 (0), T2 (0.5%), T3 (1%) and T4 (2%).

Figure 2. The relative expression of immune and antioxidant-related genes in the intestine of common carp fed four experimental diets

According to our findings, dietary MB improved various growth indices (WG, FCR, and SGR) of common carp. According to the literature review, there was no previous study evaluating to what extent MB affects fish growth. However, there are frequent similar experiences implementing other herbal immunostimulant diets, such as supplementation of diet with thyme in rainbow trout (*Oncorhynchus mykiss*) (Ahmadifar et al., 2011; Sönmez et al., 2015), combined herbs extracts (oak acorn, coriander, and common mallow) in common carp (Raissy et al., 2022 a), savory (*Satureja hortensis*) essential oil in Caspian roach (*Rutilus caspicus*) (Ghafarifarani et al., 2022), coriander seed extract (*Coriandrum sativum*) in rainbow trout (Farsani et al., 2019). Bilen et al. (2020) reported that the inclusion of lemon balm (*Melissa officinalis*) in rainbow trout's feed resulted in higher weight gain and SGR. The enhanced growth performance of MB may be related to the stimulatory effect of the plant on digestive enzymes. It is also proven that phytotherapy improves the microbial community of the host gastrointestinal tract toward fermenting bacteria (Xu et al., 2019 b). Moreover, it has frequently been emphasized that phytochemicals and their acquired compounds prevent the development of undesirable bacteria (Khalil et al. 2020), help to gain more nutrients in digestion and assimilation (Ahmadifar et al., 2011), as well as stimulate appetite. Undoubtedly, more research is necessary to explain the mechanisms through which these compounds act. It should be mentioned that MB did not significantly affect SR of carp.

Immunostimulants can increase non-specific immunity and protect fish from infectious diseases by promoting immune factors. Evaluation of routine immunological parameters sheds light on the advantages of herbal compounds as promising food additives in the fish farming industry. LYZ is a bactericidal enzyme produced by leukocytes and is known as a factor, which hydrolyzes gram-positive bacteria (Saurabh and Sahoo, 2008). Results indicated that dietary administration of MB resulted in no statistical changes in LYZ levels. In contrast to our study, significant improvement of LYZ was observed in fish that were fed plant supplements: coriander seed extract (*Coriandrum sativum*) in rainbow trout (Farsani et al., 2019), ginger (*Zingiber officinale*) extract in common carp (Mohammadi, 2020 a), and lemon balm in rainbow trout (Bilen et al., 2020). Meanwhile, LYZ displayed no remarkable variation in common carp treated with dietary herbal supplements (*Ferula assafoetida*) (Safari et al., 2016). These differences are probably due to the different sources of herbal supplementation, their medical form, dosage, fish species, and study methods.

ACH50, synthesized by the fish liver, is known for having bactericidal, inflammatory, and opsonization activities (Holland and Lambris, 2002; Rajabiesterabadi et al., 2020). In this study, the high inclusion of dietary MB supplementation improved ACH50 level, which can be attributed to the stimulatory effect of 2% MB on the liver (Zhang et al., 2013; Farsani et al., 2019). Similarly, Mo-

hammad et al. (2020 b) reported that lemon balm administration in Nile tilapia (*Oreochromis niloticus*) improved TP, total Ig, and ACH50 activities. Besides, Yousefi et al. (2019) reported a significant difference in ACH50 levels in common carp that received rosemary leaf powder compared to the control.

Immunoglobulins are glycoproteins that opsonize invading bacteria by phagocyte cells and are produced by plasmablasts and plasma cells (Esteban, 2012; Devi et al., 2019). In the present study, dietary 0.5% MB increased the total Ig level. The increase in total Ig could be attributed to B lymphocytes and the total protein level rise (Ercal et al., 2000). In line with the obtained results, total Ig activity was regulated in fish that received dietary plant supplements, including some ethanol extracts on striped catfish (*Pangasianodon hypophthalmus*) (Nhu et al., 2019), lavender (*Lavandula angustifolia*) (Yousefi et al., 2020), and a mixture of plant extracts (*Coriandrum sativum*, *Malva sylvestris*, *Quercus brantii*) on common carp (Raissy et al., 2022 a).

Total protein, including albumin and globulin, is a suitable indicator of injury or infection adaptation response (Dorojan et al., 2015). The increase in TP level may result from a post-injury or infection adaptation response (Dawood et al., 2016). In this work, TP exhibited an increase in T4 supplemented with 2% MB. A similar finding was also obtained in dietary herbal supplementation of *Thymus vulgaris* and *Foeniculum vulgare*, which increased TP and albumin activities of rainbow trout (Gulec et al., 2013). Also, TP, albumin, and globulin levels showed a significant increase in common carp treated with combined herbs extracts (Raissy et al., 2022 a). In another study, lemon balm aqueous extracts increased the serum TP level of rainbow trout (Jafarpour and Fard, 2016).

The fish's antioxidant system plays a vital role in supporting living cells against free radicals, hyperinflammatory reactions, and apoptosis (programmed cell death) (Eid et al., 2017). The effects of MB on fish antioxidant status have not been studied yet. However, the enhancing effects of herbal immunostimulants on antioxidant defense have been focused on in various publications. For example, the administration of sinol (Hoseini et al., 2018), curcumin (Yonar et al., 2019), ginger extract (Zargar et al., 2020), and thyme (*Zataria multiflora*) (Mirghaed et al., 2020) boost the antioxidant status of fish. CAT, SOD, and GPx are the primary antioxidant defense enzymes that act as the system's first line of defense. These enzymes convert superoxide into molecules of hydrogen peroxide and water and play a vital role in maintaining cellular integrity in response to oxidative stress (Andriamialinirina et al., 2020; Yousefi et al., 2021; Ghafarifarani et al., 2022). GPx is responsible for hydrogen peroxide and lipid hydroperoxide decomposition (Hoseini and Yousefi, 2019). In the present findings, the experimental diet had no effects on SOD and CAT activities. In contrast to our result, ginger extract (*Zingiber officinale*) in common carp (Mohammadi et al., 2020 a)

and savory essential oil in Caspian roach (Ghafariarsani et al., 2022) elevated SOD and CAT levels. Moreover, the present study indicated that fish that received diets supplemented with 2% MB had a higher GPx level, which may be due to flavonoids and other components of MB that have antioxidant and anti-inflammatory activities. Flavonoids have been proven to improve intracellular glutathione levels by enhancing their expression (Khalili et al., 2020). The boosting effect of medicinal plants on GPx level has been reported in common carp (Raissy et al., 2022 a), Indian major carp (*Labeo rohita*) (Harikrishnan et al., 2020), and rainbow trout (Sönmez et al., 2015).

Fish skin mucus acts as a strong barrier between fish and the surrounding environment and is considered the first line of defense against pathogens (Ángeles Esteban, 2012; Esteban and Cerezuela, 2015; Dash et al., 2018). Total Ig, LYZ, ACH50, and ALP are among the main innate immunity parameters in the skin mucosa, with a critical role in mucus bactericidal activity. Skin mucus LYZ is involved in the protective host defense against bacterial infection (Modanloo et al., 2017). Besides, mucus ALP has a significant role in promoting non-specific innate immunological defense due to its hydrolytic function (Adel et al., 2016). Mucus total Ig regulates mucosal homeostasis (Esteban et al., 2012). Skin mucus immune parameters are affected by physiological and nutritional status (Azimirad et al., 2016). In the current study, the level of the above-mentioned parameters, except for TP, was highest in T2, compared to other treatments. Flavonoids in MB diet are known for their antioxidant and anti-inflammatory properties, decreasing oxidative stress reactions (Shin et al., 2010; Yousefi et al., 2023). Quercetin which exists in MB, also modulates the activity of enzymes by stimulating the receptors to which estrogen attaches and also protecting tissues from inflammatory cells (Pawlikowska-Pawłęga et al., 2007; Xu et al., 2019 a). Although the literature review signified no previous report describing the effects of MB supplements on fish antioxidant status, our results are along the same line as those studies aimed to investigate the effects of other herbal materials on mucus immunity in fish (Hoseinifar et al., 2019; Srirachaiyo et al., 2020; Hoseinifar et al., 2020; Ghafariarsani et al., 2021). In a study by Safari et al. (2016), *Ferula* resulted in an increase in skin mucus LYZ in common carp; however, mucus total Ig and protease activity revealed no significant difference. Furthermore, Yousefi et al. (2021) reported that common carp treated with a marjoram extract (*Origanum majorana*) supplementation diet could significantly improve Ig, ACH50, ALP, and LYZ levels. Similarly, the inclusion of 0.5% lemon balm extract in the Nile tilapia diet elevates mucus ACH50, LYZ, and protease activities (Mohammadi et al., 2020 b).

Medicinal herbs up-regulate pro-inflammatory cytokines secreted by immune cells (Safari et al., 2019). Interleukins (*IL*) and tumor necrosis factors (*TNF*) are classified as pro-inflammatory cytokines with undeniable

effects on the immune system (Zou and Secombes, 2016). *TNF-α* induces phagocytosis and triggers macrophages and lymphocytes to secrete other cytokines in stressful conditions (Hoseinifar et al., 2019). *IL-1β* has an undeniable role in the production of monocyte, leukocyte, lysozyme, and tissue macrophages (Moustafa et al., 2020). According to the results, the expression of *TNF-α* increased in T4, while *IL-1β* and *TLRs* expression experienced a significant decline in the fish that were fed with Moldavian balm. Similarly, Zemheri-Navruz et al. (2019) reported that *TNF-α* in common carp spleen was elevated when they were supplemented with olive leaf extract. The *IL-1β* expression reduction after MB feeding showed the ability of this immunostimulant to reduce the inflammation of fish. In a similar study, Hoseinifar et al. (2015) revealed that *IL-1β* decreased in common carp treated with date palm fruit extracts (Hoseinifar et al., 2015). Giri et al. (2019) also proved the down-regulation of *IL-1β* in common carp that were fed with turmeric-supplemented food. The results also represented that the expression of *LYZ* experienced a decrease in the supplemented fish compared to the control. A similar finding has been reported by Hoseinifar et al. (2017), indicating that the fish fed with 1% loquat (*Eriobotrya japonica*) leaf extract showed a reduction in *LYZ* gene expression. Similarly, Zargar et al. (2019) reported a significant decrease in the expression of the *LYZ* gene when the fish received *Thymus vulgaris* oils at the dosage of 2 ml. The expression of antioxidant-related genes, *SOD* and *CAT*, were significantly increased in T2 fish while showing a decrease in T3 and T4 compared to the control. A similar trend of the elevation of antioxidant-related gene expression in fish receiving medicinal plants has been reported in several studies (Salomón et al., 2020; Silva-Jara et al., 2020; Wang et al., 2020). Sometimes increased amounts of plants' active ingredients may affect liver's function which results in changes in serum enzyme levels (Raissy et al., 2022 b).

Conclusion

According to the results of the present study, using MB herbal powder enhances fish immunity and growth. The study of different levels of MB showed that 1% MB elevated fish growth performance, 2% MB had the highest ability to enhance the serum immune parameters and antioxidant status, and the use of 0.5% MB increased mucosal immune parameters. Therefore, MB could be suggested as a feed supplement for the fish farming industry; however, further research is recommended.

Conflicts of interest/competing interests

There is no conflict of interest to declare.

Animal care

All experiments were performed following the protocol approved by the Committee of Ethics of the Faculty of Sciences, University of Tehran (357; 8 November 2000).

Compliance with ethical standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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