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Effects of dietary methanolic extract of hyssop, *Hyssopus officinalis*, on growth performance, hepatic antioxidant, humoral and intestinal immunity, and intestinal bacteria of rainbow trout, *Oncorhynchus mykiss*

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The aims of the present study were to assess the effects of Hyssop, *Hyssopus officinalis*, methanolic extract (HE) on growth performance, hepatic oxidative status, humoral and intestinal immunity, and intestinal bacteria of rainbow trout, *Oncorhynchus mykiss*. Fish were allocated into twelve tanks for four treatments, receiving diets containing 0, 100, 250, and 500 mg/kg HE for eight weeks. The results showed that dietary HE supplementation induced no significant differences in the growth performance, feed efficiency, and hematological parameters ($P > 0.05$). HE supplementation significantly increased total leukocyte count and the highest count was observed in 250 mg/kg HE treatment ($P < 0.001$). Fish in 250 and 500 mg/kg HE treatments exhibited significantly lower lymphocyte ($P = 0.001$) and higher neutrophil ($P = 0.002$) percentages; the former exhibited a significantly higher monocyte percentage ($P = 0.021$). Hepatic superoxide dismutase (100 and 250 mg/kg HE; $P < 0.001$), glutathione peroxidase (100 and 250 mg/kg HE; $P = 0.001$), glutathione reducatse (all HE treatments; $P < 0.001$), and reduced glutathione (250 mg/kg HE; $P = 0.046$) significantly increased, whereas hepatic malondialdehyde levels (250 and 500 mg/kg HE; $P = 0.007$) significantly decreased in HE-treated fish. Plasma total protein, albumin, globulin, lysozyme, and alternative complement significantly increased in 250 and 500 mg/kg HE treatments and plasma total Ig significantly increased in 250 mg/kg HE treatment. Quantitative real time PCR found no *Streptococcus iniae*,

Lactococcus garvieae, *Aeromonas hydrophila*, *Yersinia ruckeri*, and *Vibrio anguillarum* in the fish intestines in any treatments. *Lactobacillus* sp. was detected in the fish intestinal samples, but there were no significant differences among the treatments (P = 0.352). Intestinal defensin (P = 0.044) and interleukin-1 beta (P = 0.0.035) expressions were significantly up-regulated in 100 mg/kg HE; intestinal interleukin-10 (P < 0.001) and tumor necrosis factor-alpha (P < 0.001) expressions were significantly up-regulated in 100 and 500 mg/kg HE; whereas, intestinal interleukin-6 expression was significantly (P = 0.009) up-regulated in 250 mg/kg HE treatments. It is concluded that HE is able to stimulate humoral and intestinal immune responses and hepatic antioxidant capacity. HE effective concentration in rainbow trout may be in the range of 100-250 mg/kg.

KEYWORDS

herbal additives, fish nutrition, intestinal health, intestinal genes, hepatic antioxidant capacity

Introduction

The rapid growth in global demand for aquatic products has resulted in shifting the rearing strategy form the traditional extensive to a modern intensive one (Harikrishnan et al., 2021). Rearing fish at high intensities increases annual yield of a farm and yield per unit of space, but has certain negative drawbacks. Physiological stress due to crowding and water quality deterioration due to elevation in fish wastes are crucial threats in the modern aquaculture industry that decrease fish growth rate, health, and disease resistance (Martos-Sitcha et al., 2020). Under such conditions, opportunistic pathogens may cause disease outbreak and economic loss. Chemical drugs and antibiotics have been extensively used to control disease outbreaks in aquaculture, but environmental concerns and rise of resistance pathogens oriented the global authorities to restrict the use of chemical drugs and antibiotics (Serrano, 2005), focusing on improving fish health and disease resistance. One technique used to boost fish immunity and health is dietary supplementation with different feed additives including herbal additives (Lee et al., 2015). Herbal additives are known for their growth-promoting, immunostimulant, antioxidant, and anti-microbial properties and dietary supplementation with these additives has been found to boost host immunity and health (Hoseinifar et al., 2020a; Elumalai et al., 2021).

Plants are rich sources of antioxidant compounds; as a result, one of the main benefits of dietary herbal additives is the improvement of the antioxidant system and a reduction of oxidative stress (Mohiseni, 2017; Abdel-Latif et al., 2020a; Abdel-Latif et al., 2020b). The liver has high metabolic rate and is the main organ of detoxification; hence, it possesses high antioxidant capacities (Hoseini et al., 2022b). Modern aquaculture is accompanied by various stressors such as high stocking density and inferior water quality that induce oxidative conditions in fish; so that dietary herbal additives may benefit the host by suppressing such drawbacks. Studies have shown that dietary herbal additives improve hepatic antioxidant enzymes' activities and suppress oxidative stress (Hamed et al., 2021; Adeyemi et al., 2022; He et al., 2022).

Use of medicinal herbs as feed additives also maintains healthy intestinal microflora by dominating beneficial bacteria and limiting harmful bacteria populations, thereby improving immune status (Ganguly and Prasad, 2012; Foysal et al., 2019; Ashry et al., 2021). For example, *Lactobacillus* sp. are known for their health benefits in fish and herbal feed additives have been found to increase their proportion in fish gut (Adel et al., 2015; Adel et al., 2016). Besides, herbal additives have critical roles in intestinal immunity by stimulating transcriptions of various genes including cytokines and antimicrobial peptide (Gora et al., 2018; Sun et al., 2018; Hoseinifar et al., 2020c; Bilen et al., 2021); thereby these additives increase disease resistance (Adeshina et al., 2021; Lumsangkul et al., 2022).

Although various plants have been used as feed additives in aquaculture (Hoseinifar et al., 2020a; Elumalai et al., 2021), there are still other plants that need to be studied in this field. Hyssop, (*Hyssopus officinalis*) is a popular medicinal, aromatic, and culinary herb. It is one of the most important medicinal plants, grown in central and southern Europe, such as Russia, Spain, France, and Italy (Omidbaigi, 2005). Hyssop extract has been studied for several biological and pharmaceutical applications (Fathiazad et al., 2011). It has been shown that Hyssop has antioxidant (Kizil et al., 2010; Rezaei Savadkouhi et al., 2020) and immune-boosting (Akbarizadeh et al., 2020) properties, and antagonistic effects on harmful bacteria and

fungi (Kizil et al., 2010; Michalczyk et al., 2012). Despite such potentials, there are no data about the benefits of Hyssop extract in fish, indicating the need for further studies.

Rainbow trout (*Oncorhynchus mykiss*) is one of most important aquaculture fish species worldwide and intensification of this species can lead to a remarkable increase in stress-related infectious diseases. Among the opportunistic bacterial pathogens, *Streptococcus iniae* (Lahav et al., 2004), *Lactococcus garvieae* (Pérez-Sánchez et al., 2011), *Aeromonas hydrophila* (LaPatra et al., 2010), *Yersinia ruckeri* (Raida and Buchmann, 2008), and *Vibrio anguillarum* (Croxatto et al., 2007) have been commonly reported to induce disease in rainbow trout. Research has shown that dietary herbal additives are capable of increasing rainbow trout immunity and antioxidant strength and resistance of the fish to these bacteria (Nya and Austin, 2011; Baba et al., 2015; TerzİoğIu and DİIer, 2016; Saeidi Asl et al., 2017; Rufchaei et al., 2020). However, there are no data available regarding the use of methanolic extract of Hyssop (HE) on this species. Hence, the current study was performed to evaluate the impact of dietary methanolic extract of Hyssop on growth performance, antioxidant, and immunological parameters, and gut bacterial populations of rainbow trout.

Materials and methods

Herbal material and extraction

Hyssop flowers were dried by a fan in the shade (24 h), and crushed by a mill. One hundred grams of the crushed materials were mixed with 1 L of methanol and kept at room temperature for 48 h with occasional shaking. After that, the mixture was passed through a mesh (500 μ) followed by filter paper (Whatman No. 1). The resultant solution was then dried at 37°C for 48 h. The residual was collected and preserved in a capped bottle at 4°C. To explore the composition of HE, 1 g of the dried HE was dissolved in methanol and used for GC-MS analysis (supplementary material).

Preparation of diets

HE was added to the diets at 100, 250, and 500 mg/kg. A control (CTL) diet without HE supplementation was included. The composition of the diets are presented in Table 1. Five grams of the dried HE were mixed with 10 mL methanol and the resultant mixture was set to 80 mL by adding distilled water.

TABLE 1 Feedstuffs' proportion and chemical composition of the experimental diets.

	Dietary HE concentration (mg/kg)			
Ingredients (g/kg)	0	100	250	500
Corn meal	50	49.9	49.75	49.5
Wheat meal	230	230	230	230
Soybean meal	143	143	143	143
Soybean oil	36.3	36.3	36.3	36.3
Fish process byproduct[1]	120	120	120	120
Poultry byproduct[2]	400	400	400	400
Vitamin premix[3]	5	5	5	5
Mineral premix[4]	5	5	5	5
Methionine	5.80	5.80	5.80	5.80
Lysine	4.40	4.40	4.40	4.40
HE	0	0.10	0.25	0.50
Proximate composition				
Moisture	91.2	89.6	89.0	90.0
Crude protein	395	399	393	398
Crude fat	152	155	156	150
Crude ash	76.5	75.6	75.0	76.9
Crude fiber	34.1	35.0	34.9	35.0

1 crude protein 54%; crude fat 18%.
2 crude protein 54%; crude fat 22%.
3 Amineh Gostar Co. (Tehran, Iran). The premix provided the following amounts of vitamin to the diets (per kg): A: 1600 IU; D3: 500 IU; E: 20 mg; K: 24 mg; B3: 12 mg; B5: 40 mg; B2: 10 mg; B6: 5 mg; B1: 4 mg; H: 0.2 mg; B9: 2 mg; B12: 0.01 mg; C: 60 mg; Inositol: 50 mg.
4 Amineh Gostar Co. (Tehran, Iran). The premix provided the following amounts of minerals to the diets (per kg): Se: 0.15 mg; Fe: 2.5 mg; Co: 0.04 mg; Mn: 5 mg; Iodate: 0.05 mg; Cu: 0.5 mg; Zn: 6 mg; Choline: 150 mg.

Then, 1.6, 4, and 8 mL of the mixture were added to diets to have final HE levels of 100, 250, and 500 mg per kg diet. The feedstuffs were finely milled, mixed and moisturized by adding a desired amount of water. The resultant paste was pelleted using a meat-grinder and dried against a fan blower. Standard protocols were followed to determine dietary chemical compositions (AOAC, 2005).

Experimental protocol

Two hundred rainbow trout (~ 60 g) were purchased and transported to a laboratory, where they were kept in a 1500-L tank and fed the CTL diet for one week. After that, 144 fish with similar size and without external abnormalities/lesions were allocated to twelve plastic tanks (150 L water) at the density of 12 fish per tank. The tanks were assigned to four treatments called: CTL (fed CTL diet), 100E (fed diet supplemented with 100 mg/kg HE), 250E (fed diet supplemented with 250 mg/kg HE), and 500E (fed diet supplemented with 500 mg/kg HE). The fish were fed daily based on 3% of biomass, divided to two meals. The feed amounts were corrected every other week, after measuring the tanks' biomasses. Water temperature, dissolved oxygen, pH, and total ammonia nitrogen were 13.1 ± 0.07°C, 8.55 ± 0.40 mg/L, 7.77 ± 0.31, and 0.12 ± 0.02 mg/L [measured by Hach Co. (Colorado, USA) Digital probe and kits]. After 8 weeks of rearing, the fish growth performance and feed efficiency were determined and the blood, hepatic, and intestine samples were collected for further analysis.

Blood sampling and analysis

Two fish were caught from each tank and anesthetized in a eugenol bath (100 µL/L). Blood samples (1.5 mL) were taken from the fish caudal vein using heparinized syringes and divided into two aliquots; one for hematological analysis and the other for plasma separation. Plasma separation was done by centrifugation at 4°C (3000 g; 7 min) and the obtained materials were kept at -70°C until analysis.

The blood erythrocyte (RBC) and leukocyte (WBC) counting was done using the dacie diluting solution based on Dacie and Lewis (1996). Hemoglobin levels were measured using a commercial kit (Zistchem Co., Tehran, Iran) and a spectrophotometer; whereas, hematocrit percentages were calculated by micro-centrifuging according to Dacie and Lewis (1996). Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated based on Blaxhall (1972). Differential leukocyte count was performed after preparing the blood smear and staining with Giemsa according to Sheikh and Ahmed (2020).

Plasma total protein and albumin were measured using commercial kits and a spectrophotometer. Plasma globulin levels were measured by subtracting total protein and albumin levels. Plasma lysozyme activity was determined based on lysis rate of *Micrococcus luteus* over 5 min at 550 nm, as suggested by Ellis (1990). Briefly, 30 µL of the samples were mixed 1 mL of the bacterial suspension (made in 0.05 M phosphate buffer, pH 6.2) and a decrease in absorbance was recorded over 5 min. Each 0.001 decrease in absorbance per min was considered as one unit of lysozyme. Plasma alternative complement activity (ACH50) was determined based on hemolytic activity against sheep erythrocyte as described by Yano (1992). Briefly, the plasma samples were diluted by 0.303-10%. Hemolytic activity of each dilution was determined against sheep erythrocyte for 2 h, and the reaction medium consisted of veronal buffer containing magnesium and gelatin. Plasma total immunoglobulin (Ig) levels were determined after precipitation with polyethylene glycol according to Siwicki and Anderson (1993). Briefly, 100 µL of the plasma samples were mixed with equal volume of polyethylene glycol 12% and shaken for 2 h. Then, the mixture was centrifuged for Ig precipitation. Difference in the total protein before and after precipitation was considered as total Ig level.

Hepatic sampling and analysis

Hepatic samples were taken from the same fish that the blood samples were collected from. A piece of ~1 g was dissected from each fish liver and immediately frozen in liquid nitrogen. Then, the samples were homogenized in five volumes of phosphate buffer (pH 7.0), centrifuged at 4°C (9500 g; 15 min), and the supernatants were collected in separate tubes.

Hepatic antioxidant parameters were determined using commercial kits provided by Zellbio Co. (Deutschland, Germany) as validated for this species (Taheri Mirghaed et al., 2020). Superoxide dismutase (SOD) activity was determined based on the reduction rate of the cytochrome C. Glutathione peroxidase (GPx), glutathione reductase (GR), and reduced glutathione (GSH) were determined based on the conversion rate of GSH to glutathione disulfide. Malondialdehyde (MDA) content was determined based on the reaction with thiobarbituric acid at 95°C.

Intestinal sampling and analysis

After dissecting the hepatic samples, the fish intestine samples (the posterior parts) were dissected and frozen in liquid nitrogen. The intestinal sample of one fish per tank was used for gene expression analysis and the sample of the other fish was used for *Lactobacillus* sp., *Streptococcus iniae*, *Lactococcus*

garvieae, *Aeromonas hydrophila*, *Yersinia ruckeri*, and *Vibrio anguillarum* populations examination.

For gene expression analysis, RNA was extracted from the intestinal samples using a commercial kit (Denazist Co., Tehran, Iran). DNAse I (Thermo Fisher Scientific, Waltham, MA, USA) treatment was applied to avoid the product contamination. cDNA was synthetized using a commercial kit (SMOBIO Technology Co.; Hsinchu City 30075, Taiwan). Relative gene expression was assessed by qRT-PCR method using an apparatus provided by Applied Bioscience (USA). Amplification of the target genes along with a reference gene (*gadph*) was performed using specific primers (Table 2) and SYBR Green (Ampliqon A/S, Stenhuggervej 22, 5230 Odense M, Denmark) kit. Relative gene expressions were calculated based on ΔΔCt method (Livak and Schmittgen, 2001).

Populations of *Lactobacillus* sp., *S. iniae*, *L. garvieae*, *A. hydrophila*, *Y. ruckeri*, and *V. anguillarum* were examined in the intestinal samples. Procedures of the sample digestion and DNA extraction (phenol-chloroform using washing kit provided by GeneAll Co., Seoul, Korea) have been described before (Hoseini et al., 2022a). The bacterial populations were determined based on total bacteria in the samples. Specific primers for the target bacteria groups are presented in Table 3. The universal 16s primer was used for total bacteria population examination. qRT-PCR was used to amplified the target genes and bacterial population was calculated based on ΔΔCt method (Hoseini et al., 2022a).

Statistical analysis

Means of the treatments were compared using one-way ANOVA and Duncan tests, except percentile data (leukocyte differential counts) that were analyzed by Kruskal-Wallis and Mann-Whitney U tests. For ANOVA execution, the data were first subjected to Shapiro-Wilk and Levene tests aiming at confirming normal distribution and variance homogeneity, respectively. Accordingly, *Lactobacillus* sp., *il10* and *tnfa* data were log-transformed as they failed to meet the ANOVA assumptions. SPSS v.22 was used for analysis and significance has been checked at $\alpha = 0.05$.

Results

No fish mortality was observed during the study. There were no significant differences in final weight (P = 0.774), weight gain (P = 0.838), SGR (P = 0.841), and FCR (P = 0.701) among the treatments (Table 4).

RBC (P = 0.970), hemoglobin (P = 0.849), hematocrit (P = 0.981), MCV (P = 0.916), MCH (P = 0.093), and MCHC (P = 0.233) exhibited no significant differences among the treatments (Table 5).

Hepatic antioxidant parameters are shown in Figure 1. HE supplementation significantly affected hepatic SOD (P < 0.001), GPx (P = 0.001), GR (P < 0.001), GSH (P = 0.046), and MDA (P = 0.007). Hepatic SOD and GPx activities in 100E and 250E treatments were significantly higher than in the CTL treatment. All HE-treated fish exhibited significantly higher GR activities, compared to CTL treatment and the highest activity was observed in the 100E treatment. Hepatic GSH level significantly increased in 250E treatment, compared to CTL; whereas, both 250E and 500E treatments exhibited significantly lower MDA levels, compared to CTL.

All HE-treated fish exhibited significant increases in WBC, compared to CTL, and the highest level was related to 250E (Figure 2). Lymphocyte percentage decreased as neutrophil percentage increased in 250E and 500E treatments, compared to CTL (Figure 2). There were no significant differences in eosinophil percentages among the treatments, however, monocyte percentage exhibited a significant elevation in 250E, compared to CTL treatment (Figure 2).

TABLE 2 Sequence, amplicon, and accession number of specific primers used for intestinal transcriptomic analysis.

Gene	Primer name	Sequences	Amplicon	Accession number
def	Defensin-F	GCGTTTCTAACCTGGCATGAT	145	NM_001195168.1
	Defensin-R	AACGGGATCCTCATAGCAGTT		
tnfa	TNFa-F	CAGGCTTCGTTTAGGGTCAAG	186	NM_001124357.1
	TNFa-R	AACTGCATTGTACCAGCCTTC		
il1b	IL1b-F	GGGTCTGGATCTGGAGGTATC	137	AJ223954.1
	IL1b-R	GAAGTTGAGCAGGTCCTTGTC		
il10	IL10-F	TCCACGAGCTGAAGAAAGAGA	146	AB118099.1
	IL10-R	GAAGAGTAGGTCCAGCTCTCC		
il6	IL6-F	TTTCATCGTTCTCACAGCACC	171	NM_001124657.1
	IL6-R	GGAGTAGGGTTGATTGAGGGT		
gadph	GAPDH-F	GAGGGTCTGATGAGCACAGTTC	150	XM_021623341.2
	GAPDH-R	GATGACCTTGCCGACAGCC		

TABLE 3 Primer sequences used for detecting different bacteria in the fish intestine.

Bacterium	Name	Sequences	Reference
Lactobacillus sp.	Lacto-F	TGGAAACAGRTGCTAATACCG	(Frank et al., 2008)
	Lacto-R	GTCCATTGTGGAAGATTCCC	
V. anguillarum	VirA	CATACGCAGCCAAAAATCAA	(Chapela et al., 2018b)
	VirB	GCACTGTCCGTCATGCTATC	
L. garvieae	ITS region	ACTTTATTCAGTTTTGAGGGGTCT	(Chapela et al., 2018a)
	ITS region	TTTAACGTCTTCGTTGACCAGA	
A. hydrophila	gcat-F	CTCCTGGAATCCCAAGTATCAG	(Tsai et al., 2019)
	gcat-R	GGCAGGTTGAACAGCAGTATCT	
Y. ruckeri	Y.rkr-F	TCTGGACATCGCTCTGG	(Bastardo et al., 2012)
	Y.rkr-R	AGTTTTTTTGCGTAGATAGGA	
S. iniae	S.iniae-F	ACACAGGTGAGCACGCTAAA	(Torres-Corral and Santos, 2021)
	S.iniae-R	CGTCACCATCGTCTTGGTCA	
All bacteria	338F	ACTCCTACGGGAGGCAGCAG	(Wei et al., 2020)
	518R	ATTACCGCGGCTGCTGG	

Plasma total protein, albumin, globulin, lysozyme, ACH50, and total Ig showed significant differences among the treatments (Figure 3). Plasma total protein, albumin, lysozyme, and ACH50 in 100E and 250E treatments were significantly higher than CTL and 250E showed the highest levels in the case of plasma lysozyme and ACH50. Plasma globulin and total Ig in 250E treatment was significantly higher than CTL treatment.

Among the bacterial groups examined, only *Lactobacillus* sp. was detected in the samples (Figure 4). However, HE treatment had no significant effects on *Lactobacillus* sp. population ($P = 0.352$)

Dietary HE supplementation significantly affected the intestinal *def* ($P = 0.044$), *il1b* ($P = 0.0.035$), *il10* ($P < 0.001$), *il6* ($P = 0.009$), and *tnfa* ($P < 0.001$) expression (Figure 5). Expressions of *def* and *il1b* were up-regulated in 100E treatment, compared to CTL. Intestinal expressions of *il10* and *tnfa* were up-regulated in 100E and 500E treatments, compared to CTL. The highest expressions of *il10* and *tnfa* were observed in 500E and 100E treatments, respectively. Expression of the intestinal *il6* was significantly up-regulated in 250E treatment, compared to CTL.

Discussion

Intensification of aquaculture has led to several issues including poor fish growth and health. This has made it hard for fish farmers to turn the biological benefits associated with intensive farming systems into economical gain. In addition, the usage of chemotherapeutic drugs to maintain fish growth and health seems to be less production-oriented, hence, unsustainable (Gabriel, 2019). Thus, development of aquaculture should also rely on environmentally friendly and sustainable practices. Recently, much attention has been paid to the use of herbal additives as alternatives to chemical agents, with an aim of increasing yield in aquaculture (Gabriel, 2019). Growth-promoting effects of dietary herbal additives relate to improvements in digestion and absorption of nutrients (Bilen et al., 2020; Xu et al., 2020; Phukan et al., 2022; Wangkahart et al., 2022) and/or stimulation of somatotropic pathways in the fish (Midhun et al., 2016; Zemheri-Navruz et al., 2020). Therefore, it is speculated that HE might fail to have such effects on rainbow trout under the conditions of the present study. Similarly, other herbal

TABLE 4 Growth performance and feed efficiency of rainbow trout fed diets supplemented with 0-500 mg/kg HE over eight weeks (mean ± SE; n = 3).

	Dietary HE levels (mg/kg)				
	0	100	250	500	P-value
Initial weight (g)	69.9 ± 0.92	69.8 ± 0.72	69.8 ± 0.74	69.9 ± 1.12	0.953
Final weight (g)	199 ± 7.12	198 ± 8.13	202 ± 5.83	193 ± 1.82	0.774
Weight gain (%)	184 ± 12.7	184 ± 14.3	190 ± 6.19	176 ± 4.95	0.838
Specific growth rate (%/d)	1.86 ± 0.08	1.86 ± 0.09	1.90 ± 0.04	1.82 ± 0.03	0.841
Feed conversion ratio	1.35 ± 0.07	1.34 ± 0.07	1.31 ± 0.03	1.40 ± 0.03	0.701
Total feed intake (g/fish)	173 ± 2.18	171 ± 3.54	174 ± 2.81	173 ± 1.19	0.914
Survival rate (%)	100	100	100	100	–

TABLE 5 Hematological parameters of rainbow trout fed diets supplemented with 0-500 mg/kg HE over eight weeks (mean ± SE; n = 6).

	Dietary HE levels (mg/kg)				
	0	100	250	500	P-value
RBC ($\times 10^6$ cell/μL)	1.44 ± 0.04	1.44 ± 0.05	1.42 ± 0.03	1.42 ± 0.03	0.970
Hemoglobin (g/dL)	8.27 ± 0.21	8.08 ± 0.37	8.38 ± 0.25	8.38 ± 0.23	0.849
Hematocrit (%)	53.3 ± 1.45	53.3 ± 1.71	53.0 ± 1.06	52.7 ± 0.95	0.981
MCV (fL)	370 ± 2.92	371 ± 2.83	373 ± 2.73	371 ± 3.03	0.916
MCH (pg)	57.3 ± 0.79	56.2 ± 1.22	58.9 ± 0.76	59.0 ± 0.47	0.093
MCHC (mg/dL)	15.5 ± 0.24	15.1 ± 0.38	15.8 ± 0.26	15.9 ± 0.18	0.233

additives have failed to improve growth performance in rainbow trout (Hernández et al., 2016; Baba et al., 2018).

Living cells are under constant threat of oxidation by pro-oxidants that are normally produced during the cell respiration or as a result of adverse ambient conditions. The antioxidant enzymes (SOD, CAT, GPx, and GR) protect cells from oxidative damage (Yousefi et al., 2020) and it is one of the most crucial benefits of using plant extracts in fish diets. Plant extracts have

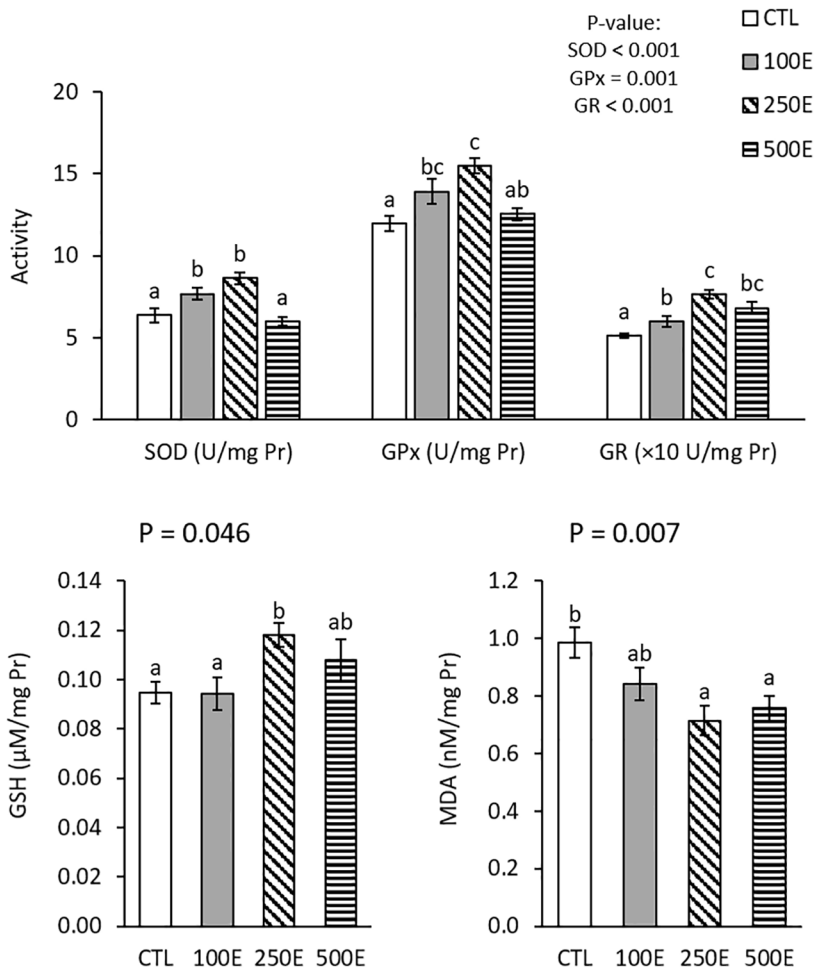

FIGURE 1
Hepatic antioxidant parameters (mean ± SE) of rainbow trout fed diets supplemented with 0-500 mg/kg HE over eight weeks. Different letters above the bars indicate significant differences among the treatments (Duncan test; n = 6).

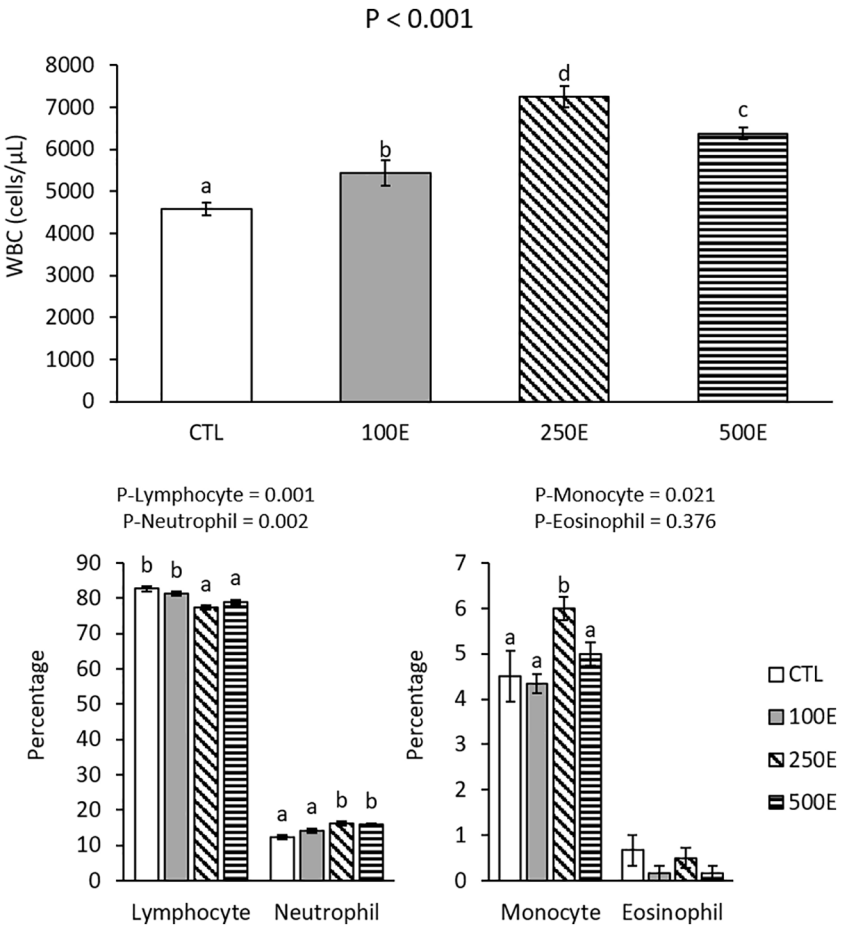

FIGURE 2
WBC and differential leukocyte count (mean ± SE) of rainbow trout fed diets supplemented with 0-500 mg/kg HE over eight weeks. Different letters above the bars indicate significant differences among the treatments (Duncan test for WBC and Mann-Whitney U test for differential counts; n = 6).

antioxidant capacity and high capability to donate hydrogen atoms to electrons and free electrons and this property is due to the existence of phenolic compounds in herbs (Jeney et al., 2015). Moreover, plant extracts have been found to stimulate the activity of the antioxidant enzymes in fish, although the exact underlying mechanisms are not clear yet (Mohiseni, 2017). These capabilities make plant extract supplementation one of the functional approaches to reduce oxidative stress in animals. SOD is an early-functioning antioxidant enzyme that neutralizes superoxide ions and has a crucial role in detoxifying the respiration-produced superoxide ion in living cells (Shi et al., 2022). GPx is responsible for neutralizing hydrogen peroxide using GSH as a co-factor. After neutralizing hydrogen peroxide, GSH is oxidized and loses its function. GR reduces the oxidized glutathione to GSH, which can be used again in hydrogen peroxide neutralization (Rocha-Santos et al., 2018). According to the present results, the lowest lipid peroxidation was observed in 250E and 500E treatments. This could be due to improved antioxidant enzymes' activity and radical scavenging activity of HE in 250E treatment (as evidenced by high GSH reserves). However, lower lipid peroxidation in 500E suggests radical scavenging activity of HE is the main reason for mitigation of lipid peroxidation. This is supported by the results of 100E treatment, where improvement in the activity of the antioxidant enzymes failed to mitigate lipid peroxidation. Supporting this, radical scavenging activity of HE has been reported under *in vitro* conditions (Soleimani et al., 2011; Alinezhad et al., 2012).

Hematological parameters provide valuable information about fish health. Studies on different fish species have indicated herbal supplements such as *Spirulina platensis* (Adel et al., 2016), garlic (Nya and Austin, 2011), and *Echinacea purpurea* (Oskoii et al., 2012) have hematopoietic effects in fish and increase blood RBC, hematocrit, and hemoglobin. However, in the present study, RBC, hemoglobin, hematocrit, MCV, MCH, and MCHC

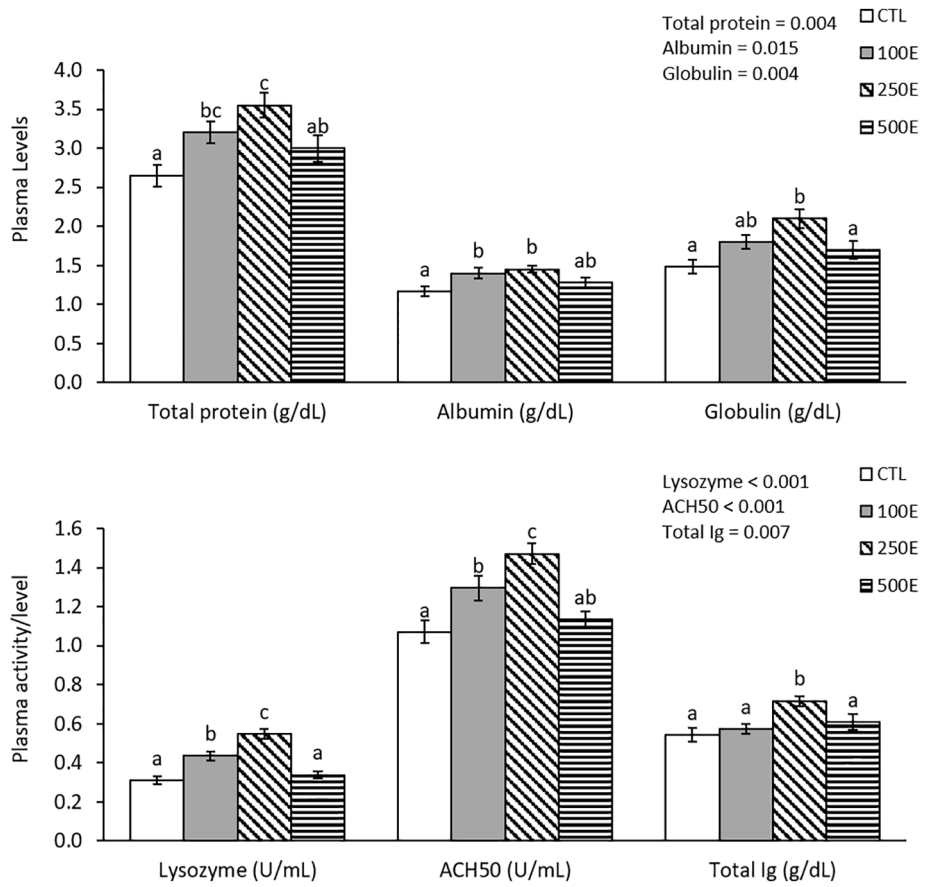

FIGURE 3
Blood plasma immunological parameters (mean ± SE) of rainbow trout fed diets supplemented with 0-500 mg/kg HE over eight weeks. Different letters above the bars indicate significant differences among the treatments (Duncan test; n = 6).

exhibited almost no change among the different treatments suggesting no hematopoietic ability of HE in this fish species, which is similar to the findings in previous studies on other herbal supplements (Adel et al., 2015; Soltanian and Fereidouni, 2016).

Total blood leukocytes are known as one of the most important cellular defenses in fish (Newaj-Fyzul and Austin, 2015). Differential leukocyte counting is a reliable method of assessing fish health and cellular immunity (Machado et al., 2015). Depending on type, leukocytes have various roles in host defense. Lymphocytes are the most abundant blood leukocyte and have roles in adaptive immunity (antibody production) and killing tumor cells (Shoemaker et al., 2015). Neutrophils are the next abundant leukocytes that are responsible for early responses to infection; they are phagocytic and involve in respiratory burst activity and lysozyme production (Shoemaker et al., 2015). Monocytes reside in the blood or migrate to the body tissues and transform to macrophages to find and destroy germs (viruses, bacteria, fungi, and protozoa) (Shoemaker et al., 2015). According to the present results, HE administration shifted leukocyte composition from late-response (lymphocyte) to early-response (phagocytes; i.e. neutrophils and monocytes). Such changes in leukocyte composition have been previously observed, when fish fed supplemented diets and helped the fish to resist against a pathogenic challenge (Lin et al., 2012). Similar results have been obtained, when rainbow trout was treated with dietary Ginger, *Zingiber officinale*; where increase in neutrophil and monocyte proportions and decrease in lymphocyte proportion after ginger administration helped the fish to better resist against *A. hydrophila* infection (Nya and Austin, 2009b). A higher neutrophil percentage (with no changes in lymphocyte and monocyte percentages) after oral administration of *Azadirachta indica* leaves has resulted in maximum survival in *Lates calcarifer* challenged with *Vibrio harveyi* (Talpur and Ikhwanuddin, 2013).

The non-specific immune system of fish is an important defense line against invading pathogens. Plasma protein levels and fractions are indicators of fish health as most of them are produced in the liver. Healthier liver and/or good rearing conditions may lead to higher plasma protein levels (Hoseini

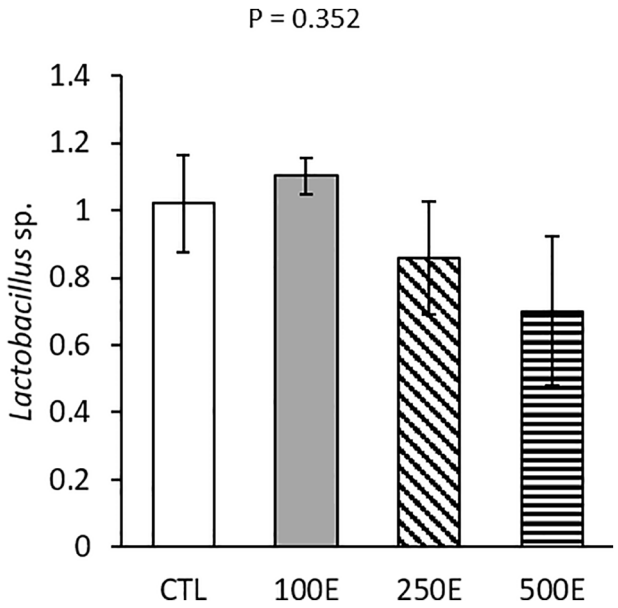

FIGURE 4
Intestinal *Lactobacillus* sp. population (mean ± SE) of rainbow trout fed diets supplemented with 0-500 mg/kg HE over eight weeks (n = 3).

and Tarkhani, 2013; Sayed and Hamed, 2017). Ig encompass a fraction of plasma protein and it has been found that basal plasma Ig may be sensitive to dietary supplementation (Jinendiran et al., 2019; Lim et al., 2019). Complement proteins are another fraction of plasma protein that are produced in the liver and have different immune roles such as cell lysis and opsonization (Holland and Lambris, 2002). According to the present results, HE supplementation seems to improve hepatic health, which leads to higher protein synthesis and innate immunity improvement. The increased levels of complement, Ig, albumin, and globulins in fish are linked with a strong innate immune response and studies on fish have shown dietary herbal supplementation increases in plasma proteins, Ig, and complement that leads to higher disease resistance (Nya and Austin, 2009a; Nya and Austin, 2009b; Nya and Austin, 2011; Talpur and Ikhwanuddin, 2013; Talpur et al., 2013). Lysozyme is another immune-related enzyme that acts as a bactericidal agent (Zhang et al., 2018). Lysozyme is produced by neutrophil and the increase in the plasma lysozyme in the present study may be due to a higher number of neutrophil and/or lysozyme production by these cells. Other studies have found an increase in plasma lysozyme activity after

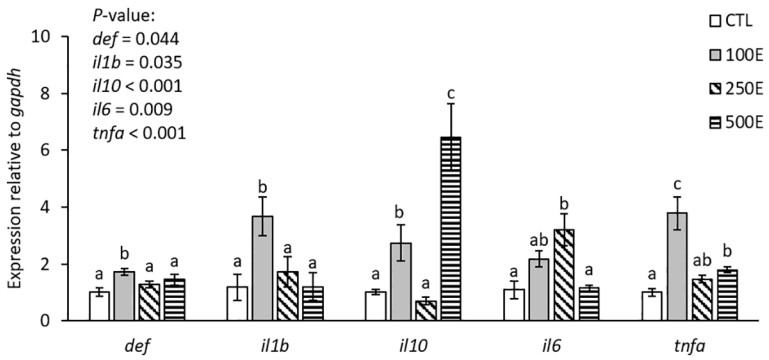

FIGURE 5
Intestinal gene expressions (mean ± SE) of rainbow trout fed diets supplemented with 0-500 mg/kg HE over eight weeks. Different letters above the bars indicate significant differences among the treatments (Duncan test; n = 3).

herbal treatment led to higher disease resistance in fish (Nya and Austin, 2009a; Nya and Austin, 2009b; Nya and Austin, 2011).

The fish intestine is a unique organ that plays important roles in digestion/absorption and immunity (Hoseini et al., 2021). The fish intestine is a site of various microbial populations, which can alter the host immunity (Hoseinifar et al., 2019). A healthy intestine is characterized by low density of harmful microbes and high density of beneficial ones (Hoseinifar et al., 2019). In this study, none of the tested pathogenic bacteria were detected in the samples, suggesting that the fishes' intestines were in good health. Moreover, *Lactobacillus* sp. was detected in the samples, which was in line with the previous studies on rainbow trout (Wang et al., 2020; Hoseini et al., 2022a). However, HE induced no significant changes in *Lactobacillus* sp. population, which is not in line with studies evaluating other herbal additives in fish (Adel et al., 2015; Adel et al., 2016; Meng et al., 2019). Such variations in the results can be due to the differences in fish species and feed additives.

Defensin is an antimicrobial peptide, known for its roles in combating bacteria, fungi, and viruses (Guo et al., 2012). It is found in fish mucosal tissues such as intestine (van der Marel et al., 2012). Similar to the present results, intestinal *def* expression has been found elevated in different fish following dietary additive supplementations such as *Sargassum wightii* extract (Gora et al., 2018), mannan oligosaccharide (Fernández-Montero et al., 2019), microalgae mixture (Cerezuela et al., 2012). Such an up-regulation in the intestinal *def* expression may help the fish to resist against bacterial and viral disease, as Casadei et al. (2009) have shown bacterial and viral stimulation significantly up-regulates intestinal *def* expression rainbow trout.

Cytokines have numerous roles in inflammation and immune responses. *il1b* is a pro-inflammatory cytokine with wide physiological roles, including increasing the number of macrophages and phagocytosis (Hong et al., 2003), antibody production (Taechavasonyoo et al., 2013) and lysozyme activity (Hong et al., 2003). It also stimulates *tnfa* expression (Bo et al., 2015), which is involved in increased phagocytosis, inhibiting bacterial growth in infected macrophages, and necrosis of tumor cells (Zou and Secombes, 2016). *il6* is another cytokine with both anti-inflammatory and pro-inflammatory roles, which is involved in macrophage growth and expression of antimicrobial peptides (Zou and Secombes, 2016). On the other hand, il10 is an anti-inflammatory cytokine that suppresses the immune responses in fish (Piazzon et al., 2015). Intestinal cytokines have an important roles in fish immune defense. Herbal additives potentiate to alter cytokine expression in fish intestine (Hoseinifar et al., 2020b; Hoseinifar et al., 2020d). It has been demonstrated that up-regulation in the intestinal pro-inflammatory cytokines following herbal additive administration has been accompanied by better disease resistance after a pathogenic challenge (Noor-Ul et al., 2020; Bilen et al., 2021; Ibrahim et al., 2021). The present results suggest that 100 mg/kg HE induces several immune genes in the fish intestine, which may be interpreted as improved intestinal health. However, it is not clear why *il6* patterns differed from those of *il1b* and *tnfa*, based on the present data. It is also speculated that *il10* up-regulation in 100 mg/kg HE treatment is a protective response to up-regulation of *il1b* and *tnfa* to protect the host from negative consequences of inflammation. But, higher up-regulation in *il10* gene expression in 500 mg/kg HE may be an indication of immunosuppression. Moreover, the patterns of blood, hepatic, and intestinal responses to HE must be considered, as HE concentration is a determinant of its effects on different tissues.

It is concluded that dietary HE supplementation has no growth-promoting effects in rainbow trout. However, HE is able to stimulate humoral and intestinal immune responses and hepatic antioxidant capacity. However, effective concentration of HE must be selected with caution, as its benefits were observed at different concentrations, based on the fish tissue examined. Therefore, HE effective concentration in rainbow trout may be in the range of 100-250 mg/kg.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Peoples' Friendship University of Russia (RUDN University) ethical committee (EC1/351, 05/06/2021).

Author contributions

MY: conceptualization, supervision, grant acquisition, methodology, and editing. SMH: conceptualization, supervision, methodology, and drafting. BA: conceptualization, study design, and drafting. YV: conceptualization and data analysis. EK: conceptualization and study design. NR: methodology, and data analysis. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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