Effects of dietary thyme (Zataria multiflora) extract on antioxidant and immunological responses and immune-related gene expression of rainbow trout (Oncorhynchus mykiss) juveniles

Ali Taheri Mirghaed a, Seyyed Morteza Hoseini b, Seyed Hossein Hoseinifar c, Hien Van Doan d,e,*

a Department of Aquatic Animal Health, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
b Inland Waters Aquatics Resources Research Center, Iranian Fisheries Sciences Research Institute, Agricultural Research, Education and Extension Organization, Gorgan, Iran
c Department of Fisheries, Faculty of Fisheries and Environmental Sciences, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran
d Department of Animal and Aquatic Sciences, Faculty of Agriculture, Chiang Mai University, Chiang Mai, 50200, Thailand
e Innovative Agriculture Research Center, Faculty of Agriculture, Chiang Mai University, Chiang Mai, 50200, Thailand

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ABSTRACT

Effects of dietary hydroalcoholic extract of Zataria multiflora (ZE) on growth performance, plasma and hepatic antioxidant capacities, and humoral and skin mucus immune parameters were evaluated in rainbow trout (Oncorhynchus mykiss) juveniles. In vitro tests showed that ZE had antioxidant property comparable to butylated hydroxytoluene (BHT) at 100–200 μg/mL concentrations, although its antioxidant property was lower than BHT at concentration below 100 μg/mL. Moreover, ZE had anti-bacterial activity against Aeromonas hydrophila, which was 30–50% lower than that of tetracycline. After feeding the fish with diets supplemented with 0 (CT, 1 (ZE1), 2 (ZE2), and 3 (ZE3) g/kg ZE for eight weeks, there were no significant differences in growth performance and feed efficiency among the treatments; however, the fish in ZE2 and ZE3 treatments showed significantly higher survival than the fish in CT treatment. Blood leukocyte counts, plasma globulin, total immunoglobulin, lysozyme and bactericidal activity against A. hydrophila in ZE2 and ZE3 groups were significantly higher than that of CT group. All the ZE-treated groups had higher plasma complement activity compared to the CT group. Mucosal lysozyme and bactericidal activities of the ZE2 fish were significantly higher than the other treatments. Expression of tumor necrosis factor alpha, interleukin-1 beta, interleukin-6, and lysozyme genes increased in head kidney of the fish treated with ZE; the highest increases were related to the ZE2 treatment. Plasma total antioxidant (TA) activities of ZE2 and ZE3 treatments were significantly higher than that of the CT treatment. Plasma and hepatic superoxide dismutase (SOD) and catalase (CAT) activities of ZE2 group were significantly higher than the other treatments. Plasma malondialdehyde (MDA) levels were significantly lower in ZE2 treatment, compared to the other treatments. However, hepatic MDA level of ZE2 treatment was significantly lower than those of the ZE1 and CT treatments. In conclusion, dietary ZE supplementation level of 2 g/kg is suggested for rainbow trout feed supplementation to augment fish survival, antioxidant and immune strength.

1. Introduction

Global production of rainbow trout (Oncorhynchus mykiss) was above 811000 tons in 2017, with economic value of higher than 3600000 USD (FAO, 2020). This makes rainbow trout an important aquaculture species throughout the world with necessity of researches on its growth and health promotion. Aeromonas hydrophila is a worldwide pathogenic bacterium, infecting fish including rainbow trout [1,2]. Therefore, it is of interest to present methods for augmenting rainbow trout resistance to this widespread pathogen. Similar to mammals, fish have innate and adaptive immune systems; however, adaptive immune system takes a relatively long time to be activated, but keeps immune memory for a relatively short period [3]. Thus, fish vaccination is not as efficient as mammalians. Consequently, it is necessary to augment fish innate immune system (as the fast-responding immune system) to prevent opportunistic pathogen infections; moreover, activation of innate

* Corresponding author. Department of Animal and Aquatic Sciences, Faculty of Agriculture, Chiang Mai University, Chiang Mai, 50200, Thailand.
E-mail addresses: hien.d@cmu.ac.th, hienqbuni@gmail.com (H. Van Doan).

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immune system involves in activation of adaptive immune system [4].

Application of herbal additives in fish diets is an efficient and common way to promote the fish growth and innate immune systems [5]. They are beneficial in mitigating diseases because of their direct anti-pathogen activity and/or by augmenting fish health and stamina to combat the pathogens. For instance, most of the herbal additives are antioxidant agents that may help fish against oxidative stress during fighting with pathogens. For instance, dietary supplementation of garlic, Allium sativum [2], ginger, Zingiber officinal [1], black cumin, Nigella sativa seed oil [6], nettle, Urtica dioica [7] and oyster mushroom, Pleurotus ostreatus [7] improved rainbow trout survival against A. hydrophila by augmenting innate immune components (e.g. leukocyte count, lysozyme, phagocytic and bactericidal activities); among them, ginger, nettle and oyster mushroom promoted the fish growth rate, as well. Moreover, dietary administration of oregano, Origanum onites essential oil [8], sage, Salvia officinalis [9], and thyme, Thymus vulgaris [9] significantly improved growth performance and antioxidant system of rainbow trout; oregano essential oil prevented the fish mortality during bacterial challenge, too.

There are evidences showing that dietary supplementation with different immunostimulants was capable to augment immune system at transcriptional levels. In this case, tumor necrosis alpha (tnfa), interleukin-1 beta (il1b), and interleukin-6 (il6) were studied as pro-inflammatory cytokines [10,11]. Lysozyme gene expression (lys) was also found to be sensitive to immunostimulants [12,13]. Up-regulation of these genes was reported in several studies using different immunostimulants; such up-regulations were accompanied with higher resistance to pathogens [13–15]. Therefore, it is important to investigate the effects of dietary herbal immunostimulants on immune-related gene expressions.

Zataria multiflora is a plant native to Iran, Afghanistan and Pakistan, which contains high concentrations of thymol and carvacrol [16]. Z. multiflora essential oil was reported to have in vitro anti-bacterial activity against A. hydrophila [17] and another fish pathogen, Lactococcus garvieae [18]. Moreover, it down-regulated virulence factor gene expression (lys) [19] and Streptococcus iniae [20]. An in vivo study has shown that dietary administration of Z. multiflora (21 days) significantly increased leukocyte count and serum bactericidal activity against A. hydrophila in common carp, Cyprinus carpio; moreover, the fish showed higher antibody titer after challenge with the bacterium [21]; although the study was conducted at sub-optimal temperature that may weaken immune responses of the fish to the dietary manipulation.

Moreover, there are no reports about the growth promoting potential of Z. multiflora; although the study was conducted at sub-optimal temperature that Z. multiflora fish showed higher antibody titer after challenge with the bacterium promoting, antioxidant and immunostimulant effects of dietary supplemenation of this medicinal plant of both growth and health of fish following a long period of administration.

According to above, the aim of this study was to evaluate the growth-promoting, antioxidant and immunostimulant effects of dietary supplementation of hydroalcoholic extract of Z. multiflora (ZE) in rainbow trout.

2. Materials and methods

2.1. ZE preparation

Z. multiflora leaves, originating from Fars province, were purchased from a local shop and washed with distilled water and allowed to be dried against a fan blow. Then they were pulverized and mixed with 80% ethanol at proportion of 1:10 (w/v). The mixture remained at room temperature for 72 h, during with it was frequently mixed. Then, the mixture was filtered through and 500 μm mesh and the resultant solution was placed in an oven (40 °C, 48 h) for alcohol evaporation. Then, it was concentrated in freeze-drier (Beta LDplus, Martin Christ Gefriertrocknungsanlagen GmbH, Germany) for 72 h. The ZE was kept at –20 °C before use.

2.2. In vitro anti-bacterial and antioxidant activities of ZE

Anti-bacterial activity of ZE was compared to that of tetracycline, using A. hydrophila. Broth dilution method was used and concentrations of 1, 3, 6 and 10 μg/mL of the extract and tetracycline were compared, as suggested by Harikrishnan and Balasundaram [22]. A suspension of the bacterium (0.5 McFarland) was prepared in Mueller Hinton Broth, then, it was diluted by 100 folds. To 1 mL of this dilution was added 1 mL of extract or tetracycline solutions at the above mentioned concentrations. The mixture incubated for 24 h; then, it was diluted by 100 fold. 200 μL of this dilution was cultured on nutrient agar plate for 24 h. After that, the number of colony forming units was counted on the plates. Antioxidant capacity of ZE was assessed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method as suggested by Kamkar, Javan [23]. For this, concentrations of 12.5, 25, 50, 75, 100 and 200 μg/mL of ZE were compared to butylated hydroxytoluene (BHT) as control antioxidant.

2.3. Diet preparation and feeding trial

Four diets with 0 (CT), 1 (ZE1), 2 (ZE2), and 3 (ZE3) g/kg ZE were prepared according to Table 1. The feedstuffs, excluding the dietary oil sources, were dry-mixed for 30 min; then ZE was mixed with oil and added to the feedstuff mixture. The mixture was moisturized by adding 300 mL/kg water and pelleted by a meat grinder.

Rainbow trout juveniles (~20 g) were stocked in 12 tanks (50 L), with 25 fish per tank. The tanks were aerated and received water flow rate of 0.5 L/min kg fish. Water temperature, dissolved oxygen, pH, and unionized ammonia nitrogen were measured using a portable apparatus (Hach HQ40d, Loveland, Colorado, USA) or based on a photometric method [24], being 15.3 ± 1.00 °C, 6.98 ± 0.79 mg/L, 7.65 ± 0.65, and 0.03 ± 0.006 mg/L, respectively. The fish were fed the CT diets for seven days for acclimation; then, the tanks assigned into four treatments (three tanks per treatment) receiving either of CT, ZE1, ZE2, and ZE3 diets for 56 days. Feeding rate was 35 of the tanks’ biomass per day. At the end of

Table 1

<table>
<thead>
<tr>
<th>Ingredients (g kg⁻¹)</th>
<th>Diets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean meal</td>
<td>CT</td>
</tr>
<tr>
<td>Fish meal</td>
<td>200</td>
</tr>
<tr>
<td>Wheat gluten</td>
<td>100</td>
</tr>
<tr>
<td>Poultry by-product</td>
<td>225</td>
</tr>
<tr>
<td>Wheat meal</td>
<td>166</td>
</tr>
<tr>
<td>Fish oil</td>
<td>50</td>
</tr>
<tr>
<td>Soybean oil</td>
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</tr>
<tr>
<td>Phytase</td>
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</tr>
<tr>
<td>Lysine</td>
<td>7</td>
</tr>
<tr>
<td>Methionine</td>
<td>4</td>
</tr>
<tr>
<td>Mineral premix</td>
<td>5</td>
</tr>
<tr>
<td>Vitamin premix</td>
<td>5</td>
</tr>
<tr>
<td>ZE</td>
<td>0</td>
</tr>
</tbody>
</table>

CT ZE1 ZE2 ZE3
Moisture 87.0 86.4 88.8 85.9
Crude protein 416 419 421 414
Crude fat 182 185 183 186
Crude fiber 30.2 30.9 31.0 31.4
Crude ash 76.5 74.6 76.0 73.9

a Soybean Co., Gorgan, Iran (crude protein 45.2%).
b Peygir Co., Gorgan, Iran (crude protein 58.8%).
c Shahdineh Aran Co., Isfahan, Iran (crude protein 78.3%).

The premix provided following amounts per kg of feed: A: 1000 IU; D3: 5000 IU; I: 20 mg; B5: 100 mg; B2: 20 mg; B6: 20 mg; B1: 20 mg; H: 1 mg; B9: 6 mg; B12: 1 mg; B4: 600 mg; C: 50 mg; Mg: 250 mg; Fe: 13 mg; Co: 2.5 mg; Cu: 3 mg; Zn: 60 mg; Se: 0.3 mg; I: 1.5 mg; Mn: 10 mg.)

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the feeding trial, the fish blood, mucus and liver samples were taken as described below; then the fish growth performance were calculated as follow:

Weight gain percentage (WG) = 100 × [(FW – IW)/IW]

Feed conversion ratio (FCR) = FI/(FW – IW)

Specific growth rate (SGR) = 100 × [(ln FW – ln IW)/d]

where, FW, IW, FI, and d were fish final weight, fish initial weight, feed intake, and rearing period, respectively.

3. Sampling and analysis

3.1. Blood

At the end of the feeding trial, six fish were sampled per treatment. For this, the fish were fasted for 24 h and anesthetized by eugenol (100 mg/L) [25] and blood samples were collected via caudal vein by heparinized syringe at 0800. An aliquot of whole blood was used for leukocyte count according to Dacie and Lewis [26]. The remaining bloods were centrifuged (1300 g; 10 min; 4 °C) to obtain plasma. The plasma were stored at −70 °C for further analysis. Plasma bactericidal activity was measured against A. hydrophila according to a previously described method [5], using nutrient agar plate counting. Plasma lysozyme activity was measured by lysis of Micrococcus luteus in phosphate buffered saline (pH 6.2) as described by Ellis [27]. Plasma total antioxidant (TA), superoxide dismutase (SOD), and catalase (CAT) activities, were measured based on the ferric ion reduction, cytochrome C oxidation, hydrogen peroxide decomposition methods, respectively, using a commercial kit (Zellbio, Berlin, Germany). Plasma malondialdehyde (MDA) levels were measured based on thiobarbituric acid reaction method, using Zellbio (Berlin, Germany) commercial kit. Plasma total immunoglobulin (Ig) levels were measured by polyethylene glycol precipitation methods as previously described [28]. Plasma globulin levels were calculated by subtracting the plasma total protein and albumin levels. Plasma alternative complement activity was determined based of heparinized syringe principle. Plasma lysozyme activity was measured against Micrococcus luteus, using Zellbio (Berlin, Germany), as described above.

3.2. Mucus

Six fish per treatment were caught and anesthetized, as described above. The fish skin mucus was collected by a cell scraper from upper part of lateral line as suggested by Guardiola, Cuesta [30]. The collected mucus samples were poured into plastic tubes, homogenized with 50 mm NaCl (1:1 v:v) and centrifuged for 10 min at 4 °C (1500 g) [19]. The supernatant was used for determination of bactericidal and lysozyme activities as described above.

3.3. Liver and head kidney

Liver sample of six fish were taken for determination of antioxidant parameters. For this, the fish were anesthetized as described above and killed by spinal cord cutting. Then, liver and head kidney sample was taken and frozen in liquid nitrogen and transferred to −70 °C freezer. Hepatic extract was obtained by homogenization with phosphate buffered saline (pH 6.2) at a proportion of 1:10 (w:v) as described by Yu, Wen [31]. The homogenate was centrifuged for 30 min at 4 °C (3000 g) and the supernatant was used for hepatic TA, SOD, and CAT activities, using Zellbio (Berlin, Germany), as described above.

Expressions of tumor necrosis alpha (tnfa), interleukin-1 beta (il1b), interleukin-6 (il6), and lysozyme (lys) genes were in the fish head kidney were assessed as described before [15]. Specific primers for rainbow trout were designed according to Genelink (Table 2). RNA of the head

| Table 2
| Primer sequences and accession number of gene selected for real time-PCR. |
|-----------------|-----------------|-----------------|-----------------|
| Gene            | Sequences       | Accession no.   |
| beta actin      | F: TCACCCACACGTGCCATCTACGA | AC006483.3     |
|                 | R: CAGCAGAGCGCTGTATGCAAATGG |                 |
| il1b            | F: ACATTGCCAACCTCATG    | AJ278240        |
|                 | R: TTGAGCAGGTCCTGTCTGCTG |                 |
| il6             | F: ACTGCCCTTGTCAACACCC  | DQ866150        |
|                 | R: GCCAGAGGTGTCCTGACTA  |                 |
| lys             | F: ACAGGCCGCTACTGGTGTGAC | X59491.1        |
|                 | R: GCTGCTGCCGACATAGAC   |                 |
| mfa             | F: TGAGGGGATGGGAGGACTAC | AJ249755.1      |
|                 | R: TGAGGGCTTTCTCAGGACAGC |                 |

Table 3

Growth performance, feed efficiency and survival rate of rainbow trout after an 8-week rearing with diets supplemented with ZE. Different letters in front of survival rate data indicate significant difference (n = 3; Kruskal-Wallis and Mann-Whitney tests).

<table>
<thead>
<tr>
<th></th>
<th>CT</th>
<th>ZE1</th>
<th>ZE2</th>
<th>ZE3</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IW (g)</td>
<td>21.0</td>
<td>21.5</td>
<td>21.0</td>
<td>21.3</td>
<td>0.905</td>
</tr>
<tr>
<td></td>
<td>0.57</td>
<td>0.29</td>
<td>0.57</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>FW (g)</td>
<td>85.9</td>
<td>91.2</td>
<td>87.1</td>
<td>88.7</td>
<td>0.803</td>
</tr>
<tr>
<td></td>
<td>2.13</td>
<td>6.78</td>
<td>2.98</td>
<td>1.93</td>
<td></td>
</tr>
<tr>
<td>WG (%)</td>
<td>310</td>
<td>324</td>
<td>315</td>
<td>319</td>
<td>0.981</td>
</tr>
<tr>
<td></td>
<td>20.9</td>
<td>34.6</td>
<td>21.2</td>
<td>21.7</td>
<td></td>
</tr>
<tr>
<td>SGR (%)</td>
<td>2.52</td>
<td>2.57</td>
<td>2.54</td>
<td>2.55</td>
<td>0.987</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0.15</td>
<td>0.09</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>FCR</td>
<td>1.26</td>
<td>1.24</td>
<td>1.25</td>
<td>1.24</td>
<td>0.991</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>0.07</td>
<td>0.05</td>
<td>0.04</td>
<td></td>
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<tr>
<td>Survival</td>
<td>86.7</td>
<td>90.5</td>
<td>100</td>
<td>99.1</td>
<td>0.029</td>
</tr>
<tr>
<td></td>
<td>0.02a</td>
<td>0.04</td>
<td>0.00</td>
<td>0.1bc</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. in vitro antibacterial (A) and antioxidant (B) activities of ZE. Asterisks indicate significant differences between ZE and BHT/tetracycline at each concentration (n = 6; r-test).
kidney samples were extracted using commercial extraction kit (Sina-clon, Tehran, Iran). The RNA quality was checked on agarose gel, then treated with DNaseI (Sinaclon, Tehran, Iran). cDNA was synthetized using commercial kit provided by Genet Bio cDNA synthesis kit (Seoul, South Korea). Beta-actin was used as the housekeeping gene and RT-PCR was used to determine the genes’ expression. Relative gene expressions were quantified by DDCt method.

3.4. Statistical analysis

Comparison of in vitro ZE anti-bacterial and antioxidant capacity with the reference materials (BHT and tetracycline) was conducted by t-test, as each concentration separately. Fish survival percentages among different treatments were compared via Kruskal-Wallis and Mann-Whitney tests. Normality and homoscedasticity of the other data were confirmed by Shapiro-Wilk and Levene tests, respectively. Then, the data were subjected to one-way ANOVA and Duncan tests. Data are presented as mean ± SE and SPSS v.22 was used for all statistical analyses.

4. Results

Feeding the rainbow trout with ZE-supplemented diets had no significant effects on FW, WG, SGR and FCR of the fish; however, the ZE2 and ZE3 groups had significantly higher survival rate (P = 0.029) compared to the CT group (Table 3).

In vitro antibacterial examinations showed that ZE had antibacterial activity against A. hydrophila; however, its activity was 30–50% of tetracycline at 1–10 μg/L concentrations (P < 0.001; Fig. 1). Moreover, ZE showed in vitro antioxidant activity, which was significantly (P < 0.001) lower than that of BHT at 12.5–75 μg/L concentrations, but comparable at 100–200 μg/L concentrations (Fig. 1).
Humoral immune responses, including blood leukocyte count (P = 0.015), plasma globulin (P = 0.016) and total Ig (P = 0.005) levels, and plasma lysozyme (P = 0.035) and bactericidal (P = 0.014) activities of the ZE2 and ZE3 groups were significantly higher than those of the CT group. These parameters were statistically similar between the CT and ZE1 groups, and between ZE1 and ZE3 groups. Plasma ACH50 activity of the ZE2 and ZE3 groups were significantly higher than those of the CT treatment; highest expression was related to the ZE2 treatment with no significant difference with that of the ZE3 treatment. Moreover, the ZE2 and ZE3 showed similar bactericidal activities in the fish body. Similar to the present results, Nya and Austin [2] showed that dietary garlic administration significantly increased blood leukocyte count, plasma globulin levels, lysozyme and bactericidal activity against A. hydrophila [17]. Moreover, the essential oil was found to suppress virulence factors of another gram-positive fish pathogen, S. iniae [20].

The present study showed that dietary ZE administration was able to improve humoral and mucosal immune parameters. Such improvements may increase fish resistance to pathogens. Leukocytes have important roles in cellular immune responses to pathogens; thus higher leukocyte count may benefit host to counteract pathogens [35]. Lysozyme is a bactericidal enzyme and plasmatic lysozyme source is blood neutrophils [36]. Plasma globulins have diverse roles including immune and anti-oxidant ones; decline in plasma globulin is an indicator of deteriorated fish health and immune function [37]. Ig are important in antibody molecules that are produced by lymphocyte and have important roles in detecting pathogens [10]. Complement proteins are secreted by the liver with a variety of immune-related roles, including opsonization and killing foreign cells [4]. Higher bactericidal activity of body fluid may be indicator of higher resistance against certain bacterium [5]. Increase in plasma lysozyme activity and total Ig levels in the ZE2 treatment might be due to elevation in leukocyte count (and consequently, neutrophil and lymphocyte count), which indicate improved immune functions of the fish. The present results indicated that ZE contains anti-A. hydrophila compounds, which might be transferred to the fish body. However, further studies are needed to illustrate if higher bactericidal activity of the fish plasma and mucus is directly related to ingredients presented in ZE, or is an indirect effect of ZE on production of bactericidal compound in the fish body. Similar to the present results, Nya and Austin [2] showed that dietary garlic administration significantly increased blood leukocyte count, plasma globulin levels, lysozyme and bactericidal activity against A. hydrophila in rainbow trout; these effects were accompanied by higher resistance of the fish against A. hydrophila infection. Saeli et al. [11] found improved blood leukocyte count, plasma lysozyme, total Ig, and complement, followed by higher resistance against V. ruckeri in rainbow trout fed nettle, Urtica dioica, supplemented diet. Dietary ginger administration significantly increased blood leukocyte count, plasma lysozyme activity, and serum bactericidal activity against A. hydrophila in rainbow trout [1]. Taee, Hajimoradloo [38] reported that myrtle, Myrtus communis, administration to rainbow
trout diet had no significant effects on mucosal lysozyme activity, but improved mucosal bactericidal activity against *A. hydrophila* and *Y. ruckeri*. The present results suggested that dietary ZE was capable to create immune responses at transcriptional levels. Up-regulation of *lys* gene was in line with higher plasma lysozyme activity in the fish treated with ZE2 and ZE3 diets. The results are in line with previous studies using different immunostimulant feed additives in different fish. Dietary commercial prebiotic supplementation significantly up-regulated *lys* gene expression in head kidney of rainbow trout; these fish showed higher resistance against *A. hydrophila* infection [13]. Dietary supplementation with *Ferula assafoetida* significantly up-regulated intestinal *lys* gene expression in *C. carpio* [12]. Inflammatory cytokines have important role in immune system by mediating different immune responses. *tnfa*, *il1b* and *il6* are among the pro-inflammatory cytokines in fish that have connected functions and induce Ig production, cell differentiation, macrophage activation, etc [39]. Such up-regulations in pro-inflammatory cytokine genes may be indicators of stronger immune power, which helps the fish to resist against pathogens. For example, dietary supplementation with *Olea europaea* leaf extract significantly up-regulated *tnfa*, *il1b* and *il6* gene expression in rainbow trout and improved the fish survival against *Y. ruckeri* [14]. Similarly, dietary supplementation with *Aloe vera* significantly increased *tnfa*, *il1b*, *il6*, and *il8* gene expressions in rainbow trout and augmented the fish resistance against a fungal infection [15].

Similar to the present results, Sharififar, Moshafi [40] found that essential oil of *Z. multiflora* [40], as the two compounds were found to be strong antioxidants [42]. The present results showed that antioxidant compounds of ZE may transferred to the fish body that augmented antioxidant power and mitigated lipid peroxidation. TA capacity derives from plasma components with reducing power. Higher Plasma TA capacity in the ZE-treated fish might be due to increase in concentration of antioxidant compounds in plasma. Moreover, ZE stimulated plasma and hepatic SOD and CAT activities, two pioneer antioxidant enzymes. These, cumulatively, led to suppressed lipid peroxidation, and indicator of boosted health. Similar to the present study, essential oil of *T. vulgaris* increased hepatic SOD and decreased hepatic CAT activities and MDA levels in rainbow trout [9]. Teimouri, Yeganeh [43] showed that dietary *Spirulina platensis* meal administration significantly increased serum TA activity and suppressed serum lipid peroxidation in rainbow trout. Moreover, the herbal treatment significantly up-regulated SOD and CAT gene expressions in the fish liver.

It is concluded that ZE has antibacterial and antioxidant properties that can be transferred to fish body. ZE, although is not growth promoter in trout, stimulated antioxidant enzymes and subsidized oxidative stress during the experiment. Moreover, the extract improved innate immune responses, up-regulated cytokine gene expression and augmented humoral and mucosal bactericidal power, that may help the fish to resist against *A. hydrophila*. Dietary supplementation with 2 g/kg ZE is beneficial for rainbow trout as it improves several immune and antioxidant parameters in the fish blood, skin mucus, liver and head kidney.

**CRediT authorship contribution statement**

**Ali Taheri Mirghaed:** Conceptualization, Writing - original draft.

**Seyyed Morteza Hoseini:** Conceptualization, Methodology, Investigation, Writing - original draft.

**Seyed Hossein Hoseinifar:** Methodology,
Fig. 6. Hepatic SOD (A) and CAT (B) activities and MDA (C) content of rainbow trout fed diets supplemented with different levels of ZE. Different letters above the bars show significant differences among the treatments (n = 6; ANOVA and Duncan tests).

Acknowledgement

Hien Van Doan: Investigation, Funding acquisition.

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