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Immune responses to methanolic extract of black cumin (*Nigella sativa*) in rainbow trout (*Oncorhynchus mykiss*)

Yasemin Celik Altunoglu [a], Soner Bilen [b, *], Ferhat Ulu [a], Gouranga Biswas [c]

[a] Kastamonu University, Faculty of Engineering and Architecture, Department of Genetics and Bioengineering, Kastamonu, Turkey
[b] Kastamonu University, Faculty of Fisheries and Aquaculture, Department of Basic Sciences, Kastamonu, Turkey
[c] ICAR- Central Institute of Brackishwater Aquaculture, Kakdwip Research Centre, Kakdwip, South 24 Parganas, West Bengal 743347, India

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ABSTRACT

The immune stimulating effects of the methanolic extract of black cumin (*Nigella sativa*) in rainbow trout (*Oncorhynchus mykiss*) was evaluated. Variable concentrations of black cumin methanolic extract [0 (Control), 0.1 and 0.5 g kg^{-1} of feed] were individually added to the basal diet and rainbow trout was fed for 30 days to assess the innate immune responses and growth performance. Feed conversion ratio significantly decreased in the group fed with 0.5 g kg^{-1} black cumin extract. Respiratory burst activity was observed to be the highest in the 0.5 g kg^{-1} black cumin extract fed group. Lysozyme and myeloperoxidase activities were significantly increased in fish of experimental groups compared to control ($P < 0.05$). TGF-β gene expression increased in black cumin 0.5 g kg^{-1} treated group. IL-1β and TGF-β gene expressions decreased in black cumin 0.1 g kg^{-1} administered group. Expression of IL-12 gene diminished in both the experimental groups. There was no significant difference in survival rates between black cumin extract treated fish groups and control ($P > 0.05$) after challenged with *Aeromonas hydrophila*. The results indicate that the methanolic extract of black cumin is a stimulator of some innate humoral immune responses, but it is ineffective for cytokine-related gene trancriptions in rainbow trout.

1. Introduction

Aquaculture is the fastest growing food industry in the world [1]. However, disease occurrences are the most limiting factors in this sector. In intensive fish culture, in order to combat pathogenic organisms, use of antibiotics or chemicals is the most preferable method to fish farmers [2]. However, it is a well-known fact that these substances lead to antibiotic resistance in bacteria and cause residual effects in the environment [3] and in cultured fish species [4]. Although, vaccination is one of the most referred prophylactic measures to prevent fish diseases, sometimes vaccines are ineffective due to low specificity and there are only few vaccines available against several important pathogens causing economic loss [5]. Therefore, it is of utmost necessity to develop more environment-friendly measures and products to prevent or limit disease outbreaks. Thus, experiments are being conducted on finding environment-friendly, sustainable and cost-effective products.

Recently, herbal immunostimulants, such as tetra *Cotinus coggyria* powder and extract [6–8], Indian lettuce *Lactuca indica* extract [9], oyster mushroom *Pleurotus ostreatus* and nettle *Urtica dioica* extracts [10] and caper *Capparis spinosa* extract [11] have been found effective in fish against important pathogens. These reports demonstrated the potential effects of some medicinal plants on growth promotion, survival and activation of immune system in fish. Turkey has a rich bio-diversity of medicinal herbs and traditional medicines prepared from those are becoming popular. Black cumin (*Nigella sativa*) is one of the important medicinal herbs cultivated in Turkey and until now many beneficial effects, namely bronchodilatatory and anticholinergic [12], antiviral [13], and anti-diabetic [14] properties of this plant have been described. Moreover, in fish, beneficial effects of black cumin seeds on growth and immunity in red tilapia [15] and Nile tilapia [16] have been reported.

Cytokines are the important regulators of the immune system, and they are produced and transported by immune cells to the site of pathogenic invasion. Cytokines induce inflammatory responses that control the capacity of resident and newly arrived phagocytes

* Corresponding author. Kastamonu University, Faculty of Fisheries and Aquaculture, Department of Basic Sciences, Kuzeykent, 37150, Kastamonu, Turkey.
E-mail address: sbilen@kastamonu.edu.tr (S. Bilen).

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to eliminate the invading pathogens [17]. Previously, a few studies examined the effect of the black cumin (*N. sativa*) on immunity in rainbow trout (*Oncorhynchus mykiss*) [18,19]. However, there is lack of adequate knowledge on cytokine mediated and non-specific immune responses to methanolic extracts of black cumin in rainbow trout. Therefore, this experiment aimed at evaluating the effects of dietary methanolic extracts of black cumin on different cytokine mediated immune responses by examining transcription of pro-inflammatory (IL-1β, IL-8, TNF-α1), anti-inflammatory (IL-10), lymphocyte regulatory (TGF-β) and cell mediated immune inducing (IL-12p40) cytokines. Moreover, non-specific immune responses such as respiratory burst activity, lysozyme activity, myeloperoxidase activity and phagocytic activity were assessed in rainbow trout administered with black cumin extract.

2. Material and methods

2.1. Experimental fish and experimental design

Rainbow trout, *O. mykiss* was procured from Kastamonu University, Fisheries Faculty and kept for acclimatization for 3 weeks. Fish (average body weight 15.02 ± 0.01 g) were distributed into 9 aquaria (300 L) at 20 number/aquarium for the experimental trial. Each treatment group had three triplicate glass aquaria assigned randomly. Black cumin extract was added to the experimental diets at 0.1 (BC0.1) and 0.5 g kg^{-1} (BC0.5). The control diet did not include any supplementation [0 g kg^{-1} (BC0)]. Two treatment and control diets were designated as BC0.1, BC0.5 and BC0. The fish were fed with the experimental diets *ad libitum* twice daily for thirty days. Six fish from each experimental unit were anaesthetized using 0.01 mg L^{-1} of Fenoxyethanol at the end of the study. The blood samples of the fish were obtained from the caudal vein and transferred to blood collection tubes containing K3-EDTA for determination of non-specific immune activities. Blood serum was prepared as per our previous protocol [11]. After blood sampling, fish were sacrificed and head kidney was aseptically removed from all the groups' fish and plunged directly in RNAlater solution (Ambion, Austin, TX, USA) for overnight, and after that preserved at −80 °C. Over the experimental period, water quality parameters were recorded as follows: temperature 16 ± 0.1 °C, pH 7.1 ± 0.1, dissolved oxygen 8.10 ± 0.3 mg L^{-1}, conductivity 414 ± 12 uS, total NH_4-N 0.01 ± 0.001 mg L^{-1}, NO_2-N 0.04 ± 0.002 mg L^{-1}, and NO_3-N 0.2 ± 0.01 mg L^{-1}.

All experimental animals were directed according to the relevant international guidelines. Study protocol was approved in advance by the local Ethics Committee for Animal Research Studies at the Kastamonu University (KUHADYEK-18.04.2016-2016.16).

2.2. Preparation of black cumin extract

Black cumin (*N. sativa*) was purchased from a herbalist in Kastamonu. The seeds were cleared with sterilized pure water and dried under natural conditions. A hundred g sample was isolated by percolation with 0.6 L methanol (40%) and filtered. After evaporation of the solvent by a rotary vacuum evaporator product was freeze-dried [20]. Lastly, 7.25 g concentrate was dissolved in 50 mL deionised water. Then, it was mixed with the pellet diet at a concentration of 0,0.1 and 0.5 g kg^{-1} in diet. The pellets were stored at −20 °C.

2.3. Growth parameters

Each fish was weighed after the 30-day trial. Specific growth rate (SGR) was determined by the formulae SGR = 100 × [(Ln final fish weight)-(Ln initial fish weight)]/days fed. Feed conversion ratio (FCR) was estimated as: FCR = feed intake (g)/weight gain (g) × 100. Weight gain (%) = (final fish weight-initial fish weight)/initial fish weight × 100 and protein efficiency ratio (PER) = wet body mass gain/crude protein intake were also calculated.

2.4. Non-spesific immune parameters

2.4.1. Respiratory burst activity

Respiratory burst activity (NBT) was determined according to Siwicki and Anderson [21]. Briefly, heparinized blood sample (0.1 mL) was inserted to the bottom of a glass tube to investigate NBT reduction activity. After that, 0.2% NBT (Sigma Aldrich, Germany) was added and incubated for 30 min at 25 °C. One mL of *N,N*-dimethyl formamide was added to the reaction to terminate activation and the solution was centrifuged at 3000 *g* for 10 min. The optical density (OD) was calculated at 540 nm wavelength by a spectrophotometer.

2.4.2. Lysozyme activity test

Lysozyme activity (LA) was achieved according to Ellis [22] utilizing the turbidimetric assay with minor modifications. In brief, 160 μL suspensions of *Micrococcus lysodeikticus* (MP Biochemicals, Illkirch, France) was mixed with 40 μL of fish serum. Prepared sample was measured after 0 and 4 min at 530 nm wavelength by a microplate reader.

2.4.3. Myeloperoxidase activity test

Serum total myeloperoxidase (MPO) activity was determined according to Sahoo et al. [23]. Thirty microlitre serum was added to 370 μL of Hank's Balanced Salt Solution without Ca^{2+} or Mg^{2+} (Sigma Aldrich, Germany). Hundred μL of 0.1 mg mL^{-1} 3, 3′, 5, 5′ - tetramethylbenzidine dihydrochloride and 0.006% fresh hydrogen peroxide were mixed with the diluted serum. The reaction was evaluated kinetically by determining the increase in absorbance ratios. Reaction velocities were measured as IU, defined as the amount of enzyme required to produce a 0.001 increase in absorbance per min for 0.5 mL mixture (ΔA 450/min/mL).

2.4.4. Phagocytic activity test

The phagocytic activity (PA) was determined according to Siwicki et al. [24]. Fifty microlitre of inactivated *Escherichia coli* (ThermoFischer Scientific, USA) was added to 50 μL of blood sample and incubated for 30 min at 27 °C in a carbon dioxide incubator. After that, this mixture was smeared onto glass slides and stained using Giemsa stain. The phagocytic activity was determined using the following formulae: PA (%)= (number of phagocytic cells/number of total cells) × 100.

2.5. Analysis of cytokine gene expression

2.5.1. RNA extraction

Approximately 20 mg of kidney sample was used for RNA extraction after dissecting out the rainbow trout from all experimental groups. Total RNA isolation was carried out using RNeasy Plus Micro RNA isolation kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. Quantity and quality of all RNA samples were checked using a NanoDrop spectrophotometer, ND-1000 (Thermo Scientific, Wilmington, DE, USA).

2.5.2. Complementary DNA (cDNA) synthesis

Extracted RNAs were subjected to treatment with 1 U DNase I (ThermoFischer Scientific, USA) in order to remove genomic DNA completely. cDNA was synthesized from 1 μg total RNA using Revert Aid RT synthesis kit (Thermo, Lithuania). cDNA reaction mixture included 1 μg of template RNA, 1 mM of dNTP mix, 15 pmol/μL oligo

dT primer, 20 U/μL Revert Aid M-MuLV RT enzyme, 4 μL 5× reaction buffer and 6 μL of nuclease free water. The reaction mixture incubated for 60 min at 42 °C for cDNA synthesis in thermal cycler (ThermoFischer Scientific).

2.5.3. Quantitative real-time PCR (qRT-PCR) analysis

After cDNA synthesis, qRT-PCR analysis was performed by utilizing Rotor-Gene qPCR detection system (Qiagen, Germany) and Rotorgene SYBR Green PCR kit (Qiagen, Germany). Gene specific primer sequences and references are enlisted in Table 1. qRT-PCR mixture included 12.5 μL of 2× SYBR Green Master Mix, 0.1 μg of template DNA, 0.4 μM of each gene specific forward and reverse primer (IL-1β, IL-8, IL-12p40, TNF-α1, TGF-β, IL-10 and β-actin as reference) and distilled water to the final volume of 25 μL. qRT-PCR steps involved were: denaturation at 95 °C for 10 s, then annealing and extension steps together at 60 °C for 40 s. Then, samples were denatured at 95 °C and held at 65 °C. Fluorescence signals were picked up at 530 nm wavelength from 60 °C to 95 °C at every 0.5 °C per second to implement melting curve analysis. qRT-PCR was achieved with three different samples from experimental and control groups, and three technical replicates were evaluated for each sample in all experiments. The ΔCT and ΔΔCT were estimated by $\Delta CT = CT_{\text{target gene}} - CT_{\text{reference}}$ and $\Delta\Delta CT = \Delta CT_{\text{treated sample}} - \Delta CT_{\text{control sample}}$. The results were analyzed by $2^{-\Delta\Delta\ CT}$ method to estimate relative gene expression pattern [25]. The standard errors of mean between replicates were computed simultaneously.

2.6. Challenge test

Challenge test was performed as described in our previous study [10] (Bilen et al., 2016). Briefly, *Aeromonas hydrophila* (ATCC 20662) with 1×10^7 CFUs mL^{-1} mixed in 100 μL PBS was injected to all fish intraperitoneally at the end of dietary feeding trial (after 30 days) and survival of groups was observed during 14 days post injection. Survival rate was determined using this formulae: SR (%)= (number of fish survived/number of fish injected) × 100.

2.7. Statistical analysis

Duncan's multiple range test and one-way analysis of variance (ANOVA) were employed to assess significant differences in growth parameters, non-specific immune parameters and cytokine gene expressions and survival at $P < 0.05$. All statistical analyses were performed using the statistical software package SPSS for Windows 22 program (SPSS Inc., Chicago, IL, USA).

3. Results

Growth promoting results were presented in Table 2. There were no differences among the experimental groups for final weight, weight gain, SGR or PER ($P > 0.05$). Feed consumption significantly decreased in BC0.1 and BC0.5 groups ($P < 0.05$) compared to control. However, FCR was significantly low only in case of BC0.5 group ($P < 0.05$).

NBT reduction activity was elevated in two treatment groups. The highest significant NBT reduction activity was recorded in BC0.5 group (1.86 ± 0.23), followed by BC0.1 group (0.80 ± 0.33) ($P < 0.05$) (Fig. 1).

Lysozyme activity increased significantly in both the black cumin extract administered groups compared to that of the control ($P < 0.05$) (Fig. 2).

MPO activity levels were also in the line with lysozyme activity results. MPO activity in black cumin extract treated groups increased significantly when compared to the control group ($P < 0.05$) (Fig. 3).

Phagocytic activity was not affected in any treatment groups when compared to the control group ($P > 0.05$) (Fig. 4).

Expression of six cytokine genes, such as IL-1β, IL-8, TNF-α, TGF-β, IL-10 and IL-12 was analyzed using qRT-PCR. Cytokine gene expression results are illustrated in Fig. 5. Transcription of pro-inflammatory cytokine (IL-1β, IL-8, TNF-α1) genes was not affected by the treatment of black cumin extract in rainbow trout. TGF-β gene expression elevated only in the BC0.5 group compared to the control ($P < 0.05$). Moreover, there was a down-regulation of IL-10 and IL-12 genes in black cumin extract treated fish.

In Fig. 6, survival rates of different experimental fish groups which were challenged with *A. hydrophila* are illustrated. Survival rate was not significantly different ($P > 0.05$) among different treatment groups and control.

4. Discussion

In this study, methanolic extract of black cumin was tested as a feed additive for growth promotion and immune stimulation in rainbow trout. Although, many researchers found the stimulating or regulating effects of different application methods of black cumin [18,19,26], our results demonstrate that, methanolic extract of black cumin is not effective for induction of immune responses related to cytokines in rainbow trout.

Dietary application of medicinal plant has positive effects on fish growth. In this study, growth was not affected by administration of black cumin extract. However, FCR and feed consumption decreased significantly. Similar to our result, Bilen et al. [27]

Table 1
Gene specific primers with their sequences and references used for qRT-PCR in the study.

Gene	Primer sequence	Reference
β-actin	F: 5′- ATGGAAGGTGAAATCGCC- 3′ R: 5′- TGCCAGATCTTCTCCATG- 3′	Sigh et.al. 2004 [50]
IL-1β	F: 5′- ACCGAGTTCAAGGACAAGGA- 3′ R: 5′- CATTCATCAGGACCCAGCAC- 3′	Awad et. al. 2011 [51]
IL-8	F: 5′- CACAGACAGAGAAGGAAGGAAAG- 3′ R: 5′- TGCTCATCTTGGGGTTACAGA- 3′	Awad et. al. 2011 [51]
TGF-β	F: 5′- AGATAAATCGGAGAGTTGCTGTG- 3′ R: 5′- CCTGCTCCACCTTGTGTTGT- 3′	Awad et. al. 2011 [51]
IL-12p40	F: 5′ GAACCCAGACGACGATGATT- 3′ R: 5′- GTTCAAACTCCAACCCTCCA- 3′	Komatsu et. al. 2009 [52]
TNF-α1	F: 5′- CAAGAGTTTGAACCTTGTTCAA- 3′ R: 5′- GCTGCTGCCGCACATAGAC- 3′	Panigrahia et. al. 2007 [53]
IL-10	F: 5′- CGACTTTAAATCTCCCATCGAC- 3′ R: 5′- GCATTGGACGATCTCTTTCTTC- 3′	Raida et. al. 2011 [54]

Table 2
Effects of different black cumin extract doses on performances of rainbow trout after the 30-day trial.

	Control (BC0)	BC0.1	BC0.5
Initial weight (g)	15.03 ± 0.82[a]	15.05 ± 0.42[a]	15.13 ± 0.57[a]
Final weight (g)	33.17 ± 0.19[a]	32.18 ± 0.01[a]	34.01 ± 0.20[a]
Total feed consumption (g)	533.32 ± 8.98[a]	508.76 ± 7.01[b]	515.42 ± 8.58[b]
Weight gain (%)	120.69 ± 12.21[a]	113.82 ± 12.98[a]	124.79 ± 11.2[a]
FCR	0.85 ± 0.12[a]	0.84 ± 0.01[a]	0.92 ± 0.03[b]
SGR (% day^{-1})	2.64 ± 0.25[a]	2.53 ± 0.02[a]	2.70 ± 0.1[a]
PER	2.27 ± 0.11[a]	2.24 ± 0.01[a]	2.44 ± 0.09[a]

BC0, BC0.1 and BC0.5 indicate black cumin extract doses at 0, 0.1 and 0.5 g kg^{-1} feed, respectively. Values are mean ± SE of 25 fish; Different superscript letters in a row show significant differences among the experimental groups ($P < 0.05$). FCR, feed conversion ration; SGR, specific growth rate; PER, protein efficiency ratio.

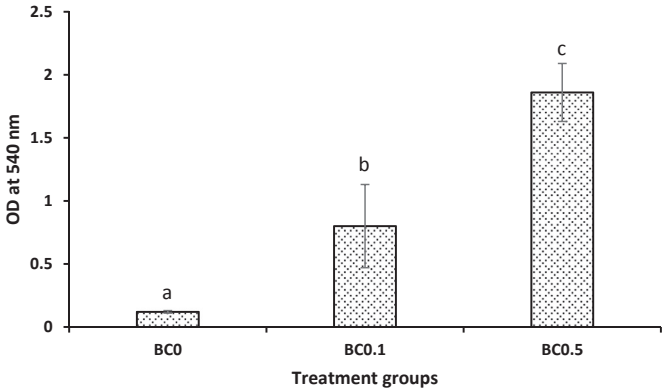

Fig. 1. Nitroblue tetrazolium (NBT) assay in rainbow trout fed with diets supplemented with different doses of black cumin extract. BC0, BC0.1 and BC0.5 indicate black cumin extract doses at 0, 0.1 and 0.5 g kg^{-1} feed, respectively. Superoxide anion production was measured as optical density (OD) (mean ± SE; n = 3) at 540 nm in the NBT assay. Different letters on bars (mean ± S.E.; n = 3) show significant differences among trial groups ($P < 0.05$).

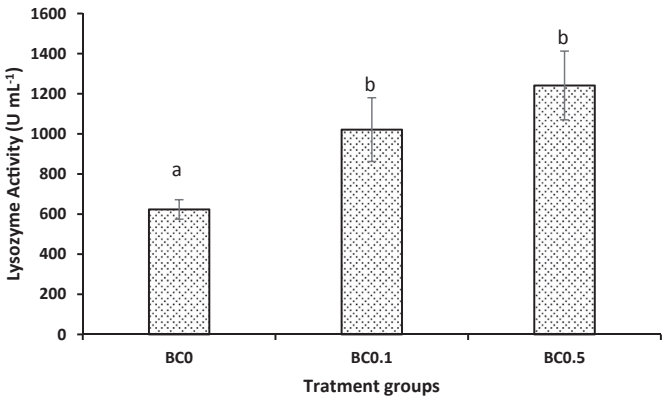

Fig. 2. Lysozyme activity in rainbow trout fed with diets supplemented with different doses of black cumin extract. BC0, BC0.1 and BC0.5 indicate black cumin extract doses at 0, 0.1 and 0.5 g kg^{-1} feed, respectively. Different letters on bars (mean ± S.E.; n = 3) show significant differences among trial groups ($P < 0.05$).

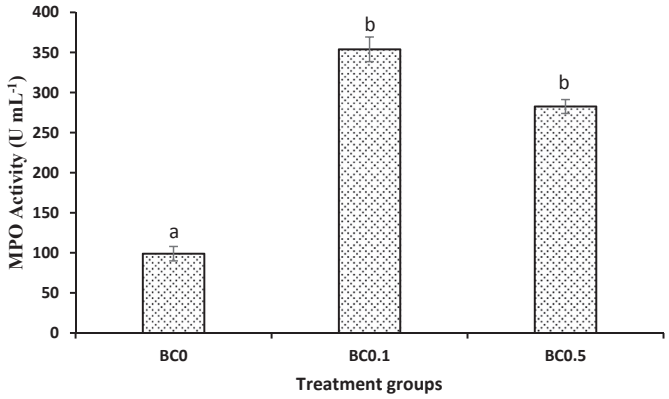

Fig. 3. Myeloperoxidase activity in rainbow trout fed with diets supplemented with different doses of black cumin extract. BC0, BC0.1 and BC0.5 indicate black cumin extract doses at 0, 0.1 and 0.5 g kg^{-1} feed, respectively. Different letters on bars (mean ± S.E.; n = 3) show significant differences among trial groups ($P < 0.05$).

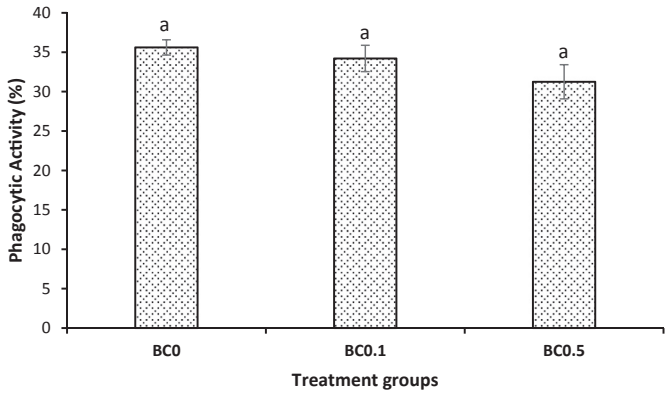

Fig. 4. Phagocytic activity in rainbow trout fed with diets supplemented with different doses of black cumin extract. BC0, BC0.1 and BC0.5 indicate black cumin extract doses at 0, 0.1 and 0.5 g kg^{-1} feed, respectively. Different letters on bars (mean ± S.E.; n = 3) show significant differences among trial groups ($P < 0.05$).

demonstrated that when fed the rainbow trout with tetra (*Cotinus coggygria*) and laurel leaf (*Laurus nobilis*) did not cause any negative or positive effects on growth. Tetra extract treated koi carp (*Cyprinus carpio*) had no effect on growth [7,8]. On the contrary, Abdelhamid and Soliman [28] showed that doses of 1% and 2% of fenugreek seeds increased SGR and feed utilization in Nile tilapia (*O. niloticus*) fry.

Non-specific immune responses such as NBT, LA, PA and MPO activity were examined in black cumin extract treated rainbow trout. Elevated NBT was recorded in all black cumin extract treated groups when compared to control. In the BC0.5 group, the highest level of NBT reduction was determined. Bilen et al. [6] observed an increase in intra- and extracellular superoxide anion accumulation in rainbow trout leucocytes treated with tetra leaf powder but no differences were caused by laurel. Similar results were obtained in the common carp (*Cyprinus carpio* L.) induced with *Rehmannia glutinosa* [29] and Nile tilapia (*Oreochromis niloticus*) treated with mistletoe extract (*Viscum album coloratum*) [30].

Lysozyme, an important enzyme, prevents the deleterious effects of pathogenic organisms such as viruses, parasites and

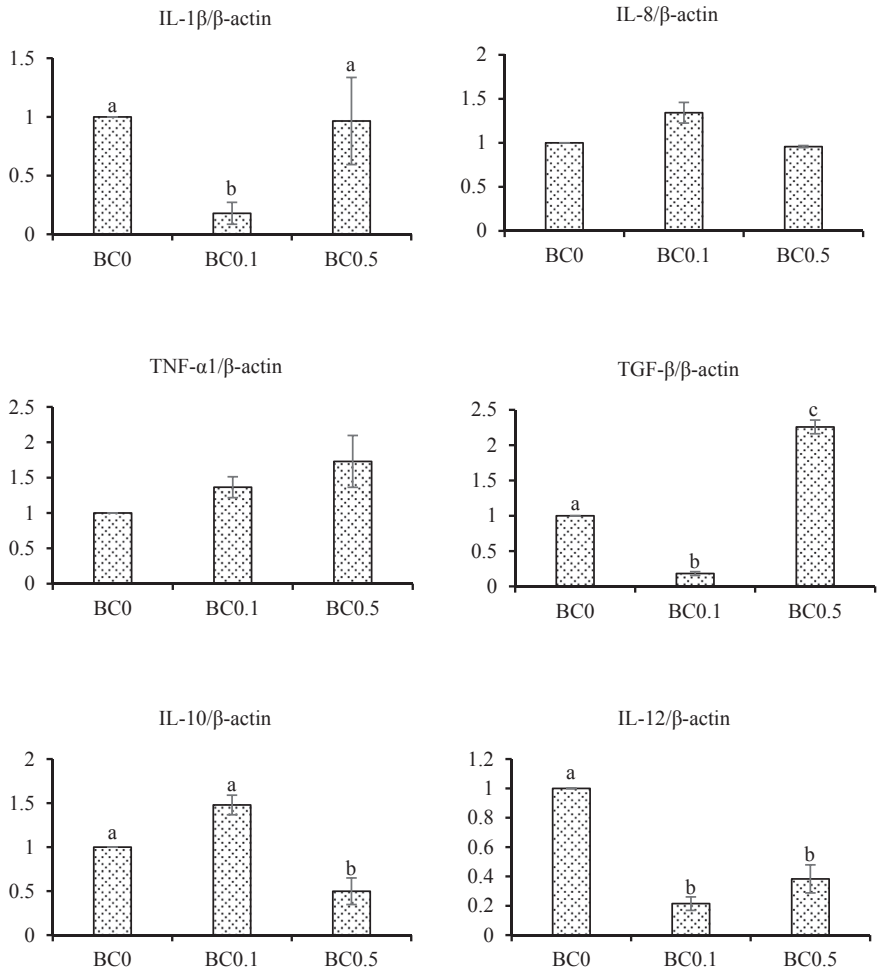

Fig. 5. Comparison of relative gene expression (mean ± SD; n = 3) levels of cytokines in the head kidney cells of rainbow trout fed with diets supplemented with different doses of black cumin extract. BC0, BC0.1 and BC0.5 indicate black cumin extract doses at 0, 0.1 and 0.5 g kg^{-1} feed, respectively. Different letters on bars denote significant differences among groups ($P < 0.05$).

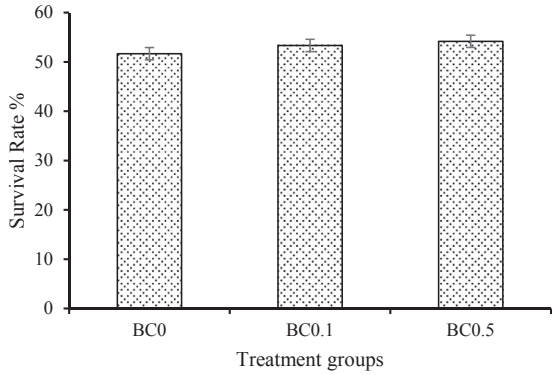

Fig. 6. Survival of black cumin extract administered rainbow trout after experimental challenge with *Aeromonas hydrophila*. BC0, BC0.1 and BC0.5 indicate black cumin extract doses at 0, 0.1 and 0.5 g kg^{-1} feed, respectively.

bacteria [31], and the concentration of this enzyme in the fish blood increases during infections or invasions of other materials [32]. In this study, similar to NBT, LA significantly increased at a similar level in both black cumin extract treatment groups compared to the control. Christybapita et al. [33] demonstrated an increased level of lysozyme in *Oreochromis mossambicus* induced with *Eclipta alba*. Moreover, Baba et al. [34] determined improved positive effect of *L. edodes* extract in *O. mykiss*.

When an invasion initiates in the fish body, several substances were oxidized by myeloperoxidase released from macrophage cells [35] and neutrophil cells [36]. MPO activity was enhanced in both BC0.1 and BC0.5 black cumin treatment groups with the highest MPO activity determined in the former group in the current study. Comparably, orally administered caper in rainbow trout (*O. mykiss*) raised the MPO activity [10]. Moreover, Alexander et al. [37] recorded an elevated MPO activity in *Oreochromis mossambicus* induced with *Tinospora cordifolia* leaf water-soluble fraction.

Phagocytic activity is an important non-specific cellular response that was not affected by black cumin extract treatment. Bilen et al. [6] established a similar result when the rainbow trout treated with different doses of laurel leaf powder. On the contrary, an enhanced phagocytic activity of blood leucocytes was reported in tilapia which was fed with different doses of *Astragalus* [38]. In addition, an improvement in the phagocytic activity levels were observed in the common carp treated with *Astragalus radix* and *Ganoderma lucidum* which are Chinese herbs [39].

IL-1β is an important pro-inflammatory cytokine and organizes immune system via activating lymphocytes [40,41]. Our study

exhibited a down-regulation in IL-1β gene expression levels in BC0.1 group and no differences were observed between BC0.5 and control groups. On the contrary, expression of IL-1β gene was elevated in head kidney of rainbow trout fed with the 0.1 g kg^{-1} caper extract [11]. TNF-α is another important pro-inflammatory cytokine that manages respiratory burst activity and phagocytosis [42]. In our study, TNF-α gene expression was not affected in any experimental groups similar to the result of PA. However, Awad et al. [43] reported an increased TNF-α gene expression in response to fenugreek (*Trigonella foenum graecum*) in gilthead seabream (*Sparus aurata* L.). Moreover, TNF-α expression was also elevated by treatment of Chinese herbal medicine sinomenine and Liang Miao San [44], and caper bud extracts [45]. The pro-inflammatory IL-8 gene was not affected by black cumin extract treatment in rainbow trout. Overall, pro-inflammatory cytokine gene (IL-1β, IL-8 and TNF-α) responses examined here could not be induced by the administration of black cumin extract in rainbow trout.

TGF-β regulates lymphocyte proliferation, differentiation, and survival rates and added to this essential role, it sustains tolerance to immune system. Furthermore, TGF-β regulates inflammatory responses by controlling activation and chemotaxis of natural killer cells, dendritic cells, lymphocytes, mast cells, macrophages, and granulocytes [46]. In the current study, up -regulated levels of TGF-β gene was determined in the BC0.5 group but a decreasing level was observed in the BC0.1 group. IL-10 is known as anti-inflammatory cytokine that regulates inflammation and acts as a suppressor [47]. There were no differences in IL-10 expression between BC0.1 and control groups. However, in the BC0.5 group, IL-10 gene expression significantly decreased. Therefore, our results suggest that black cumin extract is not an inducer of anti-inflammatory response in rainbow trout.

IL-12 is active in its heterodimeric form, which is composed of two covalently linked peptide chains: a 35-kDa chain termed IL-12p35 (or IL-12α) and a 40-kDa chain termed IL-12p40 (or IL-12β) [48]. IL-12 controls cell-mediated immune responses and ensures immune protection against to parasites, viruses and intracellular bacteria via inducing IFN-γ production from Th1 and NK cells [49]. In the present study, as a representative of IL-12, the expression of IL-12p40 gene was assessed. Our results showed that IL-12p40 gene expression decreased in head kidney cells of rainbow trout in both treatment groups. Hence, it is inferred that black cumin extract is ineffective in mounting cell-mediated immune response in rainbow trout.

Challenge test results showed no difference in survival rate in control and treatment groups. Similarly, oyster mushroom did not cause any difference in survival of rainbow trout after challenged with the *A. hydrophila* [10]. Therefore, our results suggest that administration of dietary black cumin extract to rainbow trout did not elevate fish resistance to *A. hydrophila*.

5. Conclusion

Black cumin has been proved as an effective immunostimulant or antioxidant in higher animals. On the contrary, our results suggest that the methanolic extract of black cumin could not induce cytokine-mediated immune responses in rainbow trout. Therefore, use of methanolic extract of black cumin at 0.1 or 0.5 g kg^{-1} of feed as immune inducer is not recommended for rainbow trout. Further research is needed to examine whether higher dose of black cumin methanolic extract is effective for this fish.

Acknowledgments

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