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Research Article

Effects of encapsulated garlic (*Alium sativum*), black seed (*Nigella sativa*), and black caraway (*Carum carvi*) essential oils on hematological and immunological parameters in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

This study examined the dietary impacts of essential oils from garlic (*Allium sativum*), black seed (*Nigella sativa*), and black caraway (*Carum carvi*) on the hematological and immunological parameters of rainbow trout (*Oncorhynchus mykiss*). A total of 200 fish, each with an average initial weight of 225 ± 10 g, were divided into five groups, each consisting of three replicates with 40 fish per group. The fish were fed for eight weeks with a diet supplemented with 0.2% of the herbal essential oils, while a control group received an unsupplemented diet. The hematological indices, including hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), the number of red blood cells (RBCs) and white blood cells (WBCs), and immune responses, including serum lysozyme and complement levels (C3 and C4), were studied. The analysis showed no significant differences in erythrocyte indices or leukocyte profiles between the control group and the groups receiving essential oils ($p>0.05$). The control group exhibited the highest values for RBC, Hb, MCV, MCH, MCHC, Hct, and WBC, while the highest percentages of lymphocytes and monocytes were found in the black seed and black caraway groups, respectively. On day 28 of the experiment, there were no significant differences in lysozyme activity among the control and treatments ($p>0.05$), while a significant difference was observed for black caraway essential oil on day 56 ($p<0.05$). A significant difference between the control group and all treatments was reported for C3 value on day 28 ($p<0.05$). Comparisons from days 28 to 56 indicated significant changes for the control versus mixed essential oil, garlic essential oil, and black caraway essential oil ($p<0.05$). C4 and plasma protein values showed no significant differences between the control group and treatments on both days ($p>0.05$), however, significant differences were noted when comparing values from days 28 and 56 for the control and treatments of mixed essential oil, black seed essential oil, and black caraway essential oil ($p<0.05$). The results of this study demonstrated that dietary garlic essential oil significantly enhanced immune functions and *TNF-α* expression levels in rainbow trout compared to black caraway or black seed essential oils ($p<0.05$). However, all essential oils boosted immune parameters like lysozyme activity and complement levels, but garlic essential oil showed the most promising results.

Introduction

Aquatic organisms are regarded as valuable food sources, delivering ample protein, essential amino acids, and other crucial nutrients. Consuming fish and other aquatic life regularly can provide the necessary amino acids and specific vital minerals that support overall health and growth (Mansour *et al*., 2022). Conversely, fish farming in nations with abundant water resources has the potential to generate export revenue for those countries. However, despite the promising prospects of aquaculture, several challenges persist, such as water quality issues, disease transmission, nutritional deficiencies, and occasionally stressful environmental conditions (Lieke *et al*., 2020; Ziarati *et al*., 2022).

Aquatic animals are vulnerable to various diseases and infections. These illnesses can be transmitted through water, food, infectious agents, or live carriers. To combat disease-related issues, the use of disinfectants, vaccines, continuous monitoring, and appropriate healthcare is essential (Assefa and Abunna, 2018; Dezfuly *et al*., 2020; Kumar *et al*., 2024). Providing well-balanced and appropriate nutrition for aquatic animals is equally important. Nutritional deficiencies or imbalances, including improper amino acid ratios and vitamin and mineral deficiencies, can result in stunted growth, weakened immune systems, and nutrition-related illnesses (Awuchi *et al*., 2020). Environmental factors such as temperature, light, oxygen availability, and water flow also significantly influence aquatic life. Suboptimal conditions can lead to stress, hindered growth, increased susceptibility to disease, and even mortality among aquatic animals. While various solutions have been proposed and occasionally implemented to tackle these challenges, not all have been fully effective (Reid *et al*., 2019; Menon *et al*., 2023).

The challenge of antibiotic use in the field of aquaculture is related to the excessive and inappropriate administration of antibiotics in aquatic production. This issue is recognized as a serious problem within the aquaculture industry (Santos and Ramos, 2018). Enhancing immunity and, consequently, improving the performance of the body's defense system is one of the most suitable approaches to prevent the occurrence of diseases in fish. In fish, the response to pathogens involves both innate and adaptive immunity; however, it has been observed that the activation of the innate immune response is more significant and relevant compared to the adaptive immune response (Kordon *et al*., 2018). Therefore, one of the most accepted and promising methods to strengthen and improve the immune system in aquaculture is the use of immune system stimulants (Farooqi and Qureshi, 2018; Mohanasundari *et al*., 2022).

Immunostimulants are biological compounds, either natural or synthetic, that can activate the immune system. These compounds primarily target the non-specific (innate) immune system, which is particularly beneficial for fish, and may also enhance antibody production (Vijayaram *et al*., 2022). They can be easily administered to smaller fish, and using them prior to an anticipated disease outbreak can boost immunity and resistance to infections. Although immunostimulation is not as effective as vaccination and does not provide long-term immunity or the rapid response associated with chemical treatments, it offers advantages such as minimal side effects, broader efficacy, and no risk of pathogen resistance (Kumar *et al*., 2023). Various types of immunostimulants, including synthetic, biological, animal, and plant-derived compounds, have been utilized in aquaculture. Among these, plant-based immunostimulants are particularly promising due to their cost-effectiveness, availability, and environmental safety, as they do not leave harmful residues or contribute to pathogen resistance (Aly *et al*., 2024).

Essential oils are natural compounds derived from plants that exhibit antimicrobial, antifungal, and anti-inflammatory properties. These oils serve as a valuable source of antioxidants and disinfectants (D'agostino *et al*., 2019). In aquaculture, essential oils can be used to naturally enhance the immune system of fish and improve their growth performance. They influence fish in various ways (Souza *et al*., 2019). Some essential oils possess antimicrobial and antifungal characteristics, which help fish resist infections and diseases, promoting overall health (Sutili *et al*., 2018). Others have anti-inflammatory effects that increase fish resilience to environmental stressors such as temperature fluctuations, salinity changes, and chemical exposure (Majolo *et al*., 2018). Additionally, certain essential oils have calming effects that assist fish in coping with adverse conditions. The use of these multifunctional essential oils can lead to improved growth and performance in farmed fish (Abdel-Latif *et al*., 2020). Numerous studies have demonstrated the antimicrobial, anti-stress, and immune-boosting effects of garlic, black caraway, and black seed plants (Roohi *et al*., 2017; Sutili *et al*., 2018; Souza *et al*., 2019; Karimi Pashaki *et al*., 2020; Adineh *et al*., 2020; Yousefi *et al*., 2021; Zorriehzahra *et al*., 2021; Kumar *et al*., 2023). Given the established antimicrobial properties and beneficial effects of these plants, along with the inherent instability of their essential oil compounds, this study aims to evaluate the impact of dietary supplementation with encapsulated powders of garlic, black seed, and black caraway essential oils on the hematological and immunological parameters in rainbow trout (*Oncorhynchus mykiss*).

Materials and methods

Essential oil preparation, related evaluations, and determination of essential oil compounds

The desired essential oils were purchased from Tabib Daru Company (Iran, May 2022). The effective ingredients of the herbal compounds were determined using the GC-MS (Gas Chromatography-Mass Spectrometry) method, where helium gas was used as the carrier, and a one-microliter sample of the essential oil was injected into the device's chamber for analysis (Al-Rubaye *et al*., 2017). The GC/MS analysis was performed on an Agilent GC 7890A system (Agilent Company, USA) equipped with an HP-5MS capillary column (30 m × 0.25 mm, film thickness 0.25 µm), coupled with an EI model MS 5975 C (Agilent Company, USA). The oven temperature program, carrier gas flow rate, inlet split ratio, and injector, source, and quadrupole temperatures were specified. The essential oil compounds were identified by comparing their retention times and mass spectrometry fragmentation patterns against those in the Wiley 7n.1 and NIST (National Institute of Standards and Technology) computer-stored libraries.

Preparation of essential oil capsule form

The essential oils from garlic, black seed, and black caraway were encapsulated using the Fluid Bed Dryer technique. First, lecithin was dissolved in ethanol and heated to 40°C, while thymol was dissolved in methanol. These two solutions were then mixed and poured into a balloon, which was rotated at 240 rpm and 60°C to form a thin film on the wall. Next, distilled water was added to the flask, and the mixture was rotated without a vacuum at 68°C for 30 minutes. The resulting solution was then stirred at 800 rpm and 50-60°C for 20 minutes. Finally, the solution was subjected to three cycles of ultrasonication, with each cycle lasting 3 minutes and with 1-minute intervals between the cycles. This process led to the successful encapsulation of the essential oils using the Fluid Bed Dryer method. The encapsulated essential oils were then used to evaluate their antibacterial properties against several fish pathogenic bacteria (Lammari *et al*., 2020).

Classification of fish and their feeding with encapsulated essential oils

The study was conducted at the Coldwater fish breeding and rearing farm in Firouzkoh city, Iran during the autumn of 2023. Two hundred rainbow trout with an average initial weight of 225±10 grams were randomly divided into 5 groups, each with 40 fish. The fish were reared in 5 ponds with similar water quality parameters, including a temperature range of 9.8 to 13°C, pH between 6.5 and 7.3, and dissolved oxygen concentration of 6 to 7 mg/L. The experimental groups included a control group fed the basic diet, a black caraway group (basic diet+black caraway), a garlic group (basic diet+garlic), a black seed group (basic diet + black seed), and a black seed, garlic, and black caraway group (basic diet+a combination of the three essential oils). The essential oils were added to the feed pellets at a concentration of 3 grams per 15 kg of feed. Throughout the 8-week feeding trial, the fish were manually fed twice daily at a rate of 2% of their body weight.

Hematological profile

Blood sampling was conducted on days 0, 28, and 56 to evaluate the effect of different levels of encapsulated powders on the blood indices of rainbow trout and compare between the groups. The fish were first anesthetized using clove powder, and blood was collected from the caudal vein and the heart using a 2 mm syringe. Red blood cell count and differential white blood cell count (neutrophils, lymphocytes, eosinophils, and monocytes) were determined by preparing blood smears. Hematocrit was measured using the microcentrifuge method (Witeska *et al*., 2022).

Immunological parameters

Fish blood samples were collected on days 0, 28, and 56 using 2 mL syringes to evaluate the immunological parameters. The activity of lysozyme was determined based on the method of Clerton *et al*. (2001), using the lysis of the gram-positive bacterium *Micrococcus lysodeikticus*, which is sensitive to the lysozyme enzyme. The lysozyme level was measured using the turbidimetric method, as described by Ellis (1999), with chicken egg white lysozyme (1 mg/mL) as the standard, with minor modifications. Serum (25 μL per well) was added in triplicates to a 96-well plate, and 175 μL of a 0.2 mg/mL suspension of *Micrococcus lysodeikticus* in 0.5 M phosphate-buffered saline (pH 6.2) was added. As a negative control, PBS was used instead of serum. The decrease in OD at 530 nm was recorded after 1 and 5 minutes of incubation at 22°C. One unit of lysozyme activity was defined as the decrease in absorbance of 0.001 per minute. The total immunoglobulin level was measured using the method of Siwicki *et al*. (1998). Serum was separated from the blood and subjected to cellulose acetate electrophoresis, and the immunoglobulin content was determined by densitometry. The total protein and albumin levels were measured using an auto-analyzer BT1500. The alternative complement pathway activity was determined based on the hemolysis of rabbit red blood cells, following the method of Amar *et al*. (2000). The red blood cell count was determined using a hemocytometer after washing, and different dilutions were prepared in tubes. The alternative pathway activity of the fish serum was measured by adding the serum to the diluted rabbit red blood cell samples.

TNF-α gene expression

After treating the fish with the encapsulated essential oils, 100 mg samples of the kidney, liver, and gills were collected from the fish. The total RNA from the samples was extracted using an RNA extraction kit, and the quality of the RNA was assessed using a nanodrop instrument. The extracted RNA was then converted to cDNA and stored at -20°C until the qPCR analysis. For the qPCR reaction, the β-actin gene was used as the reference gene. To analyze the qPCR results, the C_t values of the TNF-α gene for each fish (treated and control) were obtained and the gene expression level of TNF-α was calculated using the Livak or ΔΔCt method, which is reported as the Fold change (FC). The FC value represents the degree of increase or decrease in gene expression compared to the value of "one" (Hosseini *et al*., 2016).

Essential oil composition

In Table 1 the detailed chemical composition of garlic essential oil as determined by GC-MS analysis was presented. In Table 2 a detailed chemical analysis of black caraway essential oil conducted using gas chromatography-mass spectrometry (GC-MS) was provided. The GC-MS analysis of black seed essential oil, as shown in Table 3, reveals its complex chemical composition.

Table 1: Garlic essential oil composition by GC-MS analysis.

Component	Percentage (%)
Allyl sulfide	11.12
Allyl disulfide	46.15
Diallyl trisulfide	31.52
Diallyl tetrasulphide	10.60
2-Butyne, 1,4-bis(ethylthio)	0.16
1,3-Butadiene, 3-methyl-1,1-bis(methylthio)	0.09
1-Allyl-3-(2-(allylthio) propyl) trisulfane	0.20

Optical rotation: 0.8, Refractive index: 1.4714, Specific gravity: 1.0417

Data analysis

The data were analyzed statistically to assess the impact of garlic, black caraway, and black seed essential oils on hematological and immunological parameters. The Kolmogorov-Smirnov test was employed to check for normality in the data. One-way analysis of variance (ANOVA) was used to identify significant differences among treatments. To compare the means, Duncan's test was applied at a significance level of 0.05. The statistical analyses were carried out using SPSS 21 and Excel 2013 software.

Table 2: Black caraway essential oil composition by GC-MS analysis.

Composition	Percentage (%)	Acceptable Limit (%)
α-Pinene	0.31	-
Sabinene	0.21	-
β-Myrcene	0.56	0.1 - 1.0%
3-Carene	0.09	-
Limonene	44.34	30.0 - 45.0%
Linalool	0.22	-
α-Terpineol	0.08	-
Cis-Dihydrocarvone	0.93	-
Trans-Dihydrocarvone	0.18	Less than 2.5%
d-Carvone	53.10	50.0 - 65.0%

Optical Rotation: 0.74, Refractive Index: 1.485, Specific Gravity: 0.910

Results

Lysozyme activity and immunological parameters in fish serum

The comparison of the measured values of lysozyme activity on day 28 of the experiment showed no significant difference between the control group and the treatments of mixed essential oil, garlic essential oil, black seed essential oil, and black caraway essential oil (p>0.05). However, on day 56, a significant difference was observed in the lysozyme activity between the black caraway essential oil treatment and the control group as well as the other treatments (p<0.05). The comparison of the results from days 28 and 56 also showed a significant difference in the measured lysozyme activity in the control group, and the treatments of mixed essential oil, black seed essential oil, and black caraway essential oil (p<0.05).

The comparison of the measured C3 values on day 28 showed a significant difference between the control group and the treatments of mixed essential oil, garlic essential oil, black seed, and black caraway (p<0.05), while there was no significant difference among the treatment groups of mixed essential oil, garlic essential oil, black seed essential oil, and black caraway essential oil (p>0.05).

Table 3: Black seed essential oil composition by GC-MS analysis.

Composition	Percentage (%)
Myrcene	0.9
b-Pinene	1.2
3-Methyl Nonane	0.8
2(1H)-Naphthalenone	1.5
Sabinene	2.1
a-Pinene	1.8
Thymol	6.4
Limonene	3.2
1-Ethyl-2,3-dimethyl benzene	0.4
a-Phellandrene	1.9
n-Hexadecane	0.4
borneol	0.9
n-Decane	0.7
1,8-Cyneole	1.2
1-Methyl-3-propyl benzene	0.5
Carvone	1.9
Uvidine	2.4
Terpinyl acetate	0.09
Terpinolene	0.2
Fenchone	0.8
Nerol	0.8
Carvacrol	7.4
p-Cymene-8-ol	0.6
Apiole	1.7
Estragole	2.1
Dihydrocarvone	0.8
p-Mentha-2,8-dien	0.9
longicyclene	1.3
a-Longipinene	0.9
α- Thujene	5.4
Trans-Anethole	14.4
g-Terpinene	0.7
Myristicin	1.8
camphor	1.3
n-Tetradane	0.3
Longifolene	4.5
p-Cymene	8.3
β-Cyclocitral	2.1
Thymoquinone	10.9
Terpinen-4-ol	1.3
Anisaldehyde	2.4

Optical Rotation: 0.74, Refractive Index: 1.485, Specific Gravity: 0.910

The comparison of the results from days 28 and 56 also showed a significant difference in the measured C3 values between the control group and the treatments of mixed essential oil treatment, the garlic essential oil, and the black caraway essential oil (p<0.05).

The comparison of the measured C4 values on days 28 and 56 showed no significant difference between the values of the control group and the treatments of mixed essential oil, garlic essential oil, black seed essential oil, and black caraway essential oil (p>0.05), but the comparison of days 28 and 56 showed a significant difference in the measured values between the control group, mixed essential oil, and garlic essential oil treatments (p<0.05).

The comparison of the measured plasma protein values on days 28 and 56 showed no significant difference between the values of the control group and the treatments of mixed essential oil, garlic essential oil, black seed essential oil, and black caraway essential oil (p>0.05), but the comparison of days 28 and 56 showed a significant difference in the measured values between the control group and the treatments of mixed essential oil, black seed essential oil, and black caraway essential oil (p<0.05) (Table 4).

Hematological Indices

The effects of various essential oil treatments on the average erythrocyte indices of rainbow trout during trial period are presented in Table 5. Based on the results obtained, there was no significant difference in the measured erythrocyte index values including RBC, Hb, MCV, MCH, MCHC, and Hct (%) between the control group and the treatment groups of mixed extract, garlic, black seed, and black caraway (p>0.05). Additionally, the highest values of RBC, Hb, MCV, MCH, MCHC, and Hct (%) were observed in the control group.

The effects of various essential oil treatments on the average Leukocyte profile of rainbow trout during trial period are presented in Table 6. Based on the results obtained, there was no significant difference in the measured Leukocyte index values including WBC (×10^3), Neutrophil (%), Lymphocyte (%), and Monocyte (%) between the control group and the treatment groups of mixed extract, garlic, black seed, and black caraway (p>0.05).

Table 4: Dietary effect of essential oils on lysozyme activity and immunological parameters (Mean±SE) of rainbow trout during the trial period (= 3). Different lowercase letters within the same day's treatments, and different uppercase letters for similar treatments across different days, indicate statistically significant differences between the treatments. (p<0.05).

Treatment	Lysozyme Activity (u/ml/min)		C3 (mg/dl)		C4 (mg/dl)		Plasma Protein (g/dL)	
	D28	D56	D28	D56	D28	D56	D28	D56
Control	41.26±0.4 [Aa]	36.66±0.47 [Bb]	65.07±1.9 [Aa]	52.67±1.9 [Bc]	11.3±0.44 [Aa]	8.63±0.44 [Ba]	4.03±0.18 [Ba]	4.57±0.18 [Aa]
Mixed essential oils	41.83±0.35 [Aa]	38.8±0.65 [Bb]	62.66±1.9 [Ab]	54.3±1.9 [Bc]	11.1±0.44 [Aa]	9.2±0.44 [Ba]	4.33±0.18 [Aa]	4.07±0.18 [Ba]
Garlic essential oil	40.9±2.06 [Aa]	39.6±0.81 [Ab]	60.97±1.9 [Ab]	54.87±1.9 [Bc]	11.33±0.44 [Aa]	9.63±0.44 [Ba]	4.7±0.18 [Aa]	4.57±0.18 [Aa]
Black seed essential oil	39.46±1.46 [Aa]	39.86±1.25 [Bb]	56.77±1.9 [Ac]	57±1.9 [Ab]	10.66±0.44 [Aa]	9.8±0.44 [Aa]	4.2±0.18 [Aa]	4.2±0.18 [Aa]
Black caraway essential oil	38.53±0.35 [Ba]	42.46±1 [Aa]	58.67±1.9 [Bc]	63.5±1.9 [Aa]	10.26±0.44 [Aa]	11.7±0.44 [Aa]	4.2±0.18 [Ba]	4.5±0.18 [Aa]

Table 5: Dietary effect of various essential oils on average erythrocyte indices (Mean±SE) of rainbow trout during the trial period (*n*: 3). Different lowercase letters indicate significant differences between treatments (p<0.05).

Treatment	RBC (×10^6)	Hb (mg/dL)	MCV (fl)	MCH (pg)	MCHC (g/dl)	Hct (%)
Control	1.8±0.11 [a]	16.44±0.96 [a]	274.33±2.33 [a]	91±66.88 [a]	33±0.01 [a]	49.2±3.9 [a]
Mixed essential oils	1.76±0.06 [a]	16±0.66 [a]	272±1 [a]	90±33.33 [a]	33±0.01 [a]	48.2±0.01 [a]
Garlic essential oil	1.73±0.08 [a]	15.66±0.57 [ac]	271.33±4.17 [a]	90.1±66.33 [a]	33±0.01 [a]	47.1±0.073 [a]
Black seed essential oil	1.63±0.03 [a]	14.44±0.29 [bc]	265.66±1.76 [a]	88±66.66 [a]	33±0.01 [a]	43±3.88 [a]
Black caraway essential oil	1.56±0.04 [a]	14±0.39 [bc]	268.2±33.73 [a]	89±66.88 [a]	33±0.01 [a]	42.1±0.15 [a]

Table 6: The effects of various essential oil treatments on average Leukocyte profile (Mean±SE) of rainbow trout during the trial period (*n*: 3). Different lowercase letters indicate significant differences between treatments (p<0.05).

Treatment	WBC (×10^3)	Neutrophil (%)	Lymphocyte (%)	Monocyte (%)
Control	17.67±2.47 [a]	23.33±5.2 [a]	70±6 [a]	66.75±38.3 [a]
Mixed essential oils	23.47±4.48 [a]	16±4.72 [a]	79±7.09 [a]	0±0 [a]
Garlic essential oil	17.16±2.54 [a]	14.33±4.17 [a]	73.33±16.37 [a]	33.66±1 [a]
Black seed essential oil	14.96±4.57 [a]	12.66±5.17 [a]	85±5.13 [a]	33.33±1.1 [a]
Black caraway essential oil	19.95±7.09 [a]	17.66±3.75 [a]	80.33±4.33 [a]	80.57±8.6 [a]

Based on the results, the highest value of WBC (×10^3) was observed in the mixed extract treatment group, the highest value of Neutrophil (%) was observed in the control group, the highest value of Lymphocyte (%) was observed in the black seed extract treatment group, and the highest value of Monocyte (%) was observed in the black caraway extract treatment group.

TNF-α gene expression

The results of TNF-α gene expression affected by the essential oils studied are shown in Figure 1. Among these essential oils, garlic essential oil had the greatest impact on TNF-α gene expression, with the level of gene expression changes estimated to be about 4.5 times the control group. The black caraway essential oil and the combined essential oil showed no significant change in the expression of the above gene compared to the control group, and the effect of the black seed essential oil was estimated to be about 3.2 times the control group (Fig. 1).

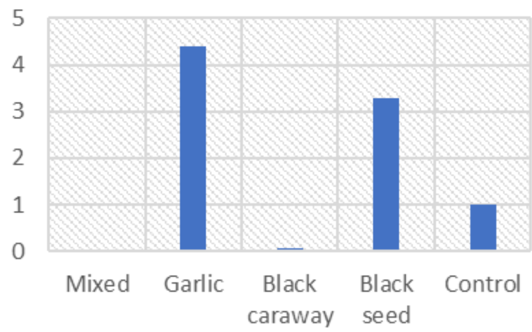

Figure 1: The effect of the essential oils studied on the mRNA expression of the *TNF-α* gene.

Discussion

The assessment of blood indices can be useful in identifying fish species, diagnosing diseases, determining nutritional status, and assessing health and sanitary conditions (Pedrazzani *et al*., 2022). One important indicator is the white blood cell count and composition, which can indicate the presence or absence of infection and the body's response (Seibel *et al*., 2021). Hematocrit, hemoglobin, and red blood cell levels reflect the oxygen-carrying capacity of the blood (Esmaeili, 2021). The present study found that hematological indices, including hematocrit and hemoglobin, significantly increased in fish fed a mixture of garlic, black seed, and black caraway essential oils, suggesting positive effects on physiological status and immune quality (Gholipour Kanani *et al*., 2014). These findings are consistent with previous reports (Shalaby *et al*., 2006; Jahanbakhshi *et al*., 2015). Additionally, the inclusion of garlic essential oil at 0.15 g/kg in the diet had a positive effect on cellular immune indices, increasing white blood cell count in juvenile beluga, *Huso huso* (Tangestani *et al*., 2011). Garlic contains strong sulfur and phenolic compounds that can stimulate the immune system and affect blood cell formation organs like the thymus, spleen, and bone marrow. The flavonoids in garlic have antioxidant effects that protect against oxidative stress, and the phenolic flavonoids possess antibiotic properties (Zaefarian *et al*., 2017; Fadeifard *et al*., 2018; Oyawoye *et al*., 2022). Studies have found that adding garlic to fish diets can significantly increase hemoglobin, red blood cells, lymphocytes, neutrophils, and white blood cell levels, enhancing the immune system (Shalaby *et al*., 2006; Tangestani *et al*., 2011; Karimi Pashaki *et al*., 2020; Aydın, 2021).

Blood parameters provide insights into fish health and physiology (Prakoso *et al*. 2017; Zorriehzahra *et al*., 2021). White blood cells play a crucial role in the immune response and tissue repair (Mokhtar *et al*., 2023). The present study found no significant differences in white blood cell counts between the essential oil treatment groups, consistent with research by Roohi *et al*. (2015) on common carp. However, Alishahi and Mesbah (2012) reported that black caraway increased white blood cells in common carp. Blood indices like red blood cells, hematocrit, and hemoglobin indicate oxygen transport capacity and overall fish health (Michael *et al*., 2019; Hosseinnia *et al*., 2021). Stress can decrease these parameters (Hastuti and Subandiyono, 2018). The lack of significant changes in white blood cells in the current study using garlic, black seed, and black caraway may be due to the compounds present in these essential oils.

The study found no significant differences in erythrocyte or leukocyte parameters between the control group and essential oil treatment groups. The control group had the highest erythrocyte index values, while the mixed extract group had the highest WBC count, the control group had the highest WBC percentage, the black caraway group had the highest lymphocyte percentage, and the black caraway group also had the highest monocyte percentage. This is consistent with research showing the positive effects of black caraway (Roohi *et al*., 2017) and Bacopa leaf extract (Pratheepa *et al*., 2010) on hematological parameters in common carp. However, other studies reported no significant differences in red blood cells, hemoglobin, and hematocrit with black caraway supplementation (Roohi *et al*., 2015) or *Tribulus terrestris* in catfish (Rezaei *et al*., 2013). No significant differences were observed in lymphocyte, monocyte, and neutrophil levels across treatments. In contrast, a separate study found decreased lymphocytes in fish fed diets containing five-finger and fennel extracts, while another reported increased neutrophils in rainbow trout fed ginger powder (Nya and Austin, 2009). The black caraway may effectively contribute to improving the innate and specific immune components in fish, which can be influenced by both nutritional and non-nutritional aspects of the diet (Dey *et al*., 2020; Mahboub *et al*., 2022).

The results showed that lysozyme activity increased in the black caraway oil group by day 56, but there was no difference between groups on day 28. C3 levels were higher in the treatment groups compared to the control on day 28, with further changes observed over time. C4 and plasma protein values did not differ between groups on individual days, but changed significantly within some treatment groups between days 28 and 56. Oral or intraperitoneal administration of medicinal plants to fish can enhance immunological parameters. Researchers have reported that the use of medicinal plants increased lysozyme activity, phagocytic activity, respiratory burst activity, and plasma protein levels, which were beneficial for boosting the immune system of aquatic organisms (Kuebutornye *et al*., 2020). Other studies examining the effect of dietary supplementation with aqueous extracts of plants like dill, nettle, and ginger have demonstrated improved growth rate, condition factor, and various immune response parameters in rainbow trout (Asadi *et al*., 2012; Karatas *et al*., 2020; Zeilab Sendijani *et al*., 2020; Korni *et al*., 2023). Ghaeni and Askary Sary (2014) reported the highest levels of albumin, globulin, and lysozyme activity in juvenile rainbow trout fed a diet containing 0.15% garlic extract. Roohi *et al*. (2016) found that black cumin and fenugreek supplements beneficially affected protein levels and antibacterial activity in the skin mucus of common carp. Karimi Pashaki *et al*. (2018) observed the highest lysozyme and IgM levels in common carp fry fed a diet with 5g/kg of garlic extract. Fadeifard *et al*. (2018) showed that black cumin enhanced immune responses in cultured rainbow trout, with higher serum lysozyme and phagocytic activity. Adineh *et al*. (2020) found that a diet containing 0.5% microencapsulated garlic extract resulted in significantly higher total serum protein and lysozyme activity in rainbow trout fry. Yousefi *et al*. (2021) found that black cumin supplements improved immune parameters in common carp, increasing defensive cells and lysozyme. Rashidian *et al*. (2023) showed that a mixture of black cumin, dill, fennel and coriander at 20g per diet improved immune parameters like lysozyme, ACH50, total Ig, C3, and C4 in rainbow trout.

The study found that garlic essential oil had the strongest effect, increasing TNF-α gene expression by around 4.5 times the control. Black caraway and the combined essential oil blend showed no significant change compared to the control. Black seed essential oil increased TNF-α expression by approximately 3.2 times the control. Garlic modulates the host immune system through the regulation of cytokine expression (Firmino *et al*., 2021). Modulating cytokine expression and targeting their receptors may have therapeutic potential. However, given the side effects experienced with chemical drugs in immune system modulation, phytotherapy using garlic may be considered for cytokine expression regulation. Arreola *et al*. (2015) examined the effects of alliin in LPS-stimulated adipocytes and found that the expression of pro-inflammatory genes IL-6, MCP-1, and Egr-1 was decreased in the cells. Additionally, the phosphorylation of ERK1/2, which plays a role in LPS-induced inflammation in adipocytes, was reduced after alliin treatment. Garlic compounds such as caffeic acid (CA), eugenol, diallyl trisulfide (DATS, also known as allitridin), diallyl sulfide (DAS), and other garlic-derived compounds can inhibit the key transcription factor NF-kB, preventing the transcription of several cytokine genes involved in pro-inflammatory responses, such as TNF-α, interleukin-1beta (IL-1β), IL-6, MCP-1, and IL-12. The polysaccharides in garlic are the main modulators of the host immune system (Valenzuela-Gutiérrez *et al*., 2021). Fresh garlic, with its higher fructan content compared to dried garlic, exhibits stronger immune-modulating effects (Delgado *et al*., 2023). Garlic polysaccharides regulate the expression of *IL-6, IL-10,* and *TNF,* leading to increased interferon-gamma expression in macrophages. Feng *et al*. (2012) demonstrated that allicin in Balb/c mice after *Plasmodium yoelii* infection led to increased interferon-gamma (*IFN-γ*), resulting in reduced parasite load and prolonged mouse survival. Garlic extract in several studies on experimental animals has been shown to stimulate the proliferation of Th2 cells, leading to enhanced antibody production. Th2 response helps generate an effective humoral immune response. Washiya *et al*. (2013) investigated the effects of garlic extract enriched with Z-ajoene in a mouse model and found that fecal IgA levels increased after 3 weeks of treatment, suggesting that garlic extract may stimulate B cells or influence interleukin secretion.

Black seed (*Nigella sativa*) is a potential source of natural metabolites such as phenolic compounds and alkaloids. The anti-inflammatory and immunomodulatory capabilities of black seed and its bioactive compounds have been studied. These compounds can modulate inflammatory and immune mediators like TNF-α, IFN-γ, NF-kB, COX, and LOX, as well as TGF-β, interleukins, and immunoglobulins (Ojueromi *et al*., 2022). Black seed can suppress the production of Th2 cytokines (IL-4, IL-5, IL-13) in animal models, thereby preventing the Th2 cell response that drives autoimmune diseases dependent on humoral immunity (Azimi *et al*., 2016). The ability of black seed to regulate the aberrant Th2 cell response and self-reactive B cell activation suggests it may be a potential therapy for autoimmune conditions (Ali *et al*., 2021). The study by Mehrabi *et al*. (2020) found that 0.5% nettle powder in rainbow trout feed improved growth, immunity, and resistance to *Saprolegnia parasitica* infection. Fish fed 0.5% nettle showed increased expression of immune genes (*TNF-α*, *IL-1b*, *IL-6*, and *IL-8*). According to the study by Gharaei *et al*. (2020), sumac supplementation at 2% and 5% levels increased the hepatic expression of the pro-inflammatory cytokines *TNF-α* and *IL-1β*, while decreasing the expression of the anti-inflammatory cytokine *IL-10* in rainbow trout. These gene expression changes may have contributed to the enhanced immune response and pathogen resistance observed in the fish fed with sumac-supplemented diets.

Conclusions

The results of this study indicate that dietary supplementation with encapsulated essential oils derived from plants like garlic, black seed, and black caraway shows great potential for enhancing aquaculture practices. In terms of hematological parameters, both the control group and the mixed essential oil group exhibited significantly higher levels of hemoglobin and hematocrit. However, no substantial differences were observed in erythrocyte or leukocyte indices among the treatment groups. Notably, lysozyme activity increased in the black caraway oil group by day

56, while no differences were detected between groups on day 28. Additionally, C3 levels were elevated in the treatment groups compared to the control on day 28, with further changes occurring over time. Conversely, C4 and plasma protein levels did not differ between groups on individual days, but significant changes were noted within certain treatment groups between days 28 and 56. Importantly, garlic essential oil demonstrated the most pronounced effect, elevating *TNF-α* gene expression to approximately 4.5 times that of the control. Black seed essential oil also increased *TNF-α* expression, reaching about 3.2 times the control level. In contrast, neither the combined essential oil blend nor black caraway alone resulted in significant changes in TNF-α compared to the control. These findings suggest that the immune-modulating effects of the essential oils varied greatly. Overall, the study highlights that dietary supplementation with specific essential oils, particularly garlic oil, can positively influence the growth performance and immune response of rainbow trout, presenting a promising strategy for enhancing aquaculture practices.

Conflicts of interest

The authors declare that they have no conflicts of interest related to this study.

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