Dietary Dracocephalum kotschyi essential oil improved growth, haematology, immunity and resistance to Aeromonas hydrophila in rainbow trout (Oncorhynchus mykiss)

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Received: 2 February 2022 | Revised: 19 February 2022 | Accepted: 27 February 2022

DOI: 10.1111/are.15829

ORIGINAL ARTICLE

Aquaculture Research WILEY

Dietary *Dracocephalum kotschyi* essential oil improved growth, haematology, immunity and resistance to *Aeromonas hydrophila* in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

In the present study, the effect of *Dracocephalum kotschyi* essential oil (DKE) was investigated on growth, haematology, immune and antioxidant defence system and resistance of rainbow trout juveniles to bacterial infection (*Aeromonas hydrophila*). For this purpose, the fish were fed a diet containing different concentrations of DKE including 0 (control), 0.2, 0.25 and 0.3 mg/kg diet in three replicates for 60 days. After feeding trial, the fish were challenged with a pathogenic dose of *A. hydrophila*. Based on the results, immune components in plasma (alternative complement activity [ACH_{50}], IgM content, lysozyme activity, total protein and total albumin) and mucus (protease activity, IgM content and lysozyme activity) significantly elevated in fish fed diet containing 0.2 and 0.25 mg DKE/kg diet compared with other groups ($p < 0.01$). The plasma bactericidal activity increased in all DKE-supplemented fish. Supplementation of fish with 0.3 mg DKE/kg diet depressed the mucosal lysozyme activity and IgM content.

The liver antioxidant enzymes, glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) showed increased activity in response to 0.25 and 0.3 mg DKE/kg diet compared with other experimental diets ($p < 0.01$). Dietary DKE changed the haematology of the fish. The haematocrit, red blood cell count (RBC), white blood cell (WBC), haemoglobin content and mean erythrocyte cell haemoglobin content (MCHC) increased in response to DKE. In contrast, the mean corpuscular haemoglobin (MCH) decreased in fish fed 0.2 and 0.25 mg DKE/kg diet compared with other diets ($p < 0.01$). The supplementation of fish with 0.2 and 0.25 mg DKE/kg diet decreased the expression of cytokine-related genes, TNF-α and IL-8. In contrast, the DKE upregulated the expression of the immune-related genes, C3 and lysozyme. DKE at concentration of 0.3 mg/kg diet depressed the C3 and lysozyme gene expressions. The DKE supplementation decreased the mortality rate of the fish after bacterial challenge compared with non-supplemented ones ($p < 0.01$). In conclusion, the findings of this study revealed the immune-stimulating effects of DKE at optimized dietary concentrations. In addition, DKE decreased the mortality induced by *A. hydrophila* infection.

KEYWORDS

bacterial, *Dracocephalum kotschyi*, essential oil, fish, immunity

1 | INTRODUCTION

Aquaculture is known as one of the most important ways to provide the protein needs of human (Pradeepkiran, 2019). However, the development of intensive aquaculture and super-intensive aquaculture has increased the risk of fish diseases as well as environmental pollutions (Cao et al., 2007; Cole et al., 2009; Jackson, 1978; Páez-Osuna, 2001; Romero et al., 2012). Today, antibiotics are widely used to treat fish diseases, but there are still some problems with these chemicals that may limit their use (Lulijwa et al., 2020; Romero et al., 2012). Continued use of antibiotics may create resistant bacterial strains, and in addition, the accumulation of these chemicals and their derivatives in fish meat can threaten human health as a consumer (Brown et al., 2021; Lulijwa et al., 2020). Furthermore, the release of antibiotics into natural aquatic environments can have a negative impact on the flora and fauna and ultimately the entire food web of that environment (Liu et al., 2017; Lulijwa et al., 2020). Owing to these problems, it seems necessary to replace antibiotics with safe alternatives. Dietary supplements, including plant-based supplements, are currently used to keep health and to treat many diseases in humans and animals (Fattepur et al., 2018; Gondo, 2021; Othman et al., 2019; Roslan & Shariff, 2021; Shalaby, 2015, 2018; Shalaby, Sakoury, Harthi, et al., 2020; Shalaby, Sakoury, Kholif, et al., 2020; You et al., 2021; Zharif et al., 2018). During the last decade, many studies have reported the medicinal, antioxidant, immunogenic and antibacterial properties of medicinal plants and their derivatives in fish (Ahmadifar et al., 2019; Farsani et al., 2019; Rashidian, Bahrami Gorji, et al., 2020; Van Doan et al., 2020; Bibi et al., 2021; DeiviArunachalam et al., 2021; Ghafarifarsani Kachuei et al., 2021; Ghafarifarsani, Hoseinifar, Adorian, et al., 2021; Ghafarifarsani, Hoseinifar, Talebi, et al., 2021; Ghafarifarsani, Rashidian, et al., 2021; Qahir et al., 2021; Rashidian et al., 2021; Yousefi, Farsani, et al., 2021; Yousefi, Ghafarifarsani et al., 2021; Raissy et al., 2022; Yousefi et al., 2022). However, the identification and introduction of the new species with immunogenic and antibacterial properties can be useful for aquaculture enhancement. *Dracocephalum* with more than 60 species is native to temperate regions of Asia and Europe, which is consumed in many parts of the world due to its high medicinal properties (Heydari et al., 2019). *Dracocephalum kotschyi* Boiss plant is one of the well-known species of *Dracocephalum*, which is used in treatment of infectious diseases due to its medicinal properties and active compounds. The chemical composition of *Dracocephalum kotschyi* includes bioactive compounds with anti-cancerous, antioxidant, antibacterial, anti-nociceptive, anti-hyperlipidaemic, antispasmodic, cytotoxic and immunostimulatory effects (Heydari et al., 2019; Moradi et al., 2020; Sadraei et al., 2017; Talari et al., 2014). In a study by Ghavam et al. (2021), the antibacterial properties of *D. kotschyi* essential oil (DKE) were attributed to some bioactive compounds in the biochemical composition of DKE including α-pinene, terpinen-4-ol, limonene, neral and geraniol. In addition, the results of Asghari et al. (2015) showed that the methanol extracts of *D. kotschyi* inhibit the growth of *Mycobacterium tuberculosis*. The extracts of *D. kotschyi* showed antibacterial effects against Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus cereus* and *Listeria Monocytogenes*) and Gram-negative bacteria (*Salmonella enterica*, *Escherichia coli* and *Enterica Aerogenes*) (Kamali et al., 2015). According to a literature review, we found no data regarding the immunogenic, antioxidant and antibacterial effects of *D. kotschyi* in fish. Therefore, the present study was conducted to evaluate the effects of DKE on immune and antioxidant defence system and resistance of rainbow trout juveniles to infection induced by *A. hydrophila*. As an opportunistic bacterium, *A. hydrophila* has cosmopolitan distribution in water bodies of the world. *A. hydrophila* causes the bacterial haemorrhagic septicaemia disease in many species of warm and cold water fish including rainbow trout (Constantino Casas et al., 1997; Harikrishnan & Balasundaram, 2005; Nya & Austin, 2010). The results of the present study may suggest a natural way to enhance the immunity and to control infection induced by *A. hydrophila* in rainbow trout.

2 | MATERIALS AND METHODS

2.1 | Fish and feeding trial

Rainbow trout fish with an average weight of 55 ± 5.6 g were purchased from a local cold water fish farm, and then transported using a tank with continuous oxygenation to lab and finally distributed into the 12 polyethylene tanks (containing 400 L disinfected and chlorinated water) for 7 days acclimation. During acclimation, the fish were fed three times a day at a rate of 4% of body weight with a commercial rainbow trout diet (Faradaneh Co., GFT1; diet form: pellet, pellet size: 5 ± 0.4 mm; diet composition: total crude protein: 40%, total crude lipid: 14%, total crude ash: 9% and total crude fibre: 3%).

After the adaptation to lab conditions, fish were distributed into the 12 tanks at density of 30 fish per tank and fed DKE at dietary levels of 0 (control), 0.2, 0.25 and 0.3 mg/kg diet in three replicates. Feeding was carried out daily at a rate of 4% of fish body weight. The dietary concentrations of the essential oil in this study were selected based on the in vitro inhibitory effect (or disk diffusion assay) of the essential oil on the growth of *A. hydrophila* according to the guidelines of Clinical & Laboratory Standards Institute (CLSI), formerly known as NCCLS (Wayne, 2007), with some modifications. Briefly, the sterile Petri dishes (9 cm in diameter) were prepared with 20 ml of Mueller–Hinton medium. Then, a 100 μl of the bacterial suspension (10^8 CFU/ml) was spread on the plates. After 6 min, a sterile filter paper disc (6 mm) containing different concentrations of DKE (0, 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 mg) was placed on the surface of the plates at 4°C for 1 h followed by incubation at 37°C for 30 h. The diameters of the inhibition zones (mm) were measured, including the diameter of the disks. Gentamycin (30 μg/disk) was used as positive control. All tests were done in three replicates. A disk diffusion assay was coducted to determine the highest antibacterial activity for DKE (Table 1). In this regard, the highest antibacterial activity was observed in treatments, 20, 25 and 30 which these concentrations were selected for feeding trial. During feeding experiment, water was daily renewed by 90% and also the water quality indices (temperature: 15.3 ± 0.3°C [using thermometer, ZEAL, UK], oxygen: 6.8 ± 0.11mg/L [using portable oxygen meter: Oxyguard Polaris Dissolved Oxygen Meter, Dynamic Aqua Supply Ltd, Canada], total ammonia: 0.008 ± 0.002 mg/L and pH: 7.5 ± 0.13 [using portable pH meter, Model AE-PH501]) were monitored daily.

2.2 | GC-Mass spectrometry of *Dracocephalum kotschyi* essential oil

The gas chromatography–mass spectrometry (Shimadzu QP2010 Ultra series instrument, follow rate 0.7 ml/min, temperature rate: 3°C/min and final temperature: 330°C) was used to analyse the chemical composition of DKE.

2.3 | Growth and survival parameters

The growth and survival parameters were measured after feeding trial by the following equations (Ghafarifarsani, Hoseinifar, Javahery, et al., 2022):

$$\text{Weight gain (WG) (\%)} = \text{final weight} - \text{initial weight}$$

$$\text{Specific growth rate (SGR) (\%/d)} = \left(\frac{(\ln \text{final wt(g)} - \ln \text{initial wt(g)})}{\text{days}}\right) \times 100$$

TABLE 1 Sequences of oligonucleotide primers for real-time PCR

Genes	Primers	Primer sequences	ID number
Lysozyme	Lysozyme Forward	TTCCCCAGGTATCCCATGAT	AB027305
	Lysozyme Reverse	GTGTCTGATGTGGCTGTGCT	
Complement	C3 Forward	AGCTTGCTGACTGGCTTTGT	100136951
	C3 Reverse	TCATAAACGGTGACCCCAAC	
TNF-α	TNF-α Forward	CATCGGCCCCTGCACCTATA	X99303
	TNF-α Reverse	TGTGGAACACAGCAGGGTTGG	
IL-8	IL-8 Forward	AGAATGTCAGCCAGCCTTGT	AJ279069
	IL-8 Reverse	TCTCAGACTCATCCCCTCAGT	
β-Actin	β-Actin Forward	ACTGCACAGCCAAGAGAGTTCA	AB039726
	β-Actin Reverse	GTTATTAAAGCGGCCGATATGC	

The gene efficacy for target and reference genes was 1.93–1.98.

$$\text{Feed conversion rate (FCR)} = \frac{\text{total feed consumed(g)}}{\text{weight gain(g)}}$$

$$\text{Survival rate (SR) (\%)} = \left(\frac{\text{final fish numbers}}{\text{initial fish numbers}}\right) \times 100$$

2.4 | Sampling

The blood, mucus and tissue samples were taken from 10 fish per tank after the feeding trial. For this purpose, fish were starved for 24 h, anesthetized with 20 mg/L clove oil and then few ($n = 3$) were placed in a nylon bag for collection of mucus based on the method of Subramanian et al. (2007). The blood samples were collected from remainders ($n = 7$) from caudal vein using 2-ml syringe. The plasmatic part of blood was separated by centrifuging at 4°C for 5 min. In addition, a part of blood was allocated for measurement of the haematological indices.

For tissue sampling, the fish were first sacrificed with high dose of ethyl 3-aminobenzoate methanesulphonate (100 g/L) to take liver sample. The plasma and liver samples were finally stored in liquid nitrogen until biochemical analysis.

2.5 | Biochemical assays

2.5.1 | Immune parameters

The activity of lysozyme in plasma and mucus was assayed using lyophilized *Micrococcus luteus* according to Ellis (1990). The immunoglobulin (IgM) content of plasma and mucus was determined using polyethylene glycol according to Siwicki and Anderson (1993). The plasma alternative complement activity (ACH_{50}) was assayed using the method of Ortuno et al. (2001) using *haemolysis* of rabbit red blood cells. The plasma bactericidal activity was evaluated by mixing plasma samples with bacterial suspension (*A. hydrophila*, OD: 0.5 at 546 nm) and finally calculating bacterial colony forming units (CFUs) created on nutrient agar plates after 24 h incubation at 36°C (Rao et al., 2006).

The total protein and albumin levels in plasma were spectrophotometrically determined using commercial assay kits (Sigma-Aldrich Co., USA) according to manufacturers' instructions. Protease activity in mucus was determined using the azocasein hydrolysis method, as described by Ross et al. (2000). Mucosal alkaline phosphatase (ALP) activity was measured using a commercial assay kit (Sigma-Aldrich Co., USA) according to manufacturers' instructions.

2.5.2 | Liver antioxidant enzymes

All antioxidant enzymes were determined using commercial assay kits, as instructed by manufacturer (*Sigma*-Aldrich CO, USA). Catalase (CAT) activity was assayed at 240 nm using catalysis of hydrogen peroxide (H_2O_2) according to Claiborne (1985). Superoxide dismutase (SOD) activity was colourimetrically determined at 440 nm through oxidation of xanthine to superoxide radicals (SR) and generation of a red formazan dye upon reaction of WST-1 reagent with the SR (Marklund & Marklund, 1974). Glutathione peroxidase (GPX) activity was measured through oxidation of glutathione by GPx and assaying oxidized glutathione at 340 nm. The lipid peroxidation was spectrophotometrically (at 532 nm) assayed upon reaction of malondialdehyde (MDA) with thiobarbituric acid (Utley et al., 1967).

2.5.3 | Haematological parameters

The total count of red and white blood cells was measured using a Neubauer haemocytometer slide at a magnification of 400× (Harikrishnan et al., 2009). Haematocrit (Hct) was measured using the microhaematocrit method (Blaxhall & Daisley, 1973). The haemoglobin (Hb) content was determined using the cyanohaemoglobin method (Oriakpono et al., 2012). The haematological indices including mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV) and corpuscular haemoglobin concentration (MCHC) were estimated according to the following equations (Blaxhall & Daisley, 1973):

$$MCHC = Hb \times \frac{10}{Hct};\ MCV = Hct \times \frac{10}{RBC(million)};$$
$$MCH = Hb \times \frac{10}{RBC(million)}$$

2.6 | Gene expression assay

2.6.1 | RNA extraction

The total RNA content was extracted from liver tissues using acid guanidinium thiocyanate–phenol–chloroform based on the method described by Awad et al. (2013) with some modifications. The quantity and quality of the extracted RNA (ER) was estimated by determining the absorbance at 260 nm, whereas the quality of ER was evaluated using electrophoresis according to Kwasek et al. (2014).

2.6.2 | RT-PCR

One microgram of ER was allocated to produce first-strand cDNAs using a Fermentas cDNA synthesis kit for reverse transcription polymerase chain reaction (RT-PCR) according to the manufacturer's protocol and a set of oligo-dT as primer. The real-time PCR primers were synthesized based on the sequences of DNA from GenBank (accessions numbers listed in Table 1) and using Gene Runner (version 6) software. The β-actin gene was considered as reference gene to evaluate the gene expressions. The real-time PCR was done using an iCycler (BioRad, USA) and commercial kit (Bio flux-Bioer Technology Co., China) following the manufacturer's protocols. The fold change in TNF-α, IL-8, complement and lysozyme relative to mRNA expression was estimated using the $2^{-\Delta\Delta Ct}$ method (Livak &

Schmittgen, 2001) and the data were analysed using iQ5 optical system software version 2.1 (Bio-Rad).

2.7 | Bacterial challenge

After 60 days of feeding experiment, the remaining fish in the tanks were exposed to *A. hydrophila* bacterium by injection. The stock of the bacterium (Strain number: IBRC-M 10814) was cultured on agar medium for 24 h at 37°C. A sub-lethal dose of bacteria (1.3×10^7 cells/ml phosphate buffered saline [PBS]) was used for bacterial challenge, which was calculated through determining the 7-day LD_{50} (3×10^7 cells/ml) in a previous exposure experiment by LaPatra et al. (2010). The cumulative fish mortality was recorded daily for 10 days. In addition, *A. hydrophila*-induced mortality was confirmed through re-isolating the organism from the liver on at least 30% of the dead fish using conventional methods.

2.8 | Statistical analysis

All statistical analysis was done using SPSS software. The normality of data (mean ± standard deviation) was evaluated using Kolmogorov–Smirnov test. The *Arcsin* transformation was used on proportional and per cent data before running analysis of variance. One-way analysis of variance was applied to find statistical differences. Finally, the comparison of means was done using Tukey test ($p < 0.01$).

3 | RESULTS

3.1 | GC-Mass spectrometry of *Dracocephalum kotschyi* essential oil

The chemical composition of DKE is presented in Table 2. δ-3-Carene (10.5%), limonene (9.8%), carvacrol (9.2%), 1,8-cineole (7.3%) and p-cymene (4.2%) composed the main compounds in the chemical composition of DKE (Table 2).

TABLE 2 Chemical compositions of *Dracocephalum kotschyi* essential oil; the compounds are listed in order of elution time

Compounds	Kovats constant	%
3Z-Hexenol	871	0.3
α-Thujene	935	0.2
α-Pinene	946	2.8
Sabinene	980	2.1
β-Pinene	985	2.7
3-Octanol	1000	1.4
δ-3-Carene	1021	10.5
p-Cymene	1026	4.2
Limonene	1035	9.8
1,8-Cineole	1041	7.3
γ-Terpinene	1065	3.1
Terpinolene	1101	3.2
p-Cymenene	1112	2.9
Linalool	1121	2.1
β-Thujone	1125	2.5
trans-Pinocarveol	1145	2.2
Camphor	1151	2.4
Pinocarvone	1172	1.8
Isopulegone	1182	1.9
Terpinen-4-ol	1186	3.1
Thuj-3-en-10-al	1189	1.9
trans-Carveol	1222	3.8
Pulegone	1245	2.5
Carvone	1250	5.3
Geraniol	1255	1.4
Limonen-10-ol	1296	1.6
Carvacrol	1305	9.2
Eugenol	1369	1.1
Neryl acetone	1441	0.2
Germacrene D	1491	1.5
Spathulenol	1585	1.4
Total		96.4

3.2 | Growth and mortality parameters

The growth parameters (weight gain per cent, specific growth rate and food conversion ratio) and mortality rate showed no significant differences between the treatments throughout 10 weeks of feeding trial (Table 3, $p > 0.01$).

3.3 | Plasma and mucus immune components

The activity of plasma complement and lysozyme and the levels of IgM, total protein and total albumin significantly increased in response to 0.2 and 0.25 mg DKE/kg diet compared with control and those fed 0.3 mg DKE/kg diet (Table 4, $p < 0.01$). However, the treatment with 0.3 mg DKE/kg diet showed higher activity in plasma complement, lysozyme and total protein compared with control (Table 4, $p < 0.01$). The DKE supplementation increased the bactericidal activity, as the number of CFUs decreased in DKE-supplemented fish (Table 4, $p < 0.01$). In mucus, the protease activity, IgM content and lysozyme activity significantly increased in response to 0.2 and 0.25 mg DKE/kg diet compared with control and fish of 0.3 mg DKE/kg diet treatment (Table 4, $p < 0.01$). However, the mucosal lysozyme activity and IgM content significantly decreased in fish fed 0.3 mg DKE/kg diet compared with other experimental groups (Table 4, $p < 0.01$).

TABLE 3 Growth and survival of the fish (rainbow trout) after 70 days of supplementation with diet containing different levels (mg/kg diet) of *Dracocephalum kotschyi* essential oil (DKE)

	Treatments			
	0 mg DKE (control)	0.2 mg DKE	0.25 mg DKE	0.3 mg DKE
FW (g)	252.5 ± 8.5	260.2 ± 11.2	255.8 ± 9.4	264.1 ± 12.3
WG (%)	358.1 ± 9.5	372.5.5 ± 11.2	365.4 ± 8.7	381.8 ± 10.9
SGR (%/d)	2.16 ± 0.05	2.21 ± 0.04	2.19 ± 0.03	2.23 ± 0.06
FCR	1.2 ± 0.04	1.18 ± 0.07	1.16 ± 0.05	1.21 ± 0.04
SR (%)	86.5 ± 2.5^{a}	90.4 ± 2.3^{a}	92.2 ± 1.5^{a}	70.2 ± 3.6^{b}

Note: Significant differences are shown as different superscripted letters (Tukey test, $p < 0.01$).
Abbreviations: FCR, Feed conversion rate; FW, Final weight; SGR, Specific growth rate (%/d); SR, Survival rate; WG, Weight gain.

TABLE 4 The plasma and mucus immune component changes in rainbow trout in response to 70 days supplementation with diet containing different levels (mg/kg diet) of *Dracocephalum kotschyi* essential oil (DKE)

	Treatments			
	0 mg DKE (control)	0.2 mg DKE	0.25 mg DKE	0.3 mg DKE
Plasma immune components				
Lysozyme activity (IU/ml)	60.6 ± 12.1^{a}	180.8 ± 15.3^{b}	241.5 ± 20.5^{c}	132.5 ± 13.2^{d}
ACH_{50} activity (IU/ml)	1.6 ± 0.3^{a}	4.9 ± 0.5^{b}	5.2 ± 0.3^{b}	3.7 ± 0.6^{c}
IgM (mg/ml)	2.33 ± 0.3^{a}	3.84 ± 0.7^{b}	4.46 ± 0.4^{b}	2.55 ± 0.11^{a}
Bactericidal activity (No. of. CFUs)	235.5 ± 22.51^{a}	152.4 ± 19.4^{b}	131.5 ± 12.5^{b}	160.6 ± 20.1^{b}
Total protein (g/dL)	2.2 ± 0.21^{a}	4.3 ± 0.12^{b}	4.5 ± 0.15^{b}	3.1 ± 0.1^{c}
Total albumin (g/dL)	0.73 ± 0.25^{a}	1.42 ± 0.14^{b}	2.65 ± 0.21^{c}	0.9 ± 0.2^{a}
Mucosal immune components				
Lysozyme activity (IU/ml)	21.5 ± 6.3^{a}	42.3 ± 10.2^{b}	50.8 ± 12.7^{b}	32.9 ± 11.5^{ab}
IgM (mg/ml)	0.42 ± 0.12^{a}	0.95 ± 0.1^{b}	1.2 ± 0.15^{b}	0.66 ± 0.18^{a}
Protease activity (IU/ml)	12.5 ± 5.3^{a}	25.2 ± 4.1^{b}	30.3 ± 7.4^{b}	14.6 ± 5.4^{a}
Alkaline phosphatase activity (IU/ml)	2.77 ± 1.5^{a}	8.22 ± 2.02^{b}	10.41 ± 1.1^{b}	4.32 ± 1.9^{a}

Note: Significant differences are shown as different superscripted letters (Tukey test, $p < 0.01$).

TABLE 5 The haematological alternations of rainbow trout after 70 days of supplementation with diet containing different levels (mg/kg diet) of *Dracocephalum kotschyi* essential oil (DKE)

	Treatments			
	0 mg DKE (control)	0.2mg DKE	0.25mg DKE	0.3mg DKE
RBC ($\times 10^6$/ml)	0.86 ± 0.07^{a}	1.43 ± 0.08^{b}	1.58 ± 0.06^{c}	0.9 ± 0.04^{a}
WBC ($\times 10^3$/ml)	3.34 ± 0.25^{a}	3.62 ± 0.15^{a}	5.12 ± 0.2^{b}	3.7 ± 0.3^{a}
Hct (%)	26.4 ± 2.5^{a}	30.2 ± 1.42^{b}	31.2 ± 1.4^{b}	28.5 ± 2.1^{a}
Hb (g/dl)	8.92 ± 1.81^{a}	9.34 ± 2.52^{a}	13.61 ± 1.13^{b}	10.12 ± 1.21^{a}
MCV (fl)	212.4 ± 13.5^{a}	221.2 ± 10.1^{a}	268.3 ± 12.4^{b}	230.5 ± 18.1^{a}
MCH (pg)	104.6 ± 6.2^{a}	65.7 ± 7.1^{b}	86.1 ± 5.2^{c}	112.5 ± 8.8^{a}
MCHC (%)	3.1 ± 0.06^{a}	3.12 ± 0.03^{a}	3.24 ± 0.05^{b}	3.06 ± 0.11^{a}

Note: Significant differences are shown as different superscripted letters (Tukey test, $p < 0.01$).

3.4 | Haematological parameters

After feeding trial, the values of haematocrit and red blood cell count significantly increased in fish fed with 0.2 and 0.25 mg DKE/kg diet (Table 5, $p < 0.01$). Meanwhile, the mean erythrocyte cell volume (MCV), haemoglobin content and mean erythrocyte cell haemoglobin content (MCHC) significantly increased in fish fed 0.25 mg DKE/kg diet than those of fish fed the other diets (Table 5, $p < 0.01$). The mean corpuscular haemoglobin (MCH) showed significant decreases in fish fed 0.2 and 0.25 mg DKE/kg diet than compared with those fed the other diets (Table 5, $p < 0.01$). The values of white blood cell (WBC) significantly increased in response to 0.25 mg DKE/kg diet compared with other diets (Table 5, $p < 0.01$). Also, there were no significant differences in WBC count between control and fish fed 0.3 mg DKE/kg diet (Table 5, $p > 0.01$).

3.5 | Liver antioxidant enzymes

The activity of glutathione peroxidase (GPX), catalase (CAT) and superoxide dismutase (SOD) significantly increased in response to 0.25 and 0.3 mg DKE/kg diet compared with control and those fed diet containing 0.2 mg DKE/kg diet (Table 6, $p < 0.01$). In the 0.2 mg DKE/kg diet treatment, only SOD showed significant increases compared with control (Table 6, $p < 0.01$).

3.6 | Lipid peroxidation index

As a lipid peroxidation marker, the levels of malondialdehyde (MDA) showed no significant differences between all experimental groups after 60 days feeding trial (Figure 1, $p > 0.01$).

3.7 | Gene expressions

After feeding trial, the expression of inflammatory genes, TNF-α and IL-8, significantly decreased in response to 0.2 and 0.25 mg DKE/kg diet (Figure 2, $p < 0.01$). In contrast, these treatments exhibited higher expression of the immune-related genes (C3 and lysozyme) in response to 0.2 and 0.25 mg dietary DKE compared with control and fish from 0.3 mg DKE/kg diet treatment (Figure 3, $p < 0.01$). The expression of C3 and lysozyme showed significant decreases in fish fed 0.3 mg DKE/kg diet compared with control (Figure 3, $p < 0.01$).

3.8 | Bacterial challenge

After 10 days of bacterial injection, the cumulative fish mortality (CFM) showed different values among the treatments (Figure 4, $p < 0.01$). The CFM was significantly lower in fish fed DKE compared with control after 10 days bacterial challenge (Figure 4, $p < 0.01$).

4 | DISCUSSION

The findings of this study revealed that dietary DKE, especially at concentrations of 0.2 and 0.25 mg DKE/kg diet, can improve the immune and antioxidant system in the rainbow trout. Therefore, DKE can be considered as an immunostimulant, although this role has not yet been reported in fish. The antioxidant properties of *D. kotschyi* are mostly attributed to the flavonoids such as terpinolene, p-Cymene, γ-terpinene, carvone, carvacrol, eugenol and limonene (Ahmad & Beg, 2013; Aydin et al., 2013; Foti & Ingold, 2003; Gülçin, 2011; de Oliveira et al., 2015; Pombal et al., 2017; Yanishlieva et al., 1999).

It is recognized that the compound luteolin plays the main role in the antioxidant activity of the plant (Fattahi et al., 2013; Gohari et al., 2003; Heydari et al., 2019). In this study, the dietary DKE also stimulated the production of antioxidant enzymes (SOD, CAT and GPx) in liver, which clearly indicates the enhancing effects of DKE on the antioxidant defense in the fish. However, we found no changes in the MDA levels as the main indicator of oxidative stress (Del Rio et al., 2005) in response to dietary DKE. In this study, DKE at dietary levels of 0.2 and 0.25 mg DKE/kg elevated immune components in plasma (ACH_{50}, IgM content, lysozyme activity, total protein and total albumin) and mucus (protease activity, IgM content and lysozyme activity) and also the expression of some immune-related genes (lysozyme and C3). However, we found no report regarding the immunogenic effects of *D. kotschyi* in the literatures. Some studies have reported a significant decline in secretion of IL-1β from the cells, which may be resulting from the presence of anti-inflammatory compounds in the biochemical composition of *D. kotschyi* (Amirghofran et al., 2011).

The results of the present study support this assumption because we observed down-regulation of the inflammatory genes, TNF-α and IL-8, after supplementation with 0.2 and 0.25 mg DKE/kg diet. The anti-inflammatory effects of *Dracocephalum* sp. has been reported in some studies (Kalantar et al., 2018; Nie et al., 2021; Sadraei et al., 2017; Toshmatov et al., 2019). Some studies have reported the antimicrobial activity of *D. kotschyi* essential oil, which may be related to some compounds such as geranial α-pinene, geraniol, neral, geraniol acetate and limonene in the biochemical composition of *D. kotschyi* (Dorman & Deans, 2000; Kamali et al., 2015; Singh et al., 2012).

In general, haematology is an important tool to evaluate the fish health in relation to diseases, stress, nutrition and changes in environmental conditions (Burgos-Aceves et al., 2019; Enayat Gholampour et al., 2020; Fazio, 2019; Ghafarifarsani, Hoseinifar,

TABLE 6 The liver antioxidant enzyme changes in the rainbow trout in response to 70 days supplementation with diet containing different levels (mg/kg diet) of *Dracocephalum kotschyi* essential oil (DKE)

	Treatments			
	0 mg DKE (control)	0.2 mg DKE	0.25 mg DKE	0.3 mg DKE
SOD (IU/mg)	0.411 ± 0.08[a]	0.693 ± 0.06[b]	0.752 ± 0.12[bc]	0.981 ± 0.09[cd]
CAT (IU/mg)	71.23 ± 13.3[a]	58.33 ± 16.45[a]	112.54 ± 8.5[b]	132.5 ± 11.2[c]
GPx (IU/mg)	0.121 ± 0.08[a]	0.163 ± 0.1[a]	0.462 ± 0.16[b]	0.714 ± 0.11[c]

Note: Significant differences are shown as different superscripted letters (Tukey test, $p < 0.01$).

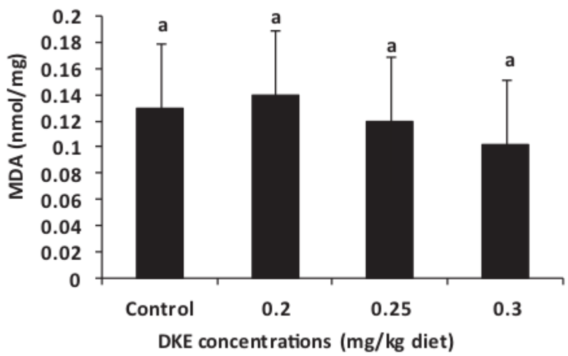

FIGURE 1 The changes in lipid peroxidation index (MDA: malondialdehyde levels) in the rainbow trout in response to 70 days of supplementation with diet containing different levels of *Dracocephalum kotschyi* essential oil (DKE). Significant differences are shown as different superscripted letters (Tukey test, $p < 0.01$)

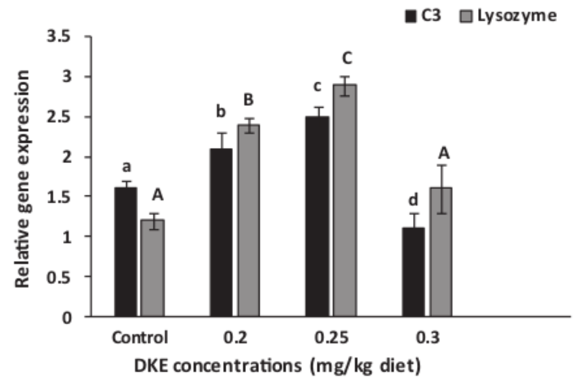

FIGURE 3 The expression of immune-related genes, complement (C3) and lysozyme in the rainbow trout in response to 70 days of supplementation with diet containing different levels of *Dracocephalum kotschyi* essential oil (DKE). Significant differences are shown as different superscripted letters (Tukey test, $p < 0.01$)

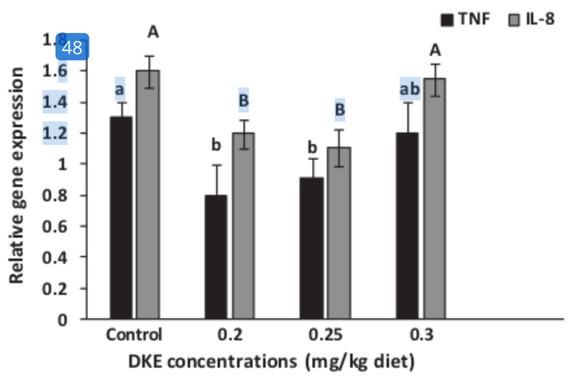

FIGURE 2 The expression of cytokine-related genes, TNF-α and IL-8, in the rainbow trout in response to 70 days of supplementation with diet containing different levels of *Dracocephalum kotschyi* essential oil (DKE). Significant differences are shown as different superscripted letters (Tukey test, $p < 0.01$)

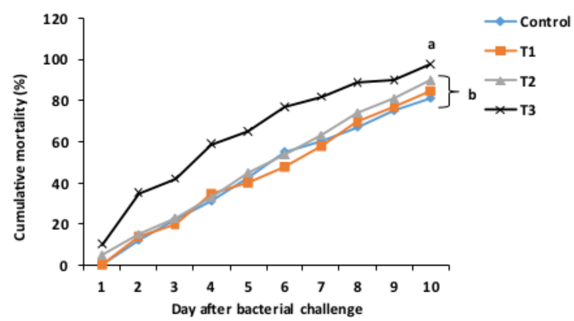

FIGURE 4 Cumulative mortality of rainbow trout, *Oncorhynchus mykiss* ($n = 15$/tank), fed different concentration of DKE in the diet throughout 10-day challenge with *Aeromonas hydrophila*. Significant differences are indicated with different lower-case letters (Tukey test, $p < 0.01$). Data: Mean ± SD

Aftabgard, et al., 2022; Rashidian, Kajbaf, et al., 2020; Sula et al., 2020). In this study, dietary DKE caused significant increases in the values of Hct, RBCs, MCV, Hb content and MCHC. However, MCH decreased in fish fed 0.2 and 0.25mg DKE/kg diet, which may be due to the depressing effect of DKE on the formation of Hb in red blood cells. In addition, the values of WBCs significantly increased in response to 0.25mg DKE/kg diet, which may be attributed to the immunostimulatory role of DKE. Generally, the effects of plants and their derivatives on fish haematology are different, which may be dependent on plant species, exposure concentration, exposure duration, experimental condition, etc.

In the present study, the supplementation of fish with 0.3mg DKE/kg diet reduced the innate immune responses compared with the 0.2 and 0.25mg DKE/kg diet treatments. Furthermore, the expression of lysozyme and C3 genes in the 0.3mg DKE/kg diet treatment was lower than control. These results may be due to the immunotoxic effects of DKE at high dietary concentrations. The cytotoxic effects of *D. kotschyi* have been previously reported by Ashrafi et al. (2017), Sani et al. (2017) and Shaabani et al. (2020). Ashrafi et al. (2017) indicated that DKE at concentrations of 26.4 μg/ml and 4266.7 μg/ml has toxic effects on Hela cells and human lymphocytes over in vitro condition. Similar results were observed in the study of Sani et al. (2017), when calu-6 and mehr-80 lung cancer cell lines were exposed to 12.5 200 Âμg/mL of methanolic extract of *D. kotschyi*. In addition, Shaabani et al. (2020) reported the cytotoxic effects of 400 μg/ml *D. kotschyi* extract on glioblastoma U87 cells over in vitro condition.

In the present study, dietary DKE reduced the mortality induced by *A. hydrophila* infection. However, this reduction was not so considerable. Generally, it is found that the antibacterial effect of DKE is stronger on Gram-positive bacteria (Ashrafi et al., 2017), as it is reported for other plant essential oils (Ballester-Costa et al., 2013; Shakeri et al., 2014). Due to the lack of cell wall in Gram-positive bacteria, the phospholipid bilayer of these bacteria is more receptive to hydrophobic compounds of essential oils than Gram-negative bacteria (Nikaido, 2003). The contact of essential oils with cell membrane results in disruptions of permeability and bacterial enzyme systems (Wendakoon & Sakaguchi, 1993). Therefore, in this study, the low antibacterial effect of DKE may be due to the Gram-negative bacteria nature of *A. hydrophila*. However, the antibacterial properties of DKE may be due to some compounds including terpinolene, β-Pinene, p-cymene, carvone, carvacrol, eugenol and limonene in its chemical composition (Devi et al., 2010; Han et al., 2020; Marchese et al., 2017; Porfírio et al., 2017; Rondon et al., 2006; Salehi et al., 2019; Xu et al., 2008).

5 | CONCLUSION

The finding of the present study indicated that DKE at dietary may improve the immunity and antioxidant defence in the rainbow trout. However, DKE had no appropriate efficiency in reducing the fish mortality induced by *A. hydrophila*.

ACKNOWLEDGEMENT

The authors are grateful to all the researchers and those who helped to conduct this research and publish the results.

CONFLICT OF INTEREST

Authors have no conflict of interest to declare for the publication of the present work.

ETHICAL APPROVAL

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

AUTHOR CONTRIBUTIONS

Hafsan Hafsan, Dmitry Bokov and Walid Kamal Abdelbasset contributed to experimental design. Mustafa M. Kadhim, Wanich Suksatan and Mahboubeh Balvardi contributed to methodology and data analysis. Hasan Sh. Majdi, Gunawan Widjaja, Abduladheem Turki Jalil and Maytham T. Qasim contributed to paper writing.

DATA AVAILABILITY STATEMENT

The datasets in this study are available from the corresponding author on reasonable request. All data and materials are available for publication.

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How to cite this article: Hafsan, H., Bokov, D., Abdelbasset, W. K., Kadhim, M. M., Suksatan, W., Majdi, H. S., Widjaja, G., Jalil, A. T., Qasim, M. T., & Balvardi, M. (2022). Dietary *Dracocephalum kotschyi* essential oil improved growth, haematology, immunity and resistance to *Aeromonas hydrophila* in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture Research*, 53, 3164–3175. https://doi.org/10.1111/are.15829

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