Aquaculture Reports 26 (2022) 101279

Contents lists available at ScienceDirect

Aquaculture Reports

journal homepage: www.elsevier.com/locate/aqrep

Can dietary *Dictyota dichotoma* powder affect performance, serum, and mucus immune parameters, and antioxidant defense in Zebrafish (*Danio rerio*)?

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ARTICLE INFO

Keywords:
Brown algae
Humoral immunity
Mucosal immunity
Antioxidant defense
Zebrafish

ABSTRACT

The present study was performed to study possible effects of *Dictyota dichotoma* which is a brown macroalgae on whole body extract (WBE) and skin mucus immune parameters, antioxidant enzyme activities as well as expression of growth, immune, and antioxidant related genes in zebrafish (*Danio rerio*). Zebrafish with an average weight of 0.3 ± 0.08 g were supplied and stocked in 12 aquariums. They were fed with different levels (0 %, 0.25 %, 0.5 %, and 1 %) of *D. dichotoma* powder for eight weeks. At the end of the trial, results revealed skin mucus immune parameters in fish treated with 1 % *D. dichotoma* were remarkably higher than control ($P < 0.05$). Similar results were obtained following evaluation of WBE total Ig and lysozyme activity ($P < 0.05$). Also, the activity of Superoxide dismutase (SOD) and glutathione S transferase (GST) were significantly increased ($P < 0.05$) when fish fed with 0.5 % and 1 % *D. dichotoma* supplemented diets. However, no significant alteration was noticed in the case of CAT ($P > 0.05$). The gene expression study revealed significant up-regulation of immune-related genes (LYZ and IL-1β) in fish fed *D. dichotoma* supplemented groups compared to the control group ($P < 0.05$). Regarding growth-related genes, *D. dichotoma* significantly increased GH and IGF1 expression in a dose-dependent manner ($P < 0.05$). Evaluation of SOD and CAT genes expression showed significant differences between the control group and treatments ($P < 0.05$). The highest increase was observed when zebrafish was fed 1 % macroalgae. In conclusion, the present findings suggested that adding 0.5–1 % of *D. dichotoma* powder to the zebrafish diet can positively affect fish antioxidant defense and immune system.

1. Introduction

In recent years, great attention has been paid to the search for inexpensive natural resources rich in bioactive compounds that can be utilized as functional components useful for fish health and performance (Dawood et al., 2020). Among these sources, macroalgae has great potential due to its valuable nutritional profiles, low caloric values, and medicinal benefits (Raposo et al., 2015; Anaya-rosas et al., 2017; Wang et al., 2017; Mekinic et al., 2021). Studies are carried out on the potential of using marine macroalgae in various industries such as medicine, biomedical, cosmetics and food (Gupta and Abu-Ghannam, 2011; Raposo et al., 2015; Sayin, 2020; Veziroglu et al., 2021).

Macroalgae change their physiology using different adaptation strategies in extreme environmental conditions and produce natural bioactive compounds and metabolites for survival (Wang et al., 2017; Chen et al., 2018; El-Shaibany et al., 2020). Species belonging to the genus Dictyota are also rich sources of bioactive secondary metabolites with various structural features. Based on the phytochemical screening of *D. dichotoma*, hundreds of bioactive natural products have been isolated from Dictyota species, including terpenes, phenols, sterols, alkaloids, tannins, flavonoids, coumarins, quinones, essential oils, fatty acids, and polysaccharides (Deyab et al., 2016; Ibraheem et al., 2017;

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https://doi.org/10.1016/j.aqrep.2022.101279
Received 15 February 2022; Received in revised form 8 July 2022; Accepted 2 August 2022
Available online 12 August 2022
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Chen et al., 2018; Siless et al., 2018; Benfares et al., 2019).

D. dichotoma has been shown to have different levels of anti-inflammatory, antioxidant, antitumor, antibacterial, cytotoxic, antifungal, antifouling, and antiviral activity due to rich sources of bioactive secondary metabolites with various structural features (Rabanal et al., 2014; Deyab et al., 2016; Ibraheem et al., 2017; Chen et al., 2018; Siless et al., 2018; Usoltseva et al., 2018; Benfares et al., 2019; Aras and Sayin, 2020).

Besides its pharmaceutical properties, it has been suggested that some brown macroalgae, including *D. dichotoma*, may have significant value in the nutrition of both humans and animals as a functional feed additive (Yoon et al., 2009; Raposo et al., 2015; Afonso et al., 2019; Benfares et al., 2019; El-Shenody et al., 2019). In a study investigating the amino acid profiles of *D. dichotoma*, *Turbinaria decurrens* and *Laurencia obtusa* macroalgae, it was stated that they have the total essential amino acid content required for food accepted by the FAO (El-Shenody et al., 2019).

Due to these unique properties of *D. dichotoma*, it is thought to have the potential to be used as a food ingredient in aquaculture. In the aquaculture sector, diversification of ingredients used in aquaculture feeds is encouraged to achieve more sustainable aquaculture production systems (Ulloa et al., 2014). Furthermore, it is now well-documented in many studies that there is a close relationship between dietary intake and fish physiology, and these functional feed additives can affect the immune response, disease resistance, and growth performance (Ahmadifar et al., 2019; Safari et al., 2019; Khalili et al., 2020; Hoseini et al., 2021).

There is limited information regarding administration of *D. dichotoma* in aquaculture. The nutrient digestibility values of shrimps fed with diets containing *D. dichotoma*, *Ulva lactuca*, and *Gracilaria vermiculophylla* meal were higher than the control diet, and higher growth performance was obtained (Anaya-rosas et al., 2017). In another study with the same algae, it was reported that better immunity was acquired when shrimp were experimentally infected with *V. parahaemolyticus* and WSSV compared to the control group. Moreover, it has also been suggested that the co-cultivation of these macroalgae and shrimp provide a stress-reducing effect (Anaya-Rosas et al., 2019).

The zebrafish (*Danio rerio*) used in this study is considered as an ideal experimental model organism for the preliminary evaluation of diets, since the evaluation of various dietary types in commercially farming species requires costly and time-consuming trials. Given such features in zebrafish, a wide range of feed additives have been tested in zebrafish and those with the highest potential were select for success in aquaculture (Ribas and Piferrer, 2014; Ulloa et al., 2014; Lee-estevez et al., 2018; Nadal et al., 2020). Moreover, zebrafish have a sequenced genome that enables new technologies such as RNA sequencing and genotyping to be used effectively to study the molecular mechanisms underlying the response of the tested organism to nutrients (Lee-estevez et al., 2018; Nadal et al., 2020).

Therefore, this study aimed to investigate the effects of *D. dichotoma* on the mucosal and WBE immune responses (total Ig and lysozyme), antioxidant enzyme activities, as well as on the expression of genes including immune-, antioxidant- and growth-related gene expression in zebrafish as model.

2. Materials and methods

2.1. Macroalgae collection and preparation

D. dichotoma was collected from 0 to 20 m depth in Iskenderun Gulf coast, Iskenderun, Hatay, Turkey (36.37.07 N 36.03.46 E) during May–June 2018. To purify materials such as epiphytes, rocks, sand, and mud, they were washed with distilled water as described by Sayin (2020). The cleaned macroalgae were dried in a shaded area in the laboratory without being exposed to the sun. In the Algal Biotechnology Laboratory, identification studies of macroalgae were carried out using an Olympus brand Ckx41sf model stereo inverted light microscope.

2.2. Preparation of diets and experimental design

The proximate analysis of the feed used as a control diet in the study is given in Table 1. This commercial diet (Biomar, France) has been considered as basal diet. To prepare experimental diets, different levels of *D. dichotoma* powder were added to the control diet at the ratios of 0 %, 0.25 %, 0.5 %, 1 % as described in our previous paper (Hoseinifar et al., 2018). Briefly, the selected amount of algae powder was sprayed on the top of feed pellets. The prepared diets were stored in sealed packages at 4 °C until the study was conducted.

2.3. Fish culture and feeding trial

Zebrafish used in the study were kindly supplied by Vakili Ornamental Fish farm (Golestan province, Iran) and transferred to the GUASNR (Gorgan University of Agricultural Sciences and Natural Resources) Zebra Lab. Before starting the study, the fish were adapted for two weeks. During this period, fish were hand-fed with a commercial diet (Biomar-France) diet three times a day. After the acclimatization, fish were stocked in 12 aquaria (100 L), assigned to control, and 3 experimental groups with 3 replicates, 50 zebrafish (0.3 ± 0.08 g) in each aquarium. Feeding was performed three times a day up to apparent satiation over eight weeks. During the experiment, Water temperature, dissolved oxygen, and pH were maintained and monitored daily at 25 ± 2 °C, 7.9 ± 0.1 mg/L and 7 ± 0.2. This study used a static culture system with continuous aeration and daily water changes (50 %).

2.4. Growth performance

The growth performance was assessed after 56 days of dietary experimentation by assessing final weight, weight gain, and survival rate using the growth performances equations (Yousefi et al., 2021).

2.5. Determination of immune parameters

2.5.1. Sampling

At the end of the feeding trial, nine fish were randomly sampled from each replicate to determine nonspecific immune parameters (lysozyme activity, total protein, and total globulin levels). Fish were anesthetized using 500 mg L of clove powder, and skin mucus was collected after Hoseinifar et al. (2021) using a polyethylene bag. Then, without delay, the samples were immediately centrifuged (5810R Eppendorf, Engelsdorf, Germany) (1500 *g*, 10 min at 4 °C), and supernatants were stored at − 80 °C for further analysis.

In addition, as the fish were too small to collect blood, the protocol suggested described by Yousefi et al. (2018) was followed to obtain whole body extracts (WBE). The samples were transferred to sterile tubes (1.5 ml) and kept at − 80 °C until analysis.

2.5.2. Evaluation of nonspecific immune parameters

In this study, total protein, globulins, and lysozyme activity were evaluated as nonspecific immune parameters in both mucus and whole-body extracts. To determine the protein concentration of WBE and mucus samples, (Bradford (1976) and Lowry et al., 1951) protocol was followed. The determination of skin mucus globulins was performed

Table 1
Composition of the basal diet used throughout the experiment.

Proximate analysis (%)	
Dry matter	93.6
Crude protein	38.9
Crude lipid	15.0
Ash	11

using the method described by (Choudhury et al., 2005). In addition, a lysozyme sensitive bacterium *Micrococcus luteus,* was used to determine WBE and skin mucus lysozyme activity as described by Esteban et al. (2014).

2.6. Antioxidant defense

At the end of feeding trial, three fish were randomly selected from each replicate (nine per treatment) and whole-body serum was obtained as described above. The antioxidant enzymes activity including Catalase (CAT), Superoxide dismutase (SOD) and Glutathione S transferase (GST) were measured using commercial kits (Zellbio, Germany) following manufacturer instructions.

2.7. Genes expression studies

2.7.1. Sampling

Nine zebrafish were randomly sampled from each aquarium at the end of the feeding trial. Fish were sacrificed using an overdose of clove powder (500 mg/l). Samples from the fish's brain, liver and intestine tissues were then immediately obtained and deep freeze in liquid nitrogen and preserved at − 80 °C until further analysis. As the fish were too small, in order to obtain enough RNA for analysis, we pooled 9 samples of each replicate (aquarium) (darvishi et al., 2022). The samples were transferred to sterile tubes (1.5 ml) and kept at − 80 °C until analysis.

2.7.2. RNA extraction, cDNA synthesis

Total RNA of samples was obtained from the tissues by using Esterabad Zistfan Pishro Azma. Then, the extracted RNA was treated with DNase I (Fermentas, Lithuania) to remove genomic DNA. The concentration and quality of RNA in each sample were checked by NanoDrop (Nanodrop Technology, Wilmington, DE, USA) and 1.5 % Agarose gel. To synthesis cDNA, a cDNA synthesis kit (Fermentas, Lithuania) was used, and the protocol suggested by the company was followed (Safari et al., 2017).

2.7.3. Real-time PCR

Table 2 shows the primers used to assess growth, immunity, and antioxidant genes expression. The primers were designed according to the GenBank sequences by Primer3 software (Safari et al., 2016). Quantitative real-time PCR (qPCR) assays were performed to study alteration in the expression of immune (IL-1β, and Lysozyme-C), antioxidant (SOD, and CAT), and growth-related genes (GH, and IGF-1). The relative real-time PCR was performed using an SYBR Green qPCR Master Mix (Fermentas, Lithuania) and iCycler (BioRad, USA) as described in our previous paper (Safari et al., 2017).

2.8. Statistical analysis

This experiment was performed in a completely randomized design. Relative gene expression data after calculating with $2^{-\Delta\Delta ct}$ and the data related to growth, antioxidant, and whole body extract and mucus immune factors were checked for normality by the Kolmogorov-Smirnov test as well as homogeneity of variances. Data were subjected to one-way ANOVA with $\alpha = 0.05$ followed by Duncan's test. Results are reported as mean ± standard deviation (X ± S.D). Statistical analysis was performed using SPSS 19 software (SPSS, USA).

3. Results

3.1. growth performance

The effects of *D. dichotoma* on growth parameters of zebrafish are presented in Table 3. At the end of feeding trial treated group should significant higher growth compared to control group ($P < 0.05$). The highest growth was noticed in those zebrafish fed with 1 % *D. dichotoma*, which was significantly higher that control and other treatments. No significant difference was noticed between 0.25 % and 0.5 % supplementation groups.

3.2. Nonspecific Immune parameters

The humoral and mucosal nonspecific immune parameters (Total protein, globulins, and lysozyme) of zebrafish fed with *D. dichotoma* are shown in Table 4. The study of mucosal immune responses, including total protein, globulins, and lysozyme activity, have shown that the highest value obtained in zebrafish fed 1 % *D. dichotoma*, which were remarkably higher than control (Table 4). The lower inclusion levels had no significant effects compared to the control.

Similar results were obtained by evaluation of WBE globulins levels and lysozyme activity. While there were no significant differences between fish fed 0.25 %, 0.5 % *D. dichotoma* and control diet ($P > 0.05$),

Table 3
Growth performance parameters and survival (mean ± SD) of the fish fed diets supplemented with 0.25 % , 0,50 % and 1 % *Dictyota dichotoma*.

	Control	0.25 %	0.5 %	1 %
Initial weight (mg)	297.8 ± 2.4^{a}	301.73 ± 1.5^{a}	300.66 ± 2.4^{a}	298.3 ± 2.9^{a}
Final weight (mg)	341.2 ± 2.57^{c}	387 ± 2.88^{b}	393.1 ± 3.4^{b}	411.3 ± 1.6^{a}
WG (mg)	45.4 ± 0.9^{c}	85.5 ± 4.1^{b}	92.468 ± 3.5^{b}	113.3 ± 5.2^{a}
Survival (%)	100	100	100	100

Data in a row assigned with different letters denote significant difference ($P < 0.05$).

Table 2
Sequence and melting temperature (Tm) primers of selected mucosal immune response and antioxidant related genes expression in zebra fish.

Primer name	Primer sequence	Tm	Application	Accession no.
IL-1 q-PCRF	CGTCTCCACATCTCGTACTCA	58	immune	AY340959.1
IL-1 q-PCRR	GTGTCTTTCCTGTCCATCTCC			
Lyz q-PCRF	GGCAGTGGTGTTTTTGTGTC	58	immune	AY340959.1
lyz q-PCRR	CGTAGTCCTTCCCCGTATCA			
SOD q-PCRF	GGGTGGCAATGAGGAAAG	58	immune	NM_139180.1
SOD q-PCRR	GCCCACATAGAAATGCACAG			
CAT q-PCRF	GCATGTTGGAAAGACGACAC	58	immune	AF210640.1
CAT q-PCRR	GTGGATGAAAGACGGAGACA			
IGF1 q-PCRF	AGTGTACCATGCGCTGTCTC	58	Growth	NM_131825.2
IGF1 q-PCRR	AATAAAAGCCCCTGTCTCCA			
GHh q-PCRF	TTGGTGGTGGTTAGTTTGCT	58	Growth	AJ937858.1
GH q-PCRR	CTCAACTGTCTGCGTTCCTC			
β- actin q-PCRF	AGCAGATGTGGATCAGCAAG	58	Housekeeping gene	NM_131031.1
β- actin q-PCRR	TACCTCCCTTTGCCAGTTTC			

Table 4
The effects of different levels of *Dictyota dichotoma* on immune parameters of zebrafish (*Danio rerio*) (n = 9). Values are presented as the mean ± S.D.

	Control	0.25 %	0.5 %	1 %	P-value
Mucosal total protein (mg/ml)	0.61 ± 0.1^{b}	0.76 ± 0.2^{ab}	0.95 ± 0.25^{ab}	1.03 ± 0.15^{a}	0.009
Mucosal immunoglobulin (mg/ml)	0.17 ± 0.28^{b}	0.22 ± 0.02^{ab}	0.38 ± 0.1^{ab}	0.35 ± 0.13^{a}	0.006
Mucosal lysozyme (U/ml)	7.56 ± 0.77^{b}	8.47 ± 1.07^{ab}	8.93 ± 0.4^{ab}	9.72 ± 1.1^{a}	0.012
Serum total protein (mg/ml)	1.82 ± 0.28^{c}	1.49 ± 0.15b^{c}	1.99 ± 0.3^{ab}	2.44 ± 0.22^{a}	0.009
Serum immunoglobulin (mg/ml)	0.63 ± 0.15^{b}	0.72 ± 0.07^{b}	0.96 ± 0.22^{b}	1.3 ± 0.2^{a}	0.007
Serum lysozyme (U/ml)	18.43 ± 0.95^{b}	18.09 ± 0.94^{b}	18.96 ± 0.75^{b}	22.1 ± 2.8^{a}	0.009

Data in a row assigned with different letters denote significant difference ($P < 0.05$).

feeding on 1 % *D. dichotoma* remarkably increased WBE globulins levels and lysozyme activity ($P < 0.05$).

The lowest WBE protein level was observed in the group with 0.25 % *D. dichotoma* supplementation. However, no significant ($P > 0.05$) difference was obtained compared to control and 0.50 % *D. dichotoma* supplementation. The highest value was recorded in the group fed 1 %, *D. dichotoma*, which was statistically significant compared to those in 0.25 % *D. dichotoma* and control ($P < 0.05$) (Table 4).

3.3. Antioxidant enzyme activities

The effects of different levels of *D. dichotoma* on antioxidant enzyme activities (SOD, CAT, GST) of Zebrafish are presented in Table 5. The SOD and GST activities significantly ($P < 0.05$) increased in 0.5 % and 1 % *D. dichotoma* treatments compared with control and 0.25 % *D. dichotoma*. In contrast, it was noticed that the CAT activity of zebrafish fed *D. dichotoma* supplemented diets were not significantly higher than control ($P > 0.05$).

3.4. Gene expressions

Comparison of mRNA levels of immune-related genes (IL-1β and LYZ), growth-related genes (GH and IGF-1), and antioxidant related genes (SOD and CAT) of zebrafish fed with different levels of *D. dichotoma* were presented in Figs. 1–3. Immune-related genes expression (LYZ and IL-1β) showed significant differences in fish fed *D. dichotoma* supplemented groups compared to the control group. The IL-1β level was statistically higher in the fish fed 0.5 %, and 1 % *D. dichotoma* compared with 0.25 % treatment (Fig. 1). However, the LYZ level was highest in the 1 % supplemented groups among fish fed with *D. dichotoma* supplementation. There was no statistical difference between the 0.25 %, and 0.5 % supplemented groups (Fig. 1).

Table 5
The effects of different levels of *Dictyota dichotoma* on antioxidant defense of zebrafish (*Danio rerio*) (n = 9). Values are presented as the mean ± S.D.

	Control	0.25 %	0.5 %	1 %	P-value
SOD (U/ml)	1870.46 ± 20.01^{c}	1922.2 ± 24.2^{c}	2070.23 ± 27.7^{b}	2244.76 ± 25^{a}	0.000
CAT (U/ml)	0.25 ± 0.02^{a}	0.27 ± 0.03^{a}	0.286 ± 0.05^{a}	0.29 ± 0.05^{a}	0.656
GST (U/ml)	133.43 ± 6.27^{b}	137.96 ± 3.43^{b}	155.78 ± 4.73^{a}	156.18 ± 8.4^{a}	0.03

Data in a row assigned with different letters denote significant difference ($P < 0.05$).

Regarding growth-related genes, *D. dichotoma* affected GH and IGF1 expression in zebrafish in a dose-dependent manner and were remarkably upregulated in *D. dichotoma* fed fish compared to fish fed the control ($P < 0.05$) (Fig. 2). The GH gene expression level in treated groups were significantly higher than control group. Also, statistical differences were obtained among groups with *D. dichotoma* supplemented. While 0.5 % and 1 % *D. dichotoma* added groups showed similar characteristics, it was higher than the 0.25 % added group. Likewise, GH gene expression, IGF-1 gene expression was extremely high in the *D. dichotoma* added groups compared to the control group ($P < 0.05$). While 0.5 % and 1 % *D. dichotoma* added groups showed similar upregulated trends, it was higher than the 0.25 % added group.

Compared to the control group, the expression of antioxidant-related genes (SOD and CAT) upregulated in all groups fed with *D. dichotoma* supplemented diet ($P < 0.05$) (Fig. 3). The statistically significant differences were found between the macroalgae added groups and the control group. In case of SOD gene expression, statistical differences were also found among treated groups. The highest increase was observed in the 1 % macroalgae group, followed by 0.5 % and 0.25 % macroalgae, respectively ($P < 0.05$) (Fig. 3).

In CAT antioxidant gene expression, groups with added showed significantly higher values than the control group. Differences were also observed among groups with added macroalgae. The 0.25 % and 0.5 % supplementation groups gave higher values than the control group, and lower values than the 1 % macroalgae added group ($P < 0.05$) (Fig. 3).

4. Discussion

Although the health benefits of *D. dichotoma* have been demonstrated in humans, there is no available information on the effects of dietary *D. dichotoma* on fish. In this context, this study was conducted to evaluate the potential impact of *D. dichotoma* powder using zebrafish as a model organism on antioxidant defense, immune response, and gene expressions following the addition in the diet.

Oxidative stress is a physiological state, within which the equilibrium between the prooxidants and antioxidants are disturbed in body, leading to formation of free radicals (Fazelan et al., 2020; Hoseini et al., 2021; Paray et al., 2020). The antioxidant system, which protects fish against oxidative stress, is also essential for fish health (Wang et al., 2016; Ahmadifar et al., 2019).

SOD, GST, and CAT are the most important antioxidant components in living organisms that are considered biomarkers for oxidative stress (Zargar et al., 2020; Rufchaei et al., 2021; Yousefi et al., 2021). These antioxidant enzymes have different roles. SOD converts superoxide ions to hydrogen peroxide, while CAT and GPx are both involved in hydrogen peroxide degradation and detoxification. On the other hand, GST is responsible for xenobiotic detoxification by binding GSH to the xenobiotic (Hoseini and Yousefi, 2019; Zargar et al., 2020). Studies in fish and other aquatic species have shown that antioxidant enzymes activities such as SOD, CAT, GST are significantly affected by feed compositions (Ashour et al., 2020; Fazelan et al., 2020; Harikrishnan et al., 2020).

To the best of our knowledge, this is the first attempt to investigate the effects of *D. dichotoma* on antioxidant response. In the current study, SOD and GST antioxidant activities were significantly increased when fed diets supplemented with 0.5 % and 1 % *D. dichotoma* compared to control and 0.25 % *D. dichotoma* supplemented diets ($P < 0.05$). However, no significant difference in CAT activity was observed ($P > 0.05$). CAT is a type of ROS scavenging enzyme that can convert H_2O_2 to O_2 and H_2O and remove H_2O_2 from the body. No change in CAT activity is attributed to the fish not having high concentrations of hydrogen peroxide, thus being in a healthy condition (Hoseini et al., 2021; Yousefi et al., 2022). Although there is no available information on the effects of dietary *D. dichotoma* on fish, in agreement with our results, increased SOD antioxidant activity has been shown in shrimp fed dietary *D. dichotoma* powder (Anaya-Rosas et al., 2019). Similar to our findings,

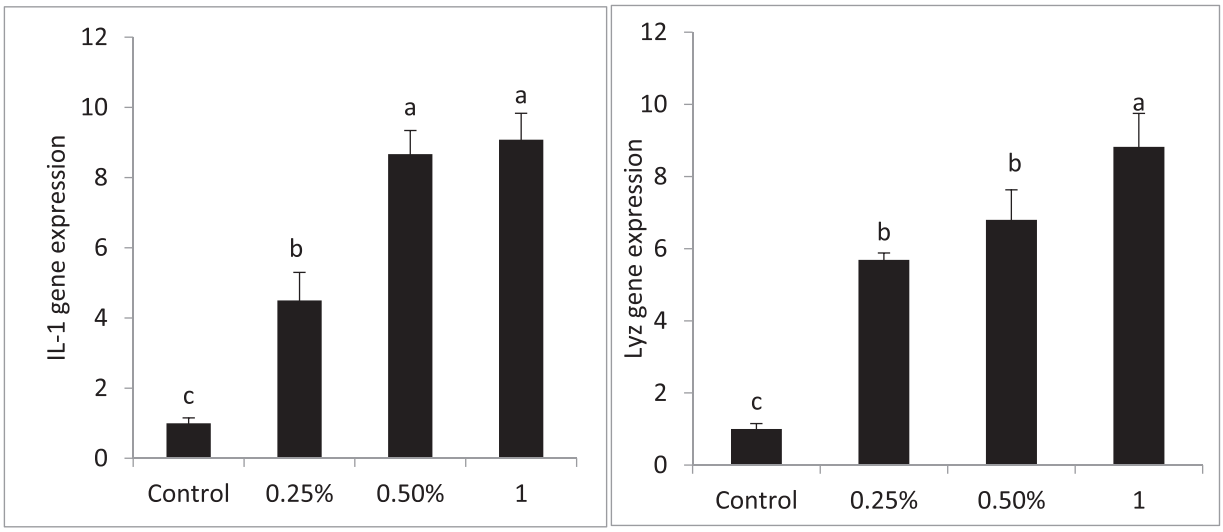

Fig. 1. The effects of different levels of *Dictyota dichotoma* on immune related genes expression in zebrafish (*Danio rerio*) intestine (n = 9). Values are presented as the mean ± S.D. Different letters indicate significant difference among groups ($P < 0.05$).

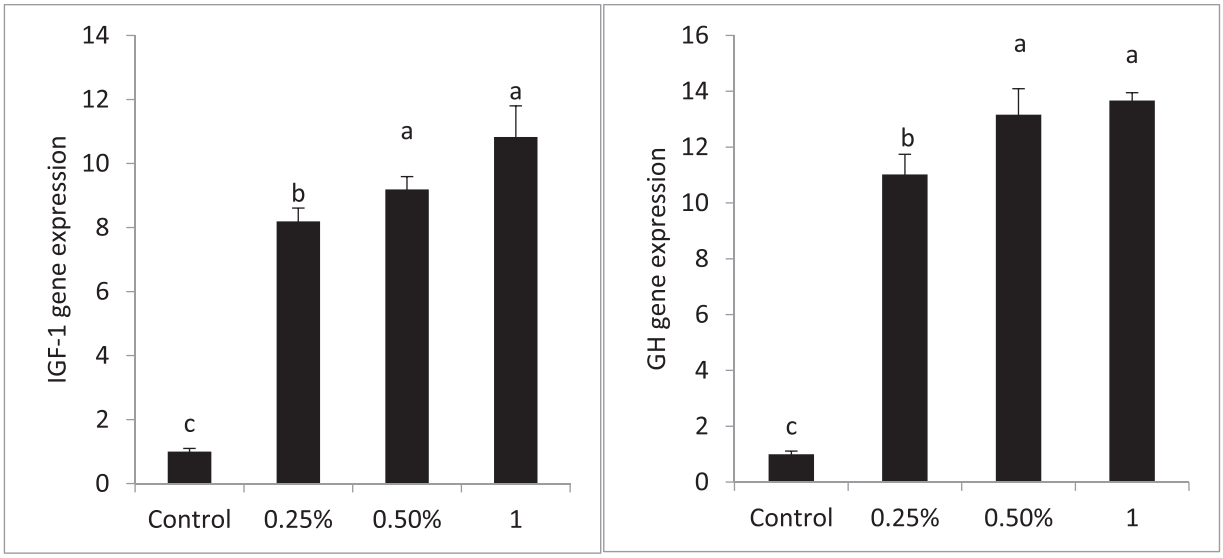

Fig. 2. The effects of different levels of *Dictyota dichotoma* on growth related genes expression in brain and liver of zebrafish (*Danio rerio*) (n = 9). Values are presented as the mean ± S.D. Different letters indicate significant difference among groups ($P < 0.05$).

the tested ethanolic extracts of *D. dichotoma* showed great antioxidant potential by measuring three in-vitro antioxidant assays (Mekinic et al., 2021). In contrast to the present results, El-Shaibany et al. (2020) reported that the different fractions of *D. dichotoma* indicated a low antioxidant activity, with petroleum ether and chloroform fractions exhibiting the least antioxidant activity.

Adding *D. dichotoma* to zebrafish diet, supporting the results obtained from antioxidant activity, also led to an increase at the transcriptional levels. The inclusion of *D. dichotoma* in the fish diets improved remarkably the SOD and CAT gene expressions compared to control. Moreover, while CAT did not show any change in antioxidant enzyme activity, it showed a significant increase at the molecular level. Such results are in line with Hoseinifar et al. (2018) stated that red algae Gracilaria powder administration elevated SOD and CAT gene expressions.

It has been stated that the antioxidant potential of *D. dichotoma* may be due to its high phenolic compound content (Zubia et al., 2009; Namvar et al., 2013; El-Shenody et al., 2019; Anaya-Rosas et al., 2019). In addition, it has been suggested that brown algae species in which *D. dichotoma* is found have higher antioxidant activity than green and red algae due to the presence of phlorotannins, which are the dominant polyphenolic secondary metabolites found only in brown algae (Namvar et al., 2013; Catarino et al., 2021; Mekinic et al., 2021). Accordingly, the enhancement of the antioxidant parameters in the current study may be attributed to phenolic compound content particularly, phlorotannins. However, the confirmation of this hypothesis also deserves studies on determining phenolic contents using different extraction methods. It has been suggested that natural antioxidants derived from various plants and seaweeds not only show great potential in improving the oxidative stability of food products but also have an essential role in maintaining health by strengthening the body's defense system and preventing a wide range of diseases (Wang et al., 2016).

Improving the immune system by including various additives from different origins in the diets of fish and crustaceans has been one of the essential goals of aquaculture (Safari et al., 2016; Hoseinifar et al., 2018; Rufchaei et al., 2021). Humoral and mucosal immunity parameters are considered essential parts of protection against opportunistic pathogens, and their measurements are considered an indicator of health status in

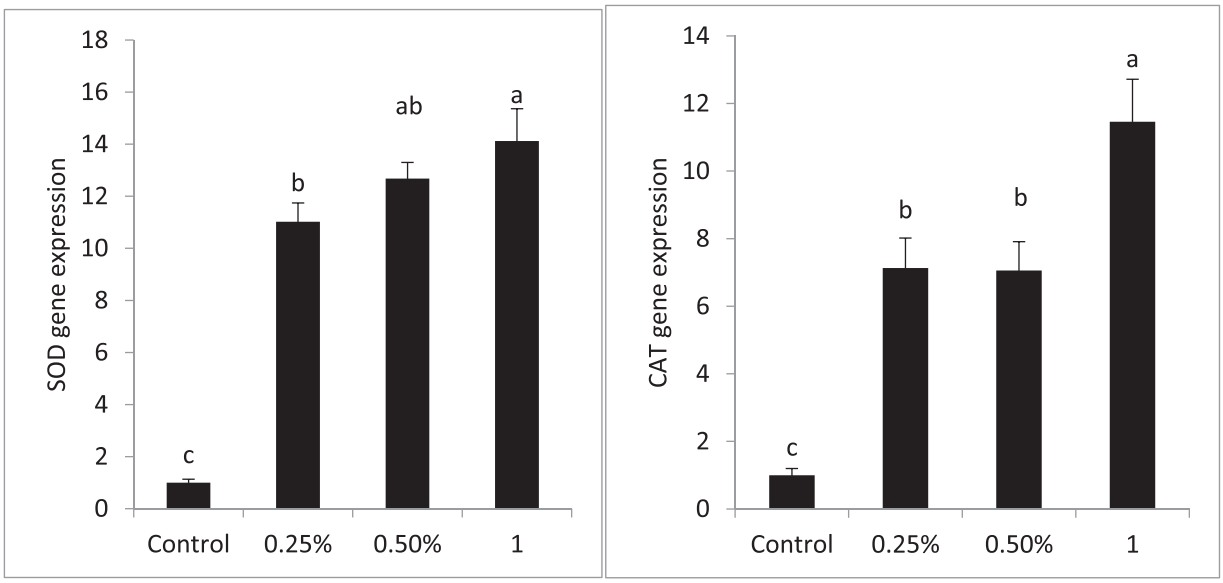

Fig. 3. The effects of different levels of *Dictyota dichotoma* on antioxidant enzyme genes expression in the liver of zebrafish (*Danio rerio*) (n = 9). Values are presented as the mean ± S.D. Different letters indicate significant difference among groups ($P < 0.05$).

fish (Esteban, 2012; Modanloo et al., 2017; Hoseinifar et al., 2020). The present study investigated lysozyme, globulins, and total protein levels as indicators of nonspecific immune system in both WBE and skin mucus.

Lysozyme, which is among the most frequently tested immune parameters in diets supported by immunostimulants, is found in mucus, plasma, and other body fluids. Lysozyme plays an essential role in both killing bacteria by breaking the peptidoglycan layer on the cell wall of bacteria and triggering other immune parameters in the innate immune system of fish (Yousefi et al., 2018; Ahmadifar et al., 2019; Devi et al., 2019). In our study, the lysozyme activity in skin mucus and whole body serum in zebras fed with *D. dichotoma* added feed was found to be relatively high compared to the control group. The highest value was observed in the groups with 1 % supplementation of *D. dichotoma*.

Globulins are also one of the main components involved in forming the immune response that protects the body against various pathogens (Hoseinifar et al., 2019; Rufchaei et al., 2021). This study showed that skin mucus and WBE globulins levels was significantly elevated in zebrafish following administration of all supplemented diet, with highest level in 1 % *D. dichotoma* fed treatment. In agreement with our findings, Hoseinifar et al. (2018) have shown that supplementing the diet with red macroalgae Gracilaria gracilis increased total immunoglobulin in skin mucus.

Regarding total protein, along with other factors such as albumin, globulin, glucose, and triglyceride levels, it is one of the parameters considered critical factors in promoting and maintaining the health of the immune system and blood functions. They are also regarded as valuable tools in assessing nutritional status (Ashour et al., 2020). It was shown that the additions of different levels of *D. dichotoma* significantly increased the total protein levels in skin mucus and WBE.

According to our literature review, there was no available information about the comparative study of *D. dichotoma* on fish innate immune parameters. However, similar to the present findings, it has been reported that the addition of fucoidan from brown algae to the diet increases the lysozyme activity and level of total protein in sea bream, *Pagrus major* (Sony et al., 2019), tilapia, *Oreochromis niloticus* (Abdel--Warith et al., 2021) and catfish, *Clarias gariepinus* (El-Boshy et al., 2014). In addition, Ashour et al. (2020) suggested that a significant increase in serum lysozyme activity was observed in tilapia fed commercial liquid seaweed extract supplemented feed called TAM. On the other hand, Yang et al. (2014) reported that adding fucoidan to yellow catfish (*Pelteobagrus fulvidraco*) feed did not cause any change in the serum total protein level, while Peixoto et al. (2016) reported that no significant increases in lysozyme activity were observed in sea bass *(Dicentrarchus labrax)* fed with three different macroalgae feeds. The increase in the amount of lysozyme, globulins, and total protein in both WBE and skin mucus can be considered an important indicator that the addition of *D. dichotoma* to the diet improves the immune system of zebrafish.

The present investigation revealed that the dietary *D. dichotoma* remarkably increased WBE and skin mucus lysozyme activity, total protein content, and globulins level. Moreover, the increase observed in the immune system was also supported by our gene expression studies at the molecular level.

The present study results revealed significant upregulation of cytokines (Lyz and IL1-β) in zebrafish fed *D. dichotoma*, and the highest amount was observed in fish fed 1 % *D. dichotoma* supplemented diet. IL-1β, as a proinflammatory cytokine, plays an essential role in the host response to microbial invasion, injury of tissue and immunological reactions, and mediate the secretion of other cytokines (Safari et al., 2016; Devi et al., 2019).

The increase in lysozyme expression supports the results obtained with lysozyme activity in WBE and skin mucus, and thus the increase in the nonspecific immune system (Ahmadifar et al., 2019). To the best of our knowledge, there is no published study regarding the effects of dietary *D. dichotoma* on fish intestinal immune-related gene expression. In this context, several previous studies have confirmed that supplementing the diet with various immunostimulants such as ferula (Safari et al., 2016), Sodium acetate (Safari et al., 2020a), Malic acid (Safari et al., 2021) increases LYZ and IL-1 β gene expressions in different fish species. On the other hand, Hoseinifar et al. (2018) reported that the addition of *Gracilaria gracilis* to zebra feed did not affect Lyz and IL-1β gene expressions. In another study, Hoseinifar et al. (2015) stated that the addition of palm extract did not cause any change in Lyz gene expression in carp but significantly down-regulated IL-1β gene expression. The contradictory results are possibly due to differences in fish species, dose level, and additives.

In fish, like mammals, growth is regulated by the interaction between Growth hormone (GH) and insulin-like growth factors (IGF-1). They are pleiotropic hormones with essential roles in lifespan (Caputo et al., 2021). GH is produced in the pituitary and triggers the release of Igf-1 from the liver (Lieke et al., 2021). GH and IGF-I gene expressions can be affected by factors such as the environment, nutrition, and

developmental stage of the organism, thus making them a valuable tool for monitoring the performance of cultured fish (Triantaphyllopoulos et al., 2020). In the present study, evaluation of growth-related genes expression in zebrafish following feeding on *D. dichotoma* macroalgae powder supplemented diet revealed significant up-regulation of GH and IGF-1 genes in a dose-dependent manner.

To our knowledge, there are no comparable studies on the effects of *D. dichotoma* on growth-related gene expression as well as growth performance in fish. However, similar to the findings obtained in our study, significant increases were reported in studies with different additives such as ferula powder, carp (Safari et al., 2016), Myrtle, zebrafish (Safari et al., 2017), Fulvic acid, zebrafish (Lieke et al., 2021) polyphenol, beluga sturgeon (Safari et al., 2020b). Contrary to our results, feeding zebrafish and carp with apple cider (Ahmadifar et al., 2019) and malic acid (Safari et al., 2021), respectively, had no significant effect on the expression growth-related genes. It is considered that the differences in the results may be due to the origins of the additives used, the dose amounts, and the differences in the fish species, as well as the experimental conditions.

These contradictory results revealed that the effects of plant-derived products are species-specific and may be related to herbal species, supplementation dose, experimental condition, and fish species (Fazelan et al., 2020; Rufchaei et al., 2021).

In conclusion, macroalgae have a variety of bioactive compounds that could be a new source of functional ingredients for human, livestock, and fish health applications (Saeed et al., 2021). Their importance increases as their rich content are revealed in a way that parallels the developing technologies. In the current study, it is suggested that adding 0.5–1 % of *D. dichotoma* powder to the feed will have positive effects on fish growth, antioxidant, and immune system. Further studies with other aquatic species should support these results. However, *D. dichotoma* was used as a powder, in this study, and derivatives such as fucoidan, phlorotannins, obtained by using different extraction methods, and its mechanism of action should be investigated.

Ethics

All experiments were performed following the protocol approved by the committee of ethics of the faculty of sciences of the University of Tehran (357; 8 November 2000).

CRediT authorship contribution statement

Negin Mahmoudi: Methodology, Data curation, Investigation. **Ali Shabani:** Conceptualization, Methodology. **Seyed Hossein Hoseinifar:** Conceptualization, Resources, Formal analysis, Writing - review & editing. **Metin Yazici:** Conceptualization, Resources. **Ehab El-Haroun:** Writing – original draft. **Roghieh Safari:** Conceptualization, Supervision, Formal analysis, Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors would like to thank the financial support of GUASNR.

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