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Antioxidant, intestinal immune status and anti-inflammatory potential of *Chenopodium ambrosioides* L. in fish: *In vitro* and *in vivo* studies

Martha Reyes-Becerril[a,*,1], Carlos Angulo[a,1], Veronica Sanchez[a], Juan Vázquez-Martínez[b], Mercedes G. López[b]

[a] Immunology & Vaccinology Group. Centro de Investigaciones Biológicas del Noroeste (CIBNOR), Av. Instituto Politécnico Nacional 195, Playa Palo de Santa Rita Sur, La Paz B.C.S, 23096, Mexico

[b] Chemistry of Natural Products Laboratory, Biotechnology and Biochemistry Department, Centro de Investigación y de Estudios Avanzados del IPN (CINVESTAV-IPN), Irapuato, Gto., Mexico

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ABSTRACT

Chenopodium ambrosioides L. has been used for centuries as traditional medicine in many clinical situations. The objectives of this study were first to assess the nutraceutical potential of *C. ambrosioides* L. extract through analyses of its chemical composition and antioxidant properties, followed by assessing toxicity and antioxidative activities on fish splenocytes. The second one was to perform an *in vivo* study using dietary *C. ambrosioides* L. extract (0.0, 0.5, 1.0 and 2.0%; w/w) for 15 and 30 days (2-week and 4-week treatments) to assess associated-intestine health status by short-chain fatty production, antioxidant enzyme activities and anti-inflammatory effects on Pacific red snapper (*Lutjanus peru*). Non-polar and polar fractions were detected by gas chromatography/mass spectrometry (GC-MS) in *C. ambrosioides*, of which the most abundant compounds were carvacrol, phytol, squalene, vitamin E and sucrose. The extract of *C. ambrosioides* L. enhanced a considerable antiradical and reducing power; fish splenocytes responded positively with higher (88%) cell viability than control. The production of nitric oxide and superoxide anion, as well as superoxide dismutase and catalase activities, were also enhanced in splenocytes treated with *C. ambrosioides* L. The *in vivo* study results showed that acetate was the major short-chain fatty acid found in fish receiving *C. ambrosioides* L. after week four. Pro-inflammatory cytokine gene expression in intestine was modulated in fish fed with *C. ambrosioides* L. at any time of the experimental trial. In addition, the histological findings suggested that its extract did not cause inflammatory damage in intestine. Overall, the results suggest that *C. ambrosioides* L. is safe for immune cells and promoting intestinal health status of fish through antioxidant and anti-inflammatory effects, making it an interesting additive in functional diets.

1. Introduction

In the last decades, antibiotics have been used against infectious diseases in finfish, but concerns about their accumulation in fish body and the appearance of resistant bacteria has led to searching for eco-friendly alternatives [1]. Vaccination is a convenient approach for prevention and treatment; nonetheless, most of the vaccines are effective against specific pathogen species, which is impractical and can be economically unviable in fish farming [2]. Prophylactic methods based on immune system stimulation are pursued in modern aquaculture [3]. Nowadays, an alternative is the use of plants or their extracts and probiotics in aquaculture industry because of the claim of organic food. Plants produce secondary metabolites as part of their physiological responses, which are also responsible for the observed pharmacological activities [4]. Recent reviews [5] have highlighted that medicinal plants can promote immune defenses and disease resistance in fish, which may have substantial positive outcomes for finfish farms. These natural products are easily obtained, usually inexpensive, and their antimicrobial effects are known to play a significant role in preventing diseases [5]. In this trial, the attention was focused on intestinal health, antioxidant and anti-inflammatory effects on fish by exposure to/supplementation of *Chenopodium ambrosioides* L., which is an annual herb that belongs to the family Chenopodiaceae and the genus Chenopodium, an herbaceous shrub known in Mexico as “epazote”. Epazote has a strong aroma and is characteristically used in Mayan cuisines of Mexico and Guatemala [6]. This species is native to Central and South

* Corresponding author.
E-mail address: mreyes04@cibnor.mx (M. Reyes-Becerril).
[1] These authors contributed equally to the study in this paper.

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America but broadly found in many regions of the world; according to the World Health Organization (WHO), *C. ambrosioides* L. is one of the topmost employed medicinal plants [7]. Leaves are used to treat viral and bacterial infections as antifungal and antiparasitic, among other uses [8]. It has well-known immunostimulatory activity on innate (macrophages) and adaptive (lymphocytes) immune cells [9,10]. Calado et al. [11] detected that crude hydroalcoholic extract of *C. ambrosioides* was effective to reduce inflammation and pain distress in a rat model of chronic osteoarthritis. Recently, Rios et al. [7] observed that *C. ambrosioides* application enhanced phagocyte activity and consequently decreased bacterial counts, as well as the systemic inflammatory response in a mouse model of sepsis. Although anti-inflammatory properties of *C. ambrosioides* L. have been validated in several animal species, surprisingly, there is still a lack of knowledge about *C. ambrosioides* L. effects on the immune system and its relationship with intestinal health in fish. Furthermore, few reports have described the possible molecules associated in the anti-inflammatory effects [11–13]. Interestingly, tea of *C. ambrosioides* or epazote made from its dried leaves is patented as a treatment for uterine fibroids [14]. Diroff [14] provided a detailed review on all aspects of *C. ambrosioides*, including its medicinal properties and biological actions.

Therefore, the main purpose of this research was to investigate the potential use of *C. ambrosioides* L. dried leaves in Pacific red snapper (*Lutjanus peru*), an important species of local, small-scale fisheries of Mexico and distributed throughout tropical and subtropical regions from the Gulf of California to Peru. First, the antioxidant properties of *C. ambrosioides* L. leaves were analyzed, followed by an *in vitro* study using splenocytes exposed to its extract, where the toxicological effect and antioxidant activity were assessed at 24 h. The second objective was to know if dietary *C. ambrosioides* L. maintained the associated-intestine health status by assessing short-chain fatty acid production, antioxidant enzyme activity and anti-inflammatory effects on fish intestine.

2. Materials and methods

2.1. Mexican Chenopodium ambrosioides L

Chenopodium ambrosioides L. leaves were obtained from a local market (La Paz, Baja California Sur (BCS), México) and washed with distilled water to remove extraneous matters. Later, leaves were desiccated in a forced-air oven (28 °C, 24 h) and pulverized using sterile pestle and mortar; acquired powders were pulverized and sieved (0.5-mm mesh).

2.2. Chemical composition (GC-MS) of Chenopodium ambrosioides L

The polar and non-polar fractions of powdered *C. ambrosioides* L. were extracted by gas chromatography and mass spectrometry (GC-MS) analyses performed on Agilent gas chromatograph/mass spectrometer model 7890A/5977 (Agilent Technologies, Inc., Santa Clara, CA, USA) using a capillary column HP5-MS (30 m × 0.25 mm x 0.25 μm). The polar phase was obtained by mixing 100 mg of *C. ambrosioides* L. with 1 ml high-performance liquid chromatography (HPLC) ethanol for 1 min and then centrifuged at 3000 × *g* for 15 min. Liquid and solid phases were separated; additionally, the solid phase was re-extracted again twice. All liquid phases obtained were mixed and dried at 45 °C with MaxyDry (Eppendorf, Inc., Hamburg, DE). The samples were derivatized using N, O-bis (trimethylsilyl)-trifluoroacetamide (BSTFA) added with 1% of TMCS (N-Methyl-N-(trimethylsilyl) trifluoroacetamide) reagent and pyridine at 85 °C for 25 min and mixed with 100 μl isooctane. Samples were injected on splitless mode with the following conditions: GC injector 280 °C; oven at 40 °C for 2 min; programmed at a rate of 4 °C/min to 240 °C; kept at 240 °C for 1 min; and finally ramped at 10 °C/min to 315 °C and kept for 15 min, helium carrier gas at 1 ml/min. The ion-source temperature operated on ionization mode at 70 eV and 150 °C. The quadrupole MS operated at 230 °C, and data were recorded on SCAN mode (*m/z* 40 to 550). The non-polar fraction was extracted with HPLC pentane, following the steps previously mentioned, and then dried under a gentle nitrogen stream. Samples were injected on splitless mode with the following conditions: GC injector 260 °C, oven at 40 °C for 5 min; programmed at a rate from 4 °C/min to 150 °C; kept at 150 °C for 5 min; finally ramped at 8 °C/min to 290 °C and kept for 5 min, helium carrier gas at 1 ml/min. The ion-source temperature operated on ionization mode at 70 eV and 150 °C. The quadrupole MS operated at 230 °C, and data were recorded on SCAN mode (*m/z* 40 to 550).

2.3. Antioxidant properties of Chenopodium ambrosioides L

One gram of powder of *C. ambrosioides* L. was mixed with 10 ml of 100% methanol and shaker incubated at 250 rpm for 24 h at 28 °C. Then the extracts were filtered through Whatman No. 1 filter paper; the volume was totally dried in a bomb vacuum. Methanolic extract was redissolved in methanol for anti-radical activity evaluation and PBS (phosphate buffer solution, 1 M, pH 7.3) (final concentration 250 μg/ml) used for the *in vitro* study.

2.3.1. Free radical scavenging activity

To determine the antioxidant activity of plant extracts, the method based on the reduction of methanolic solution of colored free radical $DPPH^-$ was used. The changes in color from deep-violet to light-yellow were measured at 515 nm in a UV/visible light spectrophotometer (Thermo Scientific, Waltham, MA, USA). Radical scavenging activity was measured according to Brand-Williams et al. [15] method. Antioxidant activity was expressed as EC50 (efficient concentration): the amount of dry extract (mg of DW) needed to obtain 50% activity per 1.0 ml of the initial solution.

2.3.2. Superoxide radical scavenging activity

The scavenging activity of the superoxide radical was assayed using the procedure as reported by Martinez et al. [16] with some modification. Each 3 ml reaction mixture consisted of 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, and various concentrations of *C. ambrosioides* L. (50–500 μg/ml), 100 μM EDTA, 75 μM NBT and 2 μM riboflavin. The reaction mixture was incubated with light for 10 min and absorbance was measured at 560 nm. Decreased absorbance of the reaction mixture indicated an increased superoxide radical scavenging activity. Butylated hydroxyanisole (BHA) was used as a positive control; the IC50 value represented the concentration of *C. ambrosioides* L. at which 50% of superoxide radicals were inhibited. The scavenging activity was calculated according to the following formula:

$$\text{Scavenging activity}\,(\%) = \{(A0 - A1)/A0\} \times 100$$

where A0 was the absorbance of the blank and A1 was the absorbance in the presence of sample.

2.4. Model of study: Lutjanus peru

Fish (*Lutjanus peru*, Pacific red snapper) (180 ± 20 g) specimens were donated from the enterprise Earth Ocean Farms (La Paz, Mexico). The fish fed (2% body weight day^{-1}) with commercial diet (Skretting, CAN) were quarantined for 15 days to guarantee that fish had no disease signs. Fish rearing conditions were continuously monitored to ensure water quality (nitrite and ammonia levels lower than 0.02 mg l^{-1}), temperature (26 °C), photoperiod (12 h dark/12 h light), dissolved oxygen (4.4–7.0 mg l^{-1}), pH (7.7–8.1. The Bioethical Committee of Centro de Investigaciones Biológicas del Noroeste (CIBNOR) authorized this study.

2.5. Experiment 1: viability and antioxidant effect of Chenopodium ambrosioides L. on fish splenocytes

To examine the viability and antioxidant effect of *C. ambrosioides* extract, Pacific red snapper splenocytes were used. Spleen leukocytes were adjusted to 10^6 cells ml^{-1} TC20 Coulter Particle Counter (BioRad, Hercules, CA, USA). Leukocyte viability was estimated using trypan blue (Sigma, Cat. T-8154), and cell suspensions with more than 95% viability were used for *in vitro* experiments [17].

One milliliter of leukocyte suspension was placed into flat-bottomed 24-well cell culture plates (Sigma, St. Louis, MO, USA) containing 1×10^6 cells per well. Then, leukocytes were incubated with 25 µl of *C. ambrosioides* extract resuspended in PBS (250 µg/ml) at 25 °C and 5% CO2 atmosphere for 24 h. As negative control, leukocytes incubated with RPMI medium were used and collected at 24 h post-incubation.

2.5.1. Cell viability

The resazurin assay was used to determine the effect of *C. ambrosioides* on viability of splenocytes according to Riss et al. [18]. Briefly, splenocytes were dispensed in 96-well plates (75 µl 1×10^6 cells/ml), incubated with 25 µl/well of *C. ambrosioides* extract (250 µg/ml) and cultured overnight (at 25 °C and 5% CO2). Then, cells were stained with 10 µl resazurin solution (Sigma, St. Louis, MO, USA) and incubated at 25 °C and 5% CO_2 for 4 h. Fluorescence was measured in Varioskan™ Flash Multimode Reader (Thermo Scientific, Waltham, MA, USA) excitation at 530 nm and emission at 590 nm. Splenocytes without *C. ambrosioides* and those incubated with methyl sulfoxide (DMSO, 10% final concentration) were used as controls.

2.5.2. Antioxidant parameters in splenocytes

Superoxide dismutase (SOD) activity was measured in skin mucus by the percentage reaction inhibition rate of enzyme with WST-1 (water soluble tetrazolium dye) substrate and xanthine oxidase using a SOD Assay Kit (Cat. 19160, Sigma, St. Lous MO, USA) according to the manufacturer's instructions. Catalase (CAT) activity was assayed by the method of Clairborne [19], and the decrease in absorbance of H2O2 was followed at 240 nm. One unit of enzyme activity was defined as the amount of enzyme required to degrade 1 mM of H_2O_2 in 1 min.

Nitric oxide production was determined according to Neumann et al. [20]. Briefly, 100 µl of leukocytes were incubated with an equal volume of Griess reagent in 96-well plate, mixed and incubated at room temperature in the dark for 15 min; OD was read at 562 nm in a microplate reader (BioRad, Model 3550 UV, Hercules, CA, USA). The data were expressed in nitrite concentration (µM).

To determine intracellular *C. ambrosioides*-induced reactive oxygen species (ROS) levels, splenocytes were incubated with 20 µM of 2′,7′-dichlorofluorescein diacetate (DCF-DA) for 1 h at 25 °C, as described previously [21]. The intracellular fluorescence levels were measured at 485 nm excitation and 538 nm emission using a Varioskan™ Flash Multimode Reader (Thermo Scientific, Waltham, MA, USA). The results were expressed as fold change in the *C. ambrosioides*-group compared with the control group.

2.6. Experiment 2. dietary Chenopodium ambrosioides L

The experimental *C. ambrosioides* L. diets were supplemented at the levels of 0 (control), 0.5, 1.0 and 2% dry food for the four experimental groups. The control diet was prepared by adding only water. To prepare them, the commercial diet was ground and mixed with *C. ambrosioides* L. powder. Mixed diets were extruded at 25 °C and pellets were desiccated as described above and stored at 4 °C for further use. A one-week adaptation period was applied using the control diet to all fish. Fish were weighted and daily diet ration was adjusted at 2% of their weights.

2.7. In vivo experimental design and sampling

Seventy-two healthy Pacific red snapper specimens were randomly placed in 12 running seawater experimental tanks (four experimental diets by triplicate/seven fish per tank). Two fish from each aquarium ($n = 6$ fish by experimental group) were examined at the end of 2-week and 4-week feeding treatments of the experiment for immune analyses. Fish were starved (24 h) before sampling and then, they were euthanized using clove oil (100 mg l^{-1} marine water). Content of the anterior intestine was collected in tubes and frozen at −80 °C for SCFA (short chain fatty acid) analysis, and another intestinal segment was fixed (10% neutral formalin) for histology. Finally, for transcriptional analysis, RNA was obtained from intestine samples (0.5 g) by the TRIzol method (Invitrogen, Carlsbad, CA, USA) and stored (−80 °C) until use.

2.8. Intestinal parameters

2.8.1. Intestine short-chain fatty acids (SCFAs) profile

The content of the anterior intestine was obtained from each sampled fish. One hundred milligrams of each sample were suspended in 1 ml of aqueous 0.5% phosphoric acid solution and mixed in vortex for 2 min. After that, samples were centrifuged at 10000 rpm for 10 min. Then, supernatant was transferred to a vial, extracted with an equal volume of ethyl acetate and centrifuged (4000 g, 10 min). Then, 200 µl of the ethyl acetate phase were transferred to a vial and immediately analyzed by Gas Chromatography (GC) using a capillary column of NUKOL (30 m × 0.25 mm x 0.25 µm) [22].

2.8.2. Preparation of crude extract for superoxide dismutase and catalase assays

SOD and CAT activities were assayed from intestine supernatants. Briefly, 0.1 g of tissue was homogenized in cold 0.5 M phosphate-buffer for 10 s and centrifuged at 12,000 g for 10 min at 4 °C. Supernatants were recovered and stored at −80 °C. Superoxide dismutase and catalase activities were previously described in part 2.5.2.

2.8.3. Gene expression

Pro-inflammatory cytokines Interleukin-1β (IL-1β, Accession number: KX137831), IL-6 (Accession number: MF136719) and IL-8 (Accession number: MF136720) mRNA expression patterns were measured in intestinal samples by real-time PCR using SYBR green fluorescent (Ssofast™ EVAGreen® Super Mix.quantitative) in a CFX96 Touch™ thermocycler (BioRad, Hercules, CA, USA). The PCR amplification program consisted of initial warming at 98 °C for 30 s, followed by 40 cycles at 98 °C for 10 s, 60 °C for 10 s, and a final extension at 65–95 °C for 4 min. Genomic contamination in samples was discarded using RNA as templates. The gene expression level of each gene was normalized to the elongation factor-1 alpha (EF1-α) gene (Accession number: KM669764.1) (Table 1). The relative mRNA expression of target genes in *C. ambrosioides* groups *versus* the control group was determined as previously described [23] and expressed as mean ± standard deviation (SD).

2.8.4. Histological analysis

Intestine sections were prepared in paraffin and then cut (5 mm) for staining with hematoxylin-eosin [24]. Slides were examined under light microscope and photographs were obtained. Thereafter, histological measurements were performed with Image Pro Plus V4.5 software. At least three slides per fish and ten pictures per slide were processed.

2.9. Statistical analysis

Data were obtained from triplicates, and the mean ± SD was calculated for each group and sampled time. One-way ANOVA was run to analyze the effects of *C. ambrosioides* L on antioxidant parameters and gene expression. When statistical differences were observed, Tukey

Table 1
Oligonucleotide primers used for real-time PCR.

Gen	Gen abbreviation	Accession No.	Length of product (bp)	Primer sequence (5′-3′)
Interleukin-1β	IL-1β	KX137831	159	AGCATCCACTGACAATGAAGAG ATTCAGACACCTCTTGCTCTTC
Interleukin-6	IL-6	MF136719	152	GCTTTTGAAAATGAATTCCAGG TTGAGAAGAACCGTGTAAGTGAG
Interleukin-8	IL-8	MF136720	155	CATCAGCAGGGACTACAACAC AAACCTTCTCCCCGCTTG
Elongation factor-1α	EF-1α	KM669764.1	139	CTGCAGGACGTCTACAAAATC GGTGCATCTCAACAGACTTAAC

multiple range test was used and considered significant at $P < 0.05$. All the statistical analyses were performed in SPSS v.19.0 software (SPSS, Richmond, VA, USA).

3. Results

3.1. Chemical composition of Chenopodium ambrosioides L

The analysis of the *C. ambrosioides* L. extract led to the identification of non-polar and polar fraction compounds (Tables 2 and 3). The non-polar fraction was enriched in NI (2,5-Dimethyl-3-hexyne-2,5-diol like) (10%), Carvacrol (5%), NI (3-Isopropyl-4-methyl-1-pentyn-3-ol like) (14%), phytol (10%), squalene (9%) and vitamin E (6.9%), which were the major components. On the other hand, the polar-fraction of *C. ambrosioides* L. contained sucrose with major percentage (32%), D-Glucose isomer 3 (7.7%), α-Linolenic acid (5.8%), phosphoric acid (2.4%), and glycerol (3.7%).

Table 2
Non-polar fraction of *Chenopodium ambrosioides* as determined using GC-MS and expressed as a percentage of the total peak area and expressed as relative %.

RT	Name	Mean	Relative %
8.217	(2E)-2-Ethylidene-1,1-dimethylcyclopentane	69381	0.121
13.871	Bicyclo[3.2.1]oct-2-ene, 3-methyl-4-methylene-	89878.5	0.156
14.662	o-Cymene	283875	0.494
14.82	α-Limonene	290784.5	0.506
18.394	trans-p-Mentha-2,8-dienol	1345196.5	2.339
18.644	NI	306312.5	0.533
18.948	4-Isopropenyl-1-methyl-2-cyclohexen-1-ol	633743.5	1.102
19.463	4,7,7-Trimethylbicyclo[4.1.0]hept-4-en-3-ol	209132.5	0.364
19.653	NI (terpene like)	173187.5	0.301
20.246	NI (terpene like)	257658.5	0.448
20.906	5-Isopropenyl-2-methylenecyclohexanol	1064467	1.851
21.891	3,4-Dimethylbenzaldehyde	366619.5	0.638
22.064	p-Mentha-6,8-dien-2-ol, cis-	503928	0.876
22.357	5-Isopropenyl-2-methylenecyclohexanol (isomer 2)	1674592.5	2.912
22.463	NI (terpene like)	306794.5	0.534
22.927	p-Mentha-6,8-dien-2-one, (R)-(−)-	168324	0.293
23.301	Benzene, m-di-tert-butyl-	1331825	2.316
23.377	1.6-Isopropyl-3-methyl-7-oxabicyclo[4.1.0] heptan-2-one	927401	1.613
23.809	2,5-Dimethyl-3-hexyne-2,5-diol	1569630.5	2.730
24.51	NI (2,5-Dimethyl-3-hexyne-2,5-diol like)	6126271	10.653
24.706	Carvacrol	2950649.5	5.131
25.043	NI (3-Isopropyl-4-methyl-1-pentyn-3-ol like)	8504680.5	14.789
25.798	NI	3264852.5	5.678
26.851	NI	446628.5	0.777
27.021	NI	652412.5	1.135
30.875	β-Ionone	1158220	2.014
32.214	Dihydroactinidiolide	962465	1.674
42.442	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	684230.5	1.190
42.561	Hexahydrofarnesyl acetone	1437681	2.500
46.653	9,12-Octadecadienoic acid, methyl ester	853246	1.484
46.752	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	1443452	2.510
46.941	Phytol	6262045	10.890
49.813	4,8,12,16-Tetramethylheptadecan-4-olide	1805070.5	3.139
54.42	Squalene	5378701.5	9.353
57.46	Vitamin E	4001612.5	6.959
		57504950.5	

NI = Unidentified.

3.2. Hydroxyl and superoxide radical scavenging activities of Chenopodium ambrosioides L

The hydroxyl and superoxide radicals are produced in cells through oxidative stress, which are toxic and harmful in excess. Therefore, the antiradical (antioxidant) capacity of *C. ambrosioides* could be relevant for protecting living organisms. The hydroxyl radical scavenging activity of *C. ambrosioides* was dose-dependent manner from 50 to 250 μg/mL, and then it was stable up to 500 μg/ml. The percentage of maximum anti-hydroxyl radical activity was 51.80% at 250 μg/ml, while BHT showed 54.23% of scavenging activity at this concentration (Fig. 1a).

The antiradical ability on superoxide radical was also dose-dependent manner from 50 to 250 μg/ml, and then it dropped drastically from 300 to 500 μg/ml. The maximum superoxide radical scavenging activity was 44.35% and 37.46% for *C. ambrosioides* and BHA, respectively, at the concentration of 250 μg/ml (Fig. 1b). In line with these findings, 250 μg/ml concentration was used for the first *in vitro* experiment.

3.3. Chenopodium ambrosioides L. not causing cell toxicity

Fish splenocytes treated (250 μg/ml) with *C. ambrosioides* had similar viability (88.50%) of control (untreated cells) group (92.05%). In contrast, DMSO (a cytotoxic positive control) reduced the viability at 9.07% (Fig. 2a).

3.4. Antioxidant parameters in splenocytes treated with Chenopodium ambrosioides L

In this study, the activity of SOD and CAT was clearly higher ($p < 0.05$) in splenocytes treated (250 μg/ml) with *C. ambrosioides* that control cells (Fig. 2bc). Similarly, nitric oxide production and intracellular ROS levels were higher ($p < 0.05$) in splenocytes incubated with *C. ambrosioides* that controlled cells (Fig. 2b–e).

3.5. Short chain fatty acids analysis on anterior intestinal content

The SCFA were determined in anterior intestinal samples at the end of 2-week and 4-week treatments in the study. Among SCFAs, acetic acid was detected although propionic or butyric acids were also analyzed. The highest acetic acid concentration was detected in fish fed with dietary *C. ambrosioides* L. at week 4 compared with the control group, indicating higher fermentation activities in these groups (Fig. 3).

Table 3
Polar fraction of *Chenopodium ambrosioides* as determined using GC-MS and expressed as a percentage of the total peak area and expressed as relative %.

Rt	Name	Mean	Relative %
14.114	Hexanoic acid	376798.5	0.111
14.303	2-Methyl-4-pentenoic acid	530158.5	0.157
14.59	l-Valine	1861588.5	0.550
15.392	l-Alanine	1176731	0.348
15.834	2-Hexenoic acid	477404.5	0.141
16.602	Oxalic acid	397111	0.117
16.91	β-Lactic acid	360420.5	0.106
17.111	l-Leucine	1238038.5	0.366
17.53	Isobutyric acid, 3-hydroxy	389356.5	0.115
17.671	l-Proline	228353.5	0.067
17.809	l-Isoleucine	779225.5	0.230
18.475	L-Valine	4618043.5	1.364
20.373	Urea	629566	0.186
20.647	NI (Hydroxy terpene like)	492698	0.146
20.832	L-Serine	642516.5	0.190
20.954	NI (Hydroxy terpene like)	298335	0.088
21.161	Ethanolamine	1885411.5	0.557
21.455	L-Leucine	2988255	0.883
21.613	Phosphoric acid	8345083.5	2.465
21.775	Glycerol	12702677	3.752
22.121	L-Proline isomer 2	2557127.5	0.755
22.177	L-Isoleucine	2591751	0.766
22.538	Thymol	1013714	0.299
22.745	Succinic acid	1721786.5	0.509
23.597	Uracil	2990144.5	0.883
23.833	Fumaric acid	716169.5	0.212
24.195	5-Hydroxyhexanoic acid	309107.5	0.091
24.566	L-Serine isomer 2	1380969	0.408
24.717	2(3H)-Furanone, dihydro-3,4-xy	467177	0.138
25.438	L-Threonine	2178378.5	0.644
26.42	β-Alanine	558873	0.165
28.05	NI (Dicarboxylic acid like)	6627813.5	1.958
28.444	NI	3578308	1.057
28.629	DL-Malic acid	903553	0.267
29.152	NI (Dicarboxylic acid like)	5727172.5	1.692
29.256	L-Proline, 5-oxo	2111370	0.624
29.575	4-aminobutyric acid	8153563.5	2.409
29.726	L-Phenyl alanine	4826826.5	1.426
30.788	NI (monosaccharide like)	10443738	3.085
31.447	L-Asparagine	1149616	0.340
32.304	L-Phenyl alanine (isomer 2)	7039966.5	2.080
36.598	Glycerol phosphate	13523936	3.995
37.702	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	10315008	3.047
38.047	D-Fructose	3827711.5	1.131
39.045	L-Tyrosine	6011141.5	1.776
39.943	D-Glucose	5110545	1.510
40.297	p-Coumaric acid	1704802	0.504
40.995	D-Glucitol	859715.5	0.254
41.068	NI (Methyl glycoside)	1682980	0.497
41.518	D-Glucose isomer 2	1576202	0.466
41.995	Pantothenic acid	306204	0.090
42.106	NI (fatty acid like)	1250385.5	0.369
42.273	D-Glucose isomer 3	26201661.5	7.740
42.818	Palmitic acid	13790580	4.074
44.201	D-Glucose isomer 4	1510232	0.446
45.745	Phytol	2667492.5	0.788
46.455	9,12-Octadecadienoic acid (Z,Z)	5477978	1.618
46.623	α-Linolenic acid	19900881	5.879
46.933	Stearic acid	6033883	1.782
55.888	Sucrose	109297229	32.287
		338513466	

NI = Unidentified.

3.6. Superoxide dismutase and catalase activities

Regarding the antioxidant enzyme activities measured in intestine, SOD and CAT activities significantly increased in those fish fed with lower doses of *C. ambrosioides* over control at any time of the experimental trial (Fig. 4ab).

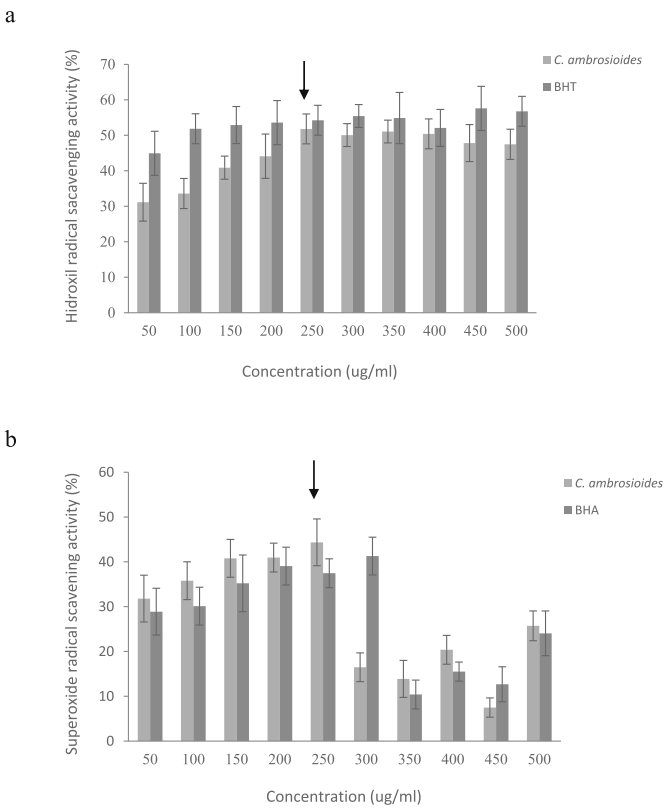

Fig. 1. (a). Hydroxyl radical scavenging activity of *Chenopodium ambrosioides* L. The results are the mean ± SD of three separate experiments, each in triplicate. (b). Superoxide radical scavenging activity of *C. ambrosioides* L. The results are the mean ± SD of three separate experiments, each in triplicate.

3.7. Pro-inflammatory cytokine gene expression in intestine

For innate immune functions, expression levels of pro-inflammatory cytokines, IL-1β, IL-6 and IL-8 genes were determined by quantitative PCR in the intestine from fish fed with *C. ambrosioides* L. at weeks 2 and 4 (Fig. 5abc). Relative expression analysis was shown as fold increase (mean ± SD) with respect to the mean control (without *C. ambrosioides* L.) at each time of the experimental trial.

IL-1β gene transcription in intestine showed the lowest expression level in fish fed with any *C. ambrosioides* L. treatments after week 2 compared with the other sampling times. However, IL-1β gene expression increased ($p < 0.05$) in 0.5% *C. ambrosioides* L. group at week 4 compared with the other treatments or control group (Fig. 5a).

The mRNA levels of IL-6 significantly ($p < 0.05$) increased in the dietary doses of 0.5% of *C. ambrosioides* L. at week 2 and 4 compared with the other treatments (Fig. 5b).

IL-8 gene expression in intestine significantly ($p < 0.05$) elevated at 0.5% of *C. ambrosioides* L. diet compared to the other doses or control after 2 weeks. After week 4 of dietary trial, IL-8 gene expression significantly up-regulated with doses of 1.0% of *C. ambrosioides* L. compared with other groups.

3.8. Histological examination in intestine

The effects of dietary *C. ambrosioides* L. on intestine were examined after hematoxylin and eosin staining under light microscopy (Fig. 6). Typical healthy morphology of the intestinal barrier was observed in fish in the control diet. The most noticeable histological finding was the presence of a huge number of melanomacrophages-like cells and intraepithelial leukocytes in those fish fed with the highest dose of *C. ambrosioides* L. (2.0%) compared with other groups; however no considerable damage or inflammation was observed with any treatment

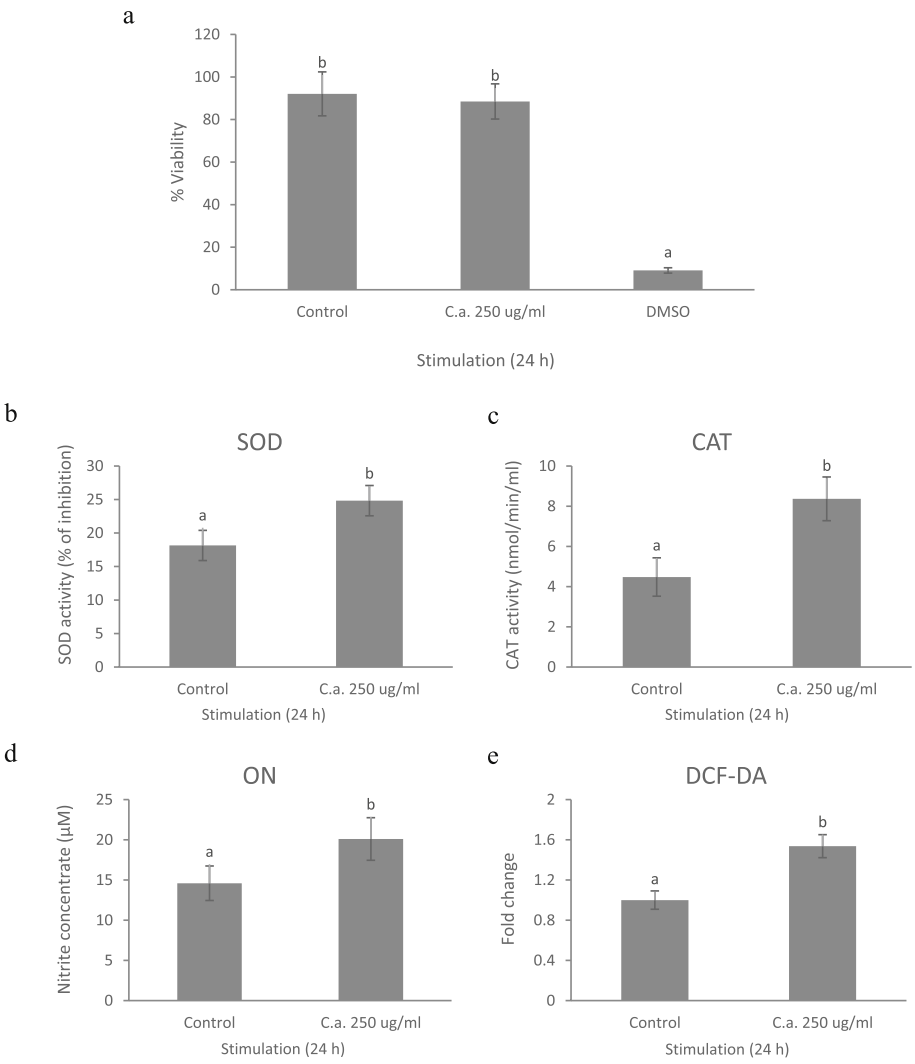

Fig. 2. (a) Alamar blue assay to assess cell viability of splenocytes stimulated with *Chenopodium ambrosioides* L. (250 µg/ml) at 24 h; (b) Superoxide dismutase activity; (c) catalase activity; (d) nitric oxide production; and (e) intracellular reactive oxygen species (ROS) levels (DCF-DA) of splenocytes stimulated with *C. ambrosioides* L. (250 µg/ml) at 24 h. Bars represent the mean ± SD (n = 9). Different letters indicate significant ($p < 0.05$) difference among groups.

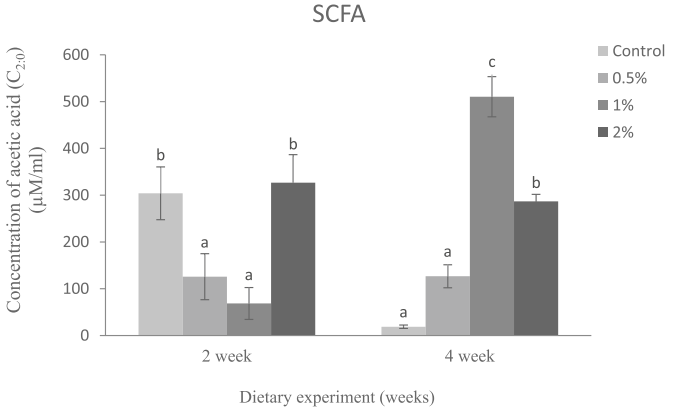

Fig. 3. Concentration of acetic (C2:0) acid determined by gas chromatography in the proximal intestinal content samples of dietary groups fed with *Chenopodium ambrosioides* L., (control, 0.5, 1.0 and 2%) sampled at weeks 2 and 4. Values are expressed as mean ± SD. Statistical differences between dietary treatments are indicated by different lower capital letters with $p < 0.05$.

compared with control diet.

4. Discussion

Because of the potential toxicity of commercial antioxidants and resistance of many strains to antibiotics, increasing attention has been directed toward natural alternative remedies to enhance immunological and antioxidant responses. Application of medicinal plants has had positive effects and new reports have demonstrated potential effects on growth promotion, survival and immune system activation [1]. Therefore, searching for suitable feed additives that could improve health status and stimulate immune response, this study was performed not only to explore the antioxidant capacity of *C. ambrosioides* L. in an *in vitro* study but also to confirm its dietary supplementation effect on intestine health and inflammatory status of Pacific red snapper juveniles. *C. ambrosioides* L. is originally native to Mexico and the tropics of Central and South America, but now this plant can be found in the oddest of places because it has been introduced to many different areas worldwide [25]. The extract of *C. ambrosioides* has several molecules, of which α-terpinene (72.7%) is the main constituent followed by p-cymene (15.3%) and ascaridole (7.2%) [26]. The GC/MS showed that the major compound of the non-polar fraction was composed principally of squalene, vitamin E, phytol and carvacrol whereas other

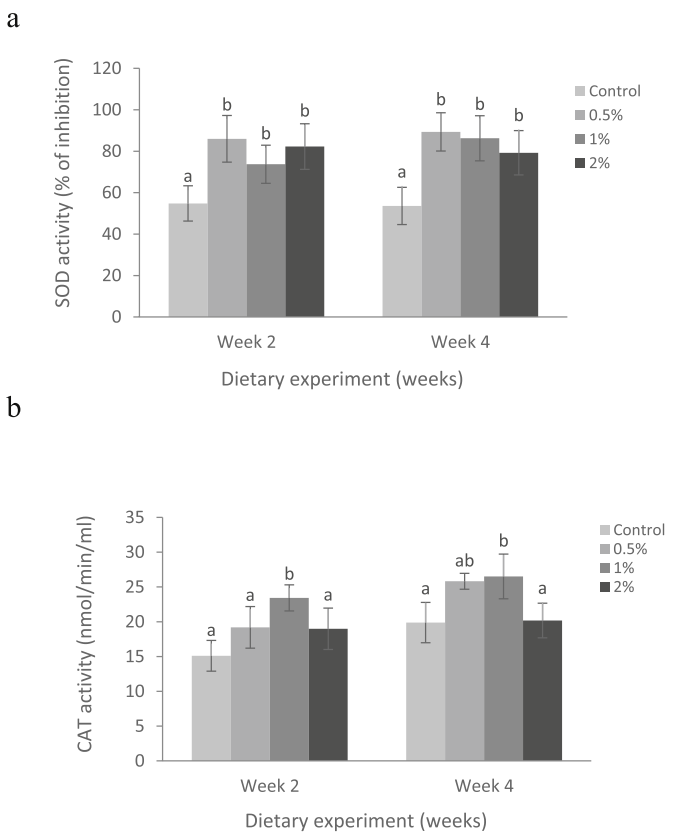

Fig. 4. Superoxide dismutase and catalase activities in intestine of dietary groups fed with *Chenopodium ambrosioides* L. (control, 0.5, 1.0 and 2%) sampled at weeks 2 and 4. Values are expressed as mean ± SD. Statistical differences between dietary treatments are indicated by different lower capital letters with $p < 0.05$.

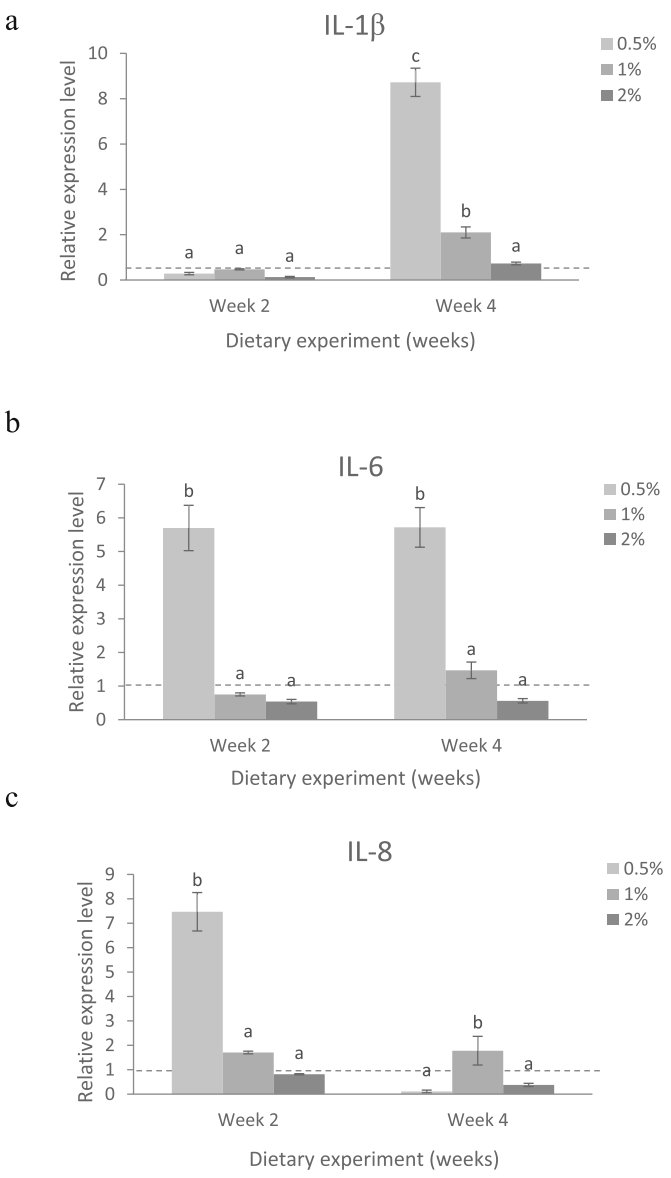

Fig. 5. Relative mRNA expression of pro-inflammatory cytokines, (a) IL-1β b) IL-6 and c) IL-8 in intestine measured by quantitative real-time PCR of dietary groups fed with *Chenopodium ambrosioides* L., (control, 0.5, 1.0 and 2%) sampled at weeks 2 and 4. Data are shown as mean ± S.D. fold increase relative to control. Different letters denote significant difference between treated groups ($p < 0.05$).

molecules were found in minimal quantities. The polar fraction was highly composed by sucrose, D-Glucose isomer 3, α-Linolenic acid and glycerol. The composition of Chenopodium could be different among plant species and geographical regions. However, dietary administration of several of those compounds, such as vitamin E, carvacrol, squalene, and α-Linolenic acid, has been reported to have immunostimulatory effects, enhancing intestinal health function capacity and/or growth promoting effects on fish [27]. Phenol carvacrol had antibacterial activity because of its property to incorporate into membranes causing disruption of ion gradients [28]. Interestingly, this *C. ambrosioides* L. is rich in squalene and vitamin E. Squalene is an isoprenoid that shares structural similarities to beta-carotene and is generated during cholesterol synthesis pathway [29]. Vitamin E is synthesized only by plants and plays key functions as antioxidant in living organisms by trapping peroxyl free radicals [30]. Overall, these characteristics are strongly related with their biological effects.

Many species of Chenopodium have been reported to possess numerous medicinal properties used in folk medicine. The study of *C. ambrosioides* L. as medicinal plant has demonstrated pharmacological activities including antitumor [31], antibacterial [32] and antiprotozoal [33]; nevertheless; studies of antioxidant capacity of *C. ambrosioides* L. are not numerous.

Several assays have been frequently used to estimate antioxidant capacities in plants and their medicinal and food properties. Most of the antioxidant potential of plants results from the redox properties of phenolic compounds [34]. In this work, two complementary tests were used to assess the antioxidant activity of *C. ambrosioides* leaf extract: DPPH free radical scavenging and superoxide radical scavenging activities. Among different reactive oxygen species, superoxide anion is generated first. Superoxide anion is also known to initiate indirectly the lipid peroxidation as a result of the formation of H_2O_2, creating precursors of hydroxyl radical. Therefore, it is of great importance to study the scavenge of superoxide anion radicals. Interestingly, *C. ambrosioides* L. extract showed higher scavenging activity than BHA at 250 μg/ml. However, *C. ambrosioides* L. extract showed weaker scavenging activity after this concentration. Nowak et al. [34] evaluated the antioxidant activity of Chenopodium L. (*Chenopodium album*, *Chenopodium hybridum*, *Chenopodium rubrum* and *Chenopodium urbicum*) where *C. rubrum* and *C. urbicum* had the best antioxidant effect of all the analyzed extracts, suggesting that it could be used as source of natural antioxidants. The effectiveness of plant extracts and natural compounds of high antioxidant activity in prevention of many cancer types is well known but the use of antioxidant agents in adjunctive cancer therapy is still controversial because of conflicting findings [34]. Stimulation of the immune system promotes a state of rapid immune response against pathogens. In this context, several traditional herbal medicines have well-known antioxidant and immunostimulatory effects to prevent or

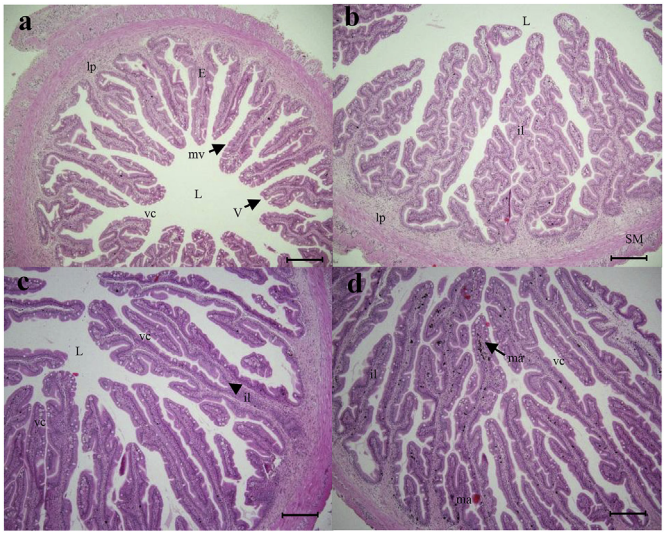

Fig. 6. Hematoxylin-eosin stain from intestine of dietary groups fed with *Chenopodium ambrosioides* L., (a) control; (b) 0.5; (c) 1.0 and (d) 2% sampled at weeks 2 and 4. General view of control (PBS) intestine with mucosa, submucosa and serosa layers with typical villi presence. E = epithelium, il = intraepithelial leucocyte, L = lumen, lp = lamina propia, M = mucosa, mv = microvilli, ma = melanomacrophages, SM = submucosa, v = vacuoles, V = villi. Scale bar = 10 μm.

treat moderate infections [35]. In our *in vitro* study, splenocytes were exposed to *C. ambrosioides* L. extract (250 μg/ml) and viability and antioxidant parameters were evaluated at 24 h. Cell viability is the proportion of cells that survive a particular situation, such as exposure to a stimulus or microorganisms. In this study, *C. ambrosioides* L. extract did not cause toxicity or death in splenocytes compared with splenocytes without treatment. Thus, *C. ambrosioides* L. must be safe to cells. The results have also shown that splenocytes exposed to *C. ambrosioides* L. extract (250 μg/ml) at 24 h could enhance antioxidant enzymes, such as superoxide dismutase and catalase activities and ROS by oxide nitric (ON), and also ROS measurement by dichlorofluorescein diacetate (DCF-DA). Antioxidant defense renders significant protective role against environmental stress in organisms and maintains homeostasis of redox balance, thereby supporting proper immune function [36]. Cruz et al. [10] found that *C. ambrosioides* promoted macrophage activation, phagocytic activity, and respiratory burst activity, associated to bactericidal mechanisms. Overall, the compounds observed in *C. ambrosioides* accounted for its promotion on respiratory burst activity and redox balance, including vitamin E that protected macrophage membranes from oxidative impairment related to respiratory burst activity in fish [37].

In a second experiment, dietary *C. ambrosioides* L. was supplemented in fish a different percentages (control, 0.5, 1.0 and 2.0%) for 4 weeks where health status, antioxidant and pro-inflammatory effects in intestine were evaluated. The homeostasis of intestinal microbiome had positive effects in the host, but disturbs between beneficial and pathogenic microorganisms had opposite outcomes. SCFAs are a product of microbial fermentation and play critical physiological functions in the gut [38]. Therefore, it was important to investigate whether the content of proximal intestine SCFA profiles changed in fish fed with *C. ambrosioides* L. As Fig. 3 shows, only was acetate detected as the major SCFAs. The concentration of SCFAs in the intestine was significantly lower in control fish after four weeks of the feeding trial compared with fish fed *C. ambrosioides* L. groups. It is known that the presence and type of carbohydrates as well as the anatomical site of the fermentation process (i.e. caecum in mice or colon in humans) affect the SCFAs profile in the intestine [39]. Acetate is the predominant short chain fatty acid in the intestine of fish and mammals [40,41]. In addition, it should be taken into account that butyrate and other SCFA are rapidly metabolized and used as energy by intestinal cells, which can remain undetected. The acetate is synthetized by enteric bacteria, particularly acetogenic bacteria that produce acetate through Wood–Ljungdahl pathway during carbohydrate fermentation [42]. Thus, the diet clearly influenced intestinal microbiome, but these effects on the fish host fed *C. ambrosioides* remain to be examined. Additionally, future determinations of fecal metabolites using LC–MS could confirm the observed effects among experimental groups.

The effect of dietary *C. ambrosioides* L. on antioxidant enzymes was evaluated also in intestine samples at weeks 2 and 4. Interestingly, superoxide dismutase and catalase activities increased in fish fed with lower doses of *C. ambrosioides* compared with control diet at any time of the feeding trial. Antioxidant enzymes play an important role in protecting against oxidative damage by eliminating free radicals, which can be naturally produced in body metabolism process and/or induced by natural immunostimulant [43]. Moreover, the free radical scavenging activity of antioxidant enzymes was demonstrated to be positively affected by the presence of dietary antioxidants [44,45]. Therefore, progressive research attempts have been made on the evaluation of different medicinal herbs as antioxidants in fish [46].

Some properties of *C. ambrosioides* have been confirmed, such as its anti-inflammatory effects [11,13]. Our group has also evaluated the possible anti-inflammatory effect of dietary *C. ambrosioides* L. extract on intestine by gene expression and histological assays. Cytokines play pivotal roles in the intestinal immune responses and modulate the normal “state of alert” in the gut [47]. Cytokine gene expressions, which were selected considering their innate immune functions were IL-1, IL-6 and IL-8 that are crucial pro-inflammatory cytokines. In general, we could observe that the expressions of IL-1β, IL-6 and IL-8 gene in the intestine up-regulated in fish fed with 0.5% *C. ambrosioides* L. after two weeks and down-regulated in those fed the diet enriched with 2.0% *C. ambrosioides* L. We deduced that 2% doses of dietary *C. ambrosioides* L. can have an anti-inflammatory effect. Overall, several studies have demonstrated that some plant extracts provoke anti-inflammatory responses on leukocytes by the down-regulation of inflammatory genes that typically can activate NFκB1 and NFκB2 pathways [48]. Although no previous report has been available in fish to our knowledge, the anti-inflammatory effect on mammals by oral administration of appropriate doses of *C. ambrosioides* extracts has been reported [12,13]. In addition, the histological findings suggested that *C. ambrosioides* L. extract did not cause inflammatory damage in the intestine. Particularly, compounds found in *C. ambrosioides*, such as p-Cymene have been known to have anti-inflammatory activity [49]. Moreover, several reports have demonstrated that *C. ambrosioides* extracts had no cytotoxic effects *in vitro* and *in vivo* experiments [50], which accounts for their use in fish aquaculture.

5. Conclusion

For the first time, our results have revealed the effect of *C. ambrosioides* L. antioxidant capacity in splenocytes and dietary effect on intestinal health status in fish. The highest amount of antioxidant activity found in dried leaves extract was observed at 250 μg/ml and no cytotoxic effect was observed in splenocytes at 24 h. Interestingly, splenocytes exposed to *C. ambrosioides* L. extract enhanced antioxidant enzyme and radical production. Moreover, we could observe that dietary *C. ambrosioides* L. can improve the percentage of acetic acid in the intestinal content, enhance antioxidant enzyme activity and that it has an anti-inflammatory effect on fish intestine.

Conflicts of interest

The authors declare no conflict of interest.

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