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Effects of dietary dandelion extracts on growth performance, liver histology, immune-related gene expression and CCl_4 resistance of hybrid grouper *(Epinephelus lanceolatus♂ × Epinephelus fuscoguttatus♀)*

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ABSTRACT

The study investigated the effects of dietary supplementation with dandelion extracts (DE) on growth performance, feed utilization, body composition, serum biochemical, liver histology, immune-related gene expression and CCl_4 resistance of hybrid grouper *(Epinephelus lanceolatus♂ × Epinephelus fuscoguttatus♀)*. A basal diet supplemented with DE at 0% (diet 0%), 0.1% (diet 0.1%), 0.2% (diet 0.2%), 0.4% (diet 0.4%) and 0.8% (diet 0.8%) were fed to hybrid grouper for 8 weeks. The results revealed that dietary DE had not a significant impact on growth performance and feed utilization ($P > 0.05$), but it could decrease the percent of crude lipids in whole body and increase the percent of crude protein in muscle ($P < 0.05$). Dietary DE increased the mRNA levels of antioxidant enzymes (catalase, glutathione peroxidase and glutathione reductase) and reduced inflammatory factor in the spleen and head-kidney of fish ($P < 0.05$), but reduced the expression of the liver antioxidant gene except for glutathione reductase ($P < 0.05$). Dietary supplementation with 0.2%–0.4% DE could effectively improve liver health. After injection of CCL_4 by 72 h, fish fed Diet0.2% and Diet0.4% showed regular hepatocyte morphology while fish fed Diet 0%, Diet 0.1% and Diet 0.8% showed hepatocyte damage. Higher survival rate and total blood cell count was observed in fish fed 0.1%–0.4% dietary DE ($P < 0.05$). In conclusion, DE could be used as a functional feed additive to enhance liver function of farmed fish. The best level of it should be between 0.2% and 0.4%.

1. Introduction

The scale and density of aquaculture are increasing following the development of aquaculture in recent years, however high density aquaculture easily led to immune suppression, which increased the susceptibility of fish to infectious agents [1]. Meanwhile, aquaculture has encountered many challenges over recent decades [2]. One of them is liver disease, which causes liver damage and even death [3]. It has become one of the major obstacles for the further development of aquaculture, especially in marine fish [4]. Liver is important in pharmacology and toxicology research because it is the main organ for detoxification and the metabolism of most chemicals [4–6]. Carbon tetrachloride (CCl_4) is a well-known compound for the induction of chemical hepatic injury including inflammation and fibrosis [7,8]. Liver damage model induced by CCl_4 is the classic model in liver protection drug screening [7,9]. It could gain an insight into the pathophysiological processes in aquaculture and find effective therapeutic strategies [4]. Various chemicals were abused to aquaculture to prevent and treat fish liver disease [10]. However, the approach led to many other problems, such as suppression the immune system of aquatic animals and environmental pollution [1,10]. Therefore, researchers are trying to find new ways to replace chemicals. It is gaining increasing attention that natural medicine and their extracts used as dietary supplements for the treatment of liver diseases in animal in recent years [4,11,12].

Dandelion (*Taraxacum officinale)* is a member of the Asteraceae *(Compositae)* family [13]. It is a perennial herb distributed throughout the world with a complex taxonomy and difficult morphology [14]. Dandelion contains various phytochemicals including oligosaccharides, polysaccharides, peptides, flavonoids, phenolic acids, alkaloids and terpenes [13,15–18]. This plant was used as a herbal medicine since

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ancient China [19]. Dandelion reveals high amounts of minerals, proteins, fiber and vitamins [13,20]. The balanced combination of micronutrients in dandelion makes it an interesting micronutrient source [13]. It has been used extensively in traditional oriental system of medicine for treatment of bladder, spleen, liver, gout and diarrhea [21–24]. Nowadays, dandelion attracts multiple attentions as a commonly available dietary supplement and a component of pharmaceutical preparations for its choleretic, diuretic, growth promoting, antirheumatic, anticancer, antimicrobial and anti-inflammatory properties [1,15,20,25]. The flower extract of dandelion could suppress both reactive oxygen species and nitric oxide and prevent lipid oxidation in vitro [22]. Dandelion root extracts could be supplemented in diet to enhance immune ability of golden pompano [1]. Dandelion leaf water extract had a protective effect against CCl_4-induced hepatic damage of Sprague-Dawley rats [25]. The mixture (consolidating probiotic with dandelion, coriander and date palm seeds extracts) contained protective factors for mercury neurotoxicity that maintained normal testosterone levels in male rats [26]. These results showed that DE had a broad application prospect in aquaculture. Nevertheless, it is still lack of information about the effectiveness of whole plants extracts of dandelion as dietary additives in aquaculture.

Hybrid grouper was first produced by fertilizing the eggs of the tiger grouper *(Epinephelus fuscoguttatus)* with the sperm of the giant grouper *(Epinephelus lanceolatus)* through the in-vitro fertilization (IVF) technique in Universiti Malaysia Sabah [10]. Recently, it has been widely cultured in the southeast coast of China for their rapid growth, high nutritional value and disease resistance [15]. Our previous research showed that ginkgo biloba leaf extract [10] and *panax notoginseng* extract [27] could improve hepatic antioxidant status, maintain normal liver histology and preserve liver function of hybrid grouper fed high lipid diets. To date, there is no report about using whole plant extracts of dandelion as supplement in hybrid grouper feed. The aims of this study are to investigate the effect of whole plant extracts of dandelion on growth performance, serum biochemical, fish composition and liver injury induced by CCl_4 in hybrid grouper.

2. Materials and methods

2.1. Diet preparation

Formulation and proximate composition of the experimental diets were provided in Table 1. The basal diet was used as control diet (Diet0). The experimental diets were supplemented with 0.1% (Diet0.1%), 0.2% (Diet0.2%), 0.4% (Diet 0.4%) and 0.8% (Diet0.8%) dandelion extracts (DE, with 20% dandelion flavonoids from the whole plant of dandelion, Nanjing DASF Bio-technology Co.,Ltd., China). All the ingredients were ground into powder with muti-function pulverizer, and then sieved through 60-mesh sieve. The diets were individually blended in a mixer and then homogenized after mixed oil was added. The water was included to achieve a proper pelleting consistency, and the mixture was further homogenized. The 2.5-mm diameter dough was wet-extruded by a pelletizer (F-26, South China University of Technology, Guangzhou, China) and air-dried to below 100 g kg^{-1} moisture of diet. Then, the diets stored at −20 °C until used [27].

Table 1
Composition and nutrient levels of experimental diets.

Ingredients Diet (%)	Diet0%	Diet0.1%	Diet0.2%	Diet0.4%	Diet0.8%
Fish meal	48	48	48	48	48
Soybean meal	20	20	20	20	20
Wheat flour	18	17.9	17.8	17.6	17.2
Beer yeast powder	3	3	3	3	3
Fish oil	3	3	3	3	3
Soybean oil	3	3	3	3	3
Soybean lecithin	1	1	1	1	1
Vitamin and Minerals mixture[a]	2	2	2	2	2
Choline chloride	0.5	0.5	0.5	0.5	0.5
Vitamin C	0.5	0.5	0.5	0.5	0.5
Monocalcium phosphate	1	1	1	1	1
Dandelion extract	0	0.1	0.2	0.4	0.8
Proximate composition (% DM)					
Crude protein	47.3	47.46	47.39	47.57	47.61
Crude lipids	10.90	10.18	10.80	10.09	10.11
Moisture	6.61	6.58	6.62	6.27	6.57
Ash	12.22	12.44	12.24	12.44	12.45

[a] Mineral and vitamin premix were provided by Guangzhou Chengyi aquatic technology co. LTD.

2.2. Experimental fish and samples collection

Hybrid grouper were obtained from Marine Fisheries Development Center of Guangdong Province (Huizhou, China) and acclimatized to the experimental condition in cylindrical tanks (500-L) with running water (the flow rate was 3 L/min) for 2 weeks and fed a basal diet. After fasting for 24 h, 300 tails of juvenile groupers of an average weight (43.32 ± 0.60 g) were randomly placed fifteen tanks and each tank contained 20 fish. The fifteen tanks were divided into five experimental groups and three replicates in each group. Each diet was randomly assigned to tanks in triplicate. All rearing tanks were provided with continuous aeration and running water. Fish were fed twice daily at 8:00 and 16:00. The number and weight of dead fish and feed consumption were recorded every day. Weigh and calculate the weight gain rate of per tank every two weeks. Water temperature, salinity, pH and dissolved oxygen were 26.5 ± 3.5 °C, 31.5 ± 1.5 psμ, 8.0 ± 0.2 and 75 ± 0.25 mg L^{-1}, respectively. Total ammonia was lower than 0.2 mg L^{-1} during the feeding trial.

All fish populations and mean body weight in each tank were determined after fasted for 24 h before sampling at the end of the feeding trial. Three fish from each tank were sampled randomly and stored at −20 °C for analysis of the whole body composition and six fish were anesthetized with 100 mg L^{-1} MS-222 and then took visceras (liver, spleen and head kidney) after drawing blood by caudal venipuncture using 2 ml syringes. The visceras were collected into 2 ml sterile tubes respectively and stored in liquid nitrogen soon. Blood samples were collected into centrifuge tubes in order to obtain serum. After collection, the whole blood was centrifuged (3000 r/min, 15 min, 4 °C) and the serum was separated. All samples were stored at −80 °C until analysis.

2.3. Growth performances

The parameters in this study were calculated as per following formula:

Weight gain rate (WGR, %) = 100 × (final body weight − initial body weight)/initial body weight;

Feed efficiency ratio (FER) = wet weight gain (g)/dry feed intake (g);

Condition factor (CF, g/cm^3) = 100 × (body weight, g)/ (body length, cm) ^{3};

Viscerosomatic index (VSI, %) = 100 × (viscera weight, g)/ (whole body weight, g);

Hepatosomatic index (HSI, %) = 100 × (liver weight, g)/ (whole body weight, g);

Intestinal fat percentage (IFP, %) = 100 × (Intestinal fat weight, g)/ (whole body weight, g).

2.4. Fish proximate composition

Crude protein, crude lipid, moisture and ash in the diets, muscles and the whole body were determined according to the established methods of AOAC (2005): moisture was determined by drying to constant weight at 105 °C; crude protein (N × 6.25) was determined by Kjeldahl method using Kjeltec (FOSS 8400, Hoganos, Sweden) after acid digestion; lipid was determined by petroleum ether (B.P. 30–60 °C for 3 h) using Soxtec™ 2055 (FOSS, Hoganos, Sweden). For ash content analysis, samples were placed in a muffle furnace (FO610C, Yamato Scientific Co., Ltd., Tokyo, Japan) at 550 °C for 6 h.

2.5. Serum biochemical measurements

The content of alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglyceride (TG), alkaline phosphatase (ALP), glucose (GLU), cholesterol (CHO), total protein (TP), globulin(GLOB) and albumin (ALB) in serum were analyzed using an automatic biochemical analyzer (HITACHI7180) by WAKO LabAssay Series of kits (Shanghai zhenzhun biotechnology co., LTD) according to the instructions.

2.6. Hepatic histological analysis

For hepatic histological analysis, three fish from each tank were sampled and dissected to obtain the hepatic (about 1 cm^3 at the same position of liver). Three liver samples per tank were fixed in 4% paraformaldehyde solution for 48 h, then washed in 70% ethanol solution, finally transferred to a 70% ethanol solution for storage until processing into histological slides. The methods were according to the describing by Tan et al. [10]. The paraffin sections and HE-staining were done by Wuhan Servicebio Technology CO., LTD, China. The pictures of livers were took by Positive fluorescence microscope at the Public Experiment Platform of School of life science, South China normal university.

2.7. RNA isolation and gene expression analysis

Total RNA of six fish head kidneys, spleens and livers from each tank were extracted using 1 ml TRIzol reagent (Vazyme Biotech Co., Ltd, China) according to the manufacturer's instructions. The final RNA was dissolved in an appropriate amount of diethyl pyrocarbonate (DEPC) treated water (SigmaeAldrich, St. Louis, MO, USA). The quantity of isolated RNA was later determined by measuring their absorbance at 260 and 280 nm using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, USA) and the quality of total RNA was detected by electrophoresis in 1.2% agarose gel. Single stranded cDNA was synthesized from 1 μg total RNA using PrimeScript RT reagent Kit With gDNA Eraser (Vazyme Biotech Co., Ltd, Nanning, China) following the manufacturer's instructions. The cDNA templates were then stored at −80 °C for later analysis.

For quantitative real-time PCR, the specific primer pairs (Table 2) were designed according to Sun et al. [27]. The β-actin gene was used as a reference gene. Real-time PCR was amplified in an ABI 7500 real-time PCR machine (Applied Biosystems, USA) using ChamQTM SYBR® qPCR Master Mix (Vazyme Biotech Co., Ltd, Nanjing, China) following the manufacturer's recommendations. Before the RT-PCR experiments, the specificity and efficiency of the primes above were detected. The standard equation and correlation coefficient were determined by constructing a standard curve using a serial dilution of cDNA. The reaction mixtures were 20 μL, containing 2 μL cDNA sample, 10 μL 2 × SYBR Premix, 0.4 μL each of the 10 μM forward and reverse primers, and 7.2 μL ddH_2O. The real-time PCR conditions were as follows: 95 °C for 5 min, then 40 cycles at 95 °C for 15 s, 60 °C for 30s. All samples were run in triplicate, and each assay was repeated three times. After finishing the program, the threshold cycle (Ct) values were obtained from each sample. Relative gene expression levels were evaluated using $2^{-\Delta\Delta CT}$ methods [28].

Table 2
Sequences of primers used in this study.

Primers	qPCR primers, forward/reverse (5′to3′)	Product size/ reference	Efficiency
CAT	F: GCGTTTGGTTACTTTGAGGTGA R: GAGAAGCGGACAGCAATAGGT	[10]	95.2%
GP X	F: TACCCTACCAAGTCCTCCAACC R: AACAAACACCCGACACCCA	[10]	106.2%
GR	F: CTTTCACTCCGATGTATCACGC R: GCTTTGGTAGCACCCATTTTG	[10]	100.9%
G6PD	F: GCTTCACATCCTTGTATCTGCTC R: GCGTTCCTTTCATTCTCCG	246bp	102.3%
PPAR-α	F: CATCGACAATGACGCCCTC R: GCCGCTATCCCGTAAACAAC	135bp	104.6%
CPT-1	F: TCCTTACCGTTGGTCCCTCT R: CTTTCCATCTGCTGCTCTATCTC	154bp	107.2%
Keap1	F: CCAGAAGGAATGTGTGGCTAAA R: TGGTTGGTCATCGGGTTGTA	[10]	106.2%
IL-8	F: AGTCATTGTCATCTCCATTGCG R: AAACTTCTTGGCCTGTCCTTTT	[10]	99.2%
IL-10	F: TTCGACGAGCTCAAGAGTGAG R: TGCCGTTTAGAAGCCAGATACA	[10]	102.5%
MHC-2	F: CCACCCGAACAAACAGACC R: TGATGCCCCCTCCAACACT	[10]	101.8%
TLR3	F: TCTCCATTCCGTCACCTTCC R: TCATCCAGCCCGTTACTATCC	[10]	107.2%
Caspase-3	F: CGCAAAGAGTAGCGACGGA R: CGATGCTGGGGAAATTCAGAC	[10]	105.2%
Caspase-8	F: TGCTTCTTGTGTCGTGATGTTG R: GCGTCGGTCTCTTCTGGTTG	[10]	98.9%
Caspase-9	F: TTTTCCTGGTTATGTTTCGTGG R: TTGCTTGTAGAGCCCTTTTGC	[10]	106.4%
β-actin	F: TACGAGCTGCCTGACGGACA R: GGCTGTGATCTCCTTCTGC	[10]	95.5%

CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; Keap1, Kelch-like-ECH-associated protein 1; CPT1: carnitine palmitoyltransferase 1; G6PD: glucose 6-phosphate dehydrogenase; PPARα: peroxisome proliferator-activated receptor alpha; TLR3, toll-like receptor 3; MHC-2, major histocompatibility complex 2.

2.8. Challenge test

After 1 week of initial sampling, all fish (10 tails per tank) were intraperitoneally injected 30% CCl_4 solution (0.05 ml/10 g body weight, CCl_4 was diluted with olive oil) according to our preliminary experiment. The grouper fed the basal diet injected olive oil was regarded as the control group. After injection, all the fish fasted for 72 h. During this time, fish were monitored carefully. Mortality was recorded twice daily over the period of 3 days. Survival rate and total blood cell counts were recorded. Liver sections were processed for histopathological investigation.

2.9. Total blood cell count

10 μL blood diluted to 20 times with the new configuration of anticoagulation and placed on a hemocytometer to count the number of cells in different experimental group with light microscope (Olympus). Each group was assigned in triplicate [29,30].

2.10. Statistical analysis

All data were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. The level of significance was set at $P < 0.05$, and the results were expressed as means ± SD of three replicates. All statistical analyses were performed using SPSS 16.0 (SPSS Inc., Michigan Avenue, Chicago, IL, USA).

Table 3
Effects of dietary Dandelion Extracts on growth performance and body Indices of hybrid grouper.

Diet (%)	Diet0%	Diet0.1%	Diet0.2%	Diet0.4%	Diet0.8%
WG	131.30 ± 0.39	133.61 ± 18.25	143.98 ± 17.78	150.96 ± 11.93	117.48 ± 14.72
FE	1.12 ± 0.02	1.08 ± 0.02	1.06 ± 0.03	1.10 ± 0.01	1.02 ± 0.03
CF	3.04 ± 0.04	2.99 ± 0.12	2.95 ± 0.14	3.07 ± 0.11	3.06 ± 0.06
VSI	7.55 ± 0.24	7.47 ± 0.11	7.21 ± 0.43	7.24 ± 0.64	7.13 ± 0.30
HSI	2.55 ± 0.05	2.52 ± 0.06	2.48 ± 0.04	2.44 ± 0.45	2.49 ± 0.35
IFP	1.42 ± 0.15^{b}	1.33 ± 0.20^{ab}	1.24 ± 0.20^{ab}	1.19 ± 0.07^{ab}	1.10 ± 0.03^{a}

Values are means ± SD of three replications. Means in the same raw with different superscripts are denoting significantly different ($P < 0.05$). WG: weigh gain; FE: feed efficiency; CF: condition factor; VSI: viscerosomatic index; HSI: hepatosomatic index; IFP: Intestinal fat percentage.

3. Results

3.1. Effect of dietary DE on growth performance and body indices of hybrid grouper

Effects of dietary DE on WG, FE, CF, VSI, HSI and IFP of hybrid grouper were shown in Table 3. Although there was no significant difference in WGR and FER ($P > 0.05$), both of them were shown the same trend over time, which were increased firstly and then decreased, and reached the peak level at the group supplemented with 0.4% DE. IFP was showed a decreased trend following the supplementation with DE ($P < 0.05$).

3.2. Effect of dietary DE on whole body and muscle composition of hybrid grouper

The effects of dietary DE on the compositions of the whole body and muscle of hybrid grouper were shown in Table 4. The dietary DE showed a significant decrease in crude lipid in the whole body of hybrid grouper ($P < 0.05$). The muscle protein contents were significantly increased following the dietary supplementation with DE ($P < 0.05$).

3.3. Effect of dietary DE on serum biochemical indices of hybrid grouper

The effects of dietary DE on serum ALT, AST, TG, ALP, GLU, CHO, TP, GLOB and ALB contents of hybrid groupers were shown in Table 5. The content of ALP and CHO decreased firstly and then increased, and reached the minimum value at the group of diet 0.4% and diet 0.2% respectively ($P < 0.05$). The content of ALT showed an increase trend and reached the maximum value in the group of diet 0.4% ($P < 0.05$). All the content of AST, TG, GLU, TP, GLOB and ALB decreased firstly and then increased, but there were all no significant difference ($P > 0.05$).

3.4. Liver histology

A higher occurrence rate of the hepatocyte swelling and nuclei shifting to the cellular periphery were observed in the hepatocytes of fish fed Diet0% and Diet0.8% (Fig. 1A and Fig. 1E). Meanwhile, the structure of liver sinusoid and cell membrane were incomplete in fish fed above diets. However, fish fed Diet0.1%, diet0.2% and Diet0.4% showed regular hepatocyte morphology (Fig. 1B, C and D).

3.5. Effect of dietary DE on antioxidant, metabolism and apoptosis-related genes mRNA levels in the liver of hybrid grouper

The mRNA levels of antioxidant-related genes in the liver of hybrid grouper were shown in Fig. 2A. It revealed that the mRNA levels of catalase (CAT), glutathione peroxidase (GPx) and Kelch-like-ECH-associated protein 1 (Keap1) in fish showed a significant decreased trend following the supplementation with DE at dose level of 0.1% and 0.2% but significantly at the dose level of 0.4% and 0.8% ($P < 0.05$). The fold change of the three genes was about 0.25 times, 0.5 times and 0.5 times compared to the control group, respectively. However, the mRNA level of glutathione reductase (GR) showed the opposite trend though there was no significant difference among all groups ($P > 0.05$).

The effects of DE on the expression of metabolism-related genes in the liver of hybrid grouper were shown in Fig. 2B. Results suggested that the relative expression of G6PD and PPAR-α showed a decreased trend following the supplemented with DE ($P < 0.05$). And the fold change of them was about 0.5 times and 0.7 times at the group of diet 0.8%, respectively. Meanwhile, the relative expression of CPT-1 increased significantly firstly and then decreased, and reached the peak level at group diet 0.1% ($P < 0.05$), the fold change of it was about 1.8 times.

The effects of DE on the expression of apoptosis-related genes in the liver of hybrid grouper were shown in Fig. 2C. It turned out that the relative expression of caspase3 showed a decreased trend following the supplemented with DE ($P < 0.05$). The fold change of it was about 0.5 times at the group of diet 0.2% and diet 0.8%, 0.7 times at a group of diet 0.4%. There was no significant difference in the relative expression of caspase8 ($P > 0.05$). The mRNA level of caspase9 was significantly increased firstly and then decreased, and reached the peak level at the group of diet0.4% ($P < 0.05$). The fold change of it was about 4 times.

Table 4
Effects of dietary dandelion extract levels on whole body and muscle proximate composition of hybrid grouper.

	Diet0%	Diet0.1%	Diet0.2%	Diet0.4%	Diet0.8%
Whole body (%)					
Crude protein	17.50 ± 0.06	17.58 ± 0.08	17.52 ± 0.23	16.74 ± 0.12	18.30 ± 1.36
Crude lipids	6.69 ± 0.51^{b}	6.81 ± 0.67^{b}	5.83 ± 0.62^{ab}	5.69 ± 0.27^{a}	5.67 ± 0.57^{a}
Moisture	71.07 ± 0.30	71.13 ± 0.60	71.27 ± 1.02	71.95 ± 1.58	70.08 ± 1.55
Ash	4.73 ± 0.25^{b}	4.18 ± 0.23^{ab}	3.96 ± 0.21^{a}	3.99 ± 0.19^{a}	4.46 ± 0.10^{ab}
Muscle (%)					
Crude protein	19.22 ± 0.02^{a}	19.47 ± 0.25^{b}	19.35 ± 0.04^{ab}	19.43 ± 0.13^{b}	19.54 ± 0.02^{b}
Crude lipids	1.77 ± 0.04	1.96 ± 0.22	1.65 ± 0.16	1.88 ± 0.10	1.85 ± 0.15
Moisture	76.70 ± 0.32	76.66 ± 0.05	76.89 ± 0.14	76.98 ± 0.12	76.81 ± 0.03

Values are means ± SD of three replications. Means in the same raw with different superscripts are denoting significantly different ($P < 0.05$).

Table 5
Effects of dietary Dandelion Extracts on plasma biochemical indices of hybrid grouper.

Diet (%)	Diet0%	Diet0.1%	Diet0.2%	Diet0.4%	Diet0.8%
ALT	8.33 ± 0.58^{a}	7.67 ± 1.53^{a}	8.33 ± 2.52^{a}	14.33 ± 3.06^{b}	11.33 ± 4.73^{ab}
AST	30.50 ± 10.61	14.00 ± 0.00	16.50 ± 7.78	15.00 ± 4.24	27.50 ± 7.78
TG	1.01 ± 0.10	1.04 ± 0.01	1.01 ± 0.11	0.95 ± 0.04	0.93 ± 0.33
ALP	157.50 ± 3.54^{b}	122.50 ± 9.19^{a}	127.50 ± 2.12^{ab}	118.00 ± 2.83^{a}	151.50 ± 7.78^{ab}
GLU	3.39 ± 0.29	3.15 ± 0.60	2.65 ± 0.26	2.97 ± 0.16	3.01 ± 0.28
CHO	11.10 ± 0.02^{ab}	9.93 ± 1.17^{a}	9.67 ± 1.06^{a}	10.86 ± 0.08^{ab}	12.14 ± 0.18^{b}
TP	42.87 ± 1.03	39.90 ± 3.62	40.27 ± 1.05	40.87 ± 0.78	41.97 ± 1.76
GLOB	35.50 ± 0.98	33.03 ± 3.16	33.30 ± 0.92	33.87 ± 0.84	34.93 ± 1.69
ALB	7.37 ± 0.06	6.87 ± 0.47	6.97 ± 0.15	7.00 ± 0.17	7.03 ± 0.25

Values are means ± SD of three replications. Means in the same raw with different superscripts are denoting significantly different ($P < 0.05$). ALT: alanine aminotransferase, AST: aspartate aminotransferase; TG: triglyceride; ALP: alkaline phosphatase; GLU: glucose; CHO: cholesterol; TP: total protein; GLOB: globulin; ALB: albumin.

3.6. Effect of dietary DE on antioxidant and immune-related genes mRNA levels in the head kidney and spleen of hybrid grouper

Transcription levels of antioxidant and immune-related genes mRNA levels in the head kidney and spleen of fish were presented in Fig. 3 and Fig. 4. Results showed that the relative expression of CAT and GPX in the head kidney significantly increased at the group of diet0.4% and diet0.8% ($P < 0.05$). The fold change of them was 40 times and 10 times, respectively. The relative expression of GR showed an increased trend though there was no significant difference among all groups ($P > 0.05$). However, there was no significant difference in the relative expression of the three genes in the spleen. The relative expression of Keap1 showed a decreased trend in the head kidney (the fold change of it was about 0.25 times) but an increased trend in the spleen (the fold change of it was about 1.85 times) following the supplemented with DE ($P < 0.05$). It was significantly decreased in the relative expression of IL-8, IL-10, MHC-1 and TLR3 in the head kidney at the group of diet0.4% and diet0.8% ($P < 0.05$). The fold changes of them were about 0.4 times, 0.2 times, 0.1 times and 0.15 times, respectively. In the spleen, except the expression level of MHC-2 was opposite to that of the head kidney, which fold change was more than 10 times. The expression trend of the other three genes in the spleen were the same as that of the head kidney even though the fold changes were not identical.

3.7. Effect of dietary DE levels on survival and total blood cell count of hybrid grouper after 72 h CCl_4 challenge

As was shown in Fig. 5, there was no fish died in the control group (fed Diet 0% and injected olive oil). The survival of group Diet0.1%, Diet0.2% and Diet 0.4% was higher than group Diet 0% and Diet 0.8% after injection CCL_4 72h ($P < 0.05$). As was shown in Fig. 6, the total blood cell count of fish fed Diet0.1%, Diet0.2% and Diet 0.4% was significantly higher than fish fed Diet 0% after injection CCL_4 72 h ($P < 0.05$).

Liver sections of test fish fed diets supplementation with DE levels after 72 h CCL_4 (0.05 mL 30% CCL_4/10 g fish) injection were showed in Fig. 7. Higher incidence of the incomplete cell membrane and nuclei shifting to the cellular periphery were showed in the hepatocytes of fish fed Diet0%, Diet0.1% and Diet0.8% (Fig. 7A, B and E). The rate of hepatocyte swelling was higher in the hepatocytes of fish fed Diet0% and Diet0.8% than other groups (Fig. 7A and E).

4. Discussion

As traditional Chinese herbs, dandelion has been used for a long time due to its various biological properties, including antioxidant and anti-inflammatory [13,21,31–34]. Dandelion contains a large number

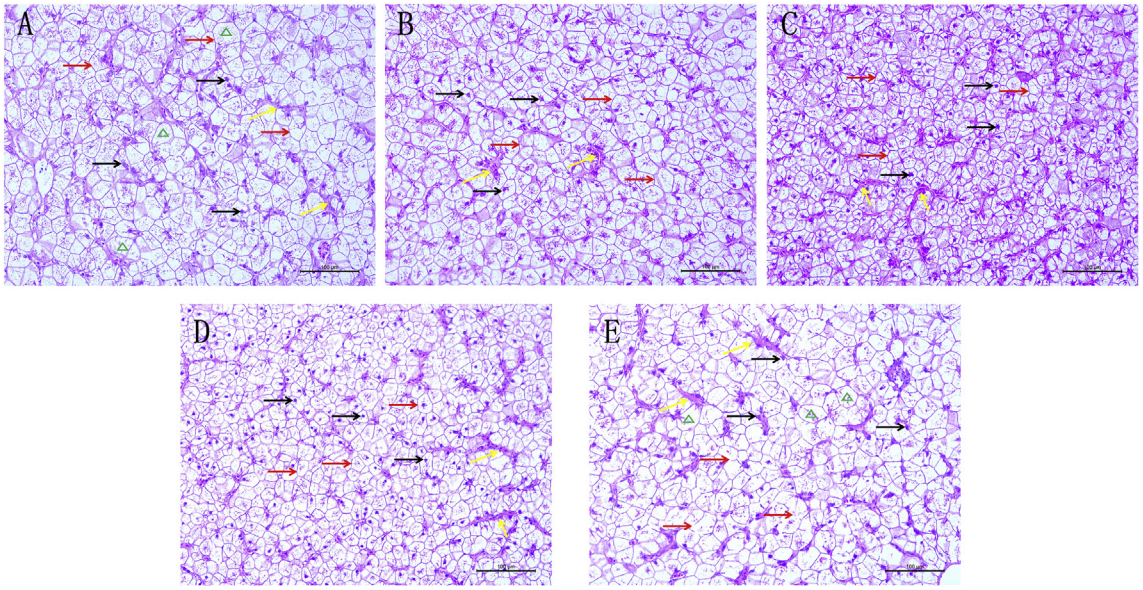

Fig. 1. Effect of dietary dandelion extracts (DE) on the liver histology (hematoxylin and eosin staining, original magnification 200 ×) of hybrid grouper after 8 weeks.
Liver from fish fed Diet 0% (0% DE); B. Liver from fish fed Diet0.1% (0.1% DE); C. Liver from fish fed Diet0.2% (0.2% DE); D. Liver from fish fed Diet0.4% (0.4% DE); E. Liver from fish fed Diet0.8% (0.8% DE); "→" nucleus; "→"nuclear membranes; "△" swelling cells; "→" venous sinus.

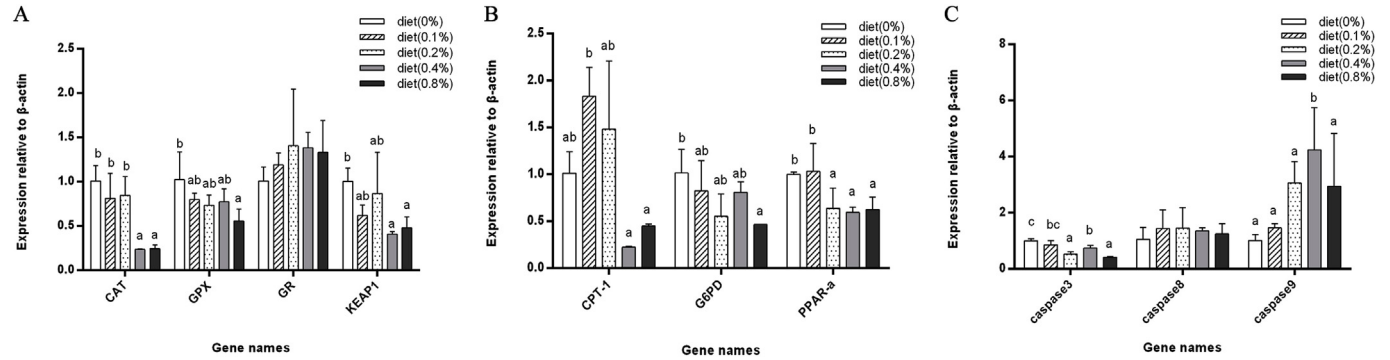

Fig. 2. Effect of dietary dandelion extracts (DE) on antioxidation(A), metabolism(B) and apoptosis(C) related genes mRNA levels in the liver of hybrid grouper. Diet0%, diet 0.1%, diet 0.2%, diet 0.4% and diet 0.8% represent the fish fed diet added different levels of DE, respectively. Values are means, error bars indicate S. D (n = 3), and different letters above a bar denote the significant difference between treatments ($P < 0.05$).
CAT: catalase; GPX: glutathione peroxidase; GR: glutathione reductase; KEAP1: Kelch-like- ECH-associated protein 1; G6PD: glucose 6-phosphate dehydrogenase; PPAR-α: peroxisome proliferator-activated receptor alpha; CPT-1: carnitine palmitoyltransferase 1; IL-8; interleukin 8; IL-10: interleukin 10; MHC-2: major histocompatibility complex 2; TLR-3: toll-like receptor 3.

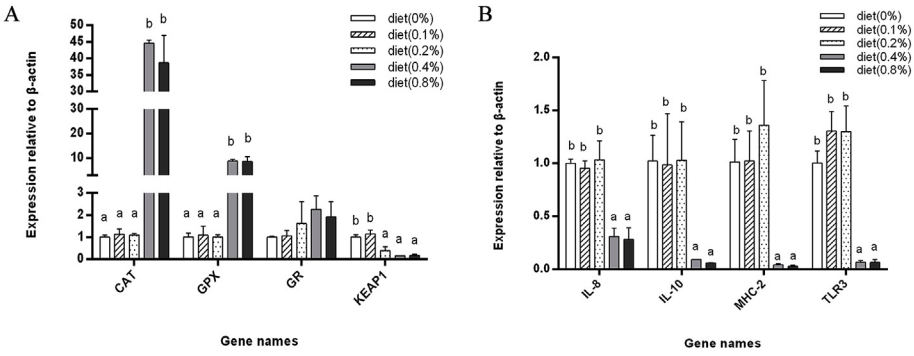

Fig. 3. Effect of dietary dandelion extracts (DE) on antioxidation (A) and immune (B) related genes mRNA levels in the head kidney of hybrid grouper. Diet0%, diet 0.1%, diet 0.2%, diet 0.4% and diet 0.8% represent the fish fed diet added different levels of DE, respectively. Values are means, error bars indicate S.D. (n = 3), and different letters above a bar denote the significant difference between treatments ($P < 0.05$).
CAT: catalase; GPX: glutathione peroxidase; GR: glutathione reductase; KEAP1: Kelch-like- ECH-associated protein 1; IL-8; interleukin 8; IL-10: interleukin 10; MHC-2: major histocompatibility complex 2; TLR-3: toll-like receptor 3.

of flavonoids, which are active ingredients of antioxidant and anti-inflammatory effects [35]. Dandelion flowers extract can effectively relieve the damage of the hematopoietic system, which caused by radiation [31]. Munkhtugs et al. confirmed that the dandelion leaf extracts may represent a promising approach for the high-fat diet induced hepatic steatosis [36]. However, a study on the application of dandelion in aquaculture is little reports up to now. Only found the report by Tan et al., which suggested that dandelion root extracts could be supplemented in the diet as an immunostimulant, growth promoter and intestine health to enhance the immune ability of golden pompano [1,15]. In the present study, even though it was not found a significant difference in the growth rate and feed efficiency of hybrid grouper, these two indicators showed an increase trend following the supplement of DE. On the other hand, crude lipid in the whole body and cholesterol in the serum significantly decreased following the dietary supplementation with DE. It has been proven that the enrichment in the diet of rats with dandelion leaves and roots affects the lipid profile of the body [20]. Dandelion leaf extract could inhibit hepatic lipid accumulation in high-fat diet fed mice [36]. Dandelion water extract supplement could be used to improve lipid metabolism [37]. It also could lower total cholesterol and triglyceride significantly to almost the control concentration in the STZ-induced diabetic rats [37]. In the present study, dietary dandelion extracts decreased whole body lipid content and the relative expression level of hepatic lipid metabolism related genes (G6PD, PPAR-a and CPT-1) in hybrid groupers. These results showed that the lipid content in fish might be reduced by regulating the expression level of lipid metabolism-related genes.

As is known to all, the liver is an important organ, which histological changes are considered as a good indicator in the evaluation of nutritional condition [10]. It is important in pharmacology and toxicology research [6]. Hepatocytes number, surface area and nuclear area are often used as morphometric parameters of hepatocytes [38]. Therefore, we analyzed the liver histology. Results showed that fish fed Diet0.1%, diet0.2% and Diet0.4% showed regular hepatocyte

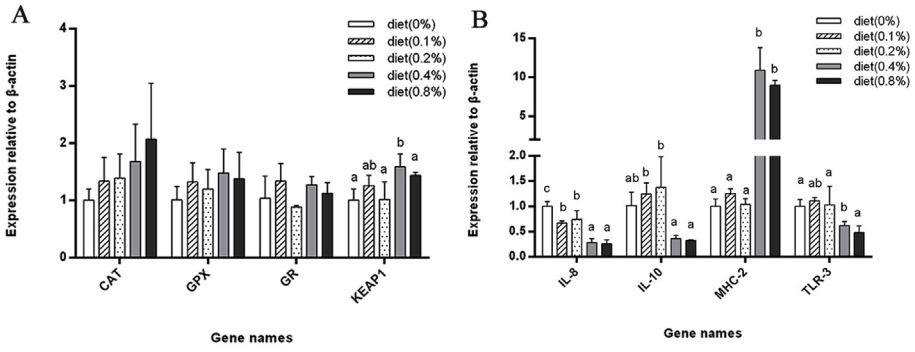

Fig. 4. Effect of dietary dandelion extracts (DE) on antioxidation (A) and immune (B) related genes mRNA levels in the spleen of hybrid grouper. Diet0%, diet 0.1%, diet 0.2%, diet 0.4% and diet 0.8% represent the fish fed diet added different levels of DE, respectively. Values are means, error bars indicate S. D (n = 3). Different letters above a bar denote the significant difference between treatments ($P < 0.05$).
CAT: catalase; GPX: glutathione peroxidase; GR: glutathione reductase; KEAP1: Kelch-like- ECH-associated protein 1; IL-8; interleukin 8; IL-10: interleukin 10; MHC-2: major histocompatibility complex 2; TLR-3: toll-like receptor 3.

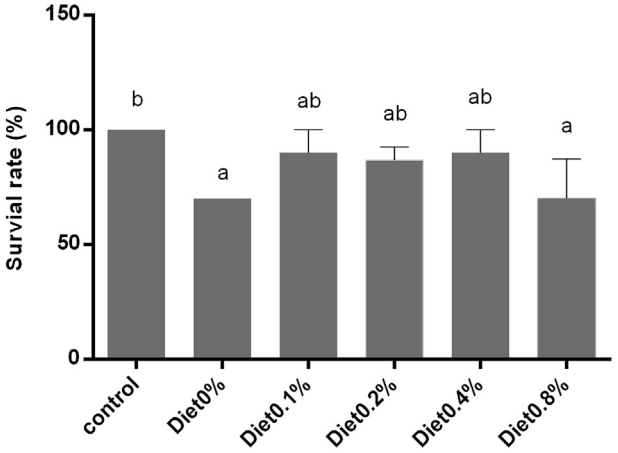

Fig. 5. Effects of dietary dandelion extracts (DE) levels on the survival rate after CCL_4 (0.05 mL 30% CCL_4/10 g fish) injection of hybrid grouper 72 h. Significant differences between the treated and control groups are indicated with different superscripts ($P < 0.05$). Each bar represents the mean ± SD, n = 3.
Control: fish fed Diet 0% (0% DE) and injected olive oil; Diet 0%: fish fed Diet 0% (0% DE) and injected 30% CCL_4; Diet0.1%: fish fed Diet0.1% (0.1% DE) and injected 30% CCL_4; Diet0.2%: fish fed Diet0.2% (0.2% DE) and injected 30% CCL_4; Diet0.4%: fish fed Diet0.4% (0.4% DE) and injected 30% CCL_4; Diet0.8%: fish fed Diet0.8% (0.8% DE)and injected 30% CCL_4.

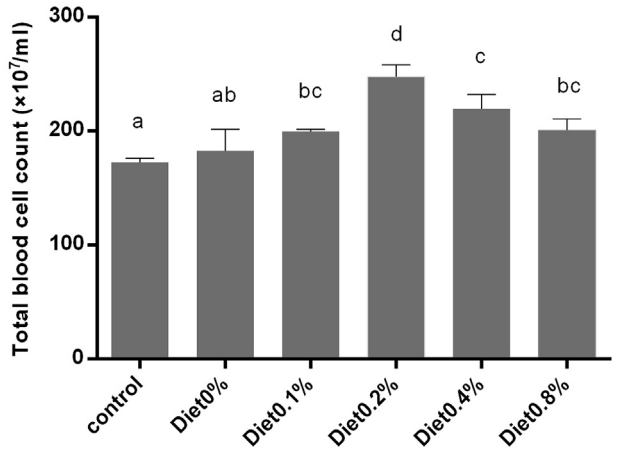

Fig. 6. Total blood cell count of hybrid grouper fed diets supplementation with dandelion extracts (DE) levels after 72 h CCL_4 (0.05 mL 30% CCL_4/10 g fish) injection. Significant differences between the treated and control groups are indicated with different superscripts($P < 0.05$). Each bar represents the mean ± SD, n = 3.
Control: fish fed Diet 0% (0% DE) and injected olive oil; Diet 0%: fish fed Diet 0% (0% DE) and injected 30% CCL_4; Diet0.1%: fish fed Diet0.1% (0.1% DE) and injected 30% CCL_4; Diet0.2%: fish fed Diet0.2% (0.2% DE) and injected 30% CCL_4; Diet0.4%: fish fed Diet0.4% (0.4% DE) and injected 30% CCL_4; Diet0.8%: fish fed Diet0.8% (0.8% DE)and injected 30% CCL_4.

morphology. However, the liver sinusoid and cell membrane were blurred and a higher occurrence rate of the hepatocyte swelling and nuclei shifting to the cellular periphery were observed in the hepatocytes of fish fed Diet0% and Diet0.8%. The result suggested that the addition of DE in the feed had a certain protective effect on hepatocyte. In addition, CCl_4 is a classic liver toxin which has been widely used in constructing liver cell damage models for screening liver protective drugs [3,6,7]. To further explore the protective effect of dandelion on the liver, we conducted a CCl_4 challenge test after the rearing experiment. The results indicated that after injection CCL_4 72 h, fish fed diet0.2% and Diet0.4% showed regular hepatocyte morphology while the fish fed Diet 0%, Diet 0.1% and Diet 0.8% showed hepatocyte damage, and the survival rate of fish fed Diet0.1%, Diet0.2% and Diet0.4% was higher than fish fed Diet0%. It was similar to the previous studies, which revealed that dandelion leaf water extract had significant protection against CCl_4 induced hepatocellular injury in rats [25,39]. This study demonstrated that dietary dandelion extract could improve liver morphology, detoxification and health, which suggested dandelion extract could be used as a functional feed additive to protect the liver and enhance liver function to improve the health of farmed fish.

The immune function of fish is closely related to growth and disease resistance [4,40]. It might be the most effective method of enhancing the immune ability for fish preventing various diseases [4,41]. In addition, the immune status of fish was closely related to the inflammation which was initiated and regulated by inflammatory cytokines [42]. Previous studies have shown that IL-8 was important pro-inflammatory cytokines, which could recruit and activate macrophages and neutrophils to remove the cell debris and invading microorganisms, and facilitate the regrowth of injured tissues [10,43]. IL-10, originally designated as a cytokine synthesis inhibitory factor, is a multifunctional cytokine that demonstrates immuno-suppressive function which can limit inflammatory response [42,44]. TLRs play an essential role in the activation of innate immunity by recognizing their cognate ligands [43]. TLR3 is considered an important pattern recognition receptor and also one of the most studied TLRs [45]. In addition, the major histocompatibility complex (MHC) molecules play an important role in the vertebrate immune system [4,10]. Tan et al. reported that dietary dandelion extract could decrease IL-8 mRNA levels in the intestine of golden pompano [15]. Giri et al. reported that the mRNA levels of IL-10 were significantly decreased in the head-kidney, intestine and hepatopancreas of *Labeo rohita* fed guava leaves supplemented diets [44]. Zou et al. showed that the mRNA expression levels of TLR3 and MHC-2 significantly increased in liver of the hybrid grouper fed *Radix Bupleuri* extracts supplement diet under D-GalN/LPS injection [4]. In the present study, the relative expression levels of IL-8, IL-10, TLR3 and MHC-2 showed a decreased trend in head-kidney of hybrid grouper fed the DE supplemented diet. However, MHC-2 showed increasing trend in the spleen and the expression trends of the other three genes were similar to the head kidney. In addition, the blood cell count is an important indicator for immune function. It is strongly correlated with the sensitivity to pathogens in aquatic organisms [21,37]. In the present study, after injection CCL_4 72 h, the blood cell count of grouper fed Diet0.2% and Diet 0.4% was significantly higher than fish fed Diet 0%. More importantly, previous studies reported that diet supplemented with dandelion root extracts could enhance the survival of fish after bacteria challenge [1]. These results indicated that dietary DE could improve the immunity of fish via regulating immune-related genes expression.

Previous studies showed that dandelion extract could inhibit inflammasome activation through ROS scavenging and anti-inflammatory activity in human intestinal epithelial cells [14,22]. The coordinated actions of various cellular antioxidants are critical for the effective detoxification of free radicals [4]. The dandelion flower represents a potentially valuable source of natural antioxidant and bioactive material [46]. It could suppresse both reactive oxygen species and nitric oxide and prevents lipid oxidation in vitro [22]. The dandelion leaf crude extract possesses the highest total content of phenolics and flavonoids and antioxidant activity [14]. Antioxidant enzymes were regarded as biomarkers to evaluate the fish health impact by stress- and immune-response [10]. The relative expression levels of antioxidant enzymes, such as CAT, GPx, GR and Keap1, partly reflect the antioxidant capacity of fish [4,10,47]. Tan et al. reported that dietary ginkgo biloba leaf extract could significantly up-regulate the mRNA levels of CAT, GPx and GR and down-regulated the mRNA levels of Keap1 in the head kidney of hybrid grouper [10]. Zou et al. showed that the mRNA levels of GPx increased whereas Keap1 decreased in the liver of hybrid grouper following the supplemented of Radix Bupleuri

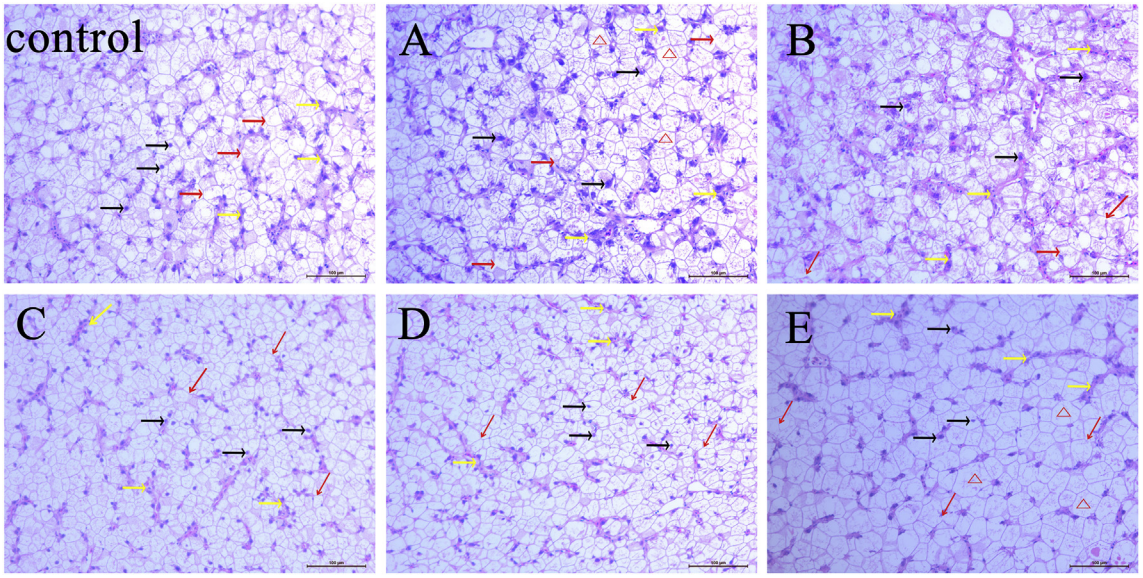

Fig. 7. Liver sections of hybrid grouper fed diets supplementation with dandelion extracts (DE) levels after 72 h CCL_4 (0.05 mL 30% CCL_4/10 g fish) injection (hematoxylin and eosin staining, original magnification 200 ×).
Control: liver from fish fed Diet 0% (0% DE) and injected olive oil; A: liver from fish fed Diet 0% (0% DE) and injected 30% CCL_4; B. Liver from fish fed Diet0.1% (0.1% DE) and injected 30% CCL_4; C. Liver from fish fed Diet0.2% (0.2% DE) and injected 30% CCL_4; D. Liver from fish fed Diet0.4% (0.4% DE) and injected 30% CCL_4; E. Liver from fish fed Diet0.8% (0.8% DE)and injected 30% CCL_4;"→" nucleus; "→"nuclear membranes; "△" swelling cells; "→" venous sinus.

extracts levels up to a certain level [4]. In the present study, dietary DE increased the relative expression levels of CAT, GPX and GR in the spleen and head-kidney of fish, and the expression of the GR in the liver. The results were similar to the above studies, which suggested that DE could improve the antioxidant capacity of fish.

In addition, caspases play a central role in apoptosis and in the proteolytic processing of pro-inflammatory cytokines [48]. Apoptosis can be triggered through two major pathways, the one is extrinsic pathway which directly activates the initiator caspase8 and the other is intrinsic pathway which is initiated by the release of cytochrome *c* activating downstream effector caspase9 and caspase3 [10,29]. Previous studies showed that activated caspase8 and caspase9 can trigger apoptotic process [4]. In both of the two apoptosis pathways, caspase3 activation is closely associated with hepatocyte apoptosis and ultimately induces liver damage [49]. The previous study showed that dietary supplementation with 0.50–4.00 g kg^{-1} ginkgo biloba leaf extracts significantly decreased caspase9, caspase8 and caspase3 mRNA levels in head kidney of hybrid grouper fed high lipid diets [10]. In our previous study, the mRNA expression levels of caspase9, caspase8 and caspase3 were significantly decreased in the head kidney of hybrid grouper fed diets supplemented with *Panax notoginseng* extract [27]. However, in this study, the mRNA expression levels of caspase9 and caspase8 showed an increase trend while and caspase3 showed the opposite trend in the liver of hybrid grouper. According to the above results, we suggested that DE could improve the health situation of the liver in hybrid grouper may be regulated by the expression of genes related to antioxidant, inflammation and apoptosis.

In conclusion, the study indicated that dietary DE had no significant impact on growth performance and feed utilization in hybrid grouper, but it could decrease crude lipids content in the whole body via regulating lipid metabolism-related genes expression and improve immunity by increasing the relative expression level of antioxidant enzymes (CAT, GPX and GR) and reducing inflammatory factor IL-8 mRNA levels in the spleen and head-kidney of fish. DE had a protective effect against CCl_4-induced liver damage in hybrid grouper and it could improve liver morphology, detoxification and health, which suggested dandelion extract could be used as a functional feed additive to protect the liver and enhance liver function to improve the health of farmed fish.

Acknowledgment

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