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Emodin ameliorates metabolic and antioxidant capacity inhibited by dietary oxidized fish oil through PPARs and Nrf2-Keap1 signaling in Wuchang bream (*Megalobrama amblycephala*)

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ABSTRACT

Dietary lipids and fatty acids are involved in cell metabolism and animal physiological regulation. However, oxidized lipids could induce oxidative stress and disorder normal growth and physiological health in fish. A 12-week rearing experiment with 6% fish oil (6F), 6% oxidized fish oil (6OF) and emodin supplemented diets (6F + E, 6OF + E) was conducted to evaluate the protective mechanism of emodin on oxidized fish oil stress in *Megalobrama amblycephala*. Results indicate that, under oxidized fish oil stress, emodin rescued the growth performance inhibition, improved special growth ratio (SGR), and reduced feed conversion ratio (FCR) and hepatosomatic index (HSI); rescued intestine histological impairment, ameliorated the structural expansion and membrane damage of mitochondria in intestine cells, and increased the length and intensity of intestinal villus. Moreover, emodin enhanced serum immune and antioxidant enzyme activity, increased metabolic activity through PPARs signaling, increased antioxidant capacity through PPARs and Nrf2-Keap1 signaling based on the transcriptional expression of specific genes. These results indicate emodin could be used as an effective immunostimulant to protect organism form oxidative stress induced by dietary oxidized lipid. This may provide insights for oxidized lipid prevention in aquaculture production.

1. Introduction

Global aquaculture industry is one of the fastest growing food production sectors, it is anticipated to play an increasingly important role in meeting the protein demand of a growing human population [1]. The rapid increase of aquaculture production worldwide will expand the demand for lipids as an essential nutrient for fish. Oils and fats are characterized by their unique fatty acid composition. Dietary lipids and fatty acids are known to be highly metabolically active, involved in controlling and regulating cell metabolism and animal physiology, which mediate inflammatory and immune responses to a variety of stresses [2]. These include digestibility, lipogenesis, lipid transport and uptake, fatty acid catabolism, and fatty acid desaturation and elongation that could influence tissue fatty acid composition [3].

In consideration of the fact that dietary fatty acid composition is mirrored in farmed fish fillet, the inclusion of essential unsaturated fatty acids in aquatic feed, especially n-3 polyunsaturated fatty acids (PUFAs) that impart health-promoting benefits to human consumers, is of great importance. However, unsaturated fatty acids (UFA) is prone to be oxidized under normal stocking condition [4]. Dietary oxidized lipid could disorder intestinal secretion, increase intestinal permeability, induce intestinal oxidative stress and disease [5], which affect growth performance, body health and even cause mortality in aquatic animals [6]. Toxic effects of oxidized lipid have been widely studied in mammals, however, the impairment mechanism of oxidized lipid on fish remains unclear, it has become a bottleneck that seriously restricted the sustainable development for aquaculture in some extent.

Peroxisome-proliferator activator receptors (PPARs) are nuclear receptors of central importance in energy homeostasis and inflammation [7,8]. Recent studies demonstrate that PPARs were implicated in

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Table 1
Formulation and proximate composition of experimental diets.

Ingredient/%	6F	6F + E	6OF	6OF + E	Nutrition value (%, dry matter)	6F	6F + E	6OF	6OF + E
casein	25.0	25.0	25.0	25.0	Dry matter, DM	92.06	92.12	92.18	92.24
Gelatin	5.0	5.0	5.0	5.0	Crude protein, CP	33.11	33.11	33.11	33.11
Fish meal	10.0	10.0	10.0	10.0	Ether extract, EE	7.01	7.01	7.01	7.01
Dextrin	10.0	10.0	10.0	10.0	Nitrogen Free Extract, NFE	1.00	1.03	1.06	1.09
α-starch	24.5	24.5	24.5	24.5	Ash	8.28	8.31	8.34	8.37
Fish oil	6.0	6.0	0.0	0.0	Ca	1.62	1.62	1.62	1.62
Oxidized fish oil	0.0	0.0	6.0	6.0	Total P	1.05	1.05	1.05	1.05
Microcrystalline cellulose	7.0	7.0	7.0	7.0	Lysine	2.49	2.49	2.49	2.49
Carboxymethylcellulose	5.0	5.0	5.0	5.0	Cysteine	0.92	0.92	0.92	0.92
Choline chloride	1.0	1.0	1.0	1.0	Methionine	0.19	0.19	0.19	0.19
Vitamin premix[a]	1.0	1.0	1.0	1.0	Threonine	1.34	1.34	1.34	1.34
Mineral premix[b]	1.0	1.0	1.0	1.0	Arginine	1.50	1.50	1.50	1.50
Calcium dihydrogen phosphate	2.0	2.0	2.0	2.0	Fe	33.70	33.70	33.70	33.70
Attapulgite	2.0	2.0	2.0	2.0	Gross Energy[c]	14.68	14.68	14.68	14.68
Ethoxyquin	0.5	0.5	0.5	0.5					
Total	100.0	100.0	100.0	100.0					
Emodin(mg kg^{-1})	0.0	30.0	0.0	30.0					

[a] Vitamin contents per kg diets : Vitamin A, 9000 IU; Vitamin B_1, 3.2 mg; Vitamin B_2, 10.9 mg; Vitamin B_5, 20 mg; Vitamin B_6, 5 mg; Vitamin B_{12}, 0.016 mg; Vitamin C, 50 mg; Vitamin D, 2000 IU; Vitamin E, 45 mg; Vitamin K_3, 2.2 mg; Niacin, 28 mg; Folic acid, 1.65 mg; Pantothenate, 10 mg; Choline, 600 mg.
[b] Mineral contents per kg diets: $FeSO_4 \cdot 7H_2O$, 250 mg; $CuSO_4 \cdot 5H_2O$, 20 mg; $ZnSO_4 \cdot 7H_2O$, 220 mg; Na_2SeO_3, 0.4 mg; $MnSO_4 \cdot 4H_2O$, 70 mg; $CoCl_2 \cdot 6H_2O$, 1 mg; KI, 0.26 mg.
[c] Energy, calculated by using standard physiological fuel values of 37.7, 16.7, and 16.7 kJ g^{-1} for protein, lipid and carbohydrate, respectively.

the oxidative stress response. Generally, PPARs may directly modulate the expression of several antioxidant and prooxidant genes in response to oxidative stress, such as catalase [9], Bcl-2 [10], SOD [11], GPx3 [12], eNOS [13], HO-1 [14], UCP2 [15], COX-2 [16], and iNOS [17]. Meanwhile, PPARs may also influence the antioxidant and anti-inflammatory responses interacting with other regulatory pathways. PPARs have shown to induce anti-inflammatory responses inhibiting proinflammatory transcription factors such as NF-κB [18], Nrf2-Keap1 [19], and Wnt/β-Catenin [20] signaling. Furthermore, Nrf2-Keap1 signaling was also reported as a defense system aimed to preserve cellular homeostasis and oxidative stress [21], there is a positive feedback loop among PPAR-γ and Nrf2, which indicates PPAR-γ may activate Nrf2 signaling directly or through its upstream pathways [22].

Emodin (6-methyl-1,3,8-trihydroxyanthraquinone) is a clinically effective drug that was approved by Food and Drug Administration (FDA) in USA. Meanwhile, emodin is also a natural anthraquinone derivative that obtained from many widely used Chinese medicinal herbs, such as *Rheum palmatum*, *Polygonum cuspidatum* and *Polygonum multiflorum* [9]. Emodin has been used as a traditional Chinese medicine for over 2000 years and is still present in various herbal preparations. Moreover, emerging evidences indicate that emodin possessed a wide spectrum of pharmacological properties, including anticancer, hepatoprotective, anti-inflammatory, antioxidant and antimicrobial activities [23–25]. Most recently, we demonstrated that emodin could increase the immune and antioxidant capacity in aquatic animals, especially *M. amblycephala* [26]. Therefore, we suppose whether emodin could rescue the oxidative stress induced by dietary oxidized fish oil.

M. amblycephala is one of the most widely cultivated freshwater species in China. It is susceptible to be affected by external stress that could induce stress response, such as hemorrhage, superficial mucus secretion decreases, and poor feed intake [27]. Meanwhile, *M. amblycephala* is vulnerable to some endogenous factors that could induce intestinal injury stress, such as dietary lipid oxidation [28]. Therefore, *M. amblycephala* is an ideal animal model to study the oxidative impairment mechanism on herbivorous fishes. In this study, we applied *M. amblycephala* as a research model to explore the ameliorative mechanism of emodin on oxidative injury stress induced by dietary oxidized fish oil. Based on the analysis of growth performance, immunity, antioxidant capacity, and metabolic and antioxidant molecular mechanisms of Peroxisome-proliferator activator receptors (PPARs) and Nrf2-Keap1 signaling, we are expecting to get deep insights into the protective effects of emodin on oxidative stress injury induced by dietary lipid peroxidation in *M. amblycephala*, which may provide a theoretical basis for the application of emodin as an immunopotentiator in aquatic feed.

2. Experimental methods

2.1. Ethics statement

This study was approved by the Animal Care and Use Committee of Nanjing Agricultural University (Nanjing, China). All animal procedures were performed according to the Guideline for the Care and Use of Laboratory Animals in China. The ethics in this experiment was the same as our previous published paper [27].

2.2. Oxidized fish oil and experimental diets

2.2.1. Oxidized fish oil preparation

Oxidized fish oil was prepared in accordance with our previously reported paper [28]. In detail, oil oxidation was conducted by intermittent oxygen injection (10 min injection and 20 min interval) through salmon oil for 14 days at 37 ± 1 °C water bath in the lab. After oxidation, ethoxyquin (150 mg kg^{-1}) was supplemented into the oil to avoid additional oxidation. The peroxide value (POV) of oxidized fish oil was monitored according to the method of National Standard of China (GB/T 5538–2005/ISO 3960:2001) daily during the oxidation period.

2.2.2. Experimental diets

Four isonitrogenous (33.11% crude protein) and isoenergetic (14.68 kJ g^{-1} energy) experimental diets were formulated with different oil source and emodin concentration. In detail, basal diets were prepared with 6% fresh fish oil (6F) and 6% oxidized fish oil (6OF), emodin enriched diets were supplemented with 30 mg kg^{-1} emodin (6F + E, 6OF + E), respectively (shown in Table 1). All ingredients were ultramicroporwderized and thoroughly mixed with oil and water, then pelleted (1.5 mm in diameter) with lab extruder (Science and Technology Industrial Factory of South China University of Technology, China). Pellets were air-dried to approximately 10% moisture, sealed in plastic bags and stored at −20 °C until use. POV of each diet (extracted

with acetone) was measured according to the methods described in section 2.2.1.

2.3. Experimental animals and rearing conditions

M. amblycephala were obtained from research station in Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences. Fish were acclimated for 14 days fed with the control diet (6F) prior to feeding experiments. Fish husbandry was conducted in an indoor freshwater recirculating system consisting of 12 fiberglass tanks (300-L each) with equal supplemental aeration and water flow (3 L min^{-1}). At the start of the trial, 300 fish with similar body weight (initial body weight, IBW 5.20 ± 0.01 g) were randomly assigned into 12 tanks (3 tanks per group, 25 fish per tank). The fish were fed to apparent satiation with respective diets four times a day (8:00, 11:00, 14:00 and 17:00) for 12 weeks, the recycling water was drawn from underground and one-third water in the tank was exchanged weekly. Aeration was also applied to maintain enough dissolved oxygen (DO). During the experimental period, water temperature was ranged from 26 ± 1 °C, the water quality was kept as follows: pH 7.6–7.8, DO > 6 mg L^{-1}, NH_3 < 0.01 mg L^{-1}, H_2S < 0.01 mg L^{-1}.

2.4. Sample collection

After 12 weeks rearing experiment, fish were starved for 24 h to evacuate the alimentary tract contents prior to sampling. Nine fish of each group (three fish from each tank) were randomly anaesthetized with MS-222 (100 mg L^{-1}) to collect samples. Blood samples were obtained from caudal vein and centrifuged at 5000 rpm at 4 °C for 10 min to extract the plasma. The plasma was stored at −80 °C for immune and antioxidant parameter measurement. Meanwhile, the sampled fish were dissected to collect the mid intestine (MI) samples on ice, frozen in liquid nitrogen immediately, and stored at −80 °C for subsequent transcriptional expression analysis. After sample collection, the remaining fish in each tank were weighed to calculate the growth performance parameters.

2.5. Growth performance

Growth performance were calculated with the following equations: Weight gain ratio (WGR) = (final body weight - initial body weight)/initial body weight × 100%; Special growth ratio (SGR) = (Ln final body weight - Ln initial body weight)/number of days; Feed conversion ratio (FCR) = (total weight gain, g)/(total feed intake, g) × 100%.

2.6. Plasma biochemical indexes

Plasma biochemical indices (AST, ALT, TP, ALB, GLB, TG, TC, and Glu) were tested by kits with BS-400 automatic biochemical analyzer (Shenzhen Mindray Ltd, China). All kits were commercial and purchased from Shenzhen Mindray Biological Medical Company. All procedures were conducted according to the manufacturer's protocol.

2.7. Antioxidant enzyme activity and cortisol concentration

Plasma antioxidant enzyme activities were measured by assay kits (Nanjing Jiancheng Bioengineering Institute, China) according to our previously described methods [28]. Total superoxide dismutase (T-SOD) was determined with hydroxylamine method, reduced glutathione (GSH) were determined with spectrophotometric method, glutathione peroxidase (GPx), anti-superoxide anion free radical (ASAFR) were determined with colorimetric method, and malondialdehyde (MDA) was determined with TBA method. Cortisol concentration was measured using radioimmunoassay (Beijing Beifang Biotech Research Institute, China) as described previously [29]. All procedures were conducted according to the manufacturer's protocol.

2.8. Mid-intestine histological ultrastructure

Histological ultrastructure was conducted according to our previous published methods [28]. In detail, 3 mid-intestine samples in each group were fixed in 2.5% glutaraldehyde for 24 h, post-fixed in 1% osmium tetroxide (OsO_4) for 1 h, and stored at 4 °C to observe histological ultrastructure. Sections were embedded in epoxy resin Epon812, cut into 70 μm thick slices with an RMC PowerTome XL microtome, stained with uranyl acetate and lead citrate, and examined under a Hitachi HT7700 transmission electron microscope (Hitachi, Tokyo, Japan).

2.9. RNA extraction and RT-PCR analysis

RNA extraction and RT-PCR analysis were conducted according to the established methods described in our previous study [26]. Total RNA was extracted from 9 mid-intestine in each group using RNAiso Plus reagent (Dalian Takara Co. Ltd., China) and was incubated with RNase-free DNase (Dalian Takara Co. Ltd., China) to remove the contaminating genomic DNA. Quantity and quality of the RNA was assessed by OD260/280 and electrophoresis in 1.5% agarose gel. Primers (shown in Table 2) for each gene were designed using primer premier 5.0 based on the mRNA sequences obtained from *M. amblycephala* intestine transcriptome sequencing database of aquatic disease and feed laboratory of Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences. All primers were synthesized by Shanghai Generay Biotechnology, Co., Ltd, China. RT-PCR was performed with the SYBR® Primix Ex TaqTM II (TliRNase Plus) Kit using ABI 7500 Real-time PCR System according to the manufacturer's protocol. The relative expression levels of the target genes were normalized to β-actin, the housekeeping gene of *M. amblycephala*. Afterwards, $2^{-\Delta\Delta CT}$ calculation was applied for statistical analysis.

2.10. Data analysis

Duncan's multiple range test was applied to compare the growth performance, immune and antioxidant enzyme activity, and cortisol concentration after one-way ANOVA in SPSS 24.0, and an independent samples *t*-test was conducted to analyze the transcriptional expression of related genes. Meanwhile, data were all validated for normality and homogeneity for variances. All results were expressed as mean ± standard error of the mean (X ± SEM).

3. Results

3.1. Emodin rescued growth inhibition induced by dietary oxidized fish oil in M. amblycephala

Our previous research demonstrated that oxidized fish oil inhibited the growth performance of *M. amblycephala* [28]. In this paper, we evaluated whether dietary emodin could rescue the growth performance inhibition induced by dietary oxidized fish oil, results were shown in Fig. 1. Under oxidized fish oil stimulation (6OF), dietary emodin (6OF + E) significantly increased final body weight (FBW), weight gain ratio (WGR) and special growth ratio (SGR) ($P < 0.05$), significantly decreased feed conversion ratio (FCR) ($P < 0.05$), indicating that emodin improved the growth performance inhibited by oxidized fish oil. Moreover, hepatosomatic index (HSI) was also significantly reduced under emodin stimulation ($P < 0.05$), revealing that emodin intended to reduce the hepatomegaly. However, FBW, WGR, and SGR in 6OF + E were significantly decreased ($P < 0.05$), while FCR in 6OF significantly increased ($P < 0.05$) compare to 6F and 6F + E group, indicating emodin could not completely rescue the growth inhibition induced by oxidized fish oil.

Table 2
Primers and sequences referred in the experiments.

	Gene	Primer	Sequence (5'→3')		Gene	Primer	Sequence (5'→3')
1	PPAR-α	F	GTGCCAATACTGTCGCTTTCAG	16	Cox-2	F	AACCCAGGACCTTACACCC
		R	CCGCCTTTAACCTCAGCTTCT			R	CCCGCAGATTTCAGAACA
2	PPAR-β	F	CATCCTCACGGGCAAGAC	17	Nox1	F	CTGGCTGCTCATCACAGAAG
		R	CACTGGCAGCGGTAGAAG			R	CCACTATCGCTGGTCTCACA
3	PPAR-γ	F	AGCTTCAAGCGAATGGTTCTG	18	Nrf2	F	AAGAGCGAACGTAGCACCAG
		R	AGGCCTCGGGCTTCCA			R	GCAGTGTGCTGAAGGGAGTAT
4	CPT1	F	TACTTCCAAAGCGGTGAG	19	Keap1	F	GAGATTCGCAGAGGAGATCGG
		R	AGAGGTATTGTCCGAGCC			R	CTGGCAATGGGACAAGCTGA
5	PI3K	F	AGTATTGAGGCGGTAGGAGAG	20	Bach1	F	CAGCCATCATTTCCAACCTT
		R	TGTGATGTGCGTGTGTATTCTTC			R	GAGACGCCTGACAAGAATCC
6	Akt	F	GGGTCAGGTTCTCTGTTGCT	21	NQO1	F	AAGCCTCTGTCCTTTGCTCC
		R	GCTGTTTTGGGTCTTTCTGG			R	TCTGGAGGAAGTGGTTTGCC
7	PTEN	F	TCAACTGTAGGGTGGCACAA	22	HO-1	F	CAGGAGCAGAATGAACAGCA
		R	CTGGTCCTGACTTCCCCATA			R	CCAAAGTGATTCCCACACCT
8	β-catenin	F	TTCTGTTCCGCATGTCTGAG	23	CAT	F	ACACCGACCCAGATTACGG
		R	TAGCTTGGGTCGTCCTGTCT			R	AGTTCACAGGGTTTCGGTTCA
9	TNF-α	F	CTGTCTGCTTCACGCTCAAC	24	GPx1	F	CCTTTATGAACGACCCCAAA
		R	GGTCCTGGTTCACTCTCCAA			R	TCTTGATGTCTCGCTCGATG
10	TGF-β	F	GAAAACGCATTGAAGCCATT	25	GSTm	F	GTGGTGACGCTCCCAACTAT
		R	GCCAACTGCTCCTGGTTTAG			R	CGCCTGATTCTCCAAGATGT
11	NF-κB	F	GGGTTTTTCATTGGTGGATG	26	IL-1β	F	CGATAAGACCAGCACGACCTT
		R	GCAGAACTGTGGCAATCTGA			R	GTTTCCGTCTCTCAGCGTCA
12	CPT2	F	CCATAGCCCACTCCGAAAC	27	IL-6	F	GTCCTCTGCCGGTCAAATC
		R	TGCCGCCATAAACCACAA			R	CAGTCGCTGGGTCTCTTCAC
13	Bcl-2	F	CGTCTACCTGGACAACCACA	28	IL-17	F	CAGGAGAGCACCAAGGACTC
		R	GCGTTTCTGTGCAATGAGTG			R	GACGAGACGCTGATTGACAG
14	UCP2	F	TGGCTACAGCACAGTTGAGG	29	β-actin	F	TCTGCTATGTGGCTCTTGACTTCG
		R	TGACCTCATCAAAGATGCAC			R	CCTCTGGGCACCTGAACCTCT
15	iNOS	F	ATTCAAGGGCAGCTTCCAGG				
		R	CAGGGGCAAAGTTTAAGGGC				

Note: The mRNA sequences for each gene was obtained from *M. amblycephala* intestine transcriptome sequencing database of aquatic disease and feed laboratory of Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences. Primers for RT-PCR were designed using primer premier 5.0.

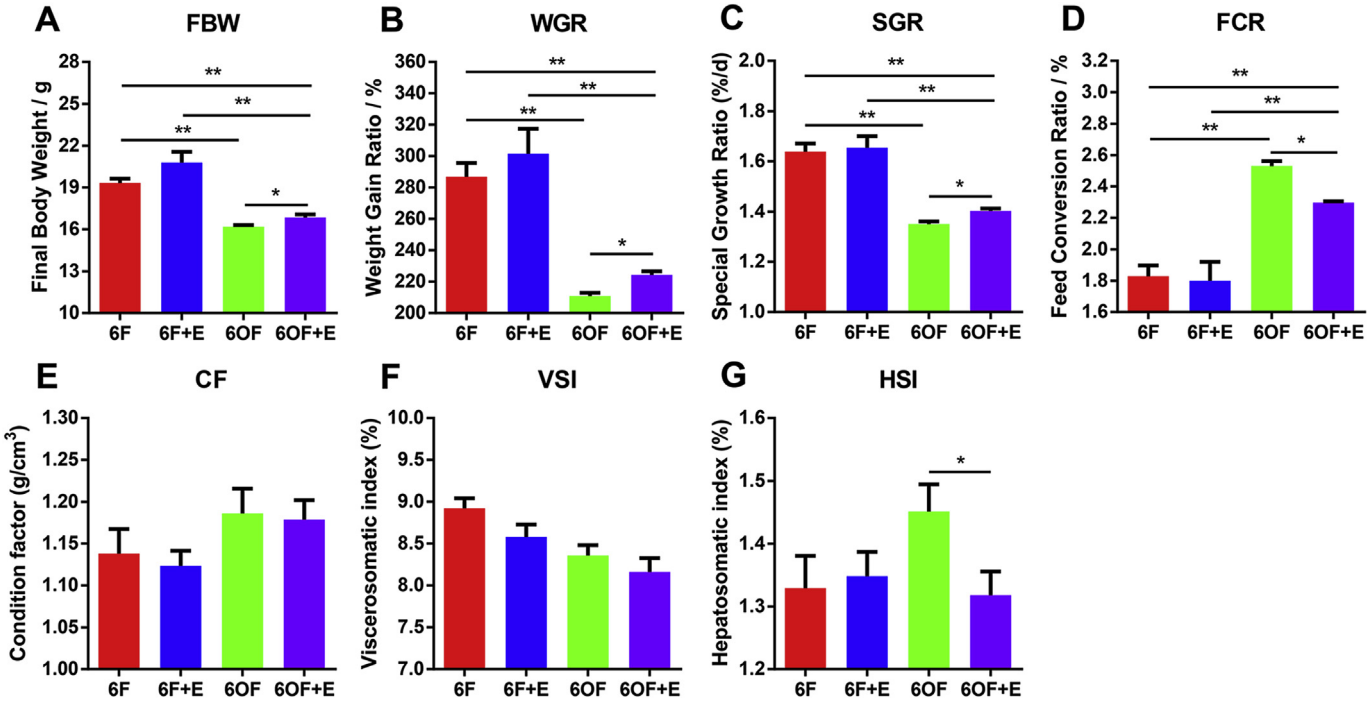

Fig. 1. Emodin rescued the growth inhibition induced by oxidized fish oil in *M. amblycephala*
(A), Final body weight, FBW; (B), Weight gain ratio, WGR; (C), Special growth ratio, SGR; (D), Feed conversion ratio, FCR; (E), Condition factor, CF; (F), Viscerosomatic
index, VSI; (G), Hepatosomatic index, HSI. Asterisk above histogram bars indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) in Students t-test, data was expressed as mean ± SEM, n = 9.

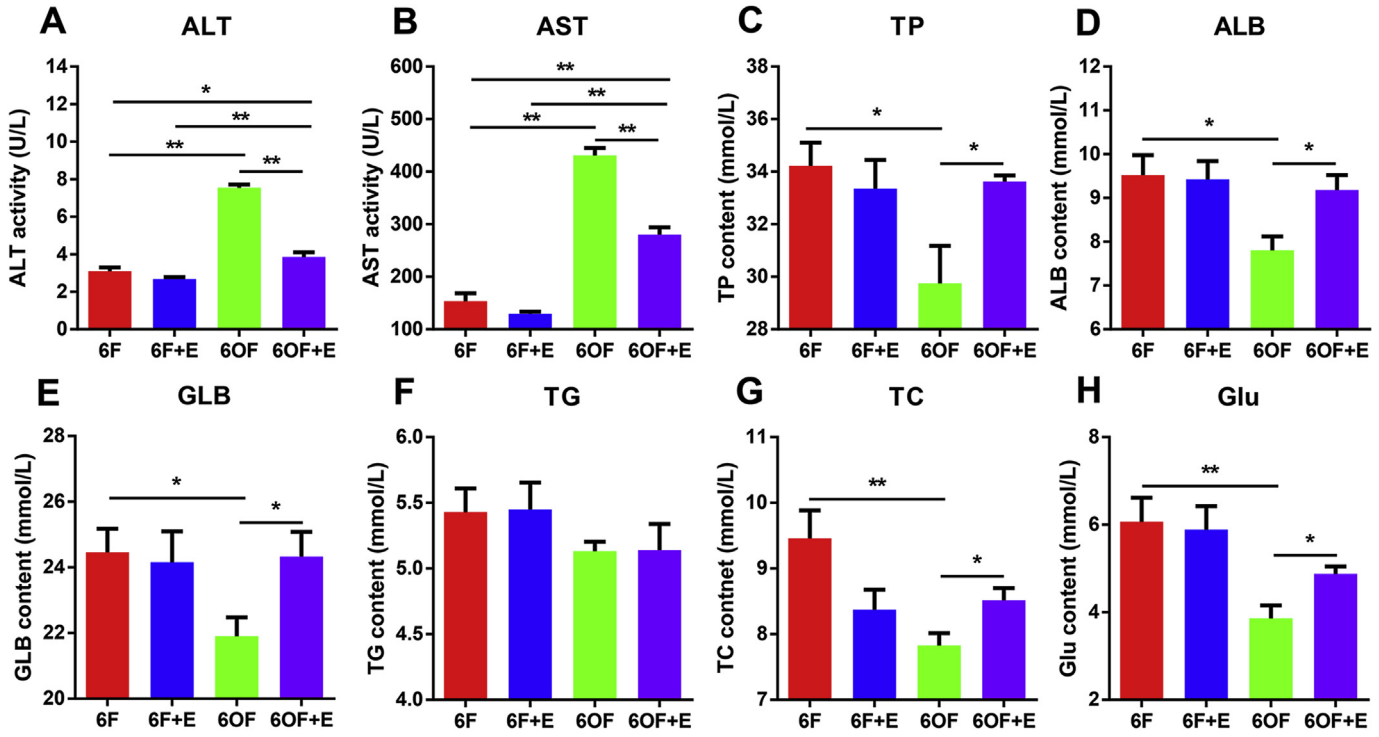

Fig. 2. Emodin enhanced plasma antioxidant biochemical indexes under oxidized fish oil administration in *M. amblycephala*
(A), Alanine aminotransferase, ALT; (B), Aspartate aminotransferase, AST; (C), Total protein, TP; (D), Albumin, ALB; (E), Globulin, GLB; (F), Triglyceride, TG; (G), Total cholesterol, TC; (H), Glucose, Glu. Asterisk above histogram bars indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) in Students t-test, data was expressed as mean ± SEM, n = 9.

3.2. Emodin enhanced plasma biochemical indexes under oxidized fish oil administration in M. amblycephala

From this study, we found that dietary emodin enhanced the antioxidant capacity of *M. Megalobrama* under oxidized fish oil stimulation (shown in Fig. 2). Emodin supplementation (6OF + E) in dietary oxidized fish oil (6OF) significantly decreased plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity ($P < 0.05$), while significantly increased plasma total protein (TP), albumin (ALB), globulin (GLB), total cholesterol (TC) and glucose (Glu) concentration ($P < 0.05$). Meanwhile, ALT and AST activity in 6OF + E were significantly higher than that in 6F and 6F + E group ($P < 0.05$). Moreover, plasma triglyceride (TG) content exhibited no statistical alteration under oxidized fish oil and emodin administration.

3.3. Emodin increased plasma antioxidant enzyme activity under oxidized fish oil administration in M. amblycephala

Emodin increased plasma antioxidant enzyme activity under oxidized fish oil stimulation (shown in Fig. 3). Under fish oil administration (6F), emodin (6F + E) significantly increased T-SOD content ($P < 0.05$), significantly decreased anti-superoxide anion free radical (ASAFR) activity ($P < 0.05$), revealing emodin could enhance antioxidant activity. Under oxidized fish oil administration (6OF), emodin supplementation (6OF + E) significantly decreased T-SOD, MDA, Cortisol content and ASAFR activity ($P < 0.05$). Moreover, results also demonstrate T-SOD and ASAFR in 6OF + E was significantly increased than that in 6F ($P < 0.05$), MDA, GPx and ASAFR in 6OF + E was significantly increased compare with 6F + E ($P < 0.05$).

3.4. Emodin rescued intestine histological impairment induced by oxidized fish oil in M. amblycephala

Fig. 4 deciphered the rescue effect of emodin on intestine histological impairment induced by oxidized fish oil. Compare with 6OF (Fig. 4C), emodin (6OF + E, Fig. 4D) ameliorated the structural expansion and membrane damage of mitochondria in intestine cells, significantly increased the length ($P < 0.01$) and intensity of intestinal villus (Fig. 4C, D and 4E). However, emodin exhibited no significant effects on lipid droplet amounts and expansion in intestine cells under oxidized fish oil stimulation (Fig. 4C and D). Moreover, intestinal mitochondria and intestinal villus in 6OF + E were extended to be damaged compare with 6F and 6F + E group.

3.5. Emodin increased lipid and glucose metabolic activity through PPARs signaling under oxidized fish oil stimulation in M. amblycephala

In this study, we evaluated the injury stress of oxidized fish oil and protection of emodin on metabolic mechanism with the transcriptional expression of PPARs signaling and down-stream target genes (shown in Fig. 5). Under oxidized fish oil (6OF) stimulation, emodin (6OF + E) significantly decreased the transcriptional expression of PPAR-α and PPAR-β ($P < 0.01$), significantly inhibited the expression of PPAR-γ, CPT1, PI3K, and Akt ($P < 0.05$), while significantly up-regulated the expression of PTEN and β-catenin ($P < 0.05$). However, PPAR-α was significantly down-regulated in 6OF + E compare with 6F and 6F + E group.

3.6. Emodin increased antioxidant activity through PPARs signaling under oxidized fish oil stimulation in M. amblycephala

Apart from the metabolic regulation, PPARs signaling also plays a vital role in antioxidant homeostasis. In this study (shown in Fig. 6), TNF-α and UCP2, the down-stream regulator of PPARs signaling were significantly down-regulated ($P < 0.05$); TGF-β, NF-κB, CPT2, and Cox-2 were significantly inhibited ($P < 0.01$); while Bcl-2 was significantly up-regulated ($P < 0.05$), indicate PPARs signaling functions importantly in the antioxidant amelioration of emodin on oxidized lipid

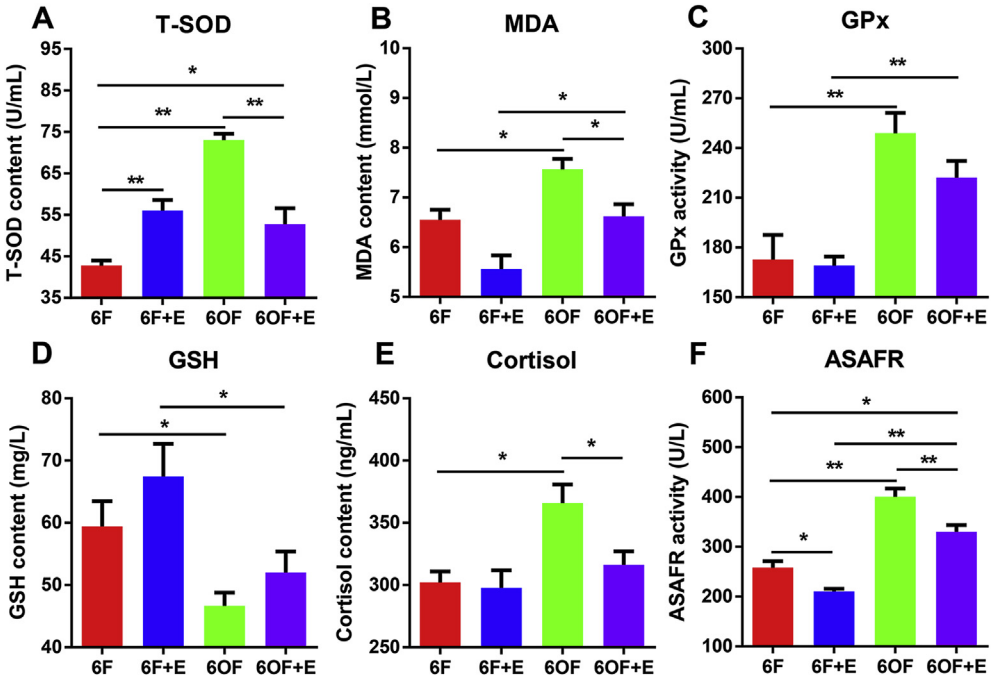

Fig. 3. Emodin increased serum antioxidant enzyme activities under oxidized fish oil administration in *M. amblycephala*Plasma antioxidant enzyme activities were measured by assay kits according to the manufacturer's protocol. Asterisk above histogram bars indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) in Students t-test, data was expressed as mean ± SEM, n = 9.

stimulation.

3.7. Emodin increased antioxidant activity through Nrf2-Keap1 signaling under oxidized fish oil stimulation in M. amblycephala

Nrf2-Keap1 is one of the most well studied antioxidant pathways in animals. In the current research, we confirm that Nrf2-Keap1 signaling participated in the antioxidant enhancement of emodin, as demonstrated in Fig. 7. Under fish oil (6F) stimulation, dietary emodin supplementation (6F + E) significantly enhanced the mRNA expression of key regulators Nrf2, Keap1, Bach1 ($P < 0.05$); and down-stream antioxidant elements NQO1, CAT, and GSTm ($P < 0.05$), which indicated emodin enhanced the antioxidant capacity. When fish were fed with oxidized fish oil (6OF), emodin (6OF + E) significantly decreased the mRNA expression of Bach1, NOX1, Nrf2 ($P < 0.05$), and down-stream of NQO1, HO-1, GPxl, GSTm, IL-1β, IL-17 and CAT ($P < 0.05$), indicating emodin exhibited protective effects on the injury stress induced by oxidized fish oil. Moreover, the transcriptional expression of Nrf2-Keap1 signaling in 6OF + E group revealed no statistical difference with 6F + E group ($P > 0.05$); while NOX1, Nrf2, Keap1, Bach1, CAT, NQO1 and GSTm were significantly up-regulated compare with 6F group ($P < 0.05$).

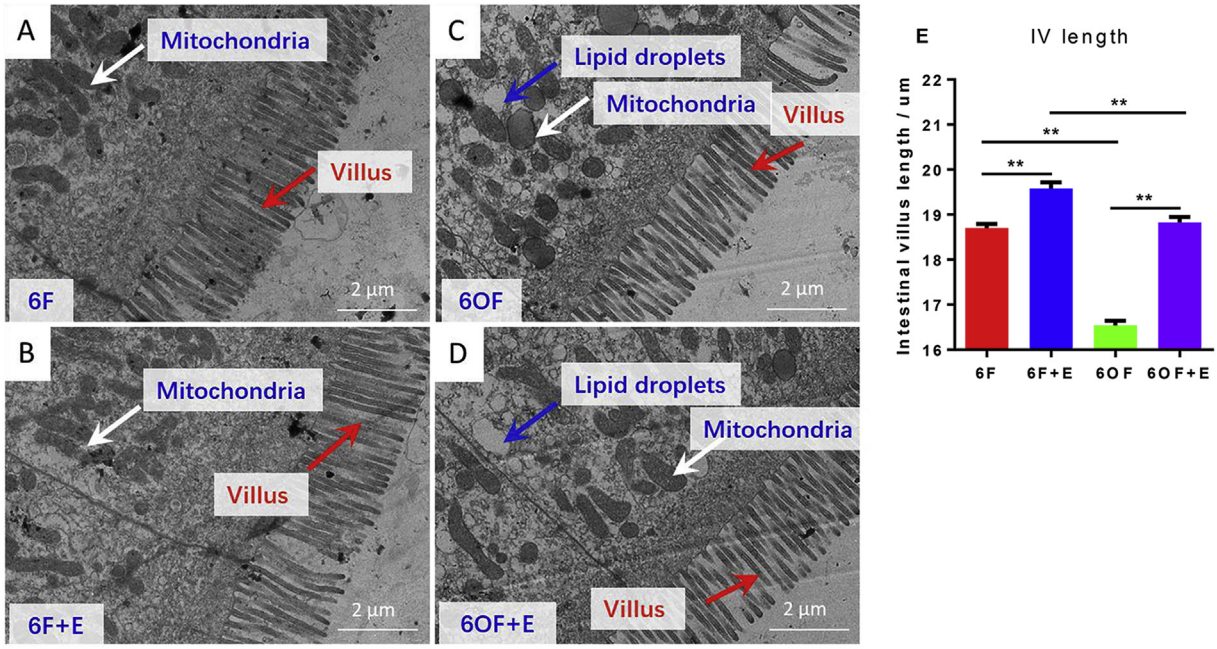

Fig. 4. Emodin rescued intestine histological impairment induced by oxidized fish oil in *M. amblycephala*
Panel A, B, C and D represents the histological structure of 6F, 6F + E, 6OF and 6OF + E, respectively; panel E represents the intestinal villus length comparation. Asterisk above histogram bars indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) in Students t-test, data was expressed as mean ± SEM, n = 3.

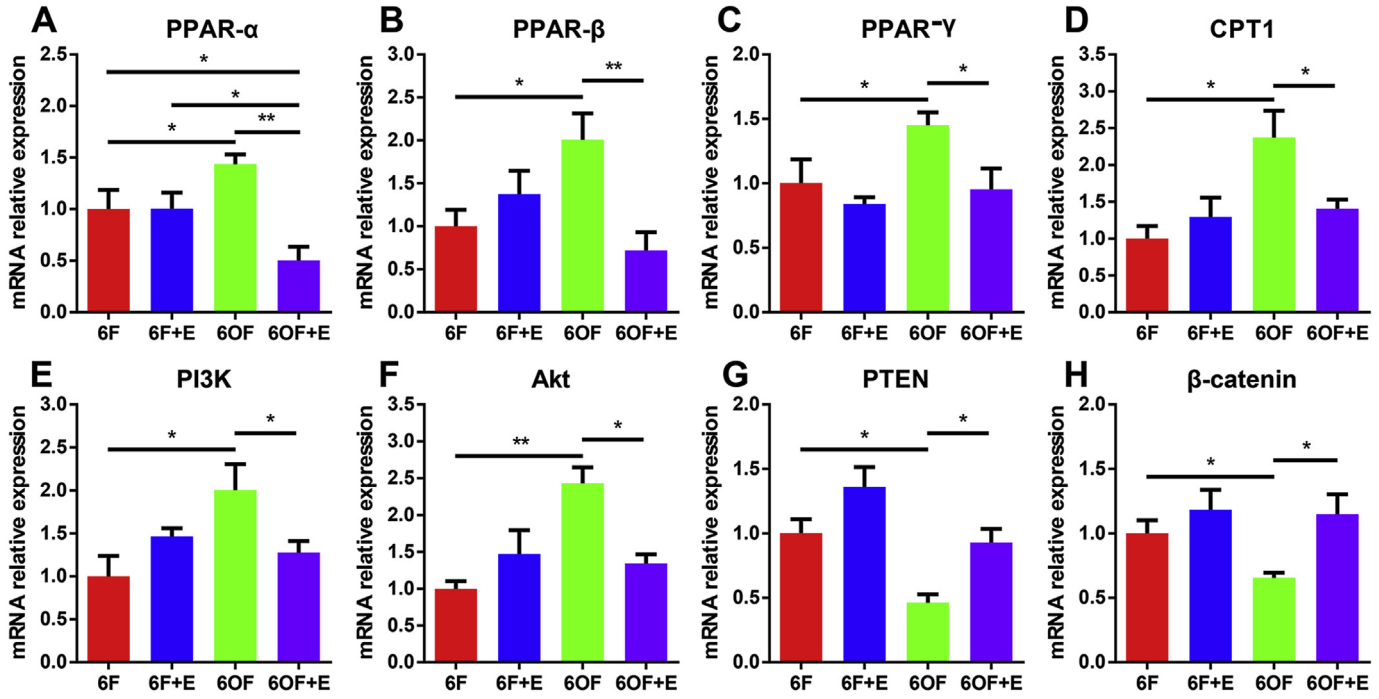

Fig. 5. Emodin increased metabolic activity through PPARs signaling under oxidized fish oil stimulation in *M. amblycephala*
Transcriptional expression of each gene was evaluated by RT-PCR. Asterisk above histogram bars indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) in Students t-test, data was expressed as mean ± SEM, n = 9.

4. Discussion

Lipids are essential nutrients and energy substances for fish, which are of great significance to maintain the normal growth, development and reproduction. Unsaturated fatty acids (UFA) is the key nutritional element for lipid requirement of fish, however, UFA is prone to be oxidized under normal condition. Dietary oxidized lipid could disorder intestinal secretion, increase intestinal permeability, induce intestinal oxidative stress and disease, which affects growth performance, body health and even cause mortality in aquatic animals [30,31]. Toxic effects of oxidized lipid have been widely studied in mammals [32], however, the underlying impairment and ameliorative mechanism in *M. amblycephala* was still unclear. Here we evaluated the protective effects of emodin, an effective component extracted from Chinese

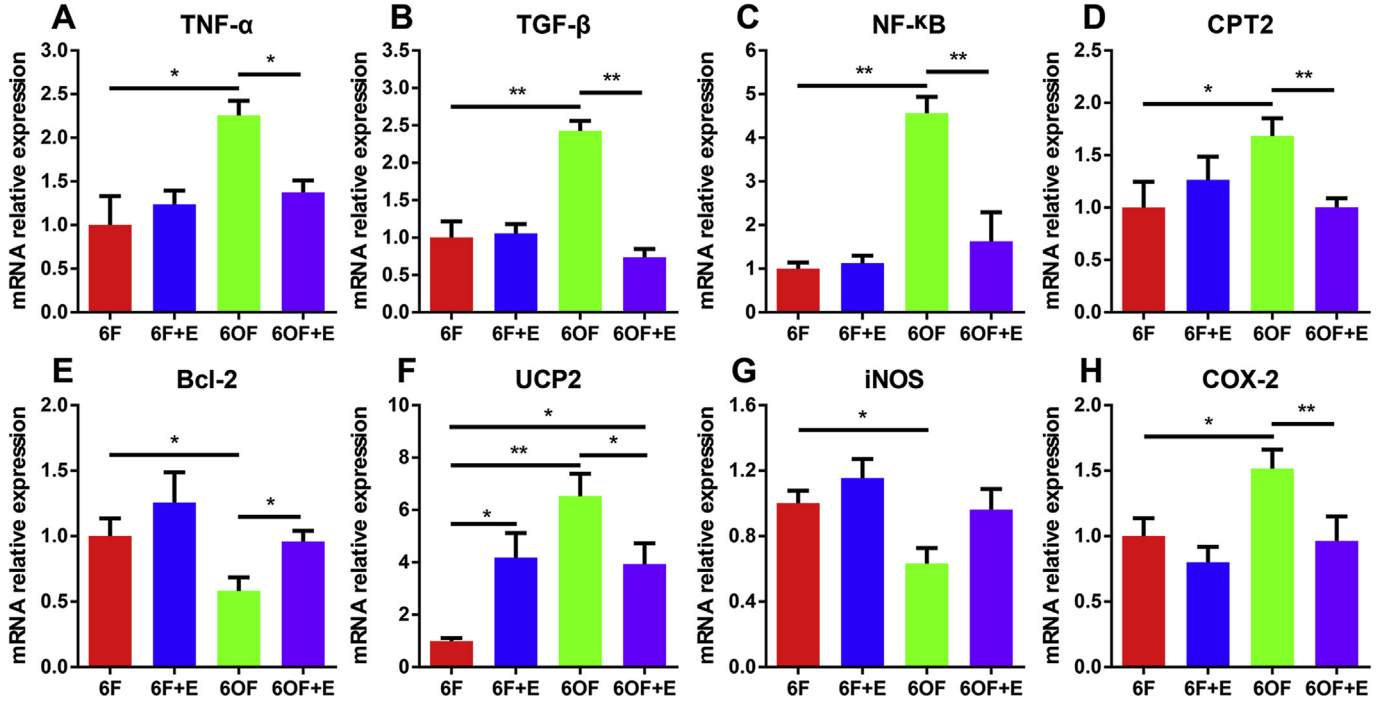

Fig. 6. Emodin increased antioxidant activity through PPARs signaling induced by oxidized fish oil in *M. amblycephala*
Transcriptional expression of each gene was evaluated by RT-PCR. Asterisk above histogram bars indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) in Students t-test, data was expressed as mean ± SEM, n = 9.

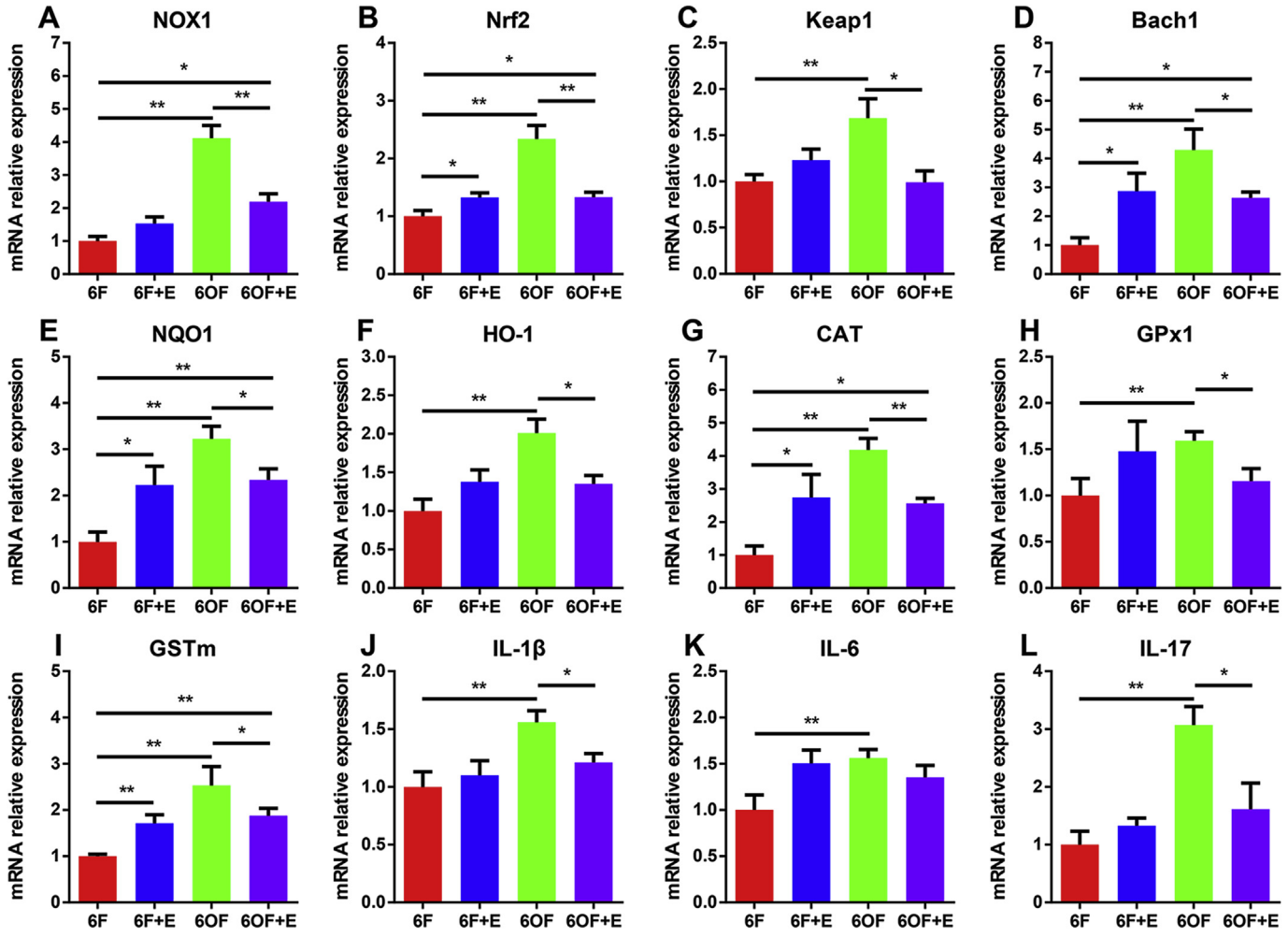

Fig. 7. Emodin increased antioxidant activity through Nrf2 signaling under oxidized fish oil stimulation in *M. amblycephala*
Transcriptional expression of each gene was evaluated by RT-PCR. Asterisk above histogram bars indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) in Students t-test, data was expressed as mean ± SEM, n = 9.

traditional herb, on oxidative stress induced by oxidized fish oil in *M. amblycephala*.

Growth performance and immunologic barrier is the endpoint of injury stress. According to our previous report, oxidized fish oil induced growth inhibition in *M. amblycephala* [28]. In the present study, emodin supplementation significantly increased the growth performance that was inhibited by oxidized fish oil. These results were in consistent with our previous results that demonstrated dietary emodin could improve the growth performance in *M. amblycephala* [29,33], and anthraquinones extracted from *Rheum officinale* Bail could improve the growth performance of *Macrobrachium rosenbergii* [34]. The underlying mechanism of emodin on growth rescue under oxidized lipid administration remains unclear, we suppose it may attribute to the improvement on lipid and glucose metabolism [35,36]. Moreover, our data also reveal that emodin reduced hepatosomatic index under oxidized fish oil stimulation, indicate emodin may decrease the lipid accumulation in hepatic tissue. This result was in accordance with Alisi's research [37], which indicated emodin prevents diet-induced intrahepatic fat accumulation in rats.

Side effects of dietary nutrient, especially lipid stress, is an important exogenous factor that leads to oxidative stress [38]. For animals, intake and absorption of oxidized lipids could continuously induce reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation in mitochondria, including the by-products of oxidative phosphorylation [39]. Excessive ROS could induce oxidative stress and impair immune system [40]. Therefore, ROS concentration needs to be precisely regulated. For animals, they have developed various enzymatic, nonenzymatic and antioxidant systems to control ROS and RNS levels [41]. In the present study, we evaluated the protective effect of emodin on immunity and antioxidant capacity based on plasma immune and antioxidant parameters. We found emodin enhanced antioxidant activity under oxidized fish oil stimulation, confirm emodin is an effective immunostimulant. These results were in consistent with previously published results shown that resveratrol and emodin could reduce oxidative stress in rat [42] and mice [35]. Moreover, as effective immunostimulants, vitamin C [43], vitamin E [44], α-lipoic acid [45,46], withaferin A [47], ginseng extracts [48], and flavonoid [49] were also exhibited protective effects on oxidative mechanism. Taken together, these results indicate the natural extract emodin exerts immune and redox protection for animals.

Fish intestine is the key digestion, absorption and immune organism for fish [50], intestinal nutrition functions vital for intestinal mucosa and immune system [51]. Our previous study revealed emodin could protect liver and intestine under thermal stress in *M. amblycephala* [52]. The intestine ultrastructure evidence demonstrate emodin protects intestine epithelial cells, mitochondria, and intestinal villus from oxidative stress induced by oxidized fish oil. We predict the underlying mechanism was that emodin enhanced immune and antioxidant capacity via improving intestinal nutrition status.

The phenotype reveals emodin rescued growth performance,

enhanced immunity and antioxidant capacity, protected intestinal structural integrity. This encourages us to uncover the underlying mechanism. The next we evaluated the metabolic and antioxidant capacity with PPARs and Nrf2-Keap1 signaling.

PPARs are a family of ligand-activated nuclear receptor transcription factors that regulates the function and expression of complex gene networks, especially involved in energy homeostasis and inflammation [7,8]. This family comprises three known members: PPAR-α, PPAR-γ, and PPAR-β/δ, also known as NR1C1, NR1C3, and NR1C2 [53]. Oxidized lipids are natural endogenous PPARs ligands [54], here we found PPARs expression were all activated under oxidized fish oil stimulation, while their expression was reverted to fish oil level when administrated with emodin. Moreover, PPARs are ligand-activated transcription factors that control lipid and glucose metabolism, as well as the inflammatory response [53]. In this study, emodin modulated the expression of downstream targets related to glucose and lipid metabolism of PPAR-α (CPT1), PPAR-β (PI3K and Akt), and PPAR-γ (PTEN and β-catenin) in response to oxidized fish oil stress. This may suggest that PPARs are implicated in emodin regulated glucose intake [55] and lipid accumulation [56]. Nonetheless, the experimental evidence also indicated PPARs modulate the expression of several antioxidant and prooxidant genes (TNF-α, TGF-β, NF-κB, CPT2, Bcl-2, UCP2, iNOS, and Cox-2) in response to oxidized fish oil stress, suggest that PPARs are implicated in oxidative stress response. This hypothesis is also corroborated by the sheer number of scientific papers that reveal the fact of PPARs regulates oxidative stress [10,13–16,57].

In addition to PPARs signaling, Nrf2-Keap1 signaling was also involved in emodin regulated redox homeostasis under oxidized fish oil stress. Nrf2-Keap1 signaling is one of the most well studied signals in cellular defense against oxidative or electrophilic stress. Nrf2 is a transcriptional factor that controls both the basal expression of genes under unstressed homeostatic conditions and the inducible expression of genes upon redox perturbation [58–60]. It directly regulates a battery of genes that involved in either cytoprotection against endogenous and environmental stressors or lipid and carbohydrate metabolism [61]. In this study, oxidized fish oil activated the expression of Nrf2-Keap1 signaling and its downstream targets, indicate oxidized fish oil induced oxidative stress in *M. amblycephala*, which was also demonstrated in our previous study [28]. Meanwhile, emodin ameliorated the expression of Nrf2-Keap1 signaling under oxidized fish oil stimulation, revealing emodin enhanced antioxidant capacity in *M. amblycephala*, which was in accordance with our previous experiment [26,62] and other scientific papers [63,64]. Interestingly, the expression levels of Nrf2-Keap1 signaling between oxidized fish oil and fish oil under emodin administration exhibited no statistical difference, revealing emodin exerts strong potential to repair the redox homeostasis impaired by dietary oxidized lipid.

5. Conclusion

Taken together, our data solidly reveled that dietary emodin supplementation rescued the growth inhibition and intestine histological impairment induced by oxidized fish oil, enhanced serum immune and antioxidant enzyme activity, increased metabolic activity through PPARs signaling, increased antioxidant capacity through PPARs and Nrf2-Keap1 signaling. These results indicate emodin is an effective immunostimulant that could protect organism form oxidative stress induced by dietary oxidized lipid, which may provide insights for oxidized lipid prevention in aquaculture production.

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Declaration of competing interest

The authors declare there have no competing interests.

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