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Effect of Quercetin on the Activity and mRNA Expression of Antioxidant Enzymes and Physiological Responses in Olive Flounder (*Paralichthys olivaceus*) Exposed to Cadmium*

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ABSTRACT : We investigated the antioxidant efficacy of quercetin (0% Diet 1, 0.25% Diet 2, and 0.5% Diet 3) pretreatment for 30 and 60 days in response to cadmium (Cd) toxicity in the olive flounder, and measured the plasma lysozyme activity to understand the immune effects of quercetin. The lysozyme activity with Diets 2 and 3 was higher than with Diet 1. Based on this result, to examine the immune ability and antioxidant role of quercetin, we exposed olive flounder fed quercetin to Cd and then measured the expression and activity of antioxidant enzymes (superoxide dismutase (SOD) and catalase (CAT)) and lipid peroxidation (LPO). With Diets 2 and 3, the expression and activity of antioxidant enzymes and the H_2O_2 concentration were lower than with Diet 1. In addition, the LPO levels were lower than with Diet 1, which protected the cell membrane. Therefore, quercetin removed the reactive oxygen species (ROS) produced by Cd, indicating that quercetin has antioxidant ability. In addition to its antioxidant ability, quercetin has immune effects.
(**Key Words :** Antioxidant, LPO, Lysozyme, Quercetin, ROS, Cadmium, Olive Flounder)

INTRODUCTION

Many environmental stress such as cadmium (Cd) increase reactive oxygen species (ROS) and provoke oxidative stress in organisms (Storey, 1996). ROS include superoxide (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical (HO^-), and singlet oxygen ($^1O_2$) (Kinnula et al., 1995). Overproduction of ROS by Cd can increase lipid peroxidation (LPO), the oxidation of nucleic acid and proteins, and DNA damage.

To protect themselves against ROS generating oxidative stress, aerobic organisms have evolved complex antioxidant defense systems. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione S-transferase (GST), and low-molecular-weight antioxidant materials such as vitamin C and vitamin E are found in the livers and kidneys of marine organisms (Basha and Rani, 2003; Hansen et al., 2006).

Previous studies (Pinho et al., 2005; Weng et al., 2007; Prieto et al., 2008) have examined systems involving natural antioxidants. These studies investigated the antioxidant effects of vitamin E supplements in the crab (*Chasmagnathus granulatus*) (Pinho et al., 2005), mouse (*Mus musculus*) (Weng et al., 2007), and tilapia (*Oreochromis niloticus*) (Prieto et al., 2008) against microcystin toxicity by analyzing CAT and GST activity. In isolated carp (*Cyprinus carpio* var. *Jian*) enterocytes, Chen et al. (2009) showed that the antioxidant effect of glutamine scavenging of ROS induced by H_2O_2 treatment occurred via the activity of the antioxidant enzymes SOD and CAT.

In this study investigated the effect of the natural antioxidant quercetin in olive flounder exposed to Cd. Quercetin is a polyphenolic flavonoid compound that is a strong antioxidant and is almost ubiquitous in plants. Broccoli and apples contain 7-110 mg/kg, while onions contain 284-486 mg/kg (Scalbert and Williamson, 2000). Quercetin chelates metal ions, has free radical scavenging activity, and reduces the concentration of metal to protect

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cell membranes (Bors and Saran, 1987). It can stop redox reactions by chelating ROS generated by toxic materials like Cd, and it inhibits LPO on the cell membrane. The phenoxy radical generated combines with another ROS and then inhibits the production of ROS (Frankle et al., 1993).

Many recent studies have examined the effects of antioxidants against oxidative stress induced by toxic materials. For example, Jayaraj et al. (2007) investigated the effects of pretreatment with three flavonoids (silybin, quercetin, and morin) on the effects of microcystins. Many studies of a variety antioxidants have been reported, including vitamin C (Qinghui et al., 2004; Weng et al., 2007), lycopene (Al-Jassabi, 2005), and tea polyphenols (Xu et al., 2007). In fish, although many studies have examined how supplementation with vitamin C (Trenzado et al., 2009), vitamin E (Prieto et al., 2008), selenium (Atencio et al., 2009), and glutamine (Chen et al., 2009) affect the antioxidant system, little is known about the antioxidant effects of supplemental quercetin.

Antioxidant improved immune effect as well as antioxidant ability, Qinghui et al. (2004) reported beneficial effects of vitamin C on immunological parameters, such as lysozyme, in the Japanese seabass (*Lateolabrax japonicus*). Increased immune ability was demonstrated by the increased lysozyme (Ortuno et al., 1999; Ai et al., 2004). Many antioxidants are reported to enhance immune capacity as well as antioxidant capacity.

Therefore, this study determined the effects of quercetin on enhancing growth and immune ability by measuring the plasma lysozyme activity. Then, we investigated the effect of quercetin pretreatment on the toxicity induced by Cd in the olive flounder by measuring the expression and activity of antioxidant enzymes (SOD and CAT), the plasma H_2O_2 concentration, and LPO as an oxidative stress parameter.

MATERIALS AND METHODS

Experimental fish and conditions

Olive flounders (n = 800, length 10±0.5 cm, weight 19.9±1.3 g) were obtained from a commercial fish farm and acclimated to the experimental conditions for 2 weeks in nine 300 L circulation filter tanks in the laboratory. During the experiments, the water temperature and photoperiod were maintained at 20±1°C and a 12L:12D cycle, respectively. The fish were fed a commercial and experimental diets feed twice daily (09:00 and 17:00). In all, 800 flounders were chosen randomly and distributed in the nine 300 L flow-through tanks.

Extraction of quercetin

The extraction of quercetin was carried out according to the methods of Velioglue and Mazza (1991) and Kang et al. (1998), with modification. The dried onion peels were blended with methanol in a Waring blender for 5 min and filtered through Whatman No. 1 filter paper, and then the filtrate was concentrated using a rotary evaporator at 40°C. The residue was washed with ether in a separatory funnel to remove lipids and other fat soluble materials. The onion extract was fractionated in the order of ethylacetate to purified glucosides type of quercetin, and then we examined quercetin content level by HPLC.

Experimental diets

Fish meal, dehulled soybean, and corn gluten meal were used as protein sources, and wheat flour and squid liver oil were used as carbohydrate and lipid sources, respectively. The ingredients of the experimental diets were mixed well with water in a ratio of 3:1 and then pelletized. The experiment diets were dried at room temperature and stored at -20°C until required. In the experimental diets, the crude protein content ranged from 55.1-56.0%, and the crude lipid content from 9.1-9.7%. The estimated energy content was 4.1 kcal/g.

Experimental diets using purified quercetin were made by jeilfeed company (Kyoungnam, Korea), and were coated for protecting soluble nutritions include quercetin in the water. Experimental diets were in the water for a while, quercetin content level was not significantly different from the content of original experimental diets. The experimental diets contained 0% (Diet 1), 0.25% (Diet 2), or 0.5% (Diet 3) quercetin, at the expense of 0, 0.25, or 0.5% wheat flour, respectively (Table 1).

Plasma lysozyme activity

To determine the lysozyme activity of olive flounder, 50 μl of plasma were added to 950 μl of a suspension of *Micrococcus lysodeikticus* (0.2 mg/ml) in 0.05 M sodium phosphate buffer (pH 6.2). The reactions were carried out at 25°C, and the absorbance at 530 nm was measured between 0.5 and 4.5 min. One lysozyme activity unit was defined as the amount of enzyme producing a 0.001/min decrease in absorbance.

Cd exposure

The experimental fish were exposed to $CdCl_2 \cdot 2.5H_2O$ (Kanto Chemical, Tokyo, Japan) dissolved in water to a Cd^{2+} concentration of 10 ppb in 50 L tanks for 0, 6, 12, 24, and 48 h. Four fish from each group (Diets 1, 2, and 3) were selected randomly for blood and tissue sampling after fed Diet 1, 2 and 3 for 30 and 60 days respectively. The fish were anesthetized with 200 mg/L tricaine methanesulfonate (MS-222, Sigma, USA) before blood collection. Blood was collected from the caudal vein using a 3-ml syringe coated with heparin. Plasma samples were separated by

Table 1. Ingredients and nutrient composition of the experimental diets

Ingredient (%)	Diet		
	Diet 1 (0%)	Diet 2 (0.25%)	Diet 3 (0.5%)
Fish meal[1] (ML)	45.00	45.00	45.00
Corn gluten ML	15.00	15.00	15.00
Wheat flour	19.90	19.65	19.40
Soybean ML	10.00	10.00	10.00
Fish oil - salmon	2.50	2.50	2.50
Squid ML	2.00	2.00	2.00
Krill ML	2.00	2.00	2.00
Lecithin	1.00	1.00	1.00
Mono-calcium	1.00	1.00	1.00
Choline	1.00	1.00	1.00
VITAMIX[2]	0.20	0.20	0.20
MINEMIX[3]	0.20	0.20	0.20
Vitamin C	0.20	0.20	0.20
QUERCETIN	0.00	0.25	0.50
Total	100	100	100

[1] Imported from Chile.

[2] Vitamin premix contained the following ingredients (g/kg mix): L-ascorbic acid, 121.2; DL-α-tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-D-pantothenate, 12.7; myo-inositol, 181.8; D-biotin, 0.27; folic acid, 0.68; p-aminobenzoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalciferol, 0.003; cyanocobalamin, 0.003.

[3] Mineral premix contained the following ingredients (g/kg mix): $MgSO_4 7H_2O$, 80.0; $NaH_2PO_4 2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4 7H_2O$, 20.0; Ca-lactate, 356.5; CuCl, 0.2; $AlCl_3 6H_2O$, 0.15; KI, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4 H_2O$, 2.0; $CoCl_2 6H_2O$, 1.0.

centrifugation (4°C, 10,000 g, 5 min), and stored at -80°C until analysis. To collect liver tissue samples, fish were euthanized by spinal transection. Immediately after collection, the samples were frozen in liquid nitrogen and stored at -80°C until total RNA was extracted for analysis.

Quantitative PCR (QPCR)

QPCR was conducted to determine the relative expression of SOD and CAT mRNA in total RNA extracted from the liver. Primers for QPCR were designed with reference to the known sequences of olive flounder as follows: SOD forward primer (5’-CGT TGG AGA CCT GGG GAA TGT G-3’), SOD reverse primer (5’-ATC GTC AGC CTT CTC GTG GAT C-3’), CAT forward primer (5’-GGC TGA GAA GTT CCA GTT CAA TCC-3’), CAT reverse primer (5’-CTC CAC CTC TGC AAA GTA GTT GAC-3’), β-actin forward primer (5’-GCA AGA GAG GTA TCC TGA CC-3’), and β-actin reverse primer (5’-CTC AGC TCG TTG TAG AAG G-3’). PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, USA) and iQ™ SYBR Green Supermix (Bio-Rad, USA) according to the manufacturer’s instructions. QPCR was performed as follows: denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 20 s. As an internal control, experiments were duplicated with β-actin, and all data were expressed as change with respect to corresponding β-actin calculated threshold cycle (CT) levels.

SOD and CAT activity analysis

Tissues were homogenized in ice-cold 0.1 M phosphate-buffered saline (PBS, pH 7.4) containing 1 mM EDTA. The homogenates were centrifuged at 10,000 g for 15 min at 4°C, the supernatant was removed, and the remaining sample was analyzed. SOD and CAT activities were determined using commercial kits supplied by Cayman Chemical (USA).

The SOD activity was assessed using a tetrazolium salt to detect superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The absorbance is read at 450 nm. Each assay was performed in duplicate, and enzyme units were recorded as U/ml.

The method for CAT activity is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced is measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen (Wheeler et al., 1990). Purpald specifically forms a bicyclic heterocycle with aldehydes and changes from colorless to purple on oxidation. The absorbance is read at 540 nm. Each assay was performed in duplicate, and the CAT activity was expressed as nmol/min/ml.

H_2O_2 assay

H_2O_2 concentrations were measured using the modified methods of Nouroozzadeh et al. (1994) and a Peroxidetect kit (Sigma, USA). 20 μl of olive flounder serum was added per well to flat bottom 96-well microtitre plates. Plates were left at room temperature for 20 min to allow serum to settle and adhere. A working color reagent was prepared by mixing 100 ml distilled water containing 100 mM sorbitol and 125 μM xylenol orange (Sigma, USA) with 1 ml of 25 mM ferrous ammonium sulphate prepared in 2.5 M sulphuric acid (Sigma-Aldrich). 200 μl of this reagent was then added to each well and allowed to incubate at room temperature for 1 h. Absorbance was read at 560 nm and concentration of H_2O_2 were interpolated from a standard curve. Concentrations are expressed as nmole peroxide/ml.

LPO assay

LPO is quantified by measuring malondialdehyde

(MDA) and 4-hydroxynonenal (4-HNE), the degradation products of polyunsaturated fatty acids (PUFAs) hydroperoxides (Esterbauer et al., 1991). The Cayman Chemical (Ann Arbor, MI, USA) lipid hydroperoxide assay kit was used to measure hydroperoxides directly, utilizing the redox reaction with ferrous ion. Hydroperoxides were extracted into chloroform and reacted with ferrous ions to produce ferric ions. The resulting ferric ions were detected using thiocyanate ion as the chromogen. The hydroperoxide concentration was determined based on the absorption at 500 nm.

Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). One way ANOVA followed by *post hoc* Duncan's multiple range test was used to compare the differences in the data ($p<0.05$).

RESULTS

Quercetin contents

The amount of quercetin (glucosides type) from onion by HPLC was about 1 g/kg onion (0.1%), and then formulated to contain three concentrations (0, 0.25, and 0.5%) of extracted quercetin by replacing wheat flour in experimental diets.

Growth performance

The weight gain of flounder fed Diet 3, which contained quercetin, for 30 and 60 days were significantly higher than those in flounder fed Diet 1, which did not contain quercetin. This result indicated that high concentration (Diet 3) of quercetin is very effective than low concentration (Diet 2) in growth (Figure 1).

Plasma lysozyme activity

In Diets 2 and 3, the lysozyme activity was significantly higher than in Diet 1 after feeding flounder quercetin for 30 and 60 days. In addition, the lysozyme activities in Diets 1, 2, and 3 fed for 60 days were significantly higher than were those fed for 30 days (Figure 2).

QPCR for SOD and CAT mRNA expression

Using a quantitative polymerase chain reaction (QPCR), we examined the changes in SOD and CAT mRNA expression when olive flounder fed a diet containing quercetin were exposed to Cd. After feeding for 30 days, for Diet 1, the SOD mRNA expression was increased significantly at 24 h and then decreased, whereas the expression levels with Diets 2 and 3 were lower than with Diet 1 (Figure 3A); the CAT mRNA was increased at 6 h and then decreased, whereas the expression levels with Diets 2 and 3 were lower than with Diet 1 (Figure 3C). After feeding for 60 days, with Diet 1, the SOD mRNA expression was increased significantly at 12 h and then decreased, whereas the expression levels with Diets 2 and 3 were lower than with Diet 1 (Figure 3B); the CAT mRNA was increased at 12 h and then decreased, whereas the expression levels with Diets 2 and 3 were lower than with Diet 1 (Figure 3D).

SOD and CAT activity

The effects of quercetin on Cd-induced antioxidant enzyme (SOD and CAT) activities are shown in Figure 3. After feeding for 30 days, with Diet 1, the SOD activity was increased significantly at 12 h and then decreased, whereas the activity levels with Diets 2 and 3 were increased significantly at 6 and 12 h and then decreased, but they were lower than with Diet 1 (Figure 4A); the CAT activity

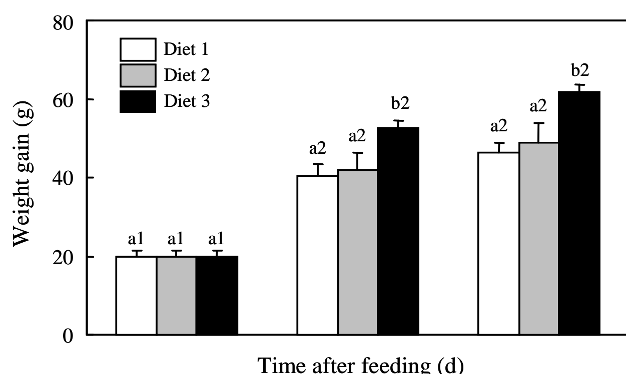

Figure 1. Weight gain of olive flounder fed experimental diets containing quercetin. Values with alphabets indicate significant differences between Diet 1, 2 and 3 within the same time after feeding. The numbers indicate significant differences from the initial weight (within the same diet) ($p<0.05$). All values are means±SD (n = 5).

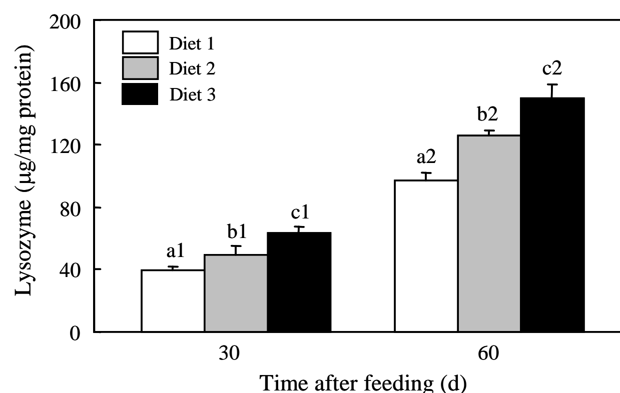

Figure 2. Lysozyme activity in plasma of olive flounder fed diets containing 0, 0.25, and 0.5% quercetin for 30 and 60 days. Values with alphabets indicate significant differences between Diet 1, 2 and 3 within the same time after feeding. The numbers indicate significant differences from the 30 days (within the same diet) ($p<0.05$). All values are means±SD (n = 5).

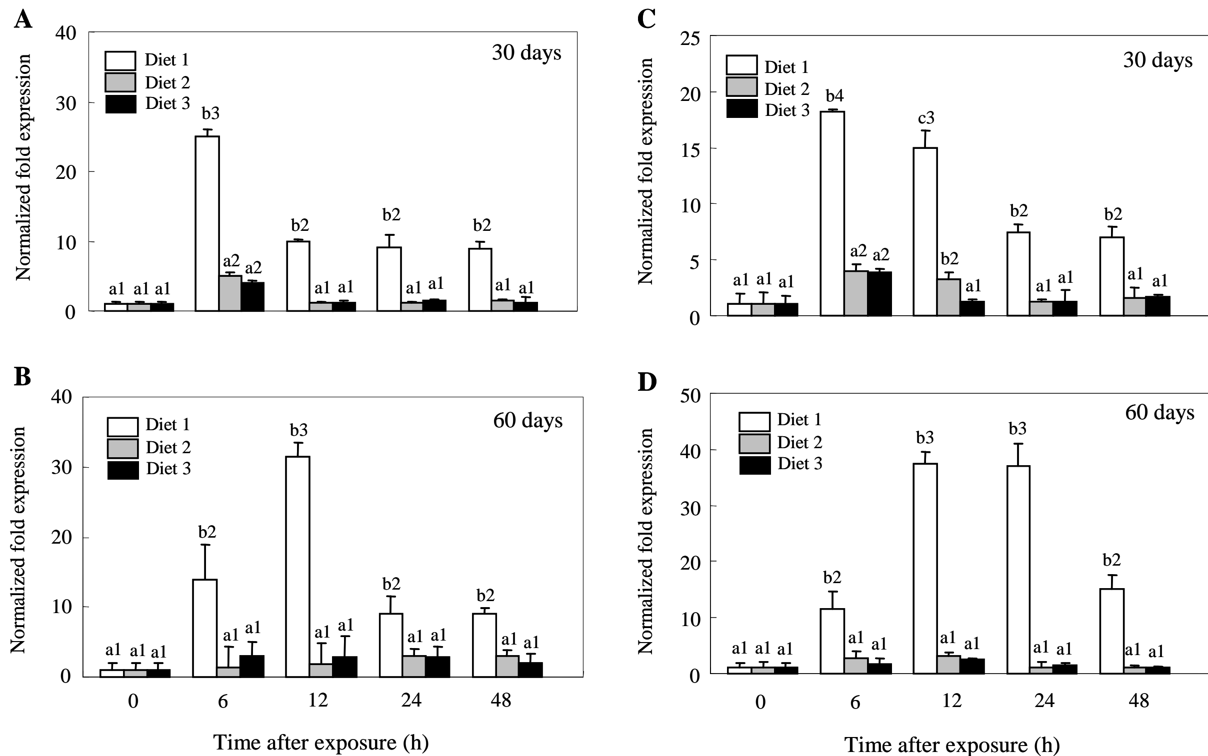

Figure 3. Expression of SOD (A) and CAT (C) in olive flounder fed a diet containing quercetin for 30 days and SOD (B) and CAT (D) in olive flounder fed a diet containing quercetin for 60 days. The mRNA levels in the livers of olive flounder exposed to Cd (0, 6, 12, 24, and 48 h) determined using quantitative real-time PCR. First, 2.5 μg of total RNA prepared from the liver was reverse-transcribed and amplified using gene-specific primers. The results are expressed as normalized fold expressions with respect to β-actin levels for the same sample. Values with alphabets indicate significant differences between Diet 1, 2 and 3 within the same time after exposure. The numbers indicate a significant difference from the control (within the same diet) ($p<0.05$). All values are means±SD (*n* = 5).

was increased at 12 h and maintained until 48 h, and the activity level with Diets 2 and 3 was increased significantly at 12 h and then decreased, but they were lower than with Diet 1 (Figure 4C). After feeding for 60 days, with Diet 1, the SOD activity was increased significantly at 6 h and maintained until 48 h, and the activity levels with Diets 2 and 3 were increased significantly at 12 h and then decreased, but they were lower than with Diet 1 (Figure 4B); the CAT activity increased gradually until 48 h, and the activity levels with Diet 2 and 3 were increased significantly at 12 h and then decreased, but they were lower than with Diet 1 (Figure 4D).

H_2O_2 assay

After feeding for 30 days, with Diet 1, the plasma H_2O_2 concentration increased significantly at 6 h and then decreased, and H_2O_2 concentrations with Diet 2 and 3 were increased significantly at 12 h and then decreased to initial levels, but they were lower than with Diet 1. After feeding for 60 days, with Diet 1, the plasma H_2O_2 concentration increased significantly until 24 h and then decreased, and the H_2O_2 concentrations with Diet 2 and 3 were lower than with Diet 1 and not significantly different from the initial levels (Figure 5).

LPO assay

After feeding for 30 days, with Diet 1, the LPO level (expressed as MDA and 4-HNE compounds) increased significantly until 48 h, and the LPO levels with Diets 2 and 3 were not significantly different from the initial levels, but they were lower than with Diet 1. After feeding for 60 days, with Diet 1, the LPO level increased significantly at 24 h; with Diet 2, the LPO level increased significantly at 6 h and was maintained until 48 h; and with Diet 3, no significant difference was observed compared with the initial levels. The levels with Diets 2 and 3 were lower than with Diet 1 (Figure 6).

DISCUSSION

To understand the antioxidant effect of quercetin on oxidative stress induced by Cd in olive flounder, we investigated the effects on lysozyme, and the expression and activity of SOD and CAT.

The weight gain of flounder fed Diet 3 for 30 and 60 days were significantly higher than those in flounder fed Diet 1 and 2 (Figure 1). This result indicated that high concentration (Diet 3) of quercetin is very effective than

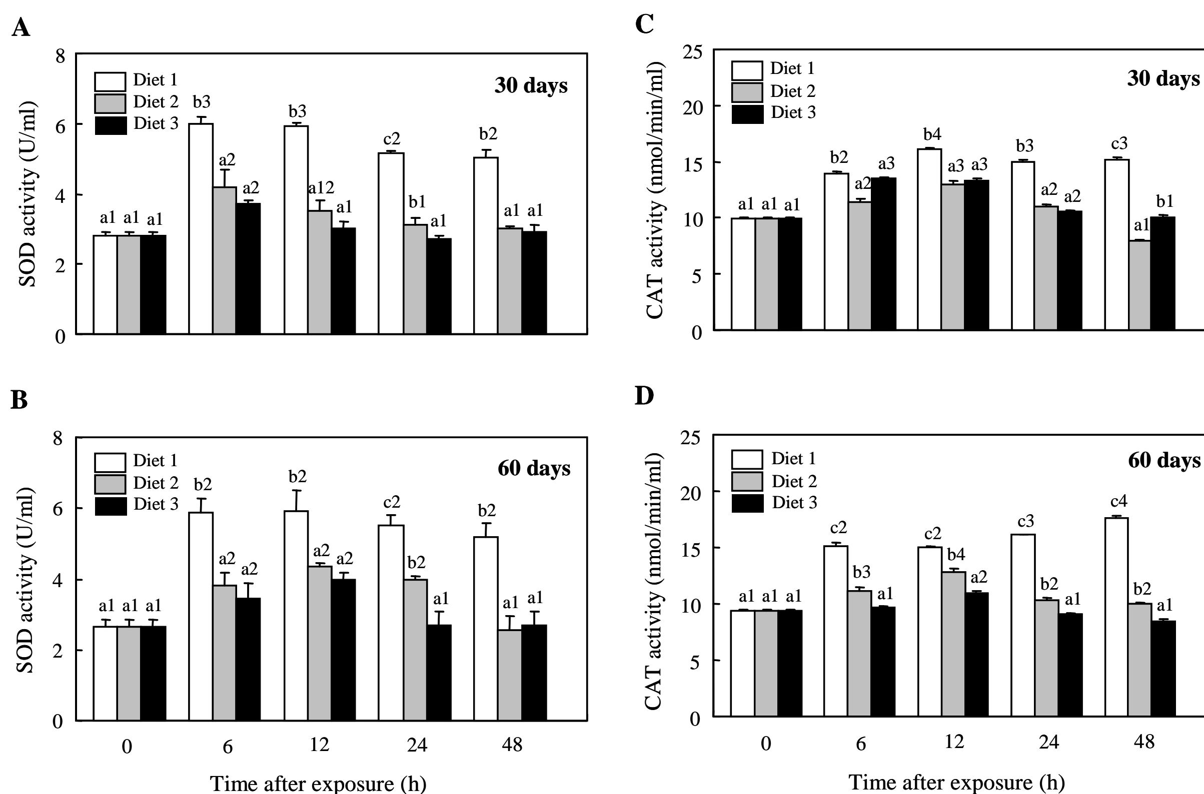

Figure 4. Activity of SOD (A) and CAT (C) in olive flounder fed a diet containing quercetin for 30 days and SOD (B) and CAT (D) in olive flounder fed a diet containing quercetin for 60 days. These activities in livers of olive flounder exposed to Cd (0, 6, 12, 24, and 48 h) determined using a microplate reader. Values with alphabets indicate significant differences between Diet 1, 2 and 3 within the same time after exposure. The numbers indicate a significant difference from the control (within the same diet) ($p<0.05$). All values are means ±SD (n = 5).

low concentration (Diet 2) in growth. Antioxidant, vitamin C could enhance the growth in fresh shrimp (*Penaeus chinensis*) (Wang and Li, 1996), African catfish (*Clarias gariepinus*) (Merchie et al., 1997a) and carp (*Cyprinus carpio*) (Gouillou-Coustans et al., 1998).

The plasma lysozyme activity with Diets 2 and 3 were significantly higher than with Diet 1 after feeding for 30 or 60 days (Figure 2). These results concur with those of Eo and Lee (2008), who reported that vitamin C improved the immune responses and disease resistance by increasing plasma lysozyme activity in Tiger puffer (*Takifugu rubripes*) fed vitamin C for 8 weeks, and with Zheng et al. (2009), who observed higher lysozyme activity in channel catfish (*Ictalurus punctatus*) fed oregano as an antioxidant.

The expression of SOD and CAT mRNA with Diets 2 and 3 was significantly lower than with Diet 1 (Figure 3). Similarly, the SOD and CAT activities were increased significantly in all groups exposed to Cd, but the activities with Diets 2 and 3 were significantly lower than with Diet 1 (Figure 4). These results are similar to those of Jayaraj et al. (2007), who reported that the CAT activity in mice exposed to microcystin after feeding with quercetin was significantly lower than in controls, suggesting that quercetin, with its strong antioxidant activity, protects hepatic cells from toxic materials that induce oxidative stress. In addition, the activities of SOD and CAT in a freshwater fish, the matrinxã (*Brycon cephalus*), exposed to methyl parathion after feeding selenium were significantly lower than in controls, indicating that selenium is an antioxidant that scavenges the ROS induced by methyl parathion (Monteiro et al., 2009). Combined with the results of previous studies, the fact that the expression and activity of antioxidant enzymes in the fish fed Diets 2 and 3 were lower than in those fed Diet 1 indicates that quercetin has antioxidant activity by scavenging the ROS induced by Cd directly. In addition, the H_2O_2 concentrations with Diets 2 and 3 were significantly lower than with Diet 1 (Figure 5), also indicating that quercetin scavenges ROS overproduced in the olive flounder.

The oxidative stress caused by ROS generates LPO and damages cells (Valavanidis et al., 2006). In this study, the LPO levels with Diets 2 and 3 were significantly lower than with Diet 1 (Figure 6). These results agree with Hiratsuka et al. (2008), who reported that LPO levels were reduced in mice fed docosahexaenoic acid (DHA), indicating that antioxidant can protect biomembranes and lipid

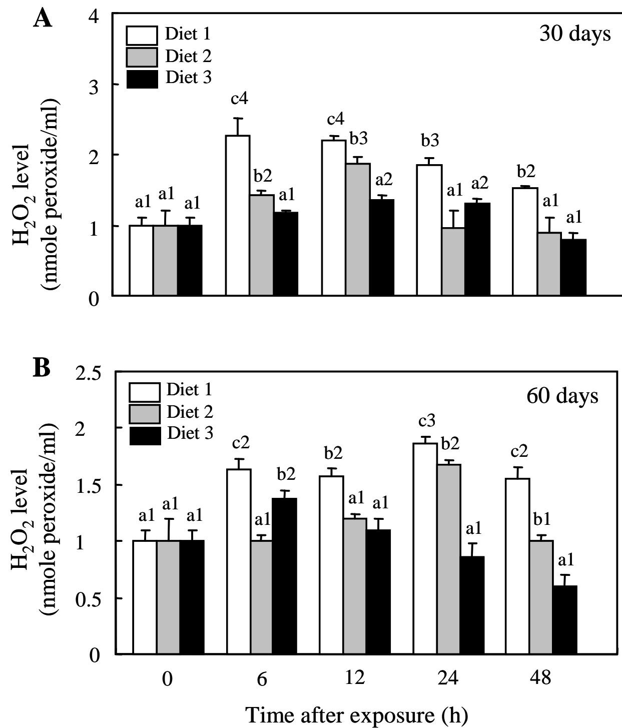

Figure 5. H_2O_2 concentrations in plasma of olive flounder fed for 30 or 60 days diets containing quercetin 0.25 or 0.5%, after 6, 12, 24, and 48 h exposure to Cd. Values with alphabets indicate significant differences between Diet 1, 2 and 3 within the same time after exposure. The numbers indicate a significant difference from the control (within the same diet) ($p<0.05$). All values are means±SD ($n = 5$).

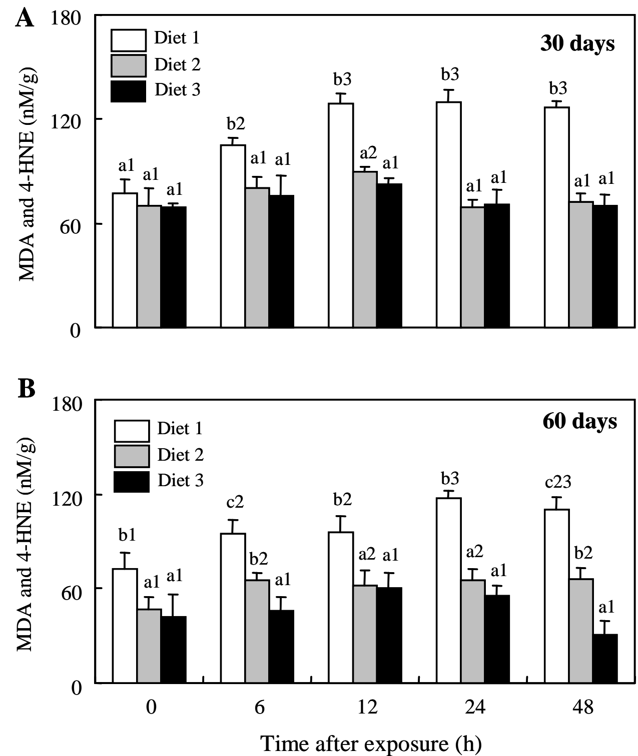

Figure 6. MDA and 4-HNE concentrations in the livers of olive flounder fed for 30 or 60 days diets containing quercetin 0.25 or 0.5%, after 6, 12, 24, and 48 h exposure to Cd. Values with alphabets indicate significant differences between Diet 1, 2 and 3 within the same time after exposure. The numbers indicate a significant difference from the control (within the same diet) ($p< 0.05$). All values are means±SD ($n = 5$).

components containing unsaturated fatty acids against the attack from ROS.

In conclusion, quercetin increases lysozyme activity enhancing immune ability in olive flounder. In addition, the mRNA expression and activity of the antioxidant enzymes SOD and CAT and the H_2O_2 concentrations in fish fed Diets 2 and 3 were significantly lower with Diet 1, indicating that quercetin scavenges the ROS induced by Cd to enhance antioxidant effects. Hence, we confirmed that quercetin was a strong antioxidant material in this study. Additional studies should examine the effects of various antioxidants on environmental stress factors and oxidative stress.

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