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The Effects of Quercetin on Physiological Characteristics and Oxidative Stress Resistance in Olive Flounder, *Paralichthys olivaceus*

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ABSTRACT : We investigated the effect of quercetin on growth and plasma cholesterol level and the effects of quercetin pretreatment (Diet 1, 0%; Diet 2, 0.25%; and Diet 3, 0.5% quercetin) for 30 and 60 days on oxidative stress induced by hypo-osmotic conditions (17.5, 8.75, and 4 psu) in olive flounder. The weights of flounder were higher with Diet 3 than with Diet 1 and 2, which indicated that a high concentration (Diet 3) of quercetin was very effective in growth. Total cholesterol levels were lower with Diets 2 and 3 than with Diet 1, leading us to hypothesize that quercetin removed low-density lipoproteins from circulation and thereby reduced total cholesterol. To understand the antioxidant role of quercetin, we measured the mRNA expression and activities of superoxide dismutase (SOD) and catalase (CAT) and the H_2O_2 concentration in quercetin-treated flounder exposed to osmotic stress. The H_2O_2 concentration and the SOD and CAT expression and activity levels were lower in flounder fed with Diets 2 and 3 than with Diet 1, suggesting that quercetin directly scavenges reactive oxygen species to reduce oxidative stress. Furthermore, the plasma lysozyme activity and osmolality were higher with Diets 2 and 3 than with Diet 1, indicating that quercetin increases immune function and helps to maintain physiological homeostasis. Plasma cortisol was lower with Diets 2 and 3 than with Diet 1, suggesting the quercetin protects against stress. These results indicate that quercetin has hypocholesterolemic and antioxidant effects, increases immune function, and acts to maintain physiological homeostasis. (**Key Words :** Quercetin, Antioxidant, ROS, Salinity, SOD, CAT)

INTRODUCTION

Environmental salinity is an important factor affecting metabolism, growth, survival, osmolality, and immune function in fish (Britoa et al., 2000). Specifically, changes in salinity can generate physiological stress via changes in hormones, energy metabolism, and ion status in marine organisms (Barton and Iwama, 1991). Furthermore, stress induced by salinity changes has been associated with enhanced reactive oxygen species (ROS) generation, which may seriously affect immune function and lead to oxidative stress (Fisher and Newell, 1986). Sea cucumbers (*Apostichopus japonicus*) exposed to hypoosmotic conditions showed decreased lysozyme activity, suggesting that stress induced by salinity changes can suppress immunity (Wang et al., 2008). ROS, including superoxide, hydrogen peroxide (H_2O_2), hydroxyl radicals, and singlet oxygen, are produced naturally during oxidative metabolism (Roch, 1999). Overproduction of ROS in response to environmental stress can lead to increased lipid peroxidation and may affect cell viability by causing membrane damage and enzyme inactivity. Subsequently, cell senescence and apoptosis, and the oxidation of nucleic acids and proteins may be accelerated. The resultant DNA damage may provoke a variety of physiological disorders such as accelerated aging and reduced disease resistance and reproductive ability (Kim and Phyllis, 1998; Pandey et al., 2003).

Complex antioxidant defense systems maintain homeostasis and protect aerobic organisms against ROS and the subsequent damage of oxidative stress. Antioxidants may be enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, and glutathione S-transferase, or compounds such as metallothionein, quercetin (Jayaraj et al., 2007), vitamin C, and vitamin E (α-tocopherol) (McFarland et al., 1999). Antioxidant defense systems are found in the livers and kidneys of

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marine organisms (Basha and Rani, 2003; Hansen et al., 2006).

This study investigated the effects of the natural antioxidant quercetin, which is almost ubiquitous in plants. Quercetin is a polyphenolic flavonoid compound, behaves as powerful antioxidant and free radical scavenger (Rice-Evans et al., 1996) and is able to interact with several key enzymes (Di carlo et al., 1999). Quercetin has shown to be an effective inhibitor of xanthine oxidase and lipoxygenase, enzymes involved in processes such as inflammation, atherosclerosis, cancer and ageing (Da silva et al., 1998). Broccoli and apples contain 7 to 110 mg/kg of quercetin, and onions contain 284 to 486 mg/kg (Scalbert and Williamson, 2000). Quercetin chelates ROS induced by lipid peroxidation and metal ions, provides H^+ ions to prevent lipid peroxidation in the cell membrane, and scavenges free radicals. Furthermore, quercetin converts ROS to energy and reduces metal concentrations to protect cell membranes (Bors and Saran, 1987). Previous studies have investigated antioxidant systems via xenobiotic exposure after natural antioxidant materials have been supplied (Pinho et al., 2005; Weng et al., 2007; Prieto et al., 2008; Chen et al., 2009). Few studies about quercetin in fish have been performed (Weber et al., 2002; Trischitta and Faggio, 2006; Park et al., 2008), but studies on antioxidant systems by environmental stress (e.g., changes in salinity) in fish have not been performed.

Here, we used olive flounder fed a diet containing quercetin for 30 or 60 days as an experimental model for studying the effects of quercetin on the growth, plasma cholesterol level and on oxidative stress induced by hypoosmotic conditions. We measured the plasma total cholesterol concentration to determine the ability of quercetin to reduce plasma cholesterol, and the mRNA expression and activities of the antioxidant enzymes SOD and CAT, the plasma H_2O_2 concentration, lysozyme activity, plasma cortisol concentration, and plasma osmolality to elucidate the effects of quercetin on the oxidative stress induced by changes in salinity.

MATERIALS AND METHODS

Experimental fish and conditions

Olive flounder (n = 800; length, 10±0.5 cm; weight, 19.9±1.3 g) were obtained from a commercial fish farm and were allowed to acclimate 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 12-h light: 12-h dark, respectively. The fish were fed a commercial and experimental diets feed twice daily (09:00 and 17:00). In all, 800 flounders were randomly chosen, distributed among the nine 300-L flow-through tanks, and divided into three experimental groups (Diets 1, 2, and 3).

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. The filtrate was concentrated with a rotary evaporator. The residue was washed with hexane in a separatory funnel to remove lipids and other fat soluble materials.

Experimental diets

In the three 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 diet ingredients (Table 1) were mixed well with water, at a 3:1 ratio of ingredients to water, and then pelletized, dried at room temperature, and stored at -20°C until needed. In the experimental diets, the crude protein content ranged from 55.1 to 56.0%, the crude lipid content was 9.1 to 9.7%, and the estimated energy content was 4.1 kcal/g. Experimental diets using purified quercetin were made by jeilfeed company (Kyoungnam, Korea). The experimental diets also 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 total cholesterol analysis

Cholesterol ester in plasma was hydrolysised to cholesterol and fatty acid by Cholesterol Esterase (CE), and Cholesterol was oxidized to generate hydrogen peroxide by Cholesterol Oxidase (CHO). Hydrogen peroxide provoked a condensation reaction by peroxidase (POD) under 4-AAP and phenol, and then we measured cholesterol amount through measuring absorbance of chelate dye which was formated by condensation reaction (HBi, Korea).

Osmotic stress produced by changes of salinity

The experimental fish were acclimated in seawater (35 psu) and then transferred sequentially to tanks with salinities of 17.5, 8.75, and 4 psu, which already made by diluting underground water, respectively. The fish were maintained at each salinity for 24 h. Four fish from each group (Diets 1, 2, and 3) were randomly selected 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, St. Louis, MO, USA) prior to blood collection. Blood was collected from the caudal vasculature using a 3-ml syringe coated with heparin. Plasma samples were separated by centrifugation (4°C; 10,000 rpm; 5 min) and stored at -80°C

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 phosphate	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): $MgSO_47H_2O$, 80.0; $NaH_2PO_42H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_47H_2O$, 20.0; Ca-lactate, 356.5; CuCl, 0.2; $AlCl_36H_2O$, 0.15; KI, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4H_2O$, 2.0; $CoCl_26H_2O$, 1.0.

until analysis. To collect liver tissue samples, the 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; 40 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

The 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 rpm for 15 min at 4°C, and removed supernants and then remaining sample for analyses. SOD and CAT activities were determined using commercial kits supplied by Cayman Chemical (USA).

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

For CAT activity, the method 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 hetero cycle with aldehydes, which upon oxidation changes from colorless to a purple color. Absorbance read at 540 nm. Each assay was performed in duplicate, and CAT activity was expressed as nmol/min/ml.

H_2O_2 assay

H_2O_2 concentrations measured using the modified methods of Nouroozzadeh et al. (1994) and a Peroxidetect

Table 2. Weight gain of olive flounder fed experimental diets containing quercetin

Quercetin concentrations (%)	Time after feeding		
	Initial (g)	30 days (g)	60 days (g)
0		40.20±3.40[b]	46.25±2.62[b]
0.25	19.96±1.38[a]	42.00±4.24[b]	48.75±5.12[b]
0.5		52.50±2.00[c]	61.75±2.00[c]

The different letters indicate a significant differences from the initial weight of fish ($p<0.05$). All values are means±SD ($n = 5$).

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.

Plasma lysozyme activity analysis

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

Plasma osmolality and cortisol analysis

Plasma osmolality was measured with a vapor pressure osmometer (Vapro 5520; Wescor Inc., Logan, UT, USA) and plasma cortisol was analyzed using a radioimmunoassay kit (Diagnostic Systems Laboratories, Atlanta, GA, USA).

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

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 (Table 2).

Feed efficiency ratio (FER)

The FER of flounder fed Diet 3, which contained quercetin 0.5%, for 30 and 60 days were significantly higher than those in flounder fed Diet 1, which did not contain quercetin. And FER of flounder fed Diet 2, which contained 0.25%, for 60 days was significantly higher than those in flounder fed Diet 1. This result indicated that Diet 2 and 3 are effective by side of FER than Diet 1 (Table 3).

Plasma total cholesterol

Total cholesterol levels in flounder fed Diets 2 and 3, which contained quercetin, for 30 and 60 days were significantly lower than those in flounder fed Diet 1, which did not contain quercetin. In addition, total cholesterol levels in flounder fed Diets 2 and 3 were significantly lower after 60 days compared with 30 days (Figure 1).

SOD and CAT mRNA expression

Using a quantitative polymerase chain reaction assay, we examined changes in SOD and CAT mRNA expression in flounder that were fed the experimental diets and then exposed to osmotic stress. After 30 days of feeding with Diet 1, SOD mRNA expression was significantly increased in the fish at 17.5 and 8.75 psu, followed by a decrease at 4 psu. The SOD mRNA level was lower with Diets 2 and 3 than with Diet 1 (Figure 2A). CAT mRNA expression increased gradually with changes in osmotic pressure, and similar to SOD mRNA expression, the CAT mRNA level was lower with Diets 2 and 3 than with Diet 1 (Figure 2C). After 60 days of feeding with Diet 1, the SOD mRNA expression level was significantly increased at 17.5 psu, followed by decreases at 8.75 and 4 psu, and the SOD mRNA level was lower with Diets 2 and 3 than with Diet 1

Table 3. Feed efficiency ratio (FER) of olive flounder fed experimental diets containing quercetin

Quercetin concentrations (%)	Time after feeding	
	30 days	60 days
0	1.10±0.021[a]	1.20±0.040[a]
0.25	1.12±0.032[a]	1.35±0.012[b]
0.5	1.40±0.024[b]	1.58±0.022[c]

The different letters indicate significant differences from the control (0%) FER of fish ($p<0.05$). All values are means±SD ($n = 5$).

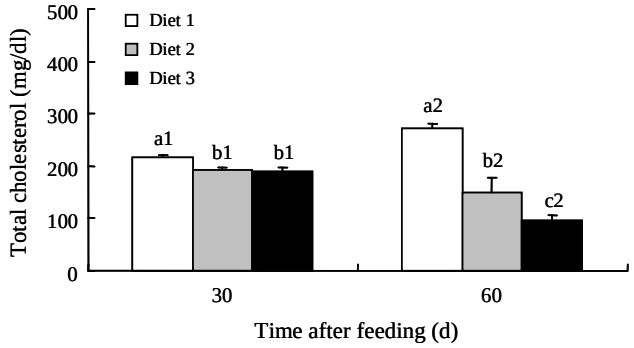


Figure 1. Total plasma cholesterol in olive flounder fed a diet containing quercetin (Diet 2, 0.25%; Diet 3, 0.5%) or no quercetin (Diet 1, 0%) for 30 or 60 days. Values with alphabets indicate significant differences between Diet 1, 2 and 3 within the same time after exposure. The numbers indicates significant differences from the control (within the same diet) ($p<0.05$). All values are means±SD ($n = 5$).

(Figure 2B). CAT mRNA expression was increased at 8.75 psu and then decreased at 4 psu with Diet 1. CAT mRNA levels were lower with Diets 2 and 3 than with Diet 1 (Figure 2D).

SOD and CAT activities

To further examine the effect of quercetin on antioxidant activity in flounder, the osmotic stress-induced activities of the antioxidant enzymes SOD and CAT were measured in flounder fed the three experimental diets (Figure 3). After 30 days of feeding with Diet 1, SOD activity was significantly increased with decreases in osmotic pressure until 4 psu. SOD activities with Diets 2 and 3 were significantly increased, but remained lower than those with Diet 1 (Figure 3A). With Diets 2 and 3, CAT activity levels were increased at 17.5 psu and maintained until 4 psu, but all levels were lower than those with Diet 1 (Figure 3C). After 60 days of feeding with Diet 1, SOD activity was significantly increased at 17.5 and 8.75 psu, followed by a decrease at 4 psu. SOD activities with Diets 2 and 3 were significantly increased, but remained lower than those with Diet 1 (Figure 3B). CAT activity was increased at 17.5 and 8.75 psu, after which it decreased at 4 psu with Diets 2 and 3, all levels were lower than those with Diet 1 (Figure 3D).

H_2O_2 concentration

To explore the ability of quercetin to scavenge ROS, the plasma H_2O_2 concentration was determined in osmotically stressed flounder. After both 30 and 60 days, the plasma H_2O_2 concentration in flounder was significantly increased at 17.5 and 8.75 psu with Diet 1, followed by a decrease at 4 psu. Although significantly increased at 17.5 and 8.75 psu,

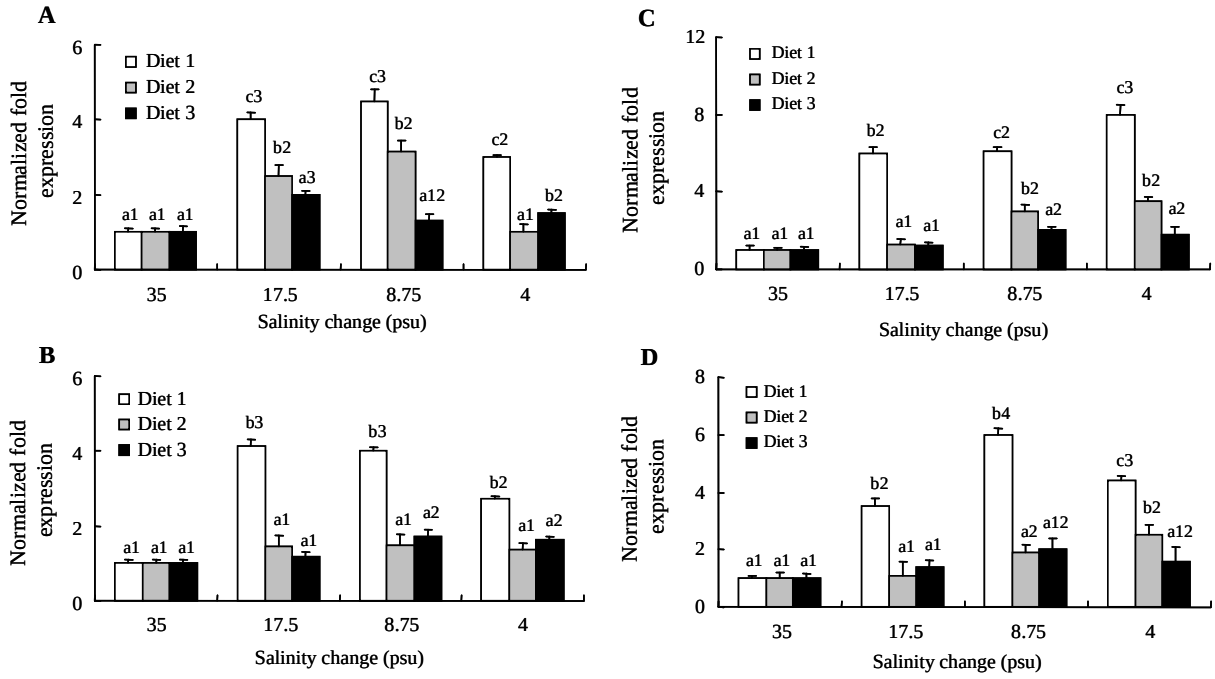


Figure 2. Expression of SOD (A and B) and CAT (C and D) mRNA, as measured by quantitative real-time PCR, in olive flounder fed a diet containing quercetin (Diet 2, 0.25%; Diet 3, 0.5%) or no quercetin (Diet 1, 0%) for 30 (A and C) or 60 days (B and D) and then exposed to seawater (35 psu) and osmotic stress (17.5, 8.75, and 4 psu). Total liver RNA (2.5 μg) was reverse-transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin in the same sample. Values with alphabets indicate significant differences between Diet 1, 2 and 3 within the same time after exposure. The numbers indicates significant differences from the control (within the same diet) ($p<0.05$). All values are means±SD ($n = 5$).

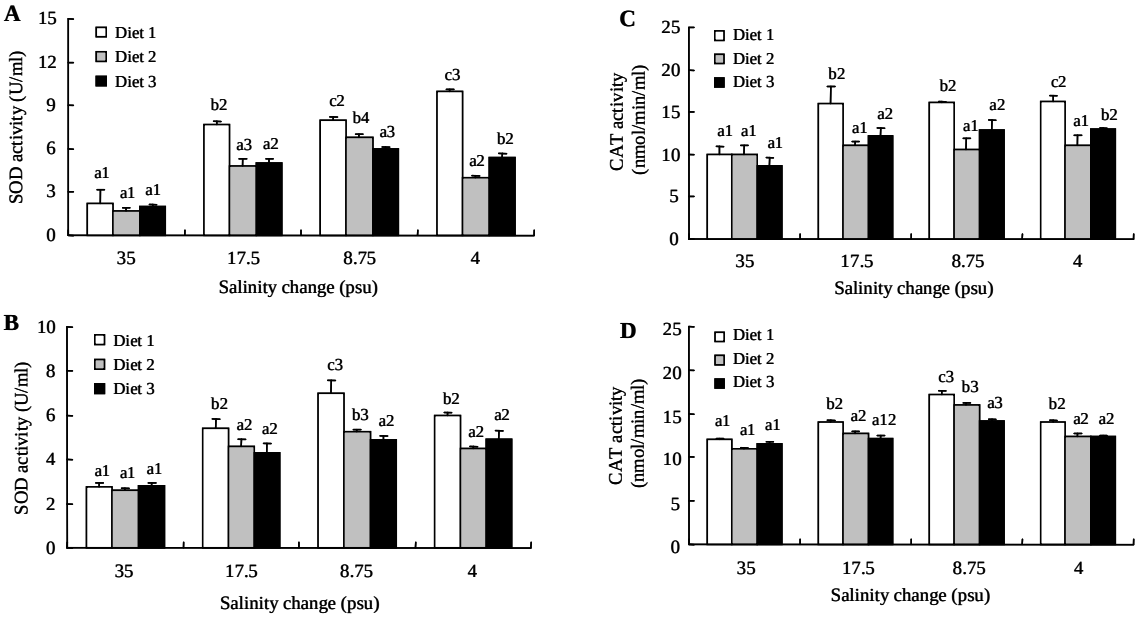


Figure 3. Activity levels of SOD (A and B) and CAT (C and D) in olive flounder fed diets containing quercetin (Diet 2, 0.25%; Diet 3, 0.5%) or no quercetin (Diet 1, 0%) for 30 (A and C) or 60 days (B and D) and then exposed to seawater (35 psu) and osmotic stress (17.5, 8.75, and 4 psu). Values with alphabets indicate significant differences between Diet 1, 2 and 3 within the same time after exposure. The numbers indicates significant differences from the control (within the same diet) ($p<0.05$). All values are means±SD ($n = 5$).

the H_2O_2 concentrations with Diets 2 and 3 were lower than those with Diet 1 after both 30 and 60 days (Figure 4).

Plasma lysozyme activity

As an indicator of immune function, the plasma lysozyme activity was measured. After both 30 and 60 days of feeding with Diet 1, the plasma lysozyme activity in flounder was significantly decreased with decreases in salinity. Plasma lysozyme activities were also significantly decreased with Diets 2 and 3 after both 30 and 60 days, but always remained higher than those with Diet 1 (Figure 5).

Plasma cortisol

As a measure of oxidative stress, the plasma cortisol levels were determined in the flounder. After both 30 and 60 days of feeding with Diet 1, the plasma cortisol concentration was increased significantly in the flounder at 17.5 psu and decreased with further decreases in salinity. Plasma cortisol concentrations with Diets 2 and 3, although significantly increased at 17.5 psu, were lower than those with Diet 1 after both 30 and 60 days (Figure 6).

Plasma osmolality

After both 30 and 60 days of feeding with Diet 1, the plasma osmolality level in flounder significantly decreased with decreases in environmental osmotic pressure. Plasma osmolality levels with Diets 2 and 3 were significantly decreased, but were higher than those with Diet 1 (Figure 7).

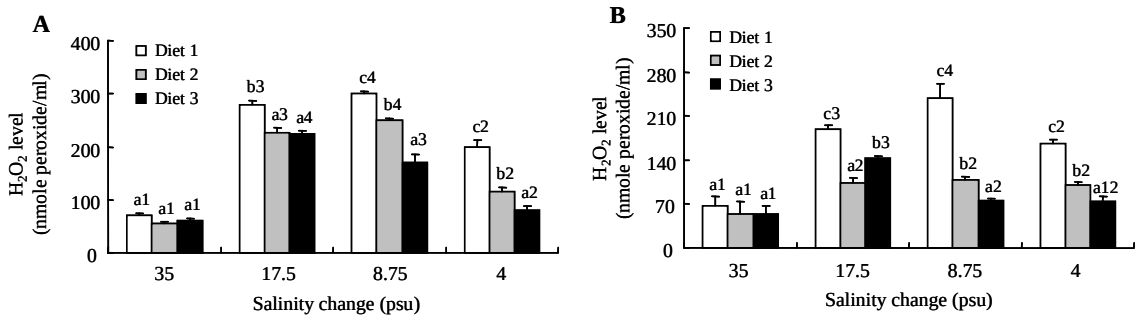


Figure 4. Plasma H_2O_2 concentrations in olive flounder fed a diet containing quercetin (Diet 2, 0.25%; Diet 3, 0.5%) or no quercetin (Diet 1, 0%) for 30 (A) or 60 days (B) and then exposed to seawater (35 psu) and osmotic stress (17.5, 8.75, and 4 psu). Values with alphabets indicate significant differences between Diet 1, 2 and 3 within the same time after exposure. The numbers indicates significant differences from the control (within the same diet) ($p<0.05$). All values are means±SD ($n = 5$).

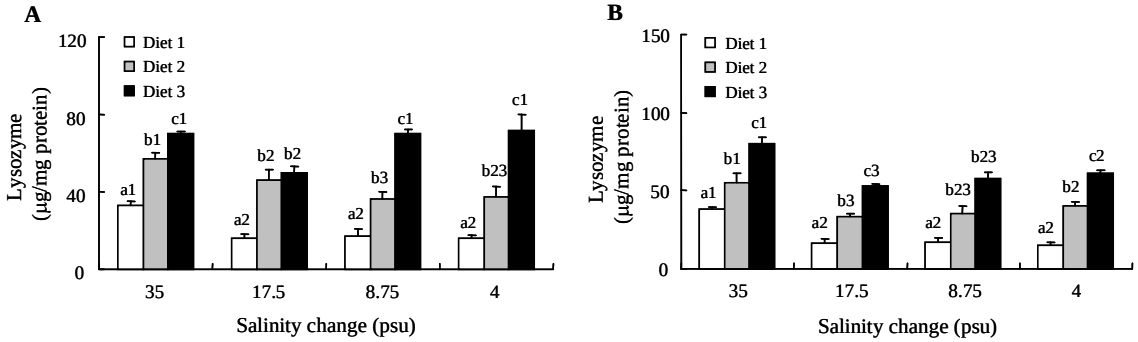
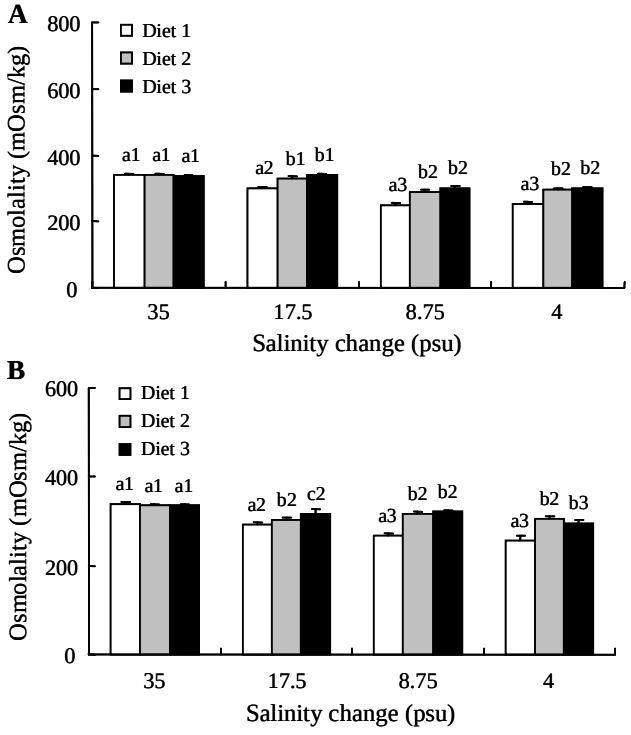


Figure 5. Plasma lysozyme activity in olive flounder fed a diet containing quercetin (Diet 2, 0.25%; Diet 3, 0.5%) or no quercetin (Diet 1, 0%) for 30 (A) or 60 days (B) and then exposed to seawater (35 psu) and osmotic stress (17.5, 8.75, and 4 psu). Values with alphabets indicate significant differences between Diet 1, 2 and 3 within the same time after exposure. The numbers indicates significant differences from the control (within the same diet) ($p<0.05$). All values are means±SD ($n = 5$).

DISCUSSION

The effects of quercetin on the plasma cholesterol level and on oxidative stress induced by hypoosmotic conditions were investigated in olive flounder. Our results demonstrated lower cholesterol levels, increased SOD and CAT mRNA levels and activities, lower H_2O_2 concentrations, higher lysozyme activity, lower cortisol concentrations, and decreased plasma osmolality in olive flounder fed diets containing quercetin compared with flounder fed a diet containing no quercetin.

In this study, plasma cholesterol concentrations in Diet 2 and 3 were significantly lower than those in flounder fed Diet 1 (Figure 1). This study about cholesterol analysis in



Figire 6. Plasma osmolality in olive flounder fed a diet containing quercetin (Diet 2, 0.25%; Diet 3, 0.5%) or no quercetin (Diet 1, 0%) for 30 (A) or 60 days (B) and then exposed to seawater (35 psu) and osmotic stress (17.5, 8.75, and 4 psu). Values with alphabets indicate significant differences between Diet 1, 2 and 3 within the same time after exposure. The numbers indicates significant differences from the control (within the same diet) ($p<0.05$). All values are means±SD ($n = 5$).

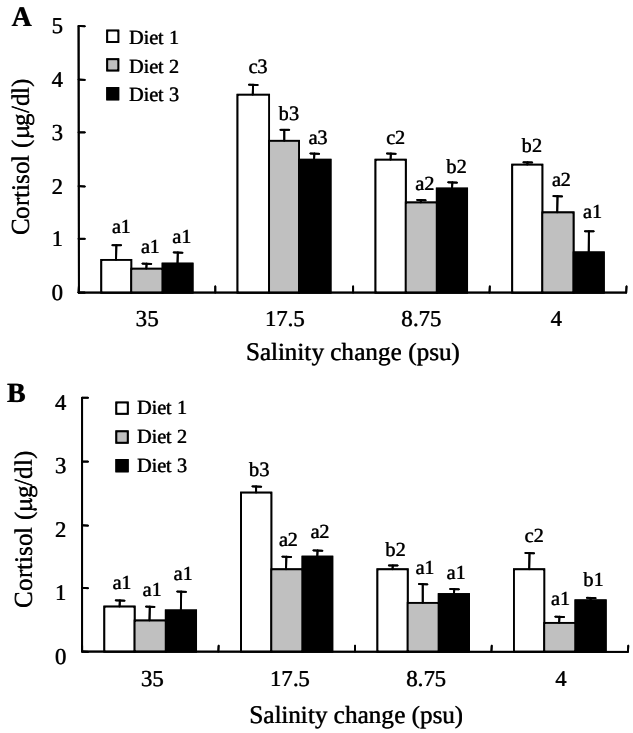


Figure 7. Plasma cortisol in olive flounder fed a diet containing quercetin (Diet 2, 0.25%; Diet 3, 0.5%) or no quercetin (Diet 1, 0%) for 30 (A) or 60 days (B) and then exposed to seawater (35 psu) and osmotic stress (17.5, 8.75, and 4 psu). Values with alphabets indicate significant differences between Diet 1, 2 and 3 within the same time after exposure. The numbers indicates significant differences from the control (within the same diet) ($p<0.05$). All values are means±SD ($n = 5$).

fish fed quercetin has not been performed, but this result is consistent with data from Hayek et al. (1997), who reported that quercetin bounds to low-density lipoprotein to suppress its oxidation and lowers plasma cholesterol concentrations in mice fed quercetin for 6 weeks. Fuhrman et al. (2000) observed lower plasma cholesterol concentrations in mice fed the antioxidant ginger for 10 weeks. Taken together, these results demonstrate a hypocholesterolemic effect of quercetin.

The SOD and CAT mRNA expression levels and activities in Diet 2 and 3 were lower than Diet 1 (Figures 2 and 3). These results indicate that quercetin possesses strong antioxidant activity that chelates ROS directly and then scavenges the ROS induced by changes in salinity. So quercetin plays antioxidant role instead of antioxidant enzymes (SOD and CAT). The H_2O_2 concentrations were also significantly lower with Diets 2 and 3 than with Diet 1 (Figure 4), further indicating that quercetin scavenges the ROS, H_2O_2 produced during osmotic stress in olive flounder. Chien et al. (2003) reported that astaxanthin suppresses singlet oxygen activity by lower SOD activity in tiger prawn (*Penaeus monodon*) after treatment (feeding) with astaxanthin. Additionally, CAT activity in mice exposed to microcystin after treatment with quercetin was significantly lower than control activity (Jayaraj et al., 2007). Collectively, these results show that strong antioxidants such as quercetin reduce oxidative stress induced by salinity changes and toxic materials.

Acute salinity changes are thought to suppress immunity (Britoa et al., 2000). Wang et al. (2008) reported that lysozyme activity was significantly decreased in sea cucumbers (*Apostichopus japonicus*) upon exposure to hypoosmotic conditions, indicating an osmotic stress-induced reduction of immune function. In the present study, the plasma lysozyme activity in olive flounder exposed to hypoosmotic conditions was significantly higher in the flounder fed Diets 2 and 3 compared with Diet 1 (Figure 5). Quercetin plays an inflammation role by increase lysozyme activity, has an excellent probability of inhibiting viruses and improving immune function (Brinkworth et al., 1992), as well as is a very strong antioxidant by remove free radical directly. In one study, the plasma lysozyme activity was increased significantly in tiger puffer (*Takifugu rubripes*) after vitamin C treatment for 8 weeks, suggesting that vitamin C enhanced the immune ability (Eo and Lee, 2008). Zheng et al. (2009) observed that treatment with the antioxidant oregano significantly increased the plasma lysozyme activity in channel catfish.

As acute salinity changes cause physiological stress in fish, we measured the plasma cortisol level as an indicator of stress due to salinity changes. Although the plasma cortisol concentrations were significantly increased in all of the diet groups, the flounder that were fed Diets 2 and 3 had significantly lower cortisol levels compared with the flounder that were fed Diet 1 (Figure 6). Kawabata et al. (2009) reported biological effects of quercetin on the hypothalamic-pituitary-adrenal axis (a major component of the stress response), leading to reduced stress to lower cortisol concentration in organisms. Belo et al. (2005) found that the plasma cortisol concentrations in Pacú (*Piaractus mesopotamicus*) maintained at high stocking density were significantly lower after treatment with vitamin E, compared with the controls. These results suggest that antioxidant preparations containing vitamin E can protect organisms from stress. Therefore, because these previous results are consistent with our results, we suggest that the antioxidant quercetin protects organisms against environmental stress.

The plasma osmolality in marine fish tends to decrease after exposure to hypoosmotic conditions (Sampaio and Bianchini, 2002). In the present study, the plasma osmolality in olive flounder exposed to hypoosmotic conditions was decreased significantly in all diet groups, but the osmolality was higher after treatment with quercetin (Diets 2 and 3) for 30 and 60 days, compared with Diet 1 (Figure 7). There have been few studies on plasma osmolality changes with salinity stress after treatment with antioxidants. We hypothesize that quercetin affects the plasma osmolality by reducing the stress induced by hypoosmotic conditions.

In conclusion, we examined the hypocholesterolemic and antioxidant effects of quercetin in olive flounder. The expression and activities of SOD and CAT and the plasma H_2O_2 concentration were significantly lower in flounder receiving quercetin (Diets 1 and 2) compared with those fed Diet 1, indicating that quercetin has antioxidant effects and scavenges ROS produced during the stress induced by acute changes in salinity. The antioxidant effects observed for Diets 2 and 3 were maintained for 30 and 60 days. Quercetin was also associated with increased lysozyme activity, which suggests that quercetin improves immune function against external stress. Additional studies should examine the effects of various antioxidants against environmental stress factors, from a molecular biological and physiological perspective.

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