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Original Article

Evaluation of oxidative stress induced by cadmium and comparative antioxidant effects of Shirazi thyme (*Zataria multiflora* Boiss) and vitamin E in common carp (*Cyprinus carpio*)

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Abstract: Shirazi thyme is an active phytobiotc contains phenolic compounds and flavonoids which have strong antioxidant properties. This study was conducted to investigate the potential protective effects of Shirazi thyme compared to that of vitamin E against cadmium toxicity. Common carp juveniles (34±3 g) were divided into four groups and fed by three different diets, including commercial diet without any additive (for control and metal only group) and supplemented with either 1% ground Shirazi thyme or 100 mg/kg vitamin E. All treatments except the control were exposed to sublethal concentration of waterborne cadmium (1.5 mg/L free ion) for 15 days and liver, kidney and gill were sampled 3, 7, 10 and 15 days after the exposure. The results showed that treatment of the fish with cadmium for 15 days resulted in a significant reduction in glutathione reductase (GR), glutathione-S-transferase (GST) and catalase (CAT) and led to liver, kidney and gills dysfunction. On the other hand, the level of malondialdehyde (MDA) significantly increased during metal exposure. Supplementation of diets with Shirazi thyme and vitamin E led to a significant protection against metal exposure in different tissues. Moreover, Shirazi thyme was found to be as effective as vitamin E. The current finding can provide a useful reference for stress protective effects of thyme and its beneficial role in aquaculture.

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Introduction

Due to industrialization, the application of heavy metals in a variety of industrial and agricultural fields has been expanded (Tan et al., 2010). Heavy metals cannot be eliminated through biological degradation, therefore, potentially can accumulate in the tissues of aquatic animals making this toxicant deleterious to the aquatic environment. Accumulation of heavy metals in the tissues may intensify the generating of reactive oxygen species (ROS) which may lead to oxidative stress resulting in lipid peroxidation, DNA damage and alteration in ion homeostasis (Kalay et al., 1999; Farombi et al., 2007). The toxicity generated by this contaminant generally involves neurotoxicity, hepatotoxicity and nephrotoxicity (Stohs and Bagchi, 1995). Cells have equipped by enzymatic systems which have an ability to convert oxidants into non-toxic molecules, thus protecting the organism from the harmful effects of the xenobiotics. Glutathione reductase (GR), Glutathione-S-transferase (GST), superoxide dismutase and catalase (CAT) are the main cell enzymatic defense systems (Gate et al., 1999).

Cadmium is one of the most biologically toxic heavy metals with a high environmental concern (Ruangsomboon and Wongrat, 2006; Mohiseni et al., 2016). Cadmium can actively be accumulated and result in toxicity to liver, kidneys, brain and heart. Several studies showed that using free radical scavengers and antioxidants can protect the organism against cadmium toxicity (Ochi et al., 1987; Fariss, 1991; Mohiseni et al., 2017a). In this context, protective effects of some natural antioxidant including vitamins (especially E and C) are well documented (Chaurasia and Kar, 1997; Gupta et al., 2004; Cinar et al., 2010; Mekkawy et al., 2011; Harabawy and Mosleh, 2014).

Phenolic compounds in phytochemicals are believed to promote optimal health relatively through

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their antioxidant and free radical scavenging effects, thereby, protecting cellular components against free radical-induced damages. But due to their diverse chemical structures, they are likely to possess different antioxidant capacities (Hamzawy et al., 2012; Soleimany et al., 2016; Mohiseni et al., 2017b). Thyme is a phytobiotic and used since ancient times as a medicinal herb. It has strong antimicrobial and antioxidant activity due to its very high contents of thymol, p-cymene, carvacrol, eugenol and 4-allylphenol (Alçiçek, 2011; Yılmaz et al., 2012). Shirazi thyme (*Zataria multiflora* Boiss) is a thyme-like plant belonging to the Lamiaceae family that geographically grows in central and southern parts of Iran, Pakistan and Afghanistan (Hosseinzadeh et al., 2000). The essential oil of Shirazi thyme contains significant quantities of phenolic compounds such as thymol, carvacrol and monoterpenes, which have an antioxidant, antibacterial and antifungal activities (Ehsani et al., 2014). There are some evidences about the positive effects of thyme on growth and feed utilization in broiler (Hosseini et al., 2013; Sadek et al., 2014) and fish (Dorojan et al., 2014; Sönmez et al., 2015), but despite the strong antioxidant capacity of these components, a little information are exist about using of thyme to improve stress resistance in fish (Antache et al., 2014).

Due to the presence of active ingredients, medicinal herbs possess antioxidant properties and can be used as anti-stress in aquaculture. This will reduce the cost and side effects of synthetic or chemical products. They are also eco-friendly compounds and hence will not affect the environment. Although various medicinal plants contain numerous antioxidant compounds, however, a few of them have been investigated so far. Therefore, this study was conducted to evaluate the potential protective effects of Shirazi thyme compared to vitamin E (a strong and well-known natural antioxidant) against cadmium exposure in juvenile common carp based on oxidative stress responses.

Materials and Methods

Diet preparation: Three experimental diets were prepared and used for feeding trial during the experiment. A commercial diet (Table 1) for carp (Naghshin Kermanshah, Iran) was milled by feed producer, feed additives were added, mixed thoroughly and finally, the diets re-pelletized with kitchen grinder using 3mm die (Montero et al., 1999). For control and metal only (MO), milled commercial diet re-pelletized without any supplementation whereas in diets 2 and 3 the commercial diet was supplemented with either 10 g/kg dry feed ground Shirazi thyme (T) (Yılmaz et al., 2013) or 100 mg/kg of dry feed vitamin E (E) (Kaushik, 1995; Ortuño et al., 2001). They were placed through natural air flow and after drying were kept in the refrigerator (4°C).

Table 1. Proximate composition of experimental diet (Naghshin, Kermanshah, Iran).

Chemical analysis	Proportion (%)
Crude Protein	32
Crude Lipid	5.5
Crude ash	10
Carbohydrate	47-49
Crude fiber	4
Total Phosphor	1.2

Acclimation condition and experimental design: In autumn 2014, 180 healthy juveniles of common carp (average weight of 34±3 g) obtained from Persian fish hatchery (Ahvaz, Iran) and transferred to the Khatam Alanbia, University of Technology (Behbahan, Iran). Two weeks before the experiment, juveniles were randomly divided into four groups (in triplicates) and transferred to the separate 300-L tanks, each containing 15 juveniles and individually equipped with air stone and heater (25±2°C, pH 7.3). The oxygen level was monitored daily (8±1.5 mg/l) and the fish were kept under photoperiod cycle of 12L:12D. Initially, all fish were fed 3% of biomass per day with the commercial diet. Water exchanging during both acclimation and the whole experiment periods was done daily at the rate of 30%. After the acclimation period, all groups were fed only by their own specially-prepared diets. Except for control, all experimental groups were exposed to the sublethal waterborne concentration of cadmium (1.5 mg/L free ion) for 15 days (Vinodhini and Narayanan, 2009).

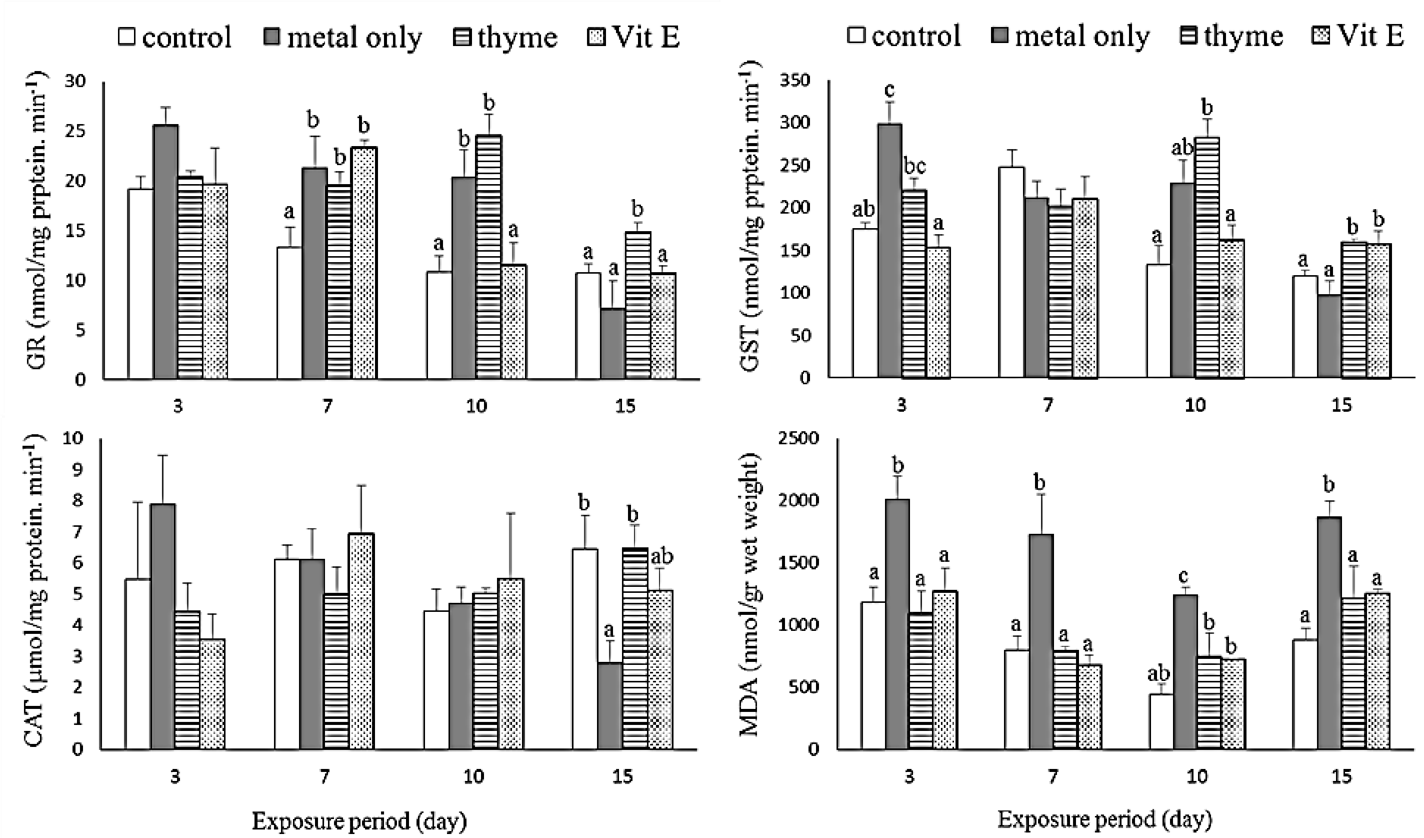

Figure 1. Effect of experimental diets on enzyme activity and lipid peroxidation in liver of common carp during different times of metal exposure. Different letters shows significant differences between groups (P<0.05).

Sampling was done on 3, 7, 10 and 15 days after cadmium exposure. 3 fish were caught from each tank (9 fish per treatment) at each sampling time. The fish were sacrificed by spinal cord dislocation, then they were dissected and liver, kidney and gills were removed and washed in an ice-cold KCl solution (1.15%). The samples were homogenized in 5 volumes of 100 mM phosphate buffer (pH=7.4). Homogenate was centrifuged at 12000×g for 15 min at 4°C. The supernatant was separated and stored at -70°C until antioxidant analysis (Oliveira et al., 2010).

Enzyme activity and MDA assay: GR activity was determined using reduced nicotinamide adenine dinucleotide phosphate (NADPH) and oxidized glutathione (GSSG) as substrates by a technique described by Cohen Duvel (1988). GST activity was evaluated according to Habig et al. (1974) using 1chloro 2,4 dinitrobenzene as substrate. CAT activity was measured spectrophotometrically based on H_2O_2 decomposition as substrate (Aebi, 1984). Total protein content of aliquots was measured according to the Bradford method (Kruger, 1994). Lipid peroxidation assay was carried out in terms of MDA by measuring the thiobarbituric acid reacting substances (TBARS) and expressed as nmol MDA/g wet weight (Ringwood et al., 2003).

Statistical analysis: All data were statistically analyzed by one-way analysis of variance (ANOVA). Turkey's test was used to evaluate the mean difference among experimental groups (P<0.05). Statistical analyses were performed using IBM SPSS Statistics for Windows (Version 19). The data presented as mean±SE.

Results

The specific activity of liver GR, GST and CAT, and MDA levels are presented in Figure 1. The results showed that cadmium exposure induced GR activity at the 7th day of exposure approximately in all treatments but after 15 days of exposure, the lowest and highest enzyme activity were recorded in the MO and T groups, respectively. Although the GST in the

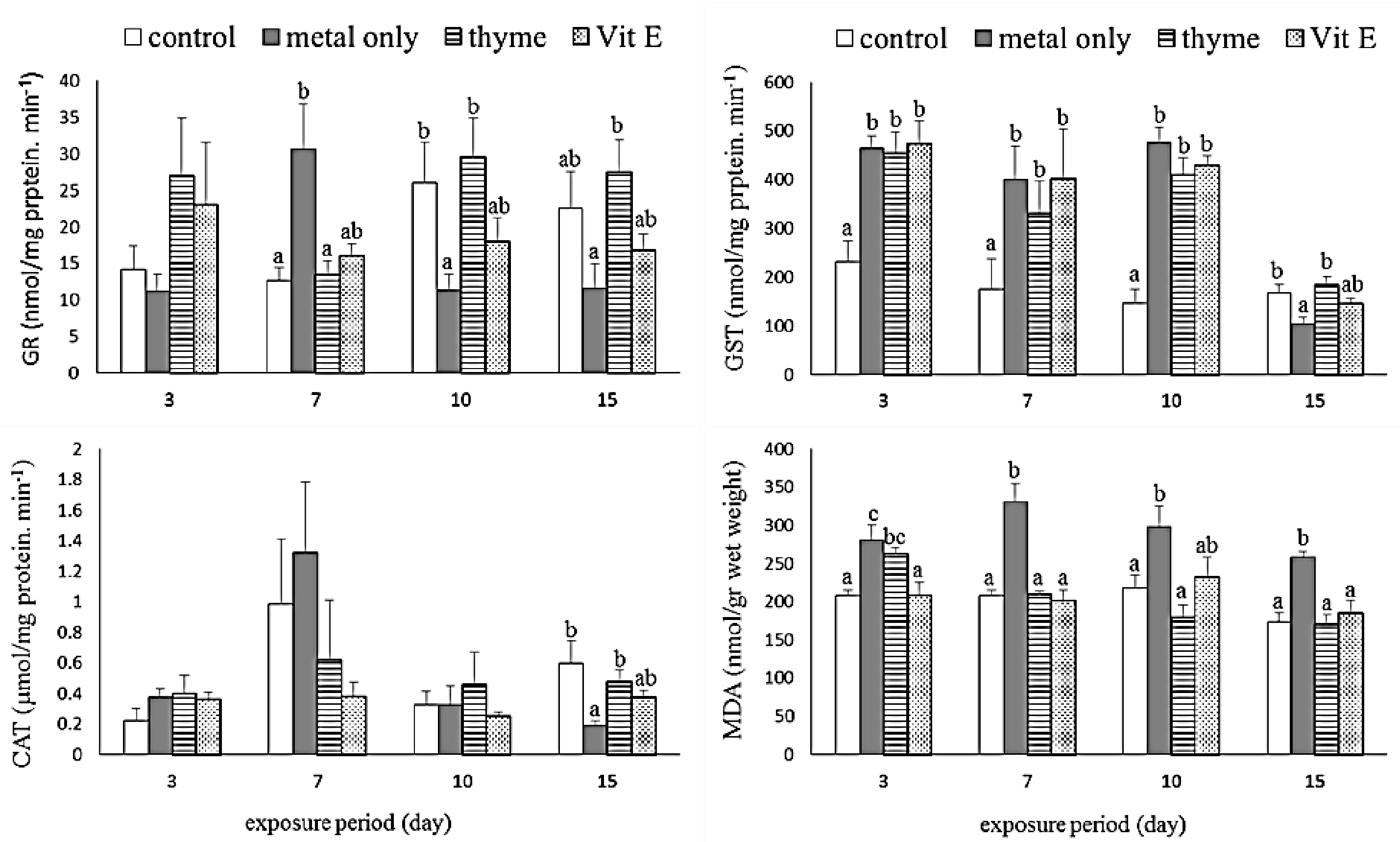

Figure 2. Effect of experimental diets on enzyme activity and lipid peroxidation in kidney of common carp during different times of metal exposure. Different letters shows significant differences between groups (P<0.05).

MO group showed the highest activity at the 3rd day, the enzyme level decreased by time and reached the lowest level at the end of the experiment. The T and E treatments had a significant higher GST activities compared to the control and MO groups. CAT showed slight changes in all treatments during the experiment, however, the enzyme activity for the MO decreased significantly compared to the other treatments (except for the E) at the end of the experiment (the 15th day). A significant elevation in MDA level was obtained for the MO group at all sampling times; however, there were not significant differences between the control and the other treatments. The increase in MDA level in the MO group was more than 200% compared to the control in the most cases. Despite the cadmium exposure, the levels of MDA in the T and E groups did not exceed the normal range (P>0.05).

Except for 7th day that the MO group showed the highest activity, kidney GR significantly decreased compared to the control and T groups at the 10th and both 10th and 15th days after cadmium exposure, respectively (Fig. 2). Cadmium led to a significant elevation in kidney GST activity at the 3rd, 7th and 10th day in all the metal exposed groups. Although the GST levels for the T and E groups tended to remain in normal range, the enzyme activity significantly decreased in the MO group at the final sampling time. The same pattern was observed in CAT at the 15th day of cadmium exposure, where the lowest enzyme activity was observed in the MO group (P<0.05), although, the CAT activity remained unchanged at the previous sampling times (3rd, 7th and 10th). Based on lipid peroxidation analysis, thyme and vitamin E kept the kidney MDA levels within the normal range in the cadmium-exposed fish. On the other hand, the MO group had the highest levels of kidney MDA during the experiment.

Figure 3 shows the gills enzyme activity and lipid peroxidation at different times of the metal exposure. There was some slight fluctuations in gill GR activity in all cadmium exposed fish during the initial times post exposure (3rd, 7th and 10th), but at the final

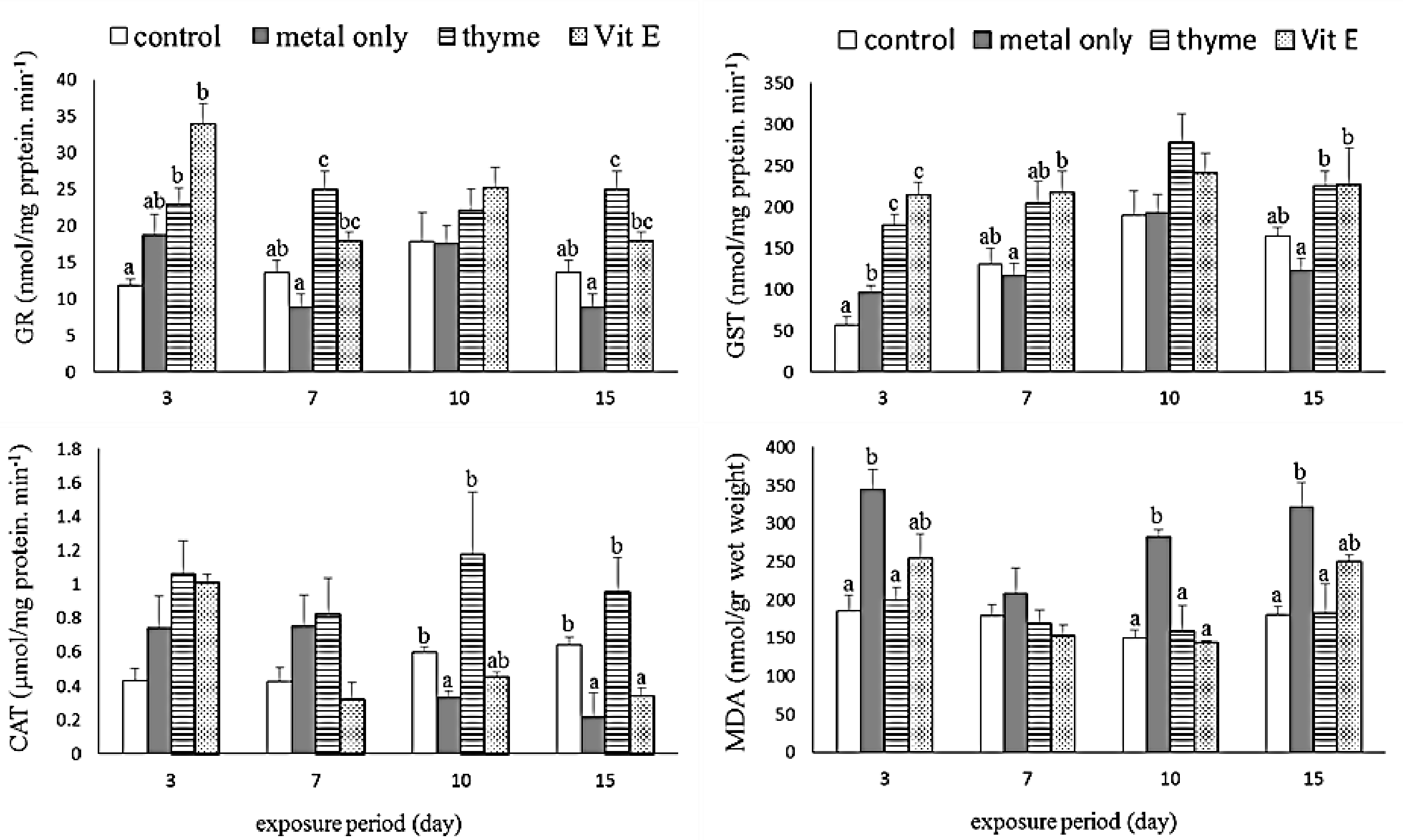

Figure 3. Effect of experimental diets on enzyme activity and lipid peroxidation in gill of common carp during different times of metal exposure. Different letters shows significant differences between groups (*P*<0.05).

sampling, the highest and lowest enzyme activity were recorded for the T and MO groups, respectively. Similar trends were obtained about GST approximately. Shirazi thyme seems to have more ameliorating effects than vitamin E on the gill CAT activity and the highest enzyme activities were related to the T group at 10th and 15th days. Similar to the MDA levels in liver and kidney (Figs. 1, 2), the gill MDA levels of the MO group were significantly higher than that of the control at the most times (3rd, 10th and 15th), while the rate of gill lipid peroxidation in the T and E groups was not exceeded the control levels (*P*>0.05).

Discussion

Cadmium is a toxic metal that can promote an oxidative stress and because of its long retention in vital tissues (such as liver and kidney), subsequently contributes to the development of serious pathological effects (El-Demerdash et al., 2004). The present study has clearly demonstrated the capability of cadmium to induce oxidative stress in different tissues of common carp.

Based on the results, although GR, GST and CAT activities initially tend to increase after cadmium exposure, finally the most enzyme activity showed a clear reduction in the MO group in all experimental tissues. Although the mechanism of the cadmium-induced oxidative stress is still not completely clarified, some hypotheses have been proposed. Cadmium potentially can inhibit the mitochondrial electron-transfer chain reaction and leading to the accumulation of semiubiquinones. This unstable component would transfer one electron to molecular oxygen to form superoxide anion, the high energy free radical. It has also suggested that cadmium could directly weaken some antioxidant enzymes including CAT, GR and superoxide dismutase. This two phenomenon will intensify the cadmium toxicity and leading to oxidative stress (Cinar et al., 2010).

Although the CAT activities showed significant decrease in the MO group at 10th and 15th days, the significant differences were only recorded at the latter day for liver and kidney. The iron deficiency may explain the dissimilar CAT activities in different tissues. Jurczuk et al. (2004) stated that cadmium has a potential effects to impair the intestinal absorption of iron which is required for the CAT activity. As the fish were challenged by waterborne cadmium in the current study, the higher recorded fluctuation in gill CAT activity for the MO group may be related to their direct exposure to the metal.

Several investigations have shown that the formation of oxygen free radicals or ROS increased as a result of metal exposure (Stohs and Bagchi, 1995; Almeida et al., 2002; Tan et al., 2010). ROS can elicit widespread damage to cell components mainly as lipid peroxidation of membrane lipids (Almeida et al., 2002). The cell's antioxidant system in response will increase the rate of antioxidant enzymes production to cope with the stressful condition. When the metal concentration or exposing duration increased and overcame the capacity of the natural detoxifying system, enzyme activities will eventually decrease and several detrimental effects may occur (Stohs and Bagchi, 1995; Sevcikova et al., 2011). In agreement with our results, the potentially harmful effects of heavy metals in cell antioxidant depletion has been previously reported in several investigations (Stohs and Bagchi, 1995; Romeo et al., 2000; El-Demerdash et al., 2004; Farombi et al., 2007; Tan et al., 2010; Sevcikova et al., 2011).

Our data on cadmium exposure in fish have shown that the rate of lipid peroxidation (MDA) in the MO group was increased in different tissues which directly results in free radical-mediated toxicity. Lipid peroxidation is one of the main indicators of oxidative damage incidence and has been found to have an important role in toxicity and carcinogenicity (El-Nekeety et al., 2011). The rate of lipid peroxidation is followed by the balance between the production of ROS and the elimination potential of those radicals by an antioxidant (Sayeed et al., 2003). The increased level of MDA along with the decreased activity of GR, GST and CAT in the MO group may be attributed to the free radical formation that initiated chain reaction of bond formation with vital macromolecules (such as nucleic acid, protein, lipids and carbohydrates), impairing crucial cellular process that may disrupt the normal metabolism of the cells and ultimately organs (El-Nekeety et al., 2011).

Similar to the vitamin E, Shirazi thyme was found to highly potentiate against cadmium toxicity. Based on the results, Shirazi thyme stimulated antioxidant enzyme activities after cadmium exposure. It can be implied that the antioxidant compounds in Shirazi thyme may enhance or reserved the existing antioxidant system. The suggested role of thyme compounds in the prevention of cadmium toxicity can be explained by their ability in radical scavenging (Miura et al., 2002) and also the promotion of antioxidant enzyme activities (Sengül et al., 2008). Flavonoids and phenolic compounds are the main components in many medicinal herbs (Kandaswami and Middleton, 1994; Rice-Evans et al., 1997). These biologically active chemicals are thought to promote optimal health via their antioxidant and radical scavenging properties, thereby keeping cellular organelles from induced damage by free radicals (Ündeğer et al., 2009). Shirazi thyme is mainly composed of monoterpenes and aromatic compounds that have antibacterial, antiviral and antifungal activities. The plants essential oil also contains considerable amounts of phenolic compounds, mainly including carvacrol, thymol and *p*-cymene (Kavoosi et al., 2012). The antioxidant activity of these compounds has been previously reported in several investigations (Miura et al., 2002; Ündeğer et al., 2009; Kavoosi et al., 2012; Sajed et al., 2013). These antioxidants are able to protect or amend the liver health and function in unfavorable environmental conditions and therefore resulted in improved metabolism and better animal growth performance. Our data on common carp support conclusions of researchers who attributed physiological protecting effects of thyme in animal diets. Accordingly, Hassan Barakat (2008) was found that thyme pretreatment in mice leads protection against nickel toxicity more effective than basil. El-Nekeety et al. (2011) also reported that the essential oil of common thyme (*Thymus vulgaris*) has potential antioxidant activities and may induce protective effects against aflatoxicosis in the male rat in dose-dependent manner. The same results on hepatorenoprotective effects of common thyme against aflatoxicosis in the rat have been previously reported (Hamzawy et al., 2012).

Vitamin E has also shown inhibitory properties over harmful effects of cadmium. Considering the lipid solubility of vitamin E, the biological function of the compound is interpreted by many reports as support for prevention of lipid peroxidation (McCay, 1985; Chow, 1991; Harabawy and Mosleh, 2014). Although this is considered as a primary role of vitamin E, however, there is some evidence that scavenging of lipid-based radicals may not be attributed the only form of its activity (Chow, 1991). The oxidation of some amino acid-based radicals including radicals of tryptophan, tyrosine, methionine and histidine is also decreased in the presence of this antioxidant (McCay, 1985). Supporting with this documentation, we also found the efficient role of vitamin E against lipid peroxidation as there was not a significant increase in liver, kidney and gill MDA levels in vitamin E treated group.

Generally, the current results revealed that cadmium induced stressful effects on liver, kidney and gills function. By inducing antioxidant enzymes activities and reduction in lipid peroxidation of the tissues, Shirazi thyme (10 mg/kg diet) showed high protective effects as those observed for vitamin E (100 mg/kg diet) in common carp juveniles. Generally, the current finding can provide a useful reference for stress protective effects of thyme and its beneficial role in aquaculture.

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چکیده فارسی

ارزیابی بروز آسیب اکسیداسیونی ناشی از فلز سنگین کادمیم و مقایسه اثر آنتی اکسیدانی آویشن شیرازی (*Zataria multiflora* Boiss) و ویتامین E در کپور معمولی (*Cyprinus carpio*)

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چکیده:
آویشن شیرازی حاوی ترکیبات فنلی و فلاوونوئیدی است که این ترکیبات خصوصیات آنتی‌اکسیدانی بالایی از خود نشان می‌دهند. این مطالعه با هدف بررسی اثر حفاظتی آویشن شیرازی در مقایسه با ویتامین E به‌عنوان یک آنتی‌اکسیدان طبیعی شناخته شده انجام شده است. ماهی‌های با میانگین وزنی (۳±۳۴) به چهار گروه تقسیم بندی شده و با سه جیره غذایی مشخص مورد تغذیه قرار گرفتند. جیره‌های غذایی با استفاده از خوراک تجاری بدون افزودنی (برای گروه کنترل و فلز به تنهایی) و حاوی مکمل ۱ درصد آویشن شیرازی و یا ۱۰۰ میلی‌گرم در کیلوگرم جیره ساخته شدند. به غیر از گروه کنترل، کلیه تیمارها به مدت ۱۵ روز در معرض غلظت زیرکشنده کادمیم قرار گرفته و در روزهای ۳، ۷، ۱۰ و ۱۵ پس از مواجهه با فلز سنگین، کبد، کلیه و آبشش مورد نمونه‌برداری قرار گرفتند. نتایج نشان داد که در طول ۱۵ روز مواجهه با کادمیم کاهش معنی‌داری در غلظت آنزیم‌های گلوتاتیون ردوکتاز، گلوتاتیون‌اس‌ترانسفراز و کاتالاز در بافت‌های مورد بررسی دیده شد. از سوی دیگر سطح مالون‌دی‌آلدهید در اثر مواجهه با فلز سنگین در بافت‌های یاد شده افزایش معنی‌داری نشان داد. استفاده از مکمل آویشن شیرازی و ویتامین E اثر حفاظتی معنی‌داری را در مواجهه با کادمیم نشان داد. در این زمینه نتایج نشان داد که اثر حفاظتی آویشن شیرازی از ویتامین E بهتر بود. یافته‌های حاصل از این بررسی بر نقش حفاظتی آویشن شیرازی در مواجهه با استرس و همچنین مزایای کاربردی آن در آبزی‌پروری تاکید می‌کند.
کلمات کلیدی: ماهی، سیستم آنتی‌اکسیدانی، فلز سنگین، گیاه دارویی، استرس.