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Protective effect of ellagic acid on oxidative stress and antioxidant status in *Cyprinus carpio* during malathion exposure

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Abstract

This study aims to determine protective efficiency of ellagic acid (EA) on malathion toxicity in carp. The fish were exposed to two sublethal concentrations of malathion (0.5 and 1 mg/L), and EA (100 mg per kg of fish weight) was simultaneously administered for 14 days. Malondialdehyde (MDA) level and superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and glutathione-S-transferase (GST) activities were evaluated in liver, kidney and gills, which were collected at the end of the experiment. In conclusion, the findings of this study demonstrated that malathion caused oxidative stress and negative alterations on the antioxidant enzyme activities of the fish. However, this toxic effect was neutralised by the administration of EA. Thus, the present results suggest that simultaneous treatment with EA (100 mg per kg of fish weight) may alleviate malathion-induced oxidative stress.

Key words: Malathion, Ellagic acid, Oxidative stress, Antioxidant enzyme, Carp.

Introduction

The extensive use by modern agriculture of different agrochemicals such as pesticides leads to alterations in the chemical composition of natural aquatic systems causing chronic toxicity to the freshwater fauna, particularly fish (1). Fish have been largely used to evaluate the quality of aquatic systems as bio-indicators for environmental pollutants (2).

Organophosphate compounds are an important insecticide class, which are widely used in agriculture and domestic purposes to control insect pests (3). Due to their rapid breakdown in water and their low environmental persistence, organophosphate compounds have largely replaced the use of organochlorides in recent years. However, there is evidence that organophosphate compounds are sufficiently persistent in reaching the river environment at concentrations high enough to affect non-target organisms such as aquatic invertebrates and fish are extremely sensitive to the neurotoxic effects of these insecticides. In this regard, fish are particularly sensitive to organophosphate compounds (2). Malathion (O,O-dimethyl-S-1,2-bis ethoxy carbonyl ethyl phosphorodithioate) is a nonsystemic, wide-spectrum pesticide in the organophosphate chemical family and is widely used throughout the world. It is one of the earliest organophosphate pesticides introduced in the 1950s and is used widely for agricultural, residential, and public health purposes because it enhances food production and provides protection from disease vectors (4). Like other pesticides, lethal and sub-lethal treatment of malathion exerts various negative effects on fish. The negative effects of malathion on growth parameters, haematological properties, swimming ability, and the depletion of some biochemical parameters (glycogen, cholesterol, and total protein content) in fishes have been documented (5,6,7) and its oxidative damage to carp and goldfish has been studied (8,9,10).

Reactive Oxygen Species (ROS), such as, hydroxyl radicals, superoxide anion, hydrogen peroxide, nitric oxide, etc., are produced during normal cellular function, particularly as a result of oxidative metabolism at mitochondrial membranes. They are very transient species due to their high chemical reactivity that leads to lipid peroxidation and oxidation of some enzymes, and a massive protein oxidation and degradation (11). High doses and/or inadequate removal of ROS result in oxidative stress that may cause severe metabolic malfunctions and finally impair the health status of animal (12). Most components of cellular structure and function are likely to be the potential targets of oxidative damage, and the most susceptible substrates for autoxidation are polyunsaturated fatty acids of the cell membrane, which undergo peroxidation rapidly (13).

Under normal physiological state, harmful effects of ROS are effectively neutralized by an antioxidant defence system that is an important mechanism to maintain the balance of ROS. Like all aerobic organisms, fish are also susceptible to the attack of ROS and, as a consequence, possess two major antioxidant defences, both the non-enzymatic system (vitamins and other molecules such as glutathione, etc.) and enzymatic system (superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GSH-Px; glutathione-S-transferase), to protect the cells from damage of ROS (12).

Ellagic acid is a polyphenolic compound present in fruits and berries such as pomegranates, strawberries, raspberries and blackberries (14). Important biological activities such as radical scavenging activities, chemopreventive, antimicrobial, estrogenic/antiestrogenic, antiinfilammatory, anticarcinogenic, antifibrosis, and antiviral activities have been ascribed to ellagic acid (15,16). It contains four hydroxyl groups and two lactone groups in which hydroxyl group is known to in-

crease antioxidant activity in lipid peroxidation and protect cells from oxidative damage (17).

The protective effect of ellagic acid against malathion-induced changes in the oxidant/antioxidant status of fish has not so far been studied. Therefore, the objectives of the study were to: (1) investigate alterations in the oxidant/antioxidant parameters induced by malathion and (2) determine possible protectiveness of ellagic acid in alleviating the negative effects of malathion.

Materials and methods

Chemicals and fish

Commercial formulation of the organophosphate pesticide malathion (190 g/L malathion, S-1,2 bis (ethoxycarbonyl) ethyl-O,O dimethyl phosphorodithioate) was used in the present work and was purchased from a commercial manufacturer (Safa agriculture companies, Konya, Turkey). All other chemicals and reagents were purchased from Sigma-Aldrich Chemical Co. and Merck.

Cyprinus carpio (total *n*= 96), weighing 51.8 ± 3.92 g (mean ± SD), were obtained from local fish culture pools (Elazig, Turkey). They were held in aquaria containing 60 L of freshwater continuously aerated to maintain dissolved oxygen values at 7.5–8.0 mg L^{-1}. Temperature was 20 ± 1 °C and pH was 7.4 ± 0.2. Photoperiod was a 12:12 light–dark cycle. Fish were acclimatized for 14 d before the beginning of the experiment and were fed commercial fish food twice daily.

All experiments were conducted in accordance with the institutional animal ethics guidelines of Firat University and were approved by the Animal Experimental Committee of Firat University.

Feed preparation

Ellagic acid is hardly dissolved under natural condition. Therefore, it was dissolved in alkaline solution (0.01 M NaOH; approximately pH 12). pH of the final solution after the addition of EA was approximately 8 (18). This final solution (pH ≈ 8) was added to diets.

A commercial basal diet was crushed and mixed with the final solution containing 100 mg of ellagic acid per kg of fish weight. The diet was reformed into pellets, spread to dry, and stored at +4 °C for the feeding experiment. The remade pellets were administered manually to the fish at a rate of approximately 2% fish body weight per day. The doses of ellagic acid used in this study was selected on the basis of the previous study (19,20).

Experimental setup

The entire experiment was independently repeated two times, and each replicate of each group contained eight fish for a total of 96 fish. The fish were divided into six groups as follows:

- Group 1 (C), the control group, was maintained in tap water and received a commercial basal diet that did not contain ellagic acid.
- Group 2 (EA) was maintained in tap water and received a diet that contained ellagic acid for 14 days.
- The fish in Group 3 (M-0.5) were exposed to 0.5 mg L^{-1} malathion for 14 days and received a commercial basal diet.
- The fish in Group 4 (M-0.5+EA) were exposed to 0.5 mg L^{-1} malathion with the simultaneous administration of ellagic acid for 14 days.
- The fish in Group 5 (M-1) were exposed to 1 mg L^{-1} malathion for 14 days and received a commercial basal diet.
- The fish in Group 6 (M-1+EA) were exposed to 1 mg L^{-1} malathion with the simultaneous administration of ellagic acid for 14 days.

The sublethal concentrations were chosen according to the malathion 96-h LC50 value previously determined for *C.carpio* (2.10 mg/L) (21). The fish were exposed to 0.5 mg/L (approximately 1/4 of the 96-h LC_{50}) and 1 mg/L (approximately 1/2 of the 96-h LC_{50}) malathion for 14 days. The experimental aquaria were aerated, and test media was renewed each day to maintain the appropriate concentration of malathion and to maintain water quality. No fish mortality occurred during these exposures.

Collection and preparation for analysis of tissues

At the end of the experiment, fish were anaesthetized with benzocaine (25 mg/l water). Then were killed by decapitation and tissue samples (liver, kidney, and gill) were collected from the individual fish. The liver, kidney, and gill washed in ice-cold physiological saline, and stored at -80 °C until the biochemical assays, which were performed within one month after extraction.

The tissue was homogenised in a Teflon-glass homogeniser in buffer containing 1.15% KCl at a 1:10 (w/v) ratio to the whole homogenate. The homogenate was centrifuged at 18,000 × g and 4°C for 30 min before the determination of the malondialdehyde (MDA) levels and the superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and glutathione-S-transferase (GST) activities.

Determination of oxidative stress and antioxidant parameters

The levels of MDA, as indices of the LPO in all tissues, were measured using the thiobarbituric acid reaction according to the method of Placer et al. (22). The thiobarbituric acid reactive substances were determined through a comparison with the standard curve of MDA equivalents generated by the acid-catalysed hydrolysis of 1,1,3,3-tetramethoxypropane.

The SOD activity was determined according to the method described by Sun et al. (23), which is based on the principle that xanthine reacts with xanthine oxidase to generate superoxide radicals that react with nitroblue tetrazolium to form a colored formazan dye. To analyse the SOD activity, 600 µL of the SOD reaction mixture containing 0.1 mM xanthine, 0.1 mM EDTA, 50 mg of bovine serum albumin, and 25 µmol of nitroblue tetrazolium per litre was added to 125 µL of the supernatant or 125 µL of an SOD standard solution. Then, 25 µL of 9.9 nM xanthine oxidase solution was added to each tube at 30-s intervals. The tube was incubated for 20 min at 25 °C, and the reaction was terminated by the addition of 0.5 mL of 0.8 mM $CuCl_2$ solution every 30 s. The amount of formazan was determined by measuring the absorbance at 560 nm with a spectrophotometer.

The results of this enzymatic assay are provided as U/mg protein, where one unit of SOD is defined as the amount of sample causing 50% inhibition of NBT reduction.

The CAT activity was determined by measuring the decrease in the hydrogen peroxide concentration at 240 nm according to Aebi (24). The reaction contained 50 mM potassium phosphate buffer (50 mM, pH 7.0; prepared by mixing 0.681 g of KH_2PO_4 in 100 mL and 1.335 g of $Na_2HPO_4{\cdot}2H_2O$ in 150 mL) and 10 mM H_2O_2 (as substrate) and was started by the addition of the sample. The decrease in H_2O_2 was assessed by measuring the absorbance at 240 nm over a period of 3 min with a spectrophotometer.

The GSH-Px activity was determined using the method developed by Beutler (25), which records the disappearance of NADPH through its absorbance at 340 nm. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM sodium azide (NaN_3), 0.2 mM NADPH, 1 EU/ml GSH-Px, 1 mM GSH, and 0.25 mM H_2O_2. An enzyme source (0.1 ml) was added to 0.8 mL of this mixture, and the resulting mixture was incubated at 25 °C for 5 min before the initiation of the reaction, which was induced by the addition of 0.1 mL of peroxide solution. The absorbance at 340 nm was recorded over a period of 5 min. The activity was then calculated from the slope of the lines as micromoles of NADPH oxidised per minute.

The glutathione-S-transferase activity was determined by the method of Habig et al. (26) by following the increase in absorbance at 340 nm due to the formation of the conjugate 1-chloro-2,4-dinitrobenzene (CDNB) using as substrate at the presence of reduced glutathione (GSH). The reaction mixture was prepared by mixing 1.5 ml sodium phosphate buffer 0.1 M pH 6.5, 0.2 ml GSH 9.2 mM, 0.02 ml CDNB 0.1 M and 0.1 ml of the sample. The absorbance was measured at 340 nm and at 20 °C spectrophotometrically. The increase in absorbance was recorded for a total 3 min.

The protein levels in the tissues were determined by the method described by Lowry et al. (27).

Statistical analyses

Means and standard errors were calculated for each experimental group. The statistical significance of the differences between the data obtained from the control and that obtained from the experimental groups was analysed via analysis of variance (one-way ANOVA) and Duncan's post-hoc test using the SPSS 21 computer program (SPSS). P-values < 0.05 were considered to be statistically significant.

Results

The MDA levels are shown in Table 1-3. In the M-0.5 and M-1 groups, the MDA levels increased significantly ($p < 0.05$) when compared to the control group. Ellagic acid supplementation inhibited the malathion-induced increases in the MDA levels in all the analyzed tissues of M-0.5+EA and M-1+EA groups ($p < 0.05$). Compared to the control group, no statistically significant ($p > 0.05$) alteration was determined in the EA group that was administered ellagic acid alone.

The antioxidant enzyme activities in the liver, kidney and gills are shown in Tables 1 to 3, respectively.

In the M-0.5 and M-1 groups, significant increases ($p < 0.05$) in the SOD, CAT and GST activities were observed in all tissues when compared to the control. However, the GSH-Px activity decreased significantly ($p < 0.05$) in all the analyzed tissues. In the M-0.5+EA and M-1+EA groups which received malathion plus ellagic acid, the activities of antioxidant enzyme were ascertained to have drawn closer ($p < 0.05$) to those of the control group. The EA group that was administered ellagic alone exhibited statistically significant difference ($p < 0.05$) in the liver SOD and GSH-Px and the kidney SOD and CAT activities compared with the control group.

Discussion

The present study indicate that the activities of SOD, CAT, GSH-Px and GST were changed during exposure

Table 1. The levels of liver MDA and SOD, CAT, GSH-Px, and GST activities in the control and experimental groups.

	Groups**					
Parameters*	**C**	**EA**	**M-0.5**	**M-0.5+EA**	**M-1**	**M-1+EA**
MDA (nmol/mg protein)	1.19 ± 0.11^{a}	1.12 ± 0.10^{a}	3.03 ± 0.12^{c}	1.42 ± 0.14^{b}	3.11 ± 0.10^{c}	1.40 ± 0.16^{b}
SOD (U/mg protein)	2.76 ± 0.12^{a}	3.20 ± 0.15^{b}	5.79 ± 0.38^{d}	3.92 ± 0.37^{c}	6.02 ± 0.57^{d}	4.00 ± 0.37^{c}
CAT (k/mg protein)	4.18 ± 0.66^{a}	4.29 ± 0.47^{a}	5.93 ± 0.55^{c}	4.41 ± 0.42^{b}	6.24 ± 0.75^{c}	4.56 ± 0.59^{b}
GSH-Px (U/mg protein)	2.21 ± 0.15^{b}	2.84 ± 0.41^{c}	1.20 ± 0.22^{a}	2.10 ± 0.62^{b}	1.14 ± 0.42^{a}	1.14 ± 0.42^{a}
GST (U/mg protein)	95.29 ± 11.80^{a}	102.51 ± 16.42^{ab}	178.33 ± 21.07^{d}	108.94 ± 15.64^{bc}	205.43 ± 33.78^{e}	114.42 ± 27.03^{c}

k: the first-order rate constant.

*MDA: Malondialdehyde level, SOD: Superoxide dismutase activity, CAT: Catalase activity, GSH-Px: Glutathione peroxidase activity, GST: Glutathione-S-transferase.

**C, control; EA, ellagic acid (100 mg/kg fish/day); M-0.5, Malathion (0.5 mg/L); M-0.5+EA, Malathion (0.5 mg/L) plus ellagic acid (100 mg/kg fish/day); M-1, Malathion (1 mg/L); M-1+EA, Malathion (1 mg/L) plus ellagic acid (100 mg/kg fish/day).

[a,b,c,d,e] The different letters in the same line are statistically significant ($p < 0.05$).

Table 2. The levels of kidney MDA and SOD, CAT, GSH-Px, and GST activities in the control and experimental groups.

	Groups**					
Parameters*	**C**	**EA**	**M-0.5**	**M-0.5+EA**	**M-1**	**M-1+EA**
MDA (nmol/mg protein)	1.54 ± 0.13^{a}	1.50 ± 0.12^{a}	3.96 ± 0.33^{c}	1.90 ± 0.15^{b}	4.12 ± 0.24^{c}	2.01 ± 0.20^{b}
SOD (U/mg protein)	2.44 ± 0.13^{a}	3.02 ± 0.18^{b}	4.98 ± 0.62^{c}	2.59 ± 0.19^{a}	5.10 ± 0.56^{c}	2.65 ± 0.28^{a}
CAT (k/mg protein)	2.39 ± 0.15^{a}	2.91 ± 0.18^{b}	4.37 ± 0.60^{c}	2.84 ± 0.34^{b}	4.63 ± 0.42^{c}	2.88 ± 0.19^{b}
GSH-Px (U/mg protein)	1.59 ± 0.06^{bc}	1.75 ± 0.10^{c}	0.72 ± 0.08^{a}	1.44 ± 0.12^{b}	0.50 ± 0.09^{a}	1.47 ± 0.09^{b}
GST (U/mg protein)	74.13 ± 14.25^{a}	71.88 ± 12.70^{a}	133.42 ± 23.56^{c}	91.43 ± 18.45^{b}	131.89 ± 22.30^{c}	95.17 ± 14.92^{b}

*MDA: Malondialdehyde level, SOD: Superoxide dismutase activity, CAT: Catalase activity, GSH-Px: Glutathione peroxidase activity, GST: Glutathione-S-transferase.

**C, control; EA, ellagic acid (100 mg/kg fish/day); M-0.5, Malathion (0.5 mg/L); M-0.5+EA, Malathion (0.5 mg/L) plus ellagic acid (100 mg/kg fish/day); M-1, Malathion (1 mg/L); M-1+EA, Malathion (1 mg/L) plus ellagic acid (100 mg/kg fish/day).

[a,b,c,d,e] The different letters in the same line are statistically significant ($p < 0.05$).

Table 3. The levels of gill MDA and SOD, CAT, GSH-Px, and GST activities in the control and experimental groups.

	Groups**					
Parameters*	**C**	**EA**	**M-0.5**	**M-0.5+EA**	**M-1**	**M-1+EA**
MDA (nmol/mg protein)	1.12 ± 0.13^{a}	1.20 ± 0.09^{a}	3.29 ± 0.21^{c}	1.56 ± 0.15^{b}	3.48 ± 0.30^{c}	1.69 ± 0.12^{b}
SOD (U/mg protein)	1.62 ± 0.10^{a}	1.70 ± 0.16^{a}	4.12 ± 0.35^{c}	2.03 ± 0.19^{b}	4.21 ± 0.48^{c}	2.14 ± 0.13^{b}
CAT (k/mg protein)	1.58 ± 0.12^{a}	1.62 ± 0.14^{a}	3.36 ± 0.51^{c}	1.90 ± 0.19^{b}	3.98 ± 0.35^{d}	2.13 ± 0.24^{b}
GSH-Px (U/mg protein)	1.37 ± 0.09^{cd}	1.45 ± 0.13^{d}	0.67 ± 0.10^{a}	1.26 ± 0.14^{c}	0.52 ± 0.08^{b}	1.39 ± 0.14^{cd}
GST (U/mg protein)	69.52 ± 9.47^{a}	72.01 ± 11.60^{a}	145.41 ± 15.51^{d}	98.63 ± 12.28^{b}	162.79 ± 24.37^{e}	116.36 ± 19.72^{c}

*MDA: Malondialdehyde level, SOD: Superoxide dismutase activity, CAT: Catalase activity, GSH-Px: Glutathione peroxidase activity, GST: Glutathione-S-transferase.

**C, control; EA, ellagic acid (100 mg/kg fish/day); M-0.5, Malathion (0.5 mg/L); M-0.5+EA, Malathion (0.5 mg/L) plus ellagic acid (100 mg/kg fish/day); M-1, Malathion (1 mg/L); M-1+EA, Malathion (1 mg/L) plus ellagic acid (100 mg/kg fish/day).

[a,b,c,d,e] The different letters in the same line are statistically significant ($p < 0.05$).

period compared to the control group. These changes demonstrate the presence of oxidative stress. Antioxidant enzymes can be induced by a slight oxidative stress due to compensatory response; however, a severe oxidative stress depresses the activities of these enzymes due to oxidative damage and a loss in compensatory mechanisms (13). In the present study, the high activities of SOD, CAT and GST showed a adaptive response to neutralise ROS induced by malathion exposure. However, the high MDA levels in the different tissues of carp revealed that malathion-induced ROS are not completely scavenged by antioxidant enzymes. This was aggravated by the decrease in the GSH-Px activity in tissues.

The oxidative damage includes the peroxidation of unsaturated fatty acids and the corresponding increase in tissue MDA levels, which is widely used as indices of lipid peroxidation. Increased level of MDA may reflect the degrees of lipid peroxidation injury in tissues (28,29,30). In this study, increased MDA production in all the analyzed tissues was found in the malathion treated groups and this result is consistent with previous literature (9,10). This increase can be attributed to the malathion-induced excessive production of free radicals and consequently elevated lipid peroxidation. However, the simultaneous treatment with EA decreased the levels of MDA in the different tissues of malathion treated fish; this decrease in the tissue MDA levels can be attributed to the antioxidant effect of EA.

The SOD–CAT system provides the first defence against oxygen toxicity. SOD catalyzes the dismutation of the superoxide anion radical to water and hydrogen peroxide, which detoxified by the CAT activity. Usually a simultaneous induction response in the activities of SOD and CAT is observed when exposed to pollutants (31).

In the present study, carp exposed to malathion displayed a increase in the SOD and CAT activities at both tested concentrations. This finding is consistent with our previous study (10) in which malathion caused sig-

nificant increase in the tissue SOD and CAT activity. However, the simultaneous treatment with EA resulted in a significant decrease in the tissue SOD and CAT activities. This decrease in the SOD and CAT activities may be due to superoxide anion radical scavenging and hydrogen peroxide scavenging properties of EA (32).

The GSH-Px catalyses the reduction of hydrogen peroxide and lipid peroxides and is considered an efficient protective enzyme against lipid peroxidation at the expense of GSH (33). The GSH-Px activity in all the analyzed tissues was a significantly lower than controls in the present study. This reduction in GSH-Px activity in a given tissues could indicate that the antioxidant capacity is exceeded by the amount of hydroperoxide products generated through lipid peroxidation (34). In addition, low activity of GSH-Px demonstrates the inefficiency of tissues in neutralizing the impact of peroxides (35). EA administration increased the tissue GSH-Px activities in malathion treated fish. This increment may be explained with possibly enhancement of antioxidant capacity in the tissues (20).

The GST is multifunctional dimeric enzymes that are involved in detoxification of endogenous (intracellular metabolites) and exogenous substances (drugs, pesticides, and other pollutants). GST is members of a multigene family present in all organisms, and the structural diversity within the GST family of isoenzymes provides the capability to conjugate a very broad range of compounds (36). GST is a cytosolic or microsomal enzyme catalyzing the conjugation of electrophilic xenobiotics to GSH, which converts a reactive lipophilic molecule into a water-soluble non-reactive conjugate. Thus, GST plays a important role in protecting tissues from oxidative stress (37,38). In the present study, malathion treatment caused significant increases in the tissue GST activity. Similar changed activity of the GST has also been reported by other authors. For example, Huculeci et al. (8) observed increased antioxidant enzyme activity of GST in kidney and intestine of the freshwater goldfish after exposure to malathion in concentrations of 0.05 mg l^{-1}. Elevated GST activity may indicate a defensive mechanism to counteract the effects of pesticide toxicity (39). Induced GST activity may reflect the role of this enzyme in protection against the toxicity of xenobiotic-induced lipid peroxidation (35) Treatment with EA decreased the tissue GST activity in malathion treated fish. This decrease in GST activity may have been caused by free radical inhibition via EA administration.

Our data here show that there were a significant increases in the liver SOD and GSH-Px and the kidney SOD and CAT activities of fish that received EA alone compared to the control group; therefore, EA had a positive effect on the antioxidant enzymes. This finding agrees with a previous observation by Mise Yonar et al. (20), who reported that feeding *Oncorhynchus mykiss* a diet enriched with EA increased the antioxidant enzyme activities in different tissues.

In conclusion, the present study that malathion caused oxidative stress in carp. EA treatment provided a protective effect against the oxidative stress induced by malathion. EA administration alone significantly modulated oxidant/antioxidant parameters. Therefore, EA may be used as an antioxidant. However, further studies are needed to elucidate the precise biological action and potential usefulness as an antioxidant of EA.

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