


# The Protective Effects of Curcumin on Organophosphate Insecticide Chlorpyrifos-Induced Oxidative Stress and DNA Damage in *Oncorhynchus mykiss*

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## Article History

Received 12 October 2018

Accepted 04 March 2019

First Online 06 March 2019

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## Keywords

Pesticide

Chlorpyrifos

Curcumin

Oxidative Stress

DNA Damage

## Abstract

The aim of this study was to determine the levels of enzymatic and nonenzymatic antioxidants and DNA damage in blood of rainbow trout (*Oncorhynchus mykiss*) exposed to chlorpyrifos (CPF) and the protective effects of curcumin (CUR) on CPF-induced oxidative stress and DNA damage. For this purpose, 0.5% and 1.0% of CUR was administered via the intraperitoneal route for 45 days during exposure to water containing 0.040 mg/L CPF in rainbow trout. We observed a significant decrease in total peroxide (TP), glutathione (GSH), GSH peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT), paraoxonase (PON), and ferric ion reducing antioxidant power (FRAP) levels, whereas there was a significant increase in TP level in CPF-exposed rainbow trout ( $P<0.05$ ). Antioxidants, depending on the dose, had relatively modest to significant effects on CPF-induced oxidative stress. Low doses of CUR also significantly increased serum FRAP levels in rainbow trout exposed to CPF ( $P<0.05$ ). We observed increased comet assay levels in blood, predominantly in rainbow trout exposed to CPF ( $P<0.05$ ). The amount of DNA damage induced by CPF exposure was more than that in the control. Overall, this study shows that CUR, depending on the dose, may modify CPF toxicity to a moderate or high degree in rainbow trout.

## Introduction

Pesticides, which are chemical materials used in agricultural pest control, can be divided into many groups based on their origins. Pesticides that are mainly used to prevent pests on food for human consumption and their residuals may have toxic effects on organisms other than those targeted for elimination.

The effectiveness of chlorpyrifos (CPF), which is an organophosphate (OP)-based pesticide/insecticide, the use of which has been banned in many countries, could be associated with the inhibition of acetylcholinesterase (AChE). Although the mechanism of CPF toxicity includes AChE inhibition, it has been documented that oxidative

stress is also induced (Ambali *et al.*, 2010; Basha & Poojary, 2011; Basha & Poojary, 2012). The combined effect of cellular changes induced by ROS could cause cellular dysfunction and final catabolism in living organisms (Basha & Poojary, 2012).

CPF is a broad-spectrum insecticide used to kill a wide variety of insects. It remains one of the most widely used agricultural OP insecticides and is currently in use in more than 100 countries worldwide, including Turkey (Olsvik, Berntssen, & Sjøteland, 2015).

In Turkey, it is widely applied to control insect pests of maize (*Zea mays* L.), potato (*Solanum tuberosum* L.), tomato (*Lycopersicon esculentum* Mill.) and other vegetable crops. The use of CPF in Turkey has increased

considerably in the amounts of 93, 94, 102, 156 and 241 tonnes in the years 1992, 1993, 1994, 1995 and 1996, respectively (Yücel, İlim, Gözek, Helling, & Sarıkaya, 1999).

Insecticides may have a direct lethal effect based on their chemical structure and the fish species. Acute effects may appear shortly after the aquatic organism is exposed to high levels of the contaminant, resulting in death of the organism. Chronic effects may be seen after the organism is exposed to low concentrations of the contaminant over long periods of time (Kayhan, Kaymak, & Yön, 2013.) It has been reported by many researchers that CPF causes oxidative stress in fish. Kavitha and Venkateswara (2007) reported that the LC50 concentration in *Gambusia affinis* that were exposed to CPF for 96 hours was 297 µg/L. Similarly, Kutluyer, Kocabaş, Erişir and Benzer (2017) analysed the negative effects of CPF exposure at three different concentrations (5 µg/L, 10 µg/L and 15 µg/L) in *Salmo coruhensis* to determine the oxidative balance. CPF inhibits the enzyme AChE and causes oxidative stress and endocrine disruption (Olsvik, Berntssen, & Sjøfteland, 2015).

*Curcuma longa* L. produces natural antioxidants, including curcumin (CUR) (1,7-bis (4-hydroxy-3-methoxyphenyl) 1,6-heptadien-3,5-dione), the main active compound, and phenolic and quinone groups (Ammon, Safayhi, Mack, & Sabieraj, 1993). CUR strongly binds many reactive oxygen species, including hydroxyl radicals. In addition, CUR inhibits lipid peroxidation (LPO) in different animal species (Reddy & Lokesh, 1994; Ak & Gülçin, 2008).

Rainbow trout are a commonly used species in toxicology studies and are sensitive to many contaminants (Decker, 2015). However, to the best of our knowledge, the toxic effects of CPF in blood of rainbow trout and the protective effects of CUR on these tissues have not been analysed for the antioxidant mechanism. In addition to evaluation these aforementioned factors, a DNA damage (comet) assay was used to determine the potential preventive effects of CUR on CPF-induced DNA damage in rainbow trout. The aim of this study was to determine the levels of enzymatic and nonenzymatic antioxidants, including total peroxide (TP), glutathione (GSH), GSH peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT), paraoxonase (PON), and ferric ion reducing antioxidant power (FRAP), in some tissues and the relationship of antioxidant enzyme levels and the DNA damage in the blood of rainbow trout exposed to pesticides.

## Materials and Methods

### Chemicals

CUR and CPF (96.5% purity (technical grade)) were purchased from Sigma Chemicals (Sigma-Aldrich, Turkey). All other chemicals were purchased from

standard commercial suppliers (St Louis, MO, USA). CPF (480 g/l CPF-ethyl) was purchased from commercial suppliers (Hektaş Ticaret A.Ş. Turkey).

### Animals, Experimental Diets and Design

In the study, the establishment and implementation of the experiment was performed in concrete fish ponds for 45 days in the Department of Fisheries and Aquaculture at Harran University, Bozova Vocational School for Higher Education. Male rainbow trout (*O. mykiss*) (age, 2 years; n = 120) (for two replicates), with an average weight of 80–100 g, were obtained from Karacalar Food, Agriculture, Aquaculture Import and Export Limited Company. During the study, water temperature, pH and dissolved oxygen were 15.0°C±1.5°C, 7.8±0.9 and 8.8±0.3 mg/L, respectively.

CPF stock solution was prepared in acetone and stored at 4°C during the study. The stock solution was added daily into the ponds at the defined concentrations. The use of fish and experimental application protocols were approved by the Dollvet A. S. Animal Experiments Local Ethics Committee (Şanlıurfa-Turkey) (Protocol No: 2014/61). Analyses of the tissues were performed in the laboratories of the Harran University Biology Department.

Rainbow trout were kept in 500-L ponds with continuous aeration for 45 days (mean water temperature, 15.0°C±1.5°C; mean dissolved oxygen, 8.92±0.3 mg/L). The stock solutions of CPF (via actual concentration) were added to the ponds where the fish was kept, and the water in the ponds was changed by 1/3 per day. CUR was adjusted to 0.5% and 1.0% of the total weight of the fish. The CUR extract was dissolved in dimethyl sulfoxide (DMSO), and stock solutions were prepared in phosphate buffered saline at pH 7.2 and administered to fish via intraperitoneal injections (IP) (Behera, Swain, Sahoo, Mohapatra, & Das, 2011; John Xie, Bell, & Liang, 2013). All experiments were conducted in duplicate (Control, CPF, CUR1, CUR2, CPF + CUR1 and CPF + CUR2). In total, 10 fishes were placed in each pond. In the first group, normal water (without CPF) was used, and the fish were fed without additives (Control). The second fish group was exposed to water containing 0.040 mg/L CPF (Doğu *et al.*, 2015). The third fish group received only 0.5% CUR (CUR1) in twelve doses (two doses per week) via IP injections, and the fourth group received only 1% CUR (CUR2) in twelve doses (two doses per week) by IP injections. The fifth group was exposed to water containing 0.040 mg/L CPF and received CUR (CPF + CUR1) at a rate of 0.5% of the total weight of the fish in twelve doses (two doses per week) by IP injections. The sixth group was exposed to water containing 0.040 mg/L CPF and received CUR (CPF + CUR2) at 1.0% of the total weight of the fish in twelve doses (two doses per week) by IP injections. Experimental conditions are summarised in Table 1.

**Table 1.** Experimental design of the study

Treatments	Experimental Groups					
	Control	CPF	CUR1	CUR2	CPF+CUR1	CPF+CUR
CPF (mg/l)	-	0.040	-	-	0.040	0.040
CUR (%)	-	-	0.5	1	0.5	1

### Sample Collection and Laboratory Analyses

At the end of the study, 0.6 mL/L 2-phenoxyethanol was applied as an anaesthetic agent before taking blood samples from all experimental groups. Subsequently, blood samples from each fish were transferred into individual heparinised test tubes. The anaesthetised fish were rinsed with distilled water prior to dissection to avoid external pesticide residue.

After centrifugation, the plasma was separated from the whole blood, and the blood plasma samples were stored at  $-20^{\circ}\text{C}$  until the determination of antioxidants.

### Antioxidant Enzyme Assays

#### Total Peroxide (Serum TP)

Serum TP concentrations were determined by an enzymatic assay as described previously (Yeni *et al.*, 2005). TP levels determined the concentration of hydroperoxide, which is the primary oxidation product. TP was expressed as  $\mu\text{mol H}_2\text{O}_2$  equivalents/L.

#### Glutathione (GSH)

The level of GSH was measured using a modification of the method by Koyuncu, Kocyigit, Gonel, Arslan and Durgun (2017). GSH was used as the standard, and GSH samples were detected using a microplate reader (Spectra max M5, USA) with excitation at 345 nm and emission at 425 nm. The results were expressed as nmol/ml in serum.

#### Glutathione Peroxidase (GPx)

GPx activity was determined by observing the change of t-butylhydroperoxide at  $37^{\circ}\text{C}$  and 340 nm for 5 minutes (Beutler, 1984). The amount of enzyme that oxidises 1 mole of NADPH per minute is the GPx activity.

#### Superoxide Dismutase (SOD)

SOD was produced using the method developed by McCord and Fridovich (1969), based on the ability of the enzyme to inhibit iodo-p-nitro tetrazolium (INT) violet; reduction was determined by monitoring the change in optical density at 505 nm for 3 minutes at  $37^{\circ}\text{C}$ . SOD activity was expressed as mU/mg protein, wherein 1 U of SOD was calculated as equivalent to the amount of

enzyme inhibiting 50 % of the reduction ratio of INT violet.

#### Catalase (CAT)

CAT activities in plasma were analysed by the Aebi (1984) test. One unit of CAT activity was defined as the amount of enzyme that degrades 1 mole of  $\text{H}_2\text{O}_2$  per minute in mU/mg protein.

#### Paraoxonase (PON)

Measurement of PON activity was determined in plasma with an autoanalyser (CobaSintegra c 800) using commercially available kits (Rel assay) by an enzymatic assay as described previously (Mackness, Arrol, & Durrington, 1991).

#### Ferric Ion Reducing Antioxidant Power (FRAP)

For the measurement of FRAP values, antioxidants using reduced oxidising zing Fe (III) were utilised in a reductive redox-linked colourimetric method, reduction of a ferric tri-pyridiazine complex to ferro-(2,4,6-tripyridyl-s-triazine) (Prior, Wu, & Schaich, 2005). The FRAP value was calculated according to the following equation:  $\text{FRAP value } (\mu\text{M}) = [\text{Abs. (Sample)} \times \text{Std } (\mu\text{M})] / \text{Abs FRAP value Std}$ .

#### DNA Damage (Comet assay)

The alkaline single cell gel electrophoresis analysis (comet assay) was used to study the potential preventive effects of CUR on CPF-induced DNA damage in peripheral blood mononuclear cell genotoxicity in rainbow trout. The comet assay was performed according to the methods mentioned by Singh, McCoy, Tice, & Schneider, (1988) with slight modifications (Kocyigit, Koyuncu, & Taskin, 2016) as follows: approximately  $2 \times 10^4$  cells were suspended in low melting-point agarose (LMA) (75  $\mu\text{L}$  of 1.0%) and stratified onto semi-frozen slides previously covered with a thin film of normal melting-point agarose (1.0%). Another 0.5% LMA layer was placed on the second layer. Cells were resuspended in buffers (0.3 M NaOH, 1 mM (pH 7.5)) for 2 hours at  $4^{\circ}\text{C}$  in a solution (100 mM EDTA, 2.5 M NaCl, 10% DMSO, 1% Triton X-100, 10 mM Tris, pH 10.0 and EDTA, pH 13.1) and were then subjected to electrophoresis. Subsequently, the slides were neutralised in another buffer (0.4 M Tris-HCl, pH 7.5).

Slides were carefully dried at 25°C in an incubator and stained with ethidium bromide (10 µg/mL in distilled water, 70 µL/slurry). The slides were read using a fluorescence microscope (Leica DM 1000, Solms, Germany) imaging system, and a hundred cells were randomly measured in each sample and rated on a scale of 0–4 based on fluorescence beyond the nucleus. The scale scores used were: 0, no tail; 1, comet tail, half of the width of the nucleus; 2, tail equal to the width of the nucleus; 3, the tail is longer than the nucleus and 4, the tail is twice the width of the nucleus. Cellular scoring was accurately and precisely analysed using computerised image analysis (Kocycigit Koyuncu, & Taskin, 2016).

### Statistical Analyses

Statistical analyses were performed using the SPSS 16.0 computer program (SPSS Inc. Chicago, Illinois, USA). One-way analysis of variance (ANOVA) was performed to determine whether there are any statistically significant differences between the means of six groups. The one-way ANOVA followed by Tukey post hoc was used to compare differences among individual means. A probability of 0.05 was utilised to account for the statistical difference among means.

### Results and Discussion

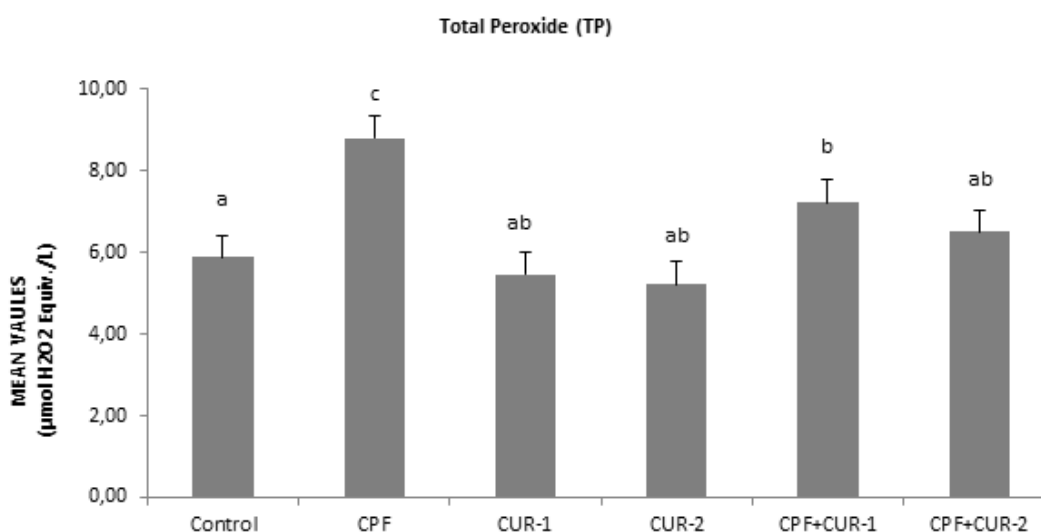
The measurements of enzymatic and nonenzymatic antioxidants, TP (H<sub>2</sub>O<sub>2</sub> Equiv./L), GSH (nmol/mL), GPx (U/g), SOD (U/g), CAT (k/g), PON (U/L), FRAP (µmol/L) and comet assay (AU), are presented in Figure 1–4. When the CPF group was compared with the control group, GSH, GPx, SOD, CAT, PON and FRAP levels decreased significantly, and TP level increased

significantly ( $P < 0.05$ ). When compared with the control, a low dose of CUR decreased the SOD levels ( $P < 0.05$ ); conversely, there was no significant effect of the other antioxidant enzymes. With the higher dose of CUR, the GSH level increased, and the GPx and the PON levels significantly decreased. The TP, SOD, CAT and FRAP levels were similar for the higher dose of CUR and the control. For the CPF-exposed group with the low dose of CUR in comparison with the control group, the TP level was high, and the GPx, SOD, CAT and PON levels were significantly lower ( $P < 0.05$ ). The GSH and FRAP levels were similar between the control and the experimental group. When the CPF-exposed group with the high dose of CUR was compared with the control group, the GPx and CAT levels were significantly lower ( $P < 0.05$ ), and the TP, GSH, SOD, PON and FRAP levels were similar.

The main effects of CPF and other OP are the inhibition of AChE and oxidative stress (Bagchi, Bagchi, Hassoun & Stohs, 1995; Yurumez, Cemek, Yavuz, Birdane & Buyukokuroglu, 2007). In this study, the TP levels were increased in the CPF-exposed trout as a result of oxidative stress (Figure 1a).

When the effects of both dosages of CUR were compared with the control group, there were no significant changes in antioxidant enzyme and TP levels. When compared with CPF, the high dosage of CUR with CPF increased SOD, CAT, PON and FRAP levels significantly; the low dosage of CUR with CPF increased only the FRAP level significantly ( $P < 0.05$ ). CUR at both dosages with CPF significantly decreased the TP levels ( $P < 0.05$ ).

It was reported that ROS, including hydrogen peroxide and superoxide, increased as a result of OP toxicity, causing disequilibrium in the production and destruction of reactive oxygen types (Hai, Varga, &



**Figure 1a.** Effect of CPF and CUR on TP levels of blood in *O. mykiss* (n=10). Data are presented as means ± SD. Superscript letters indicate significant differences among exposure groups ( $P < 0.05$ ,  $P < 0.001$ ).

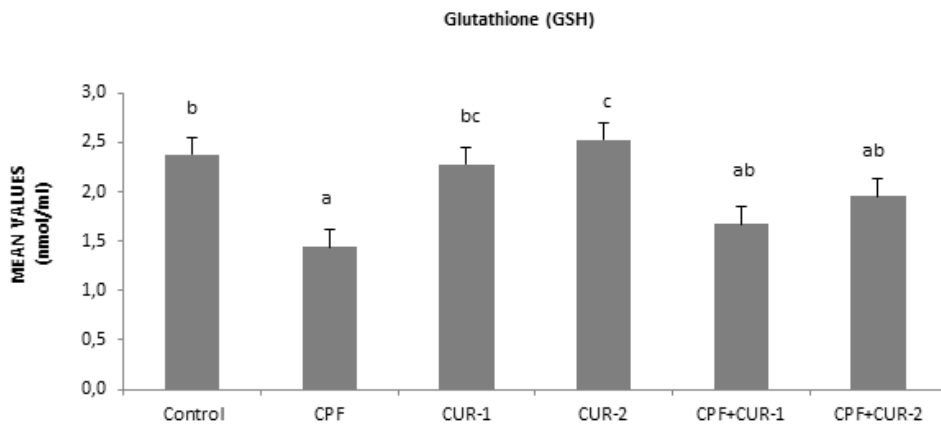
Matkovics, 1997; Peña-Llopis, Peña, Sancho, Fernandez-Vega, & Ferrando, 2001; Yurumez, Cemek, Yavuz, Birdane, & Buyukokuroglu, 2007). It was shown that dichlorvos (Hai, Varga, & Matkovics, 1997) in European eel (*Anguilla anguilla* L.) and thiocarbamate herbicides (Peña *et al.*, 2000; Peña-Llopis, Peña, Sancho, Fernandez-Vega, & Ferrando, 2001) in crabs (*Cyprinus carpio*) and cat fish (*Ictalurus nebulosus*) cause oxidative stress (Sheriff *et al.*, 2017).

CUR had two electrophilic  $\alpha$ - and  $\beta$ -unsaturated carbonyl groups, which react with nucleophiles (Scapagnini *et al.*, 2002). CUR, which was reported to have strong potential to inhibit ROS generation (Kempiah & Srinivasan, 2004), was also shown to protect renal cell lines against oxidative stress, causing both oxygenase-I (Balogun, Foresti, Green, & Motterlini, 2003) and kidney injury (Okada *et al.*, 2001).

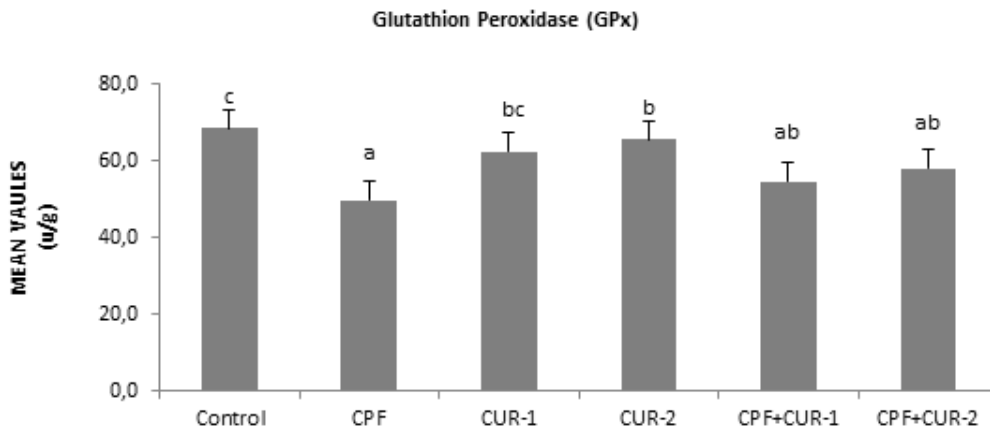
GSH is a tripeptide-involving thiol. GSH has many biological functions, such as in DNA and protein synthesis, and it also functions as an ROS scavenger. The

most important function of GSH was assumed to be the control of reduced forms of thiol groups (-SH) of enzymes at sufficient levels (Meister, 1983; Meister, 1994; Deneke & Fanburg, 1989). In the current study, a significant decrease was observed in GSH and GPx activity as a result of exposure to CPF (Figure 1b, c). GSH levels of crabs exposed to dichlorvos were decreased (Hai, Varga, & Matkovics, 1997).

Given that GSH involves the substrate GPx in preventing the increase of free radicals and H<sub>2</sub>O<sub>2</sub> after treatment with CPF, it was consumed quickly (Meister, 1983). Monteiro, de Almeida, Rantin and Kalinin (2006) showed that methyl parathion, which was an OP, caused decreases in GSH and LPO in all tissues in *Byrcon cephalus*. Both doses of CUR did not cause a significant increase in GSH and GPx. Moreover, GSH and GPx levels in the blood serum of fish that were administered CUR with exposed to CPF were similar to CPF-exposed fish. However, in previous studies, it was observed that N-acetylcysteine, a free radical scavenger and a precursor



**Figure 1b.** Effect of CPF and CUR on GSH levels of blood in *O. mykiss* (n=10). Data are presented as means  $\pm$  SD. Superscript letters indicate significant differences among exposure groups ( $P < .05$ ,  $P < .001$ ).



**Figure 1c.** Effect of CPF and CUR on GPx levels of blood in *O. mykiss* (n=10). Data are presented as means  $\pm$  SD. Superscript letters indicate significant differences among exposure groups ( $P < .05$ ,  $P < .001$ ).

of GSH, increased in vitro and in vivo GSH level (Hoffer, Baum, Tabak, & Taitelman, 1996; Yurumez, Cemek, Yavuz, Birdane, & Buyukokuroglu, 2007).

In contrast, Modesto and Martinez (2010) observed that GSH increased in *Prochilodus lineatus* exposed to Roundup® (glyphosate-based herbicide), which is an herbicide. In addition, antioxidant effects on fish species can be changed different types of species and OP. Consistent with results of this study, GSH was an indicator for environmental pollutants in rainbow trout, and further, in fish exposed to CPF minimally reduced the effects of OP oxidative stress. The group only administrated CUR did not exhibit significantly increased GSH and GPx activities; further, the group administrated CUR and exposed to CPF did not exhibit increase GSH and GPx activity. Thus, both dosages of CUR were insufficient to prevent increased H<sub>2</sub>O<sub>2</sub> caused by CPF. Similar to our study, OP insecticides increase free radical formation and cause oxidative stress by decreasing antioxidant enzymes (Kovacic, 2003, Birkhoj *et al.*, 2004).

CPF decreased SOD and CAT activity in the blood plasma (Figure 2a, b). Over production of anion radicals based on CPF could inhibit CAT activity (Kono & Fridovich, 1982). The CAT enzyme reduces hydrogen peroxidase to O<sub>2</sub> and H<sub>2</sub>O. At high concentrations of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), CAT could reduce it. Another enzyme, SOD, converts O<sub>2</sub><sup>-</sup> (superoxide radical) to H<sub>2</sub>O<sub>2</sub> as a scavenger of reactive oxygen species against O<sub>2</sub> toxicity (Cemeli, Baumgartner, & Anderson, 2009).

Continuous protein synthesis was required for CAT activity. Protein synthesis fails as a result of exposure to organic phosphorus, and thus CAT activity decreases (Uner, Sevgiler, Durmaz, Piner, & Cinkiloglu, 2009). CUR did not increase SOD and CAT activity alone. CUR, which was administered in high dosages, could reverse the negative effect of CPF on these two enzymes. CUR might increase the protein synthesis required for this antioxidant enzyme activity and might prevent the negative effect of CPF (Uner, Sevgiler, Durmaz, Piner, & Cinkiloglu, 2009). In contrast to the findings of this study, it was observed that organophosphorus insecticides increased SOD activity and LPO in the liver of *Cyprinus carpio* (L.) and tilapia *Oreochromis niloticus* (Peixoto, Alves-Fernandes, Santos, & Fontainhas-Fernandes, 2006; Hai, Varga, & Matkovic, 1997, Lima *et al.*, 2006). A significant increase in SOD, CAT and GSH S-transferase activities in *Brycon cephalus* exposed to methyl parathion was observed (Monteiro, de Almeida, Rantin, & Kalinin, 2006).

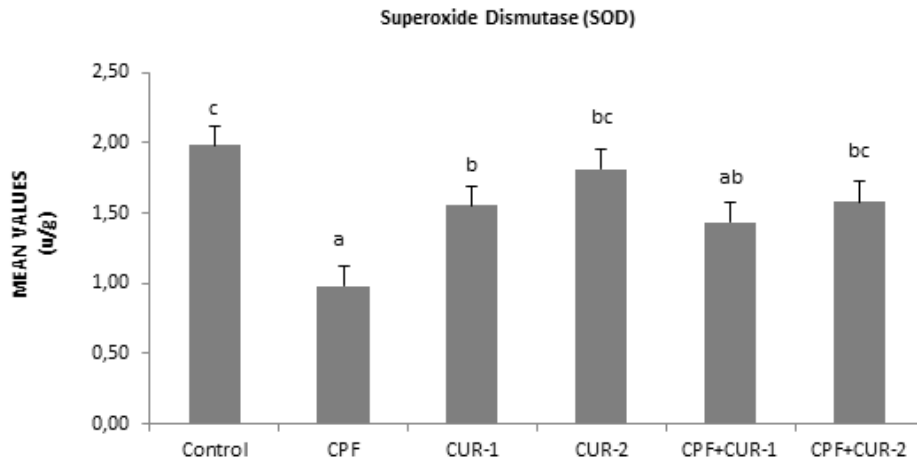
PON is an ester hydrolase that has both aryl esterase and PON activities (aryldialchil phosphatase; OP hydrolase; PON hydrolase) activity. It catalyses hydrolysis of OP compounds used commonly in the production of insecticides (Gan, Smolen, Eckerson, & La Du, 1991). It was reported that PON proteins, which have a broad distribution among mammal species, did not exist in fish, birds and invertebrates, such as those

belonging to the phylum Arthropoda (Walker & Mackness, 1987). In contrast, it was reported that PON was produced in the liver of trout (*Salmo trutta*) (Chemnitius, Losch, Losch, & Zech, 1983) and pacu [*Piaractus mesopotamicus* holmberg (Characidae)] (Bastos *et al.*, 1998), cascudo (*Hypostomus punctatus* Valenciennes, 1840, Loricariidae) and matrinxas [*Brycon cephalus* Günther, 1869, (Characidae)] (Bastos, Alves, Bernardino, Ceccarelli, & Bastos, 2004) and transferred to the blood serum.

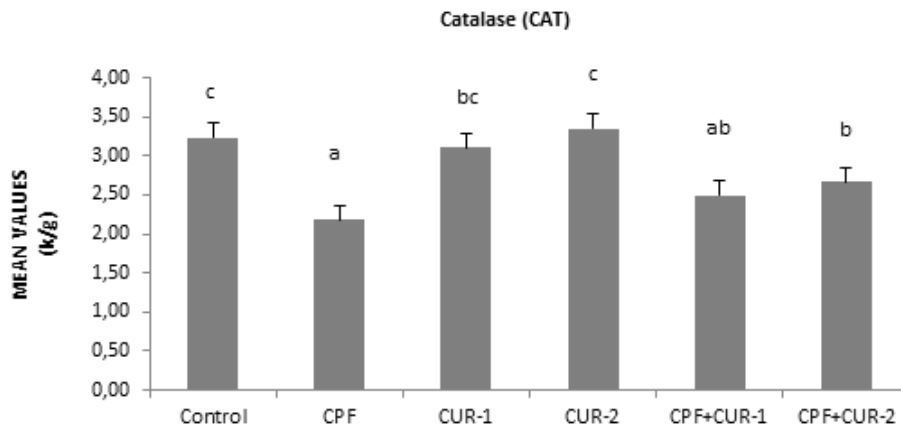
These fish could hydrolyse Ops, such as PON, CPF oxon, pyrimifos oxon and diazinon oxon (Mackness, Arrol, & Durrington, 1991). OP toxicity was higher in birds than mammals given that the serum PON activity was low (Brealey, Walker, & Baldwin, 1980). In this study, PON activity in blood plasma of rainbow trout was determined (Figure 2c). CPF reduced PON activity significantly in comparison to the control group. This situation showed that CPF activity inhibits PON. Li, Furlong and Costa (1995) reported that when rabbit PON was injected into rats, serum PON values increased and protected against CPF toxicity. When both dosages of CUR were compared to the control group, there was a significant difference in PON activity. In other words, CUR could not increase PON activity alone.

The minimal increase of PON activity in CPF and CUR groups showed that CUR protected weakly against the reduction of PON activity. During oxidative stress, PON and total antioxidant activities decreased and TP levels increased (Ece *et al.*, 2007). Vitamin C, which is an important antioxidant (Frei & England, 1989), decreased the loss of PON1 activity based on hypochloride in vitro (Kunes, Cordero-Koning, Lee, & Lynch, 2009). These results were partially in accordance with the results of current study.

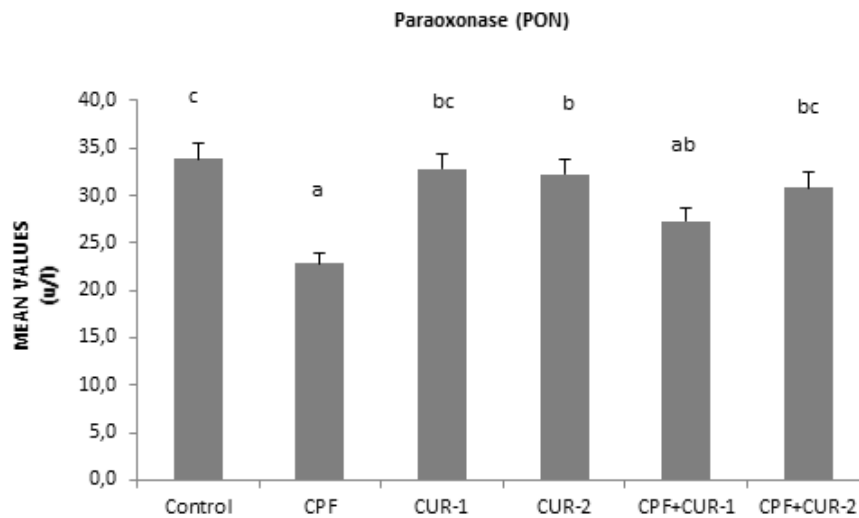
Total antioxidant capacity could be measured indirectly with plasma iron reduction capacity (FRAP) (Benzie & Strain, 1996). FRAP determines the reduction capacity of a metal complex in a sample (Bartosz, 2010). In this study, FRAP was found to be significantly lower compared to the CPF group (Figure 3). The groups in which CPF was administered, the TP level was found to be high. In this study, the similarity between FRAP and antioxidant enzyme levels was determined. FRAP values of groups, in which CUR was administered, were found to be similar to the control groups. However, FRAP measurements had a weak relationship with other antioxidant measurements (Prior, Wu, & Schaich, 2005). In addition, in some situations, high correlations between high FRAP values and polyphenols, which are pro-oxidants, were determined (Prior, Wu, & Schaich, 2005). Some flavones and flavanones were found to have high FRAP values (Cao, Sofic, & Prior, 1997). In this study, results of the CAT test did not correspond to the results of either FRAP or oxidant and antioxidant tests. Consistent with these results, for evaluating toxic stress (the toxic effect of CPF) and the effect of antioxidants (CUR), the FRAP and GSH test used in this study



**Figure 2a.** Effect of CPF and CUR on SOD levels of blood in *O. mykiss* (n=10). Data are presented as means  $\pm$  SD. Superscript letters indicate significant differences among exposure groups ( $P < .05$ ,  $P < .001$ ).



**Figure 2b.** Effect of CPF and CUR on CAT levels of blood in *O. mykiss* (n=10). Data are presented as means  $\pm$  SD. Superscript letters indicate significant differences among exposure groups ( $P < .05$ ,  $P < .001$ ).



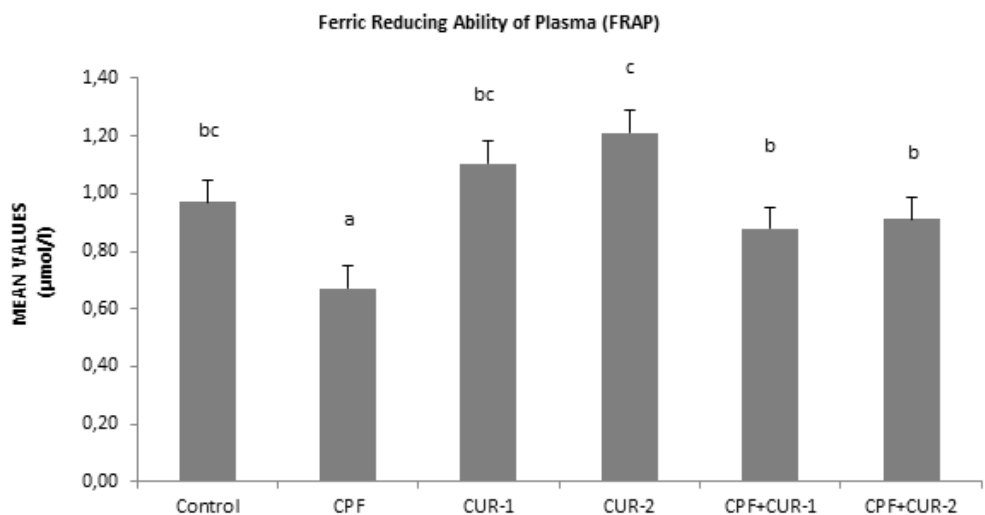
**Figure 2c.** Effect of CPF and CUR on PON levels of blood in *O. mykiss* (n=10). Data are presented as means  $\pm$  SD. Superscript letters indicate significant differences among exposure groups ( $P < .05$ ,  $P < .001$ ).

produced similar results. Despite these significant results, to evaluate the results of the antioxidant effect of the CUR, more parameters are needed. In this study, the measured DNA damage increased in CPF-exposed fishes (Figure 4).

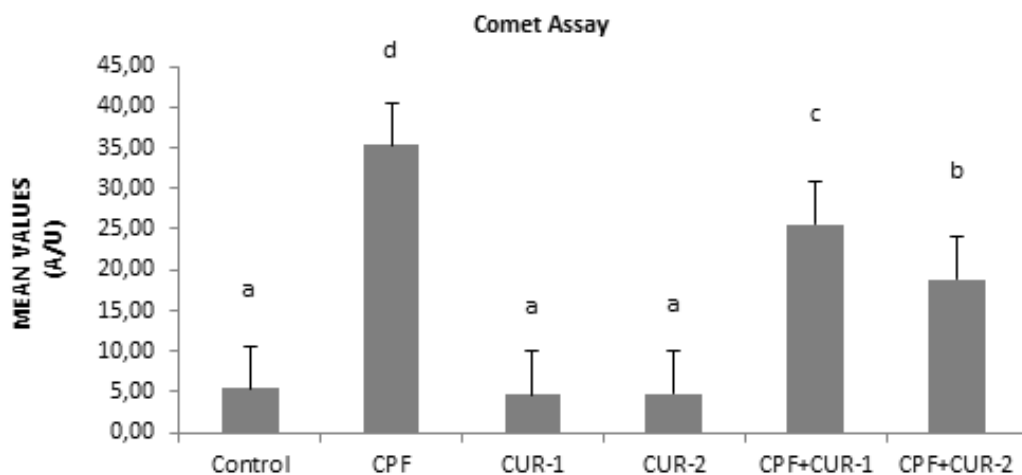
In the current study, CPF exposure caused an increase in comet assay DNA injury levels (Figure 4). CUR applied alone (at both dosages) did not show significant changes compared to the control group. The use of CUR with CPF decreased DNA damage significantly in comparison to CPF alone.

These results show that CPF is a ROS generator in rainbow trout. It was shown in that comet assay that melatonin in rat lymphocytes had antioxidant activity against anti-cancer drugs that were ROS generators (Kim *et al.*, 2005; Arabski *et al.*, 2005). The doses of the CUR did not create a significant change in DNA damage like the antioxidant enzymes analysis of the study.

However, CUR decreased DNA damage significantly in comparison to the CPF group. Hydrogen peroxide has been shown to generate single strand DNA breaks in human bronchial epithelial cells (Gabrielson, Yu, & Spannhake, 1994), human peripheral monocytes and lymphocytes (Allan, Vaughan, Milner, Lunec, & Bacon, 1988; Sandström, 1991) and bovine lens epithelial cells (Kleiman, Spector, & Wang, 1990). Hydrogen peroxide caused DNA damage in blood leukocytes. The results in the present study indicate that the DNA strand breaks caused by CPF were likely to be mediated by  $H_2O_2$  (Diaz-Llera, Gonzalez-Hernandez, Prieto-Gonzalez, & Azoy, 2002). However, clear protective effects of SOD were found against the DNA strand breaks caused by external factors, such as CPF (Cemeli, Baumgartner, & Anderson, 2009). Similarly, nonenzymatic antioxidants, such as vitamin C, vitamin E and carotenoids, were thought to provide protection by



**Figure 3.** Effect of CPF and CUR on FRAP levels of blood in *O. mykiss* (n=10). Data are presented as means  $\pm$  SD. Superscript letters indicate significant differences among exposure groups ( $P < .05$ ,  $P < .001$ ).



**Figure 4.** Effect of CPF and CUR on Comet Assay of blood in *O. mykiss* (n=10). Data are presented as means  $\pm$  SD. Superscript letters indicate significant differences among exposure groups ( $P < .05$ ,  $P < .001$ ).



quenching endogenous or exogenous ROS before they were able to damage the DNA (Duthie, Ma, Ross, & Collins, 1996; Cemeli, Baumgartner, & Anderson, 2009).

Flavonoids are responsible for inhibition of enzymes responsible for  $O_2^-$  production and reduce the effects of free radicals, such as  $O_2^-$ , RO and HO, which have high oxidation power. Kaempferol and quercetin relieve DNA damage, which resulted in the production of  $H_2O_2$  in human lymphocytes (Cemeli, Schmid, & Anderson, 2004). However, it was reported that the pro-oxidant effects of quercetin on mammalian cells were based on the concentration using the comet assay (Anderson, Basaran, Dobrzynska, Basaran, & Yu, 1997).

## Conclusion

In this study, CUR with a flavonoid active component decreased DNA damage by decreasing  $O_2^-$ , RO and HO. We focused on observing the effects of antioxidant enzymes against CPF toxicity. The protective mechanisms of antioxidant enzymes against CPF toxicity with increasing amounts of antioxidants were revealed, and CUR in different amounts could influence antioxidant enzyme activation in rainbow trout blood serum if it was administered alone or with CPF. However, more detailed studies are required for standardisation of antioxidant usage to minimise insecticide toxicity.

## Acknowledgements

We declare that we have no conflicts of interest. This work was supported by the [Harran University Scientific Research Commission (HÜBAK)] under Grant [number K-16213].

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