Protective effect of cinnamic acid on orthophenylphenol-induced oxidative stress in rats

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Abstract

This study aimed to evaluate the protective effect of cinnamic acid (CA) on orthophenylphenol (OPP)-induced oxidative stress in rats. Thirty-two Sprague-Dawley male rats were divided into four groups as control, OPP, CA and OPP + CA groups. The animals in control, OPP and CA groups were received corn oil, OPP (700 mg kg⁻¹ dissolved in corn oil) and CA (200 mg kg⁻¹) by gavage for 21 days, respectively. The animals in OPP + CA group were received CA for 3 days and from day 4; OPP and CA were applied together daily until day 25. Blood and liver samples were collected at the end of experiment for measurement of aminotransferases, creatinine (CREA), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), reduced glutathione (GSH) and malondialdehyde (MDA). The OPP-induced high serum activities of aminotransferases and level of CREA were reduced significantly by CA administration. The OPP induced significant increases of CAT activities and MDA levels in serum and liver tissue samples. Moreover, OPP significantly decreased GSH levels as well as GSH-Px and SOD activities. Pre-treatment with CA ameliorated the GSH levels along with GSH-Px and SOD activities compared to OPP-receiving rats. On the other hand, CAT activities and MDA levels significantly decreased following the pre-treatment with CA compared to OPP-receiving rats. It has been determined that OPP causes oxidative stress and lipid peroxidation in blood and liver tissues and creates changes in anti-oxidant defense enzymes. Pre-treatment with CA reduces lipid peroxidation and provides protective effect against oxidative stress.

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Introduction

Orthophenylphenol (OPP) is a broad-spectrum phenolic substance used as fungicide, anti-bacterial and disinfectant agent in a variety of agricultural, industrial and domestic uses.¹,² During the storage of fruits, especially the protection of citrus fruits, disinfection of storage materials and elimination of pathogens on the surface of fruits and vegetables are the main uses of OPP.³⁻⁵ The OPP is also used as a disinfectant in hospitals, veterinary clinics, poultry and cattle farms, homes and various workplaces.¹,²,⁶ When xenobiotics such as OPP enter the organism, they are activated by cytochrome P450 monoxygenase enzyme system and toxic intermediate are produced and these products bind irreversibly to cellular macromolecules and cause tissue damage.⁷ Compared to some other phenolic derivatives, OPP shows a highly lipophilic and electrophilic structure. These physicochemical properties are extremely important in terms of transporting from membranes and interacting with target macromolecules (e.g., DNA, proteins and lipids).⁴ The OPP is converted to phenyl-hydroquinone (PHQ) and phenylbenzoquinone (PBQ) through microsomal monoxygenase enzyme system and induces reactive oxygen species (ROS) formation. These reactive metabolites have a potential to inhibit thiol (SH)-dependent enzymes. First, they consume intercellular glutathione (GSH) stores and then interact with SH group-containing structures in cells and tissues.⁷

Cinnamic acids (CAs) are a group of aromatic carboxylic acids found naturally in plants.⁸ In the last 10 years, researchers’ interest in CA has increased significantly. Recent studies and reviews have focused on the anti-oxidant, anti-cancer, anti-malarial, anti-fungal,
Materials and Methods

The animal experiments were conducted in accordance with the ethical guidelines for investigations in laboratory animals and were approved by the Animal Experiments Local Ethics Committee of Burdur Mehmet Akif Ersoy University, Burdur, Turkey (Ethics No.: 162-22/12/2015).

Experimental animals and design. Thirty-two Sprague-Dawley male rats, weighing 200-300 g, 10-12 weeks old, were obtained from our local animal facility at Burdur Mehmet Akif Ersoy University, Burdur, Turkey. The animals were kept for 12 hr in light and 12 hr in dark period at 21.00-23.00 °C and 60.00-80.00% humidity. All animals received ad libitum pellet feed and water. The animals were randomly divided into four groups, eight animals in each. The animals in control group were given 1.00 mL of corn oil for 21 days by gavage. The OPP group was administered 700 mg kg⁻¹ OPP (dissolved in corn oil) and CA group was administered 200 mg kg⁻¹ CA (dissolved in 0.10% dimethyl sulfoxide) by gavage for 21 days. In OPP + CA group, rats received 200 mg kg⁻¹ CA 3 days prior to OPP, from day 4, 700 mg kg⁻¹ OPP + 200 mg kg⁻¹ CA were applied daily by gavage until day 25. The OPP and CA doses used in the experiment were determined as a result of conducted preliminary experiments, following literature evaluation. At the end of experiment, blood samples were withdrawn from the animals via cardiac puncture under isoflurane (2.00-3.00%) anesthesia. Blood samples were collected in tubes with and without EDTA. All animals were euthanized by cervical dislocation. The abdominal area was opened, and the liver was removed immediately by dissection and then, washed in ice-cooled isotonic saline.

Serum aminotransferases and creatinine levels determination. Blood samples taken directly into the tubes were centrifuged at 4,000 rpm for 10 min. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatinine (CREA) levels were measured by an autoanalyzer (Gesan chem 200 Gesan Production srl, Campobello, Italy). The ALT and AST activities were detected according to kinetic UV optimized international methods and CREA levels were detected according to Jaffé method.

Blood and liver tissue samples preparation. Blood samples collected into tubes containing K₂EDTA were centrifuged for 5 min at 4,000 rpm at 4.00 °C. The plasma fraction was separated and stored at −20.00 °C until analysis. The erythrocyte fraction was centrifuged at 4,000 rpm for 5 min by adding three times of its volume of saline phosphate buffer solution (pH: 7.40) and the supernatant was discarded. The same procedure was repeated three times. Then, 1:1 volume of saline phosphate buffer solution was added to erythrocytes and samples were stored at −20.00 °C until analysis. Liver tissues were washed primarily with 0.90% ice-cold isotonic saline. The tissues were homogenized (1/10 w/v) with phosphate buffer saline (prepared as: 140 mM KCl, 10.00 mM NaHCO₃, 3.00 mM KH₂PO₄ and 2.00 mM K₂HPO₄ L⁻¹; dissolved in 950 mL of deionized water, adjusted to pH: 7.20 with 5.00 N NaOH and completed to 1,000 mL). The homogenate was then centrifuged at 15,000 rpm for 45 min (4.00 °C) and the supernatant was separated. Samples were stored at −20.00 °C until analysis. The erythrocytes, plasma samples and tissue homogenate supernatants were used to evaluate the GSH and malondialdehyde (MDA) levels and superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) activities.

Anti-oxidant enzyme activities determination. The CAT activities in erythrocyte and tissue homogenate samples were measured according to the method reported by Aebi. The principle of this method is based on the degradation of hydrogen peroxide added to the medium by the CAT present in the samples. Fifty µL of hemolysate or homogenate was taken and diluted 1:5 by adding 200 µL distilled water. By taking 50.00 µL of this mixture, 4,950 µL phosphate buffer was added and a second dilution was made in the ratio of 1:100 (total dilution ratio of 1:500). Simultaneously, 2.00 mL of supernatant was taken for the blank and sample, 1.00 mL of phosphate buffer solution was added to the blank and 1.00 mL of hydrogen peroxide-phosphate buffer solution was added to the sample. Absorbances were read at 240 nm against the blank at 0 and 30 sec in the spectrophotometer (UV-1700; Shimadzu, Kyoto, Japan). The CAT activities in erythrocyte and tissue homogenate samples were expressed as the rate constant of a first-order reaction (k) g⁻¹ of Hb and (k) g⁻¹ of protein, respectively. The GSH-Px activities in erythrocyte and tissue homogenate samples were measured according to the method reported by Paglia and Valentine. The principle of method is based on the oxidation of reduced GSH using GSH reductase via NADPH. One hundred µL Tris-EDTA (prepared with 1.00 M Tris buffer and 5.00 µM EDTA), 20.00 µL 1M reduced GSH, 100 µL 2.00 µM NADPH and 100 µL 10.00 U GSH reductase were added to the
blank and sample tubes. Hemolysate or homogenate (10 µL) were added into the sample tubes and kept at 37.00 °C for 10 min. The reaction was initiated by adding 7.00 µM of t-butylhydroperoxide and absorbances were read at 340 nm against the blank at 0 and 150 sec in the spectrophotometer. The activities of GSH-Px were expressed in erythrocyte and tissue homogenate samples as U g⁻¹ of Hb and U g⁻¹ of protein, respectively.

The SOD activities in plasma and tissue homogenate samples were performed using a commercial kit (Rat super oxidase dismutase ELISA Kit EA0168Ra, Bioassay Technology Laboratory, Shanghai, China). The analysis was performed according to the kit procedure. Fifty µL of the standard solutions and plasma/tissue samples were added to the wells (in one of the wells, there was only diluted solution), then, biotinylated antigen was added, and wells were kept for 60 min at 37.00 °C. The wells were washed five times with buffer solution. Then, 50.00 µL of avidin-horseradish peroxidase was added to the wells and wells were kept at 37.00 °C for 60 min. The wells were washed five times with buffer solution again. Fifty µL of substrate A and then, 50.00 µL of substrate B were added to each well and wells were kept in darkness for 10 min. Fifty µL of stop solution was added to the wells and mixed well and absorbances were read at 450 nm. The SOD activities in plasma and tissue homogenate samples were expressed as ng L⁻¹ and ng mg⁻¹ protein, respectively.

**Reduced GSH determination.** Reduced GSH levels in erythrocyte and tissue homogenate samples were measured according to the method reported by Sedlak and Lindsay. The principle of method is based on the formation of 5-thio-2-nitrobenzoic acid by reacting 5,5’-dithio-bis-(2-nitrobenzoic acid) (DTNB) with GSH and a thiol compound and breaking the disulfide bond. Seven hundred µL 15.00% trichloroacetic acid (TCA) and 700 µL sample (distilled water for the blank) were taken and centrifuged at 2,500 rpm for 5 min. Then, 500 µL of supernatant was taken and 200 µL of Tris-EDTA and 100 µL of 0.01 M DTNB were added and kept at room temperature for 5 min. Absorbance at 412 nm was read on the spectrophotometer against the blank. The GSH levels were expressed in erythrocyte and tissue homogenate samples as µmol g⁻¹ of Hb and µmol g⁻¹ of protein, respectively.

**Lipid peroxidation determination.** The measurements of MDA in erythrocyte were done as reported by Yoshioka et al., and the determined lipid peroxide is referred as MDA. The principle of method is based on the spectrophotometric measurement of the pink product formed by MDA, the end product of thiobarbituric acid (TBA) and lipid peroxidation under acidic conditions. The hemolysate was diluted 1:5 and 0.50 mL was taken. Distilled water was used for the blank. Then, 0.50 mL of phosphate buffer and 0.50 mL of 15.00% TCA were added to diluted hemolysate and blank and kept on ice pouches for 2 hr. They were centrifuged for 10 min at 4,000 rpm at 4.00 °C. One mL of supernatants were taken, added to 75.00 µL of 0.10 M EDTA and 250 µL of 1.00% TBA solution (prepared in 0.05 N NaOH), mixed and kept in a boiling water bath for 15 min. Absorbances at 532 nm were read on the spectrophotometer against blank. The MDA level was expressed in erythrocyte as nmol g⁻¹ of Hb. The measurements of MDA in tissue homogenate samples were done as reported by Ohkawa et al., and the determined lipid peroxide is referred as MDA. One hundred µL of 8.10% sodium dodecyl sulfate and 200 µL of tissue homogenate were mixed and kept at room temperature for 10 min. Distilled water was used for the blank. After that, 750 µL 20.00% acetic acid (pH: 3.50) and 750 µL 0.60% TBA were added to samples and blank. They were left in the boiling water bath for 60 min and then, left to cool at room temperature. Then, 2.50 mL of butanol: pyridine (15:1) mixture was added. The organic (pink) layer was separated and the absorbance at 532 nm was read against the blank on the spectrophotometer. The MDA level was expressed in tissue homogenate samples as nmol g⁻¹ of protein.

**Hepatic total proteins determination.** Total protein was measured according to the Biuret method reported by Gornall et al. The principle of method is based on the formation of blue colored complexes with nitrogen atoms in the structure of proteins with Cu²⁺ in Biuret solution and measuring the absorbance of this complex at 540 nm.

**Statistical analyses.** Data were expressed as mean ± standard error of the mean. All data were edited with the SPSS (version 22.0; IBM Corp., Armonk, USA). The results were analyzed using one way analysis of variance (ANOVA) followed by Tukey’s test for comparison between different treatment groups. The p < 0.05 was considered as statistically significant.

**Results.**

In OPP group, a significant increase was observed in serum AST and ALT activities and CREA level compared to the control group (p < 0.05). In OPP + CA group, it was determined that there was a significant decrease in AST and ALT activities and CREA level compared to the OPP group (p < 0.05), (Table 1).

In blood and liver tissue samples, in OPP group, a significant increase in CAT activities and MDA levels (p < 0.05) and significant decrease in GSH levels as well as GSH-Px and SOD activities were observed compared to the control group (p < 0.05). In OPP + CA group, a significant decrease in CAT activities and MDA levels (p < 0.05) and significant increase in GSH levels and SOD and GSH-Px activities were detected compared to OPP group (p < 0.05), (Tables 2 and 3).
Table 1. Effects of orthophenylphenol (OPP), cinnamic acid (CA) or their combination on liver and kidney function. Values represent as mean ± SEM of eight rats per group.

| Parameters          | Control       | OPP          | CA            | OPP + CA       |
|---------------------|---------------|--------------|---------------|----------------|
| AST (U L⁻¹)         | 139.67 ± 2.91ᵃ | 319.52 ± 3.92ᵈ | 160.13 ± 3.41ᵇ | 245.76 ± 3.13ᶜ |
| ALT (mg dl⁻¹)       | 79.14 ± 1.18ᵃ  | 166.86 ± 5.15ᶜ | 79.88 ± 2.11ᵃ  | 116.38 ± 0.50ᵇ |
| CREA (mg dl⁻¹)      | 0.48 ± 0.01ᵃ   | 4.58 ± 0.21ᶜ  | 0.48 ± 0.01ᵃ   | 0.96 ± 0.02ᵇ   |

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase
ᵃᵇᶜᵈ Values carrying different letters on the same row are statistically significant (p < 0.05).

Table 2. Effects of orthophenylphenol (OPP), cinnamic acid (CA) or their combination on oxidative stress biomarkers in the blood. Values represent as mean ± SEM of eight rats per group.

| Parameters          | Control       | OPP          | CA            | OPP + CA       |
|---------------------|---------------|--------------|---------------|----------------|
| CAT (k g⁻¹ of Hb)   | 15.98 ± 0.27ᵃ | 143.12 ± 2.90ᵈ | 24.12 ± 0.57ᵇ | 65.62 ± 0.92ᶜ  |
| GSH-Px (U g⁻¹ of Hb)| 169.67 ± 3.32ᵃ | 34.60 ± 1.29ᵃ | 184.45 ± 2.37ᵈ | 217.70 ± 26.76 |
| SOD (ng L⁻¹)        | 2,081.30 ± 36.54ᶜ | 674.70 ± 3.88ᵃ | 2,141.00 ± 8.40ᶜ | 1,455.00 ± 34.99ᵇ |
| GSH (µmol g⁻¹ of Hb)| 2.69 ± 0.03ᵈ   | 0.31 ± 0.02ᵃ   | 2.33 ± 0.07ᶜ   | 1.77 ± 0.04ᵇ   |
| MDA (nmol g⁻¹ of Hb)| 126.85 ± 3.22ⁿ | 573.20 ± 13.65ᶜ | 151.75 ± 4.09ᵃ | 318.48 ± 2.75ᵇ  |

CAT: Catalase; GSH-Px: Glutathione peroxidase; SOD: Superoxide dismutase; GSH: Glutathione; MDA: Malondialdehyde; Hb: Hemoglobin.
ᵃᵇᶜᵈ Values carrying different letters on the same row are statistically significant (p < 0.05).

Table 3. Effects of orthophenylphenol (OPP), cinnamic acid (CA) or their combination on oxidative stress biomarkers in the liver. Values represent as mean ± SEM of eight rats per group.

| Parameters          | Control       | OPP          | CA            | OPP + CA       |
|---------------------|---------------|--------------|---------------|----------------|
| CAT (k g⁻¹ protein) | 171.68 ± 6.26ᵃ | 1,770.10 ± 8.74ᵈ | 373.18 ± 8.32ᵇ | 836.80 ± 5.54ᶜ |
| GSH-Px (U g⁻¹ protein)| 267.79 ± 6.9³ᶜ | 56.40 ± 0.41ᵃ   | 269.82 ± 4.26ᶜ | 128.22 ± 1.77ᵇ |
| SOD (ng mg⁻¹ protein)| 2,423.90 ± 46.6³ᶜ | 946.09 ± 22.20ᵃ | 2,350.50 ± 40.89ᶜ | 1,475.70 ± 21.19ᵇ |
| GSH (µmol g⁻¹ protein)| 3.197 ± 1.44ᶜ  | 6.18 ± 0.22ᵃ    | 29.43 ± 1.46ᶜ  | 17.19 ± 0.49ᵇ   |
| MDA (nmol g⁻¹ protein)| 1,087.70 ± 0.07ᵃ | 3,217.70 ± 26.76ᵈ | 1,215.40 ± 38.38ᵇ | 2,135.10 ± 4.77ᶜ |

CAT: Catalase; GSH-Px: Glutathione peroxidase; SOD: Superoxide dismutase; GSH: Glutathione; MDA: Malondialdehyde.
ᵃᵇᶜᵈ Values carrying different letters on the same row are statistically significant (p < 0.05).

Discussion

The OPP is a broad spectrum anti-microbial agent widely used in fungicides and disinfectants, especially for the protection and storage of citrus fruits after harvest.⁴⁷,²¹ Because of the high exposure risk of consumers due to its widespread use, this compound has been the subject of many studies in living organisms. The strong acute and chronic toxicity, mutagenicity, teratogenicity and genotoxicity of OPP are investigated by in vivo and in vitro studies.⁴⁷,²¹,²² Although the mechanism of action is not fully elucidated, it has been reported that OPP induces ROS formation in rats, causing damage to the liver, bladder and kidney tissues.²¹ The CA is an anti-oxidant known to have protective and/or inhibitory effects against oxidative stress, cancer and heart and inflammatory diseases in vitro and in vivo. The anti-oxidant activity of CA and analogues on the oxidative stress is based on the stabilization of the resulting phenoxy radical by giving an electron or hydrogen atom to the medium. They strongly inhibit lipid peroxidation and show their activity through radical scavenging.²³

In the present study, ALT and AST activities and CREA level were increased in OPP group. Nakagawa and Tayama⁷ have given a single oral dose of 700 or 1,400 mg kg⁻¹ OPP to rats and measured serum ALT and AST activities at 6 and 24 hr. According to the results of their study, there was a slight increase in ALT and AST activities compared to the control group; but this result was not significant. The results of the study do not match totally with our results due to the acute single dose OPP administration and the short intervals between the detection of serum ALT and AST activities. In our study, there was a significant decrease in ALT and AST activities and CREA levels in OPP + CA group compared to OPP group. Tohamy et al.²⁴ have conducted a study on the protective effect of CA on cis-platinum-induced liver injury in mice and have reported that cis-platinum elevates ALT and AST activities, and CREA level was decreased in the cis-platinum + CA group. El-Sayed et al.²⁵ have reported that there is an increase in CREA level in the cis-platinum group and a decrease in the cis-platinum + CA group. These results are similar to ours.

In this study, in blood and liver tissue samples, a significant increase in CAT activities and MDA levels and a significant decrease in GSH level decreases in the OPP group. Li et al.²² have reported that OPP causes a significant decrease in GSH levels in HepG2 cells. This result is consistent with our study, although the
experiment was in vitro. In vivo and in vitro studies have shown that OPP significantly reduces GSH levels.

Here, in OPP + CA group, SOD and GSH-Px activities and GSH levels increased and there was a decrease in MDA levels and CAT activities in blood and liver tissue samples compared to OPP group. Patra et al.,26 have investigated the protective effect of CA at different doses against cyclophosphamide-induced oxidative damage in bone marrow and spleen in mice. Researchers found a decrease in MDA level and an increase in CAT, SOD and GSH-Px activities in cyclophosphamide + CA-treated groups. Abd El-Raouf et al.,27 have investigated the protective effects of CA and cinnamaldehyde on cis-platinum-induced spleen injury in rats. They have reported an increase in MDA level and a decrease in CAT activity in the cis-platinum-treated group. Also, they found that in cis-platinum + CA group, CAT activity and MDA level were close to the levels of control group. Tohamy et al.,28 have studied the protective effects of CA against cis-platinum-induced liver damage in mice. In liver tissues, they found an increase in MDA level and a decrease in GSH level in the cis-platinum-treated group. Researchers have also indicated that in cis-platinum + CA group, there is a decrease in MDA level and an increase in GSH level. Yan et al.,29 have examined the protective effects of CA and syringic acid against ethanol-induced liver damage in their study. They have reported that decreases in GSH-Px and CAT activities and GSH level in the ethanol-treated group are improved in the ethanol + CA groups and approached to the levels of the control group. The researchers have stated that CA has protective effects against oxidative damage. In our study, we also found that CA had a protective effect against OPP-induced oxidative stress.

In the present study, due to OPP-induced oxidative stress, a depletion of GSH level, decreases in SOD and GSH-Px activities and increases in lipid peroxidation products, MDA level and CAT activity were observed. Following treating the groups with OPP plus CA, an increase in GSH, SOD and GSH-Px values and a decrease in CAT and MDA values were seen. In their study, Nakagawa and Tayama7 have reported that OPP is converted to PHQ and PBQ via in vitro microsomal monooxygenase system; these metabolites are very reactive and have potential to inhibit SH-dependent enzymes. They have also indicated that PHQ and PBQ first deplet intercellular GSH storage and then they attack against structures containing cysteine. In their in vitro study, Murata et al.,28 have reported that excessive hydrogen peroxide is formed during the conversion of OPP to metabolites and this excessive hydrogen peroxide causes DNA damage in other tissues, resulting in mutagenic and carcinogenic effects.

The living organisms try to deal with oxidative stress by showing an increase or decrease in anti-oxidant enzyme systems. On the one hand, free radicals produced by organisms are broken down by anti-oxidant enzymes and converted into less harmful or harmless metabolites; on the other hand, changes occur in the form of increase or decrease in anti-oxidant enzymes due to the production of free radicals above normal conditions. In this study, the formation of superoxide anion and its conversion to hydrogen peroxide via SOD enzyme during the metabolism of OPP (conversion to PBQ and PHQ) may be the reason of the decrease in SOD enzyme levels. This may explain the decrease in the level of GSH-Px, since when the amount of hydrogen peroxide is low, it is detoxified by the GSH-Px. On the other hand, as a consequence of oxidative damage, defense system can promote the expressions of these enzymes. The increase in the CAT enzyme activity may be due to the fact that as the amount of hydrogen peroxide continues to increase in the environment, the organism can stimulate the anti-oxidant enzyme system to deal with it.

The findings of the study showed that OPP caused oxidative damage in blood and liver tissues at the dose administered and the period indicated. The CA did not cause any harm on its own and in the study group in which it was given with OPP; CA created a regressive effect on oxidative stress parameters. It has been concluded that CA can be used as an anti-oxidant agent against oxidative damage induced by OPP or similar substances with similar structure and mechanism of action.

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Conflict of interest

The authors declare no conflict of interest.

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