

Can crocin play a preventive role in Wistar rats with carbon tetrachloride-induced nephrotoxicity?

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ABSTRACT

Objective(s): To investigate protective role of crocin by attempting to create nephrotoxicity with carbon tetrachloride.

Materials and Methods: Ethics committee approval was obtained and 50 male Wistar rats were randomly divided into 5 groups that included 10 rats each: Control, Corn oil, Crocin, Carbon tetrachloride (CCl₄), and Crocin + Carbon tetrachloride. Following the experiments, the rats were decapitated under anesthesia and incised kidney tissues were subjected to biochemical and histological examinations.

Results: In the CCl₄ administered group, MDA, TOS, Bun, and creatinine levels increased, GSH, SOD, CAT, and TAS levels decreased ($P \leq 0.05$), glomerular collapse in kidney sections, narrowing and local occlusion in Bowman's space in certain glomeruli, inflammatory cell infiltration and congestion were observed when compared to all other groups. There was a significant decrease in increased MDA, TOS, Bun, and creatinine levels, and a significant increase in decreased GSH, SOD, CAT, and TAS levels in CCl₄ + crocin administered group compared to the CCl₄ group ($P \leq 0.05$), local minimal glomerular damage, tubular damage, inflammatory infiltration, and vascular collagen symptoms were observed in kidney sections, however significant improvement was observed in damage findings when compared to the CCl₄ group.

Conclusion: At this dose and time interval, against a highly toxic chemical such as CCl₄, crocin was able to suppress oxidative stress by playing a protective role in the kidney tissue.

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Introduction

Renal toxicity occurs due to drug or chemical reagent intake and is among the most common kidney problems (1, 2). Most studies conducted on environmental toxins have focused on CCl₄. Carbon tetrachloride is a nonpolar compound due to its geometric symmetry; therefore, it can easily dissolve in non-polar compounds such as oils, fats, and iodine (3, 4). Previous studies demonstrated that CCl₄ toxicity could induce free radical production in liver, kidney, lung, testis, heart, brain, and blood cell tissues (5). Carbon tetrachloride poisoning results in rat models influenced by oxidative stress under many physiological conditions. The most significant step in CCl₄ induced tissue damage is cytochrome P450 transfer, which transfers one electron to the carbon-chlorine bond, generating an unstable intermediary anionic radical that eliminates chlorines to produce the central carbon radical and leads to the formation of trichloromethyl-chloride radical. CCl₃ radical could bond with macromolecules or attack fatty acids and lipids in the membrane. CCl₃ radical could contrast with oxygen by transformation into peroxy tri-chloromethyl (CCl₃O₂) free radicals that are more reactive when compared to CCl₃ and could lead to similar damage or

destruction (5). To prevent ROS induced damage, living organisms possess an antioxidant system that includes non-enzymatic antioxidants, catalase, superoxide dismutase, and peroxidase enzymes (6). Furthermore, other synthetic or natural ROS scavengers might reduce the prevalence of free radical-mediated diseases in addition to the abovementioned natural antioxidants. Antioxidants are increasingly used in prevention and treatment of various diseases and increasing number of studies on antioxidant molecular activities, such as polyphenols and carotenoids have been conducted (7-9). The action of antioxidants against diseases seems to be through the increase in endogenous antioxidant enzyme levels and the decrease in lipid peroxidation (9, 10).

Natural antioxidants could protect the body against the negative effects of toxins such as carbon tetrachloride (11, 12). Throughout human history, medicinal plants were used to cure several diseases, however, since the mid-20th century, use of synthetic drugs became highly popular (13). Determination of the adverse effects of synthetic drugs on public health led to an increasing trend of application of plants with medicinal properties as synthetic drug alternatives (14, 15).

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Crocus sativus L., commonly known as saffron, is a plant indigenous to various regions in the world, especially Iran, Spain, and Turkey. Crocin is an easily soluble yellow colored active ingredient in *C. sativus* L., along with safranal (16). In a number of *in vitro* and *in vivo* studies, it was reported that crocin had anti-neuropathic (17), radical scavenging (18), antitumor (19), antidepressant, and antioxidant effects (20-23). In addition to these effects, saffron and its active ingredient crocin inhibit ischemia in kidney tissue (24).

The present study aimed to demonstrate the oxidative stress due to CCl₄ induced ROS products and to investigate the changes that accompany antioxidant structures in order to detail the changes that occur in kidney tissue.

Materials and Methods

Experimental animals

In the present study, 50 male Wistar albino rats (225–250 g) were procured from Inonu University, Faculty of Medicine, Experimental Animal Breeding and Research Center (INUTF-DEHUM). The authors initiated the study after obtaining approval of the Experimental Animal Ethics Committee (2016/A-60). Drinking water was provided on a daily basis and the cage cleaning was performed on a daily basis as well. The rats were kept in cages with an ambient temperature of 21 °C, ambient humidity of 55–60%, under 12 hr light/ dark cycle (light from 08:00 to 20:00). Rats were fed standard pellet feed *ad libitum* in this study.

Experimental Design

Fifty male Wistar rats, obtained from the experimental animal unit were randomly divided into five groups of 10 animals each. These groups were;

1st group (Control group); 1 ml/kg/day physiological saline solution was administered.

2nd group (Corn oil group); 1 ml/kg/day corn oil was administered.

3rd group (Crocins group); 100 mg/kg/day crocin was administered (Sigma-17304).

4th group (CCl₄ group): 1: 1 carbon tetrachloride was dissolved in corn oil and applied 0.5 ml/kg every other day.

5th group (CCl₄+crocin group): 100 mg/kg/day crocin and 1:1 carbon tetrachloride was dissolved in corn oil and applied 0.5 ml/kg every other day.

All chemical applications were repeated regularly for 15 days at the same hour every day orally (via gavage).

Preparation of the tissues for biochemical analyses

Deep frozen (-80 °C) kidney tissues were removed from the storage unit and weighed on the day of the experiment. Phosphate buffer was added on the tissues to obtain a 10% homogenate and the product was homogenized for 1–2 min at 12,000 rpm in ice (IKA, Germany). Tissue homogenates were centrifuged at 5000 rpm at 4 °C for 30 min to obtain the supernatant.

Measurement of malondialdehyde (MDA) Level

MDA analysis was conducted in accordance with a method developed by Uchiyama *et al* (25). The MDA concentration was determined by spectrophotometry; by measuring the supernatant extracted from the

n-butanol phase of the product with pink color, which was the result of the reaction between the MDA in the supernatant and thiobarbituric acid at 95 °C at 535 and 520 nm.

Reduced glutathione (GSH) level measurement

GSH analysis was conducted with a method developed by Ellman (26). The light intensity of the greenish color at 410 nm wavelength that was produced by the reaction between the GSH and 5,5'-dithiobis 2-nitrobenzoic acid in the analysis tube was read to determine the GSH level.

Superoxide dismutase (SOD) level measurement

The total reduction of nitroblue tetrazolium by the superoxide anion that was a product of xanthine and xanthine oxidase was used to determine SOD activity (27). SOD activity unit was taken as the quantity of protein inhibiting the rate of NBT reduction by 50% and presented in units per milligram protein. The total protein content of the kidney tissue homogenate was determined according to a method by Lowry *et al* (28).

Measurement of the catalase (CAT) level

CAT activity was determined with Aebi's method (29). The constant rate k was determined by the absorbance of H₂O₂ (initial concentration 10 mM) at 240 nm.

Total oxidant status (TOS) level measurement

TOS measurements were conducted by measuring the absorbance of 500 µl reagent 1 (measurement buffer) and 75 µl serum mixture at 530 nm, adjusting the ELISA to 25 °C as indicated in the kit procedure. 25 µl reagent 2 (pro-chromogenic solution) was added and incubated for 10 min. Then, TOS levels were determined by measuring the absorbance at 530 nm once more (30).

Total antioxidant status (TAS) level measurement

TAS measurements were conducted by mixing 500 µl reagent 1 (measurement buffer) and 30 µl serum as described in the kit procedure, the ELISA was set at 25 °C and the absorbance was measured at 660 nm. Then, 75 µl reagent 2 (colored ABTS solution) was added to the mixture and incubated for 10 min. The absorbance was measured once more at 660 nm after incubation to determine the TAS levels (31).

Measurement of urea and creatine levels

Blood samples were transferred into ethylene-diamine-tetra-acetic acid tubes and moved onto ice to measure plasma urea and creatine levels. After collection, the tubes were centrifuged within a few min and stored at 70 °C until the experiment. Plasma urea and creatine levels were measured with commercial Architect c 1600 automatic analyzer kits (Abbott, Abbott Park, Illinois, USA).

Histological analysis

The kidney samples were fixed in 10% buffered formalin for routine paraffin embedding. 6 µm tissue sections were cut, mounted on slides, stained with hematoxylin-eosin (H-E), and examined using a Nikon Optiphot-2 light microscope and Nikon DS-Fi2 camera and NIS-Elements Image Analysis System (Nikon Corporation, Tokyo, Japan). Semi-quantitative

Table 1. Kidney tissue oxidant-antioxidant parameters of all groups

Groups	MDA (nmol/gwt)	GSH (nmol/gwt)	SOD (U/g protein)	CAT (U/g protein)	TAS (mmol/l)	TOS (μmol/l)
1	693.9 ± 35 ^{ab}	558.9 ± 48.4 ^a	37.3 ± 4.41 ^a	40.7 ± 2.19 ^a	1.25 ± 0.09 ^a	23.57 ± 1.48 ^a
2	700.7 ± 66.6 ^a	567.2 ± 72.9 ^a	39.09 ± 2.92 ^a	40.39 ± 2.05 ^a	1.25 ± 0.10 ^a	23.17 ± 2.65 ^a
3	629.8 ± 61.1 ^b	766.2 ± 41.9 ^b	58.16 ± 1.78 ^b	50.3 ± 2.13 ^b	2.39 ± 0.24 ^b	17.47 ± 1.73 ^b
4	916.8 ± 52.6 ^c	483.5 ± 43 ^c	31.2 ± 3.84 ^c	23.02 ± 2.98 ^c	0.67 ± 0.10 ^c	34.84 ± 2.19 ^d
5	779.1 ± 55.9 ^d	559.5 ± 62.5 ^a	43.2 ± 2.02 ^d	42.5 ± 1.98 ^a	1.76 ± 0.13 ^d	24 ± 1.96 ^a

1; Control, 2; Corn Oil, 3; Crocin, 4; CCl₄, 5; CCl₄ + Crocin; Data are expressed as mean±SD of ten animals. gwt; gram wet tissue; Different letters in columns are significant P<0.05; MDA: Malondialdehyde; GSH: Reduced glutathione; SOD: Superoxide dismutase; CAT: Catalase; TAS: Total Antioxidant status; TOS: Total Oxidant Status

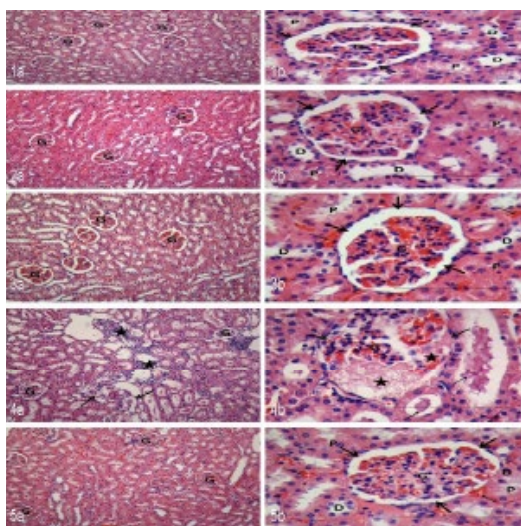


Figure 1. Histological images of kidneys tissue

- 1a: Control group: Glomerular capillary (G). H-E, x10
- 1b: Control group: Glomerular capillary (G), distal tubule (D), proximal Tubule (P), Bowman space (arrow). H-E, x40.
- 2a: Corn Oil Group: Glomerular capillary (G). H-E, x10
- 2b: Corn Oil Group: Glomerular capillary (G), distal tubule (D), proximal tubule (P), Bowman space (arrow). H-E, x40
- 3a: Crocin Group Glomerular capillary (G). H-E, x10
- 3b: Crocin Group: Glomerular capillary (G), distal tubule (D), proximal tubule (P), Bowman space (arrow). H-E, x40
- 4a: CCl₄ Group: Glomerular capillary collapse (G), inflammatory cellular infiltration (asterisk), tubular degeneration (arrow). H-E, x10
- 4b: CCl₄ Group: Glomerular capillary (G), occlusion in Bowman space (thick arrow), Glomerular capillary degeneration (asterisk), eosinophilic material accumulation in the tubule lumen (narrow arrow). H-E, x40
- 5a: CCl₄+Crocini Group: Glomerular capillary (G). H-E, x10
- 5b: CCl₄+Crocini Group: Glomerular capillary (G), distal tubule (D), proximal tubule (P), Bowman space (thick arrow), capillary congestion (narrow arrow). H-E, x40

determination of the mean glomeruli size was conducted by measuring the dimension of a minimum of 100 glomeruli in each section. A minimum of 20 fields were evaluated at 20X magnification to calculate the mean glomeruli count.

Histological error score analyses

Glomerular, tubular and interstitial changes were graded to score the kidney damage. Glomerular damage (capillary collapse, narrowing or disappearance of the Bowman's space) was evaluated as: 0, absent; 1, damage on <25% of the glomeruli; 2, damage on 25–50% of the

glomeruli; 3, damage on >50% of the glomeruli. The grading for tubular injury was scored as: 0, absent; 1, <25% of the tubules injured; 2, 25–50% of the tubules injured; 3, >50% of the tubules injured. The presence of inflammation and vascular congestion were judged as: 0, absent; 1, mild; 2, moderate; and 3, severe (maximum total score = 12).

Statistical analysis

Statistical analysis was conducted with SPSS software (v.21). Data were presented as mean and standard deviation. The homogeneity of the variances was tested with the Levene test. For homogenous group variances, one-way analysis of variance and Tukey HSD posthoc test were used, otherwise, Welch test and Games-Howell *post hoc* test were used. Level of significance was accepted as 0.05.

Results

No statistically significant difference was observed between the control group and the corn oil group biochemical parameters. In the CCl₄ group, we found that there was a statistically significant increase in MDA, TOS, BUN, and creatine levels, and decrease in GSH, SOD, CAT, and TAS levels compared to all other groups. In the crocin group, we found a statistically significant increase in GSH, SOD, CAT, and TAS levels compared to all other groups. In the CCl₄ + Crocin group, we found that MDA, TOS, BUN, and creatine levels that increased in the CCl₄ group decreased statistically significantly (P≤0.05) in the examined biochemical parameters when compared to the CCl₄ group (Tables 1 and 2).

Glomerular and tubular structures in control group kidney sections were histologically normal. Rare and minimal inflammatory cell infiltration, tubular epithelial cell damage, and congestion were observed in the corn oil and crocin groups. CCl₄ group kidney sections demonstrated glomerular collapse, narrowing of the Bowman's space in certain glomeruli, and occlusion. Partial capillary degeneration was detected in certain glomeruli. Different levels of inflammatory cell infiltration and congestion were determined in most sections. Majority of renal tubules demonstrated epithelial damage at different densities. The accumulation of eosinophilic material in the lumen of some tubules was of interest. In the kidney sections of the CCl₄ + Crocin group, local minimal glomerular damage, tubular damage, inflammatory infiltration, and vascular collagen findings were observed, but these lesion findings demonstrated a significant improvement when compared to the CCl₄ group (Figure 1).

Table 2. Serum bun and creatinine levels of all groups

Groups	Bun (mg/dl)	Creatinine (mg/dl)
1	20.8 ± 0.91 ^a	0.45 ± 0.03 ^a
2	21.8 ± 2.15 ^a	0.54 ± 0.07 ^b
3	14.8 ± 1.7 ^b	0.55 ± 0.07 ^b
4	36.6 ± 3.43 ^c	1.38 ± 0.15 ^c
5	20.9 ± 2.72 ^a	0.85 ± 0.07 ^d

1; Control, 2; Corn Oil, 3; Crocin, 4; CCl₄, 5; CCl₄ + Crocin
Data are expressed as mean±SD of ten animals

Different letters in columns are significant P<0.05

Examination of the histologic error score demonstrated that there was no difference between control, corn oil, and crocin groups, while the CCl₄ group's total score was statistically higher than that of all other groups; we found that the total score statistically significantly increased with CCl₄ + Crocin administration (P≤0.05) (Table 3).

Discussion

Previous empirical studies reported that CCl₄ induced tissue damage due to oxidative stress in several organs, mainly in the liver and kidneys (32). CCl₄ mechanism of inducing damage in tissues starts with the production of trichloromethyl free radical (CCl₃) by CCl₄'s cytochrome P450 oxygenase enzyme system. This free radical reacts very rapidly with oxygen to produce trichloromethyl peroxy (CCl₃OO.), a product with very high reactivity (33). The two free radicals have the ability to bind to proteins and/or lipids, thus allowing MDA accumulation, which is the final product of tissue lipid peroxidation and lipid peroxion (34). It is known that lipid peroxidation, which occurs due to free oxygen radicals that are produced as a result of oxidative stress is responsible for cancer, liver and kidney diseases, and pathogenesis that occurs in toxic cell damages (35). Under normal physiological conditions, there is a balance between antioxidant systems and free radicals, and as a result of this balance free radicals are rendered harmless. These antioxidant action structures include antioxidant enzymes, superoxide dismutase (SOD), catalase, glutathione S-transferase (GST), glutathione peroxidase (GPX), vitamins (vitamin A, E, and C), and other organic

and inorganic molecules including glutathione (GSH), melatonin, and selenium.

YYoshioka *et al.* reported that CCl₄ increased the MDA levels in renal tissues and Bun and creatine levels in the serum in the experimental model where Sasa veitchi extract was administered as a protective agent against carbon tetrachloride nephrotoxicity; when CCl₄ and plant extract were applied together, it increased Bun, creatinine and MDA levels, and histological examinations demonstrated that CCl₄ caused degeneration, swelling, and toxicity in the kidneys (36). In another study where the CCl₄-induced nephrotoxicity model was created, it was found that CCl₄ increased serum creatine, urea, and MDA levels when compared to the control group, decreased serum GSH levels, caused atrophy in the glomerulus, dilatation in tubules, degeneration, and necrosis of epithelium tubules (37). In a study conducted on kidney tissues of rats that were administered the mangrove extract as a protective agent, researchers reported that CCl₄ caused injured glomerulus and cell disorders in nephron tubules and these injuries were reduced with CCl₄ + mangrove administration (38). In another study, which utilized a gentamicin-induced nephrotoxicity model, the effects of crocin, which was considered a preventive were tested, it was determined that serum urea and creatine and tissue MDA levels increased when compared to other groups, the levels of abovementioned parameters statistically significantly decreased in gentamycin + crocin group, and gentamycin application caused cellular degeneration, tubular necrosis, fibrosis, epithelial edema of proximal tubules, and vascular congestion, and these damages decreased with crocin application (39).

In a study where researchers implemented an exercise test by allowing rats to swim and utilized crocin as a preventive, it was determined that crocin statistically significantly increased GSH levels and decreased MDA levels in the kidney tissue when compared to other groups, exercise was associated with hemorrhage, inflammation, degeneration in tubular cast and glomeruli, and congestion in the choroid plexus in the kidney tissue, and histopathologic events decreased with exercise + crocin application (40). In a study where ischemia-reperfusion (IR) model was established in the kidney tissue and crocin was applied as a protection agent, it was found that IR increased serum TOS, urea, bun, and creatinine levels, tissue TOS levels and increased TOS, urea, bun and creatinine levels decreased,

Table 3. Kidney tissue histopathology scores of all groups

Groups	Glomerular damage	Tubular injury	Inflammatory inflammation	Vascular congestion	Total score
1	0 (0 - 0) ^a	0 (0 - 0) ^a	0 (0 - 0) ^a	0 (0 - 0) ^a	0 (0 - 0) ^a
2	0 (0 - 0) ^a	0 (0 - 1) ^a	0 (0 - 1) ^a	0 (0 - 0) ^a	0 (0 - 1) ^a
3	0 (0 - 0) ^a	0 (0 - 1) ^a	0 (0 - 1) ^a	0 (0 - 1) ^a	0 (0 - 1) ^a
4	1 (1 - 2) ^b	2 (1 - 3) ^b	2 (1 - 2) ^b	1 (1 - 2) ^c	7 (4 - 8) ^b
5	0 (0 - 1) ^a	1 (0 - 1) ^a	1 (0 - 1) ^a	1 (0 - 1) ^{bc}	2 (1 - 3) ^{ab}

1; Control, 2; Corn Oil, 3; Crocin, 4; CCl₄, 5; CCl₄ + Crocin
Data are expressed as median (min-max) of ten animals

Different letters in columns are significant P<0.05

and decreased TAS levels increased with IR + crocin administration, IR caused renal lesions, degenerative necrotic tubule epithelium and deterioration in tubular structures, and IR + crocin treatment improved the histological findings compared to the IR group (41). A study formed a senescence model and investigated the dose-dependent and duration effects of 10, 20, and 30 mg/kg/day crocin in 10-month and 20-month long studies. They found that renal MDA levels decreased and GSH, SOD, and CAT levels statistically significantly increased in both 10-month and 20-month models, depending on the crocin dose (42).

Our results also indicated that CCl₄ application increased TOS, MDA, Bun, and creatinine levels, decreased TAS, GSH, SOD, and CAT levels, caused a glomerular collapse in kidney sections, and narrowing and occlusion in Bowman's space in certain glomeruli. Partial capillary degeneration was detected in certain glomeruli. Different levels of inflammatory cell infiltration and congestion were determined in most sections. In the majority of renal tubules, epithelial damage was detected at different densities. We observed eosinophilic material accumulation in the lumen of certain tubules. Statistically significant increase in TAS, GSH, SOD, and CAT levels in crocin group's renal tissues demonstrated the powerful antioxidant action of crocin. In the CCl₄ group, increased TOS, MDA, Bun, and creatinine levels and decreased GSH, TAS, SOD, and CAT levels caused significant reduction of MDA, TOS, Bun, and creatinine levels and increase in GSH, SOD, CAT, and TAS levels in the CCl₄ + Crocin group when compared to the CCl₄ group. In the kidney sections of CCl₄ + Crocin group, local minimal glomerular damage, tubular damage, inflammatory infiltration, and vascular collagen findings were observed but these damages significantly improved when compared to the CCl₄ group. Our results were consistent with the findings in other studies.

Conclusion

These results suggested that CCl₄ causes oxidative stress, resulting in tissue damage. Crocin increased the antioxidant capacity and suppressed the oxidative stress at this dose and time. Today, with the increasing impact of alternative medicine, we recommend that foods with sufficient antioxidants such as crocin should be consumed in adequate amounts.

Conflicts of Interest

The authors report no conflicts of interest.

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