Protective effects of *Cornus mas* fruit extract on carbon tetrachloride induced nephrotoxicity in rats

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ABSTRACT

Oxidative damage is implicated in the pathogenesis of kidney injury. *Cornus mas* is used for in renal aliments traditionally in Iran. The present study was aimed to investigate the antioxidant activity of *C. mas* fruit extract (CMFE) on carbon tetrachloride (CCl_4) treated oxidative stress in Wistar albino rats. Forty two male albino rats were divided into seven groups. Group I served as a sham; Group II served as a normal control; Group III served as a toxic control, with CCl_4 (1 ml/kg body weight; 80% in olive oil); Groups IV and V received CMFE at doses of 300 and 700 mg/kg before CCl_4 injection; Groups VI and VII received extract at same doses orally at 2, 6, 12, 24 and 48 h after CCl_4 intoxication. CCl_4 injection produced a significant rise in serum markers of oxidative stress and lipid peroxidation product malondialdehyde along with the reduction of antioxidant enzymes such as superoxide dismuta, catalase and glutathion peroxidase. Serum creatinine, urea and uric acid concentrations were increased whereas level of protein and albumin were reduced. Treatment of rats with different doses of fruit extract (300 and 700 mg/kg) significantly ($P < 0.05$) ameliorated the alterations induced with CCl_4 in lipid peroxidation, antioxidant defenses, biochemical and renal lesions. Based on these results, we conclude that CMFE protects kidney from oxidative stress induced by CCl_4.

Key words: Carbon tetrachloride, *Cornus mas*, lipid paroxidation, nephrotoxicity, oxidative stress

Introduction

Exposure to various organic compounds including a number of environmental pollutants and drugs can cause cellular damage through generation of reactive oxygen species (ROS). Carbon tetrachloride (CCl_4), a clear, colorless, volatile, heavy and nonflammable liquid, causes free radical generation and causes kidney injury in rats.[1,2] Free radicals induce lipid peroxidation and can damage cell membranes.[3] The average daily intake of CCl_4 for the general population is estimated to be 0.1 µg.

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Access this article online	
Quick Response Code:	Website: www.indianjnephrol.org
	DOI: 10.4103/0971-4065.133000

Kidney failure is frequently reported in fatal poisoning.[4,5] Furthermore, based on results of animal studies, the US Environmental Protection Agency has classified CCl_4 as a Group B2, probable human carcinogen.[5,6] In spite of the fact that the harmful effects of CCl_4 are obvious, this compound is still used as a solvent for oils, fats, lacquers, varnishes, rubber waxes and resins and as a starting material in the production of a number of organic compounds.[6,7]

It has been established that trichloromethyl (CCl_3) radical and Cl are formed as a result of the metabolic conversion of CCl_4 by cytochrome P450, which in turn, initiate lipid peroxidation process.[8-10]

Among horticultural crops, fruits are sources of diverse nutrient and non-nutrient molecules, which display antioxidant properties,[11] and can protect the human body against oxidant damage.

Cornus mas, known as the European and Asiatic Cornelian Cherry, has been used for the treatment of diarrhea, intestinal inflammation, fever and malaria.[12] Furthermore, it has been mentioned for the treatment of kidney stones,

kidney treatment and bladder infections in traditional system of medicine in Iran. Chemical characterization of *C. mas* fruit has shown that it is a rich source of phenolic and antioxidant, anthocyanins and flavonoids compounds.[12,13] Despite the favorable ethnopharmacological properties, its protective effect against CCl_4 nephrotoxicity has not been explored. In the present study, we investigated the effects of *C. mas* fruit on oxidative stress parameters in CCl_4-induced nephrotoxicity in rats.

Materials and Methods

Chemicals

Trichloroacetic acid (TCA) and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma-Aldrich Chemical Co. Ltd. (USA). Thiobarbituric acid (TBA) and CCl_4 were obtained from Merck Co. (Germany). Assay kits for the estimation of creatinine, urea and acid uric were purchased from Pars Azma (Iran) and all other chemicals used were of analytical grade were obtained from either Sigma-Aldrich or Merck (Germany).

Plant material

C. mas plant and its fruits were authenticated by the Botany Department of Tabriz University, Iran and obtained from suburbs of Arasbaran protected jungle (East Azerbaijan, Iran) at the end of spring 2012. The fruits were air-dried, protected from direct sunlight and powdered. The powder was kept in a closed container at 8°C.

Extraction

A total of 500 g of powder was extracted with a mixture of methanol: water (7:3) at 25°C. The solvent was completely removed by rotary vacuum evaporator at 50°C. *C. mas* fruit extract (CMFE) was frozen at − 20°C until use. The yield of the extract was 50% with reference to dry starting material.

Toxicity study

For toxicity studies, groups of 10 mice were administered (i.g.) the test compounds in the doses 100-1650 mg/kg. The LD50 (LD50 = 1270) was determined using the graphical methods of Litchfield and Wilcoxon.[14] Two different doses were selected to evaluate the dose dependent effect of CMFE on CCl_4-induced nephrotoxicity.

Furthermore, based on previous studies about evaluation of toxicity effects of CCl_4 like Zargar (2010), desired dose of CCl_4 was selected.

Animals and treatment

Male albino rats of Wistar strain (250-300 g) were purchased from Pasteur Institute (Tehran, Iran). The animals were housed in polypropylene cages in a temperature-controlled room (22 ± 2°C) with relative humidity (44-55%) under 12/12 h light and dark cycles for 1 week before and during the experiments. Animals were provided with a standard rodent pellet diet and clean drinking water *ad libitum*. Animals were divided into seven groups of six animals each:

- Group I served as a sham for both prophylactic and curative studies and received raw water and free access to food for 16 days
- Group II served as a normal control for both prophylactic and curative studies and received distilled water for 16 days orally and on the 16th day received olive oil (1 ml/kg body weight; i.p.)
- Group III served as a toxic control for both prophylactic and curative studies and received distilled water for 16 days orally and on the 16th day received CCl_4 (1 ml/kg body weight; 80% in olive oil)
- Groups IV and V served as pre-treatment groups (prophylactic). They received CMFE at doses of 300 and 700 mg/kg, orally for 16 days respectively and on the 16th day received CCl_4 (1 ml/kg body weight; 80% in olive oil), 2 h after administration of the last dose of extract
- Groups VI and VII served as post-treatment groups (curative). They received distilled water orally for 16 days and on the 16th day they received CCl_4 (1 ml/kg body weight; 80% in olive oil), followed by CMFE at doses of 300 mg/kg and 700 mg/kg (orally) respectively to Groups VI and VII at 2, 6, 12, 24 and 48 h after CCl_4 intoxication.

Assessment of renal functions

All animals were sacrificed 50 h after CCl_4 administration. Blood samples were collected from left ventricle. Serum was separated by centrifugation at 3000 rpm for 15 min and used for biochemical estimations and was used freshly for the assessment of kidney function tests. The urea, acid uric, creatinine and total protein levels were estimated by standard diagnostic test kits (Pars Azma, Iran).

Preparation of kidney homogenate

Renal tissues were homogenized in KCl (10 mM) phosphate buffer (1.15%) with EDTA: pH 7.4 and centrifuged at 12,000 rpm for 20 min. The supernatant was used for the measurement of malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx). Total protein contents were determined by the method of Lowry *et al*. (1951),[15] using bovine serum albumin as a standard.

Measurement of lipid peroxidation

Lipid peroxidation was measured by the TBA reaction method.[16] In brief, samples were mixed with TBA reagent consisting of 0.375% TBA and 15% TCA in

0.25-N hydrochloric acid (HCl). The reaction mixtures were placed in a boiling water bath for 30 min and centrifuged at 2500 rpm for 5 min. The absorbance of the supernatant was measured at 535 nm. MDA, a measure of lipid peroxidation, was calculated using an extinction coefficient of 1.56×10^5/M cm. The results were expressed as nmol/mg protein.

Determination of antioxidant enzymes

CAT activity was measured according to the method of Aebi (1984).[17] One unit of CAT was defined as the amount of enzyme required to decompose 1 μM of H_2O_2 in 2 min. The reaction was initiated by the addition of 1.0 ml of freshly prepared 20 mM H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically at 240 nm for 1 min. The enzyme activity was expressed as U/mg protein.

The activity of SOD was measured according to the method of McCord *et al*. (1994).[18] For the determination of SOD activity, xanthine and xanthine oxidase were used to generate superoxide radicals reacting with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyl tetrazolium chloride to form a red formazan dye. SOD activity was measured at 505 nm. Data were expressed as U/mg of protein.

GPx activity was determined by the method described by Paglia and Valentine (1967).[19] The reaction mixture consisted of 400 μl, 0.25 M potassium phosphate buffer (pH 7.0), 200 μl supernatant, 100 μl GSH (10 mM), 100 μl NADPH (2.5 mM) and 100 μl glutathione reductase (6 U/ml). Reaction was started by adding 100 μl hydrogen peroxide (12 mM) and absorbance measured at 366 nm at 1 min intervals for 5 min using a molar extinction coefficient of 6.22×10^3/M cm. Data were expressed as U/mg of protein.

Histopathology

For microscopic evaluation kidneys were fixed in a fixative (absolute alcohol 60%, formaldehyde 30% and glacial acetic acid 10%) and embedded in paraffin, sectioned at 4 μm, stained with hematoxylin/eosin and observed under a light microscope.

Animals rights and ethics

The study was cleared by the Institutional Animal Ethical Committee of Liver and Gastrointestinal Diseases Research Center (LGDRC) (No 91/232 – LGDRC).

Statistical analysis

All results are expressed as mean ± standard error of the mean one way analysis of variance followed by multiple comparison. Chi-square test with the Tukey *post-hoc* and independent samples *T*-test was used to compared different parameters. $P < 0.05$ was considered to be significant.

Results

Effect of CMFE on serum profile in rat

The effect of CCl_4 administration on serum concentration of urea, acid uric, creatinine, protein and albumin are presented in Table 1. Administration of CCl_4 to rats significantly ($P < 0.05$) increased the level of creatinine, urea and uric acid while the protein and albumin levels were reduced in comparison with the control group. Treatment of CMFE significantly ($P < 0.05$) reversed the changed levels of the above markers.

Effect of CMFE on renal antioxidant enzymes activity

Antioxidant enzymes activity in CCl_4 group was found to be lower than in the normal group. The activity of these enzymes in treatment groups (pre and post) were significantly ($P < 0.05$) increased when compared with CCl_4 group that shown in Table 2.

Effect of CMFE on renal contents of MDA

Effect of CCl_4 and CMFE on renal MDA is shown in Figure 1. Administration of CCl_4 to rats significantly ($P < 0.05$) induced lipid peroxidation as evidenced by the increased level of MDA. Treatment with CMFE protected against the CCl_4 induced oxidative stress by reducing the lipid peroxidation.

Renal histopathology

Administration of CCl_4 caused glomerular and tubular injury. Treatment with CMFE ameliorated the changes to near normal histology [Figure 2].

Table 1: Effect of CMFE on serum profile in rat

Treatment	Urea (mg/dl)	Creatinine (mg/dl)	Uric acid (mg/dl)	Total Protein (g/dl)	Albumin (g/dl)
Control	50.66±1.11	0.89±0.06	1.13±0.13	7.85±0.15	3.65±0.06
Olive oil	59.66±2.23$^+$	1.07±0.04$^+$	1.35±0.07$^+$	7.93±0.18$^+$	3.38±0.07$^+$
1 ml/kg CCl_4	1.50±3.76*	1.65±0.04*	3.61±0.16*	5.83±0.23*	1.31±0.08*
300 mg/kg CMFE+CCl_4	84.50±3.60$^+$	1.24±0.04$^+$	1.88±0.22$^+$	7.10±0.11$^+$	2.20±0.09$^+$
700 mg/kg CMFE+CCl_4	94.33±3.06$^+$	1.09±0.03$^+$	1.88±0.17$^+$	7.25±0.14$^+$	3.10±0.09$^+$
CCl_4+300 mg/kg CMFE	67.50±2.99$^+$	1.33±0.05$^+$	1.78±0.14$^+$	7.71±0.15$^+$	2.85±0.12$^+$
CCl_4+700 mg/kg CMFE	69.83±4.04$^+$	1.02±0.05$^+$	1.76±0.13$^+$	7.91±0.16$^+$	3.20±0.09$^+$

Results are expressed as mean±SE. (n=6). *Indicate significance at P<0.05 probability from control group. $^+$Indicate significance at P<0.05 probability from CCl_4 group. CMFE: Cornus mas fruit extract

Discussion

The present study demonstrated the protective potential of CMFE on CCl_4 induced nephrotoxicity. CCl_4 has been used in rat experimental models to investigate the oxidative stress induced in various organs. To the best of our knowledge, this is the first study to evaluate these effects of CMFE in an attempt to prevent kidney damage from CCl_4.

The mechanism of CCl_4 hepatotoxicity is well documented in the rat model.[14,20,21] CCl_4 intoxication generates free radicals that trigger a cascade of events resulting in organ toxicity in rats. It is well-known that the kidneys play a pivotal role in the regulation of various chemicals. CCl_4, a nephrotoxin, was used for the purpose of inducing renal damage in this study since it has previously been shown to exert its toxic effects on the kidney.[22,23] According to previous reports, CCl_4-induced toxicity is due to the conversion of CCl_4 to $CCl_3\cdot$ and $CCl_3O_2\cdot$ by the liver cytochrome P450 enzyme. These highly reactive free radicals cause cell damage.

Elevations in the serum concentrations of urea and creatinine as seen here are indicative of renal injury, which was accompanied by histological changes.[3,23,24] In addition, reduced level of serum albumin and protein in CCl_4-treated rats might have resulted from leakage in glomeruli and tubules. Similar results have been shown by Khan *et al*. The present study revealed that the treatment of CMFE to CCl_4-administrated rats ameliorated the toxic affect of CCl_4. Results obtained in this study are in agreement with earlier findings.[25]

The lipid peroxidation is an autocatalytic process and common consequence of cell death. It causes tissue damage during inflammation, cancer and aging.[26] Lipid peroxidation is reported to be a major causes of CCl_4-induced nephrotoxicity, mediated by the production of free radical derivatives of CCl_4. The renal MDA content, which is one of the end products of lipid peroxidation in the renal tissue, is used as an important indicator of CCl_4-induced oxidative stress. In the present study,

Table 2: Effect of CMFE on renal antioxidant enzymes activity

Treatment	U/mg protein		
	SOD	CAT	GPx
Control	30.69±0.77	10.91±0.49	62.19±1.64
Olive oil	29.73±0.77$^+$	9.91±0.34$^+$	58.25±1.79$^+$
1 ml/kg CCl_4	19.54±0.39*	4.46±0.24*	27.33±1.74*
300 mg/kg CMFE+CCl_4	22.35±0.47$^+$	8.90±0.33$^+$	39.20±1.10$^+$
700 mg/kg CMFE+CCl_4	23.22±0.0.35$^+$	9.60±0.22$^+$	42.36±0.95$^+$
CCl_4+300 mg/kg CMFE	25.61±0.36$^+$	10.35±0.45$^+$	51.67±1.64$^+$
CCl_4+700 mg/kg CMFE	24.30±0.36$^+$	9.49±0.23$^+$	47.14±1.64$^+$

Results are expressed as mean±SE. (n=6). *Indicate significance at P<0.05 probability from control group. $^+$Indicate significance at P<0.05 probability from CCl_4 group. CMFE: Cornus mas fruit extract

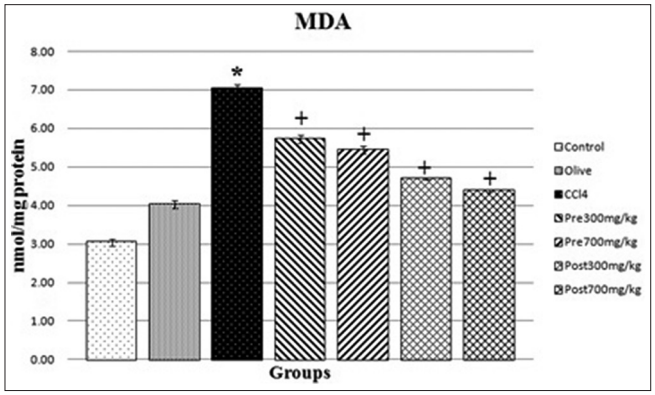

Figure 1: Effect of CMFE on renal contents of MDA in rat. *Indicate significance at P < 0.05 probability from the control group. $^+$Indicate significance at P < 0.05 probability from CCl_4 group. CMFE, Cornus mas fruit extract; MDA, malondialdehyde

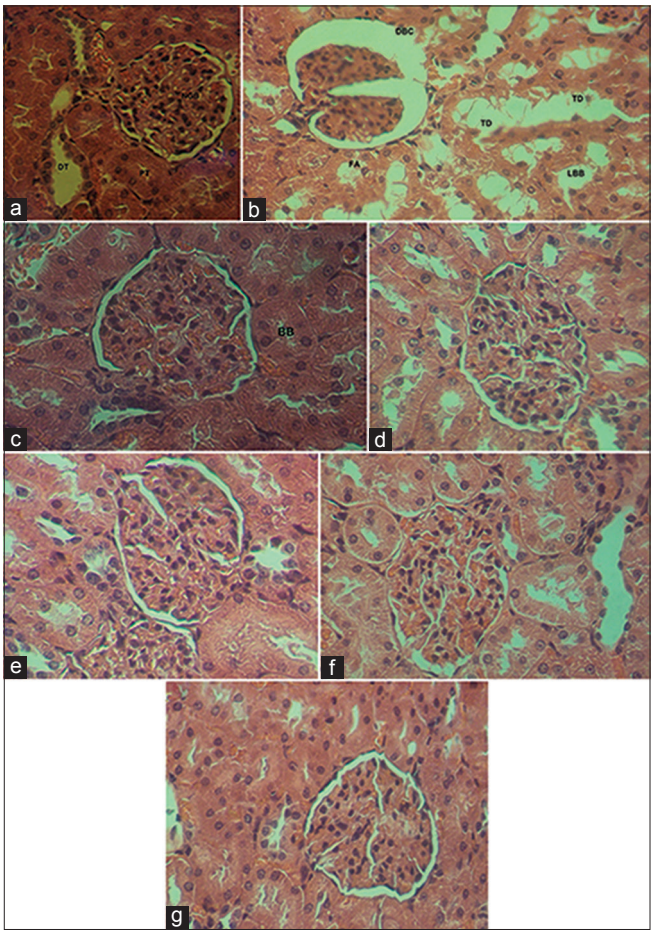

Figure 2: H and E stain: (a) Representative section from the control group. (b) CCl_4 (1 ml/kg) group. (c) Olive oil (1 ml/kg) group. (d) CMFE (300 mg/kg) + CCl_4 group. (e) CMFE (700 mg/kg) + CCl_4 group. (f) CCl_4 + CMFE (300 mg/kg) group. (g) CCl_4 + CMFE (700 mg/kg) group. CMFE, Cornus mas fruit extract; CCl4, carbon tetrachloride; DBC, damaged Bowman capsule; TD, tubular degeneration; LBB, loss of border brush; PC, proximal tubule; DT, distal tubule; NGB, normal glomerulus and Bowman capsule; BB, border brush; FA, foamy appearance

administration of CCl_4 resulted in significant elevation in MDA concentration [Figure 1] indicating elevation of lipid peroxidation along with histopathological injury [Figure 2]. Interestingly, treatment by CMFE markedly the MDA concentration.

Although there are numerous studies demonstrating that CCl_4 leads to increase in MDA levels in various tissues,[27,28] a limited number of studies have investigated the effects of CMFE. In addition, no published data has ever demonstrated the influence of CMFE on CCl_4-induced elevation in renal lipid peroxide levels.

It has been suggested that a decrease in the activities of primary antioxidant; CAT, SOD and GPx may be due accumulation of ROS. An observation that strengthens this hypothesis is that SOD activity can be inhibited by hydrogen peroxide treatment.[29] The inhibition of antioxidant system may lead to accumulation of H_2O_2 or products of its decomposition may also be aided by a decrease in CAT, SOD and GPx activities.[2,30] Measurement of these antioxidant enzymes is an appropriate indirect way to assess the pro-oxidant antioxidant status in tissues.[30,31] The level of antioxidant enzymes such as SOD, CAT and GPx decreased in CCl_4-treated group, and improved by treatment with CMFE. Results obtained in this study suggest the protective effects of CMFE against the CCl_4-induced nephrotoxicity, could be attributed to its high level of phenol[32] and other antioxidants.[33-37] These compounds could scavenge the free radicals of CCl_4 generated through P450 enzyme system thereby diminished the oxidative injuries.

In this study, the kidneys of CCl_4-treated rats have shown severe morphological abnormalities in the glomerular and tubular compartments. These changes were not observed in the groups treated with CMFE, that suggesting the protective effects of CMFE in attenuating CCl_4-induced morphological changes.

Conclusion

The present study suggests the antioxidant potential of CMFE against the toxic effects of CCl_4 in the kidney of rats. Research is needed about each of these components against CCl_4 induced nephrotoxicity.

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How to cite this article: Es.Haghi M, Dehghan G, Banihabib N, Zare S, Mikaili P, Panahi F. Protective effects of *Cornus mas* fruit extract on carbon tetrachloride induced nephrotoxicity in rats. Indian J Nephrol 2014;24:291-6.

Source of Support: Nil, **Conflict of Interest:** None declared.