Hydroalcoholic Extract of Iranian Caper Leaves Protects Hepatic Toxicity by Suppressing Oxidative Stress in Mice

Hebatullah Kalantari1, Anis Alijani2, Parvin Kheradmand3, Maedeh Goodarzian3, Leila Zeidooni4**

1Medicinal Plants Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
2Department of Toxicology, School of Pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
3Department of Pathology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
4Toxicology Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

*Corresponding author: Leila Zeidooni, Ahvaz Jundishapur University of Medical Sciences. Tel: +98-(0)6133742530, Fax: +98-(0)613332036, Email: leilazeidooni@gmail.com

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Abstract

Capparis spinosa L. (caper) is an aromatic plant, commonly used in the Mediterranean diet, possessing numerous antioxidant compounds, such as phenols, rutin, tocopherols, carotenoids, and vitamin C in its leaves. Thus, the present study investigated the effects of Iranian caper leaves extract on oxidative stress caused by CCl4 in the mice’s liver. This study was conducted on 42 male mice in seven groups. The control group, the sham group, the CCl4 group, the Iranian caper leaves extract 100, 200, and 400 mg/kg + CCl4 groups. Then, Biochemicals, oxidative stress, and hepatic histopathology parameters were evaluated. The co-administration of Iranian caper leaves extract, and CCl4 significantly decreased the levels of aspartate aminotransferase, alanine aminotransferase, and reactive oxygen species, malondialdehyde (P<0.001) and significantly increased the levels of glutathione and catalase in comparison with the group treated with CCl4 alone (P<0.01). Furthermore, Iranian caper leaves extract improved histopathological changes such as the inflammation and necrosis of hepatocytes. Iranian caper leaves extract has protective effects on hepatotoxicity induced by CCl4 mainly through suppressing oxidative stress.

Keywords: Iranian caper, Carbon tetrachloride, Oxidative stress, Liver, Mice

Introduction

Herbal medicines like plant extracts rich in flavonoids and phenolic acids have recently drawn much attention. Moreover, dietary supplements can have protective effects against ROS and consequently enhance the antioxidant system with no side effects (1).

Caper, scientifically named Capparis spinosa L., belongs to the family Capparidaceae. It is a shrub plant easily growing in poor and stony soils as well as various climatic conditions. This plant has a large natural distribution from the Mediterranean Sea to Iran. Caper’s fruit, leaves, buds, berries, roots, and seeds have been long used for medicinal purposes. This is due to its pharmacological and therapeutic effects in gout, diuretics, constipation, astringent, and tonic treatments (2-5). Caper can be effective in the treatment of paralysis, toothache (6), and edema (7). Additionally, it has potent anti-hyperglycemic (8), anti-hepatotoxic (9, 10), and anti-inflammatory activities (11). Moreover; the antioxidant, anti-carcinogenic, anti-microbial, and anti-mutagenic properties of caper can be attributable to its phenolic acids, alkaloids, glucosinolates, and flavonoid compounds (12). In this respect, Kalantari et al. argued that the administration of 100, 200, and 400 mg/kg the hydroalcoholic extract of Iranian caper leaves (Khuzestan Province) had reduced oxidative stress parameters on tetra-butyl hydroperoxide-induced acute liver damage in mice (13). Additionally, Liv-52 was considered as a medication prepared from caper leaves for the treatment of liver diseases (14, 15).

CCl4 as a solvent induced liver toxicity in experimental animals (16). Hepatotoxicity induced by CCl4 is thus characterized by hepatocellular necrosis along with fat deposition (17). The first step involves lipid peroxidation of membranes and covalent binds to cell proteins followed by cell death (18). One of the dominant strategies to prevent or treat liver injury is usually antioxidants use (19).

CCl4 toxicity can be caused by its bioactivation to trichloromethyl free radical (CCl•) using cytochrome P450 isozymes; the relevant trichloromethyl radical reacts with...
oxygen and forms a highly toxic reactive, including trichloromethyl proxy radical. Consequently, that damages endoplasmic reticulum (ER), mitochondria, cell membrane, and DNA. Eventually, this process leads to the accumulation of lipids, reduced protein synthesis, as well as the depletion of antioxidant status in tissue cells (20). Therefore, our study designed to investigate the hepatoprotective properties of Iranian caper leaves extract on biochemicals, oxidative stress, and histopathology parameters against liver injury induced by CCl₄ in mice.

Materials and methods

**Chemicals:** Ammonium molybdate, Thiobarbituric Acid (TBA), Trichloroacetic Acid (TCA), and GSH were purchased from Sigma-Aldrich (St Louis, Missouri, USA). Z’, 7’ –Dichlorofluorescin Diacetate (DCFDA) was obtained from Merck (Darmstadt, Germany). ALT and AST kits were obtained from biochemical assay kits (Pars Azmoon, Iran). CCl₄ was also purchased from Roche chemical company (Germany).

**The preparation of herbal extract:** The leaves of caper were collected from the Khuzestan Province, Iran. Caper was identified by the central herbarium of Ahvaz Jundishapur University of Medical Sciences, Iran. First, the leaves were dehydrated at ambient temperature (30–40 °C) for 25–30 days; then, they were powdered and soaked (100 g) in ethanol 90% aqueous-ethanol v/v for 72 hours. Consequently, they were filtered and dried (13).

**Animals:** Forty-two adult, NMRI male mice in the weight range of 25-30 g were achieved from the animal house of Jundishapur University of Medical Science (AJUMS), Iran. The mice were kept in polycarbonate cages under standard conditions at (25 ± 2 °C) with a 12:12 h light-dark cycle along with access to drinking water and diet ad libitum. This study was conducted according to the standard and Ethics Committee of (AJUMS) with (code: IR.AJUMS.REC.1395.02).

**Experimental design:** The plant extracts were dissolved in normal saline and administered orally for 5 consecutive days; then, CCl₄ was diluted in olive oil and mice received (1 mL/kg, IP). Forty-two male mice were divided into six groups. Group 1: control; was administrated normal saline; group 2: sham group; was fed with olive oil; group 3: CCl₄ group; received CCl₄ (1 mL/kg, IP); and groups 4, 5 and 6: received Iranian caper leaves extract (100, 200, and 400 mg/kg, respectively) orally for five days, then received CCl₄ (1 mL/kg, IP) on that day an hour after administration of extract (13).

**Sample collection:** To this end; 24 h after CCl₄ injection, the mice were anesthetized with ketamine/ xylazine (90/10 mg/kg). Then, livers were removed. The samples’ livers were divided into 2 sections. One section was homogenized for tissue oxidative stress biomarkers, and one section of the liver was located in 10% formalin for pathological examination (21).

**Serum analysis:** The serum was separated by centrifugation at 3000 rpm for 15 min. The activities of serums AST and ALT were also determined using an autoanalyzer machine (BT 3000, Italy) as well as biochemical assay kits (Pars Azmoon, Iran) (21).

**Liver homogenization:** For this purpose, 100 mg of liver tissue was homogenized, using glass homogenizer in 1 ml of phosphate buffer saline (1 mM) with (pH 7.2). Then, livers suspension centrifuged at 10,000g for 20 min at 4 °C. Next, the supernatant was isolated from the pellet and used for oxidative stress tests (22).

**The GSH assay:** Liver glutathione amount was determined by Ellman’s reagent (23, 24). Phosphate buffer saline (2 ml) was added to the liver homogenate (40 μl) and DTNB (40 μl). Then, were mixed and incubated for 10 min at 25 °C. The absorbance of specimens were measured at wavelength 412 nm. Finally, the data were reported as micromoles/g tissue.

**The CAT assay:** The CAT activity was detected by an adapted procedure developed by Goth (25). First, the liver homogenate (50 μL) along with (0.05 mmol) of tris-HCl (500 μL) and H₂O₂ (1 mL) were mixed and placed at 25°C for 10 min. Then, (500 μL) of ammonium molybdate (4%) was added to mixture. Finally, the absorbance of specimens were determined at wavelength 410 nm. The obtained results were ultimately reported as U/g tissue.

**The MDA assay:** The lipid peroxidation in liver was determined based on MDA level (26). 2 mL of TBA (0.67%) was also quickly mixed to liver homogenate (1 mL) in one test tube; then, were placed in boiling water for 30 min. After cooling and centrifuging the mixture, the supernatant was isolated. The absorbance of supernatant was read at wavelength 532 nm. In the end, the MDA amount was express as nanomoles/g tissue (27).

**The ROS assay:** The ROS intensity was determined by DCFDA in liver. In liver, DCFDA by peroxides was converted to form highly fluorescent DCF that comes out of the cell. Briefly, 100 mg of liver tissue was homogenized in 1 ml of ice-cold tris-HCl buffer (40 mM, pH 7.4). Then, DCFDA 1.25 mM was added to supernatant and incubated for 10 min. Finally, the intensity of fluorescence was measured at 488 nm excitation and 525 nm emission wavelength using a fluorimeter (Perkin-Elmer, LS-50 B, United Kingdom) (28).

**Histopathological analysis:** The livers after fixation in 10% formalin were embedded in paraffin. For histopathological studies seven slide were prepared from each group and slides were stained with Hematoxylin and Eosin. The slides were then study under a light microscope for observation necrosis and inflammatory cells (26).

**Statistical analysis:** The obtained data were performed by GraphPad Prism software and expressed as mean±SEM (N=7).
The between-group comparisons were also evaluated using one-way Analysis of Variance (ANOVA) followed by a Tukey’s posthoc test. The level of $P<0.05$ was reflected as significant.

**Results**

The effects of Iranian caper leaves extract and CCl$_4$ on serum analysis: The levels of ALT and AST were recognized as the biomarkers for hepatotoxicity studies. Figure 1 shows that 24 hours after CCl$_4$ administration, a significant increase was observed in the levels of AST, and ALT in the CCl$_4$ receiving group, in comparison with the control groups ($P<0.001$). However, treatment with Iranian caper leaves extract at both concentrations of 200, and 400 mg/kg significantly decreased AST and ALT levels, in comparison with the CCl$_4$ receiving group ($P<0.001$).

The effects of Iranian caper leaves extract and CCl$_4$ on GSH levels: The measurement of GSH level is considered among the best biomarkers for CCl$_4$ detoxification. As per Figure 2, CCl$_4$ statically reduced liver GSH amounts in comparison with the controls ($P<0.01$). Iranian caper leaves extract pre-treatment also attenuated the depletion of liver GSH levels at 400 mg/kg, in comparison with the CCl$_4$ receiving group ($P<0.01$).

The effects of Iranian caper leaves extract and CCl$_4$ on CAT activity: As shown in Figure 2, the liver CAT activity showed a significant diminished in the CCl$_4$ receiving mice, in comparison with the normal mice ($P<0.001$). Therefore, Iranian caper leaves extract pre-treatment significantly increased this variable at 200 ($P<0.01$) and 400 mg/kg ($P<0.001$), in comparison with the CCl$_4$ receiving mice.

The effects of Iranian caper leaves extract and CCl$_4$ on MDA and ROS levels: The achieved data showing that the amounts of ROS and MDA significantly increased in the CCl$_4$ receiving mice in comparison with the controls ($P<0.001$). According to Figure 3, a significant decrease ($P<0.001$) in the levels of MDA and ROS in the liver was observed in a dose-dependent manner in the pre-treated mice by Iranian caper leaves extract at all concentrations when compared with the CCl$_4$ receiving mice ($P<0.001$).
The effects of Iranian caper leaves extract and CCl4 on histopathological findings: The histopathological findings of the liver tissues revealed that hepatocyte cells, labels, and sinusoid status were healthy and without any pathological changes in the control groups (normal saline and sham) (Figures 4A and 4B). The CCl4 intoxicated group also exhibited widened sinusoids, deteriorating cells, the necrosis and inflammatory cells (Figure 4F). Pre-treatment with Iranian caper leaves extract at all doses similarly improved histological changes, including inflammation and necrosis in comparison with the CCl4-treated group (Figures 4C, 4D and 4E).

Discussion
The present study demonstrated that CCl4 could cause oxidative damages through increasing ROS generation and MDA levels. It also decreased the GSH amount and CAT activity in the liver tissue. Moreover, the serum amounts of AST and ALT increased, as the biomarkers of liver damage. The relevant histopathological findings also supported biochemical and oxidative stress parameters. Moreover, Iranian caper leaves extract had hepatoprotective effects evidenced through improving biochemical and oxidative stress as well as histological parameters induced by CCl4. The administration of Iranian caper leaves extract at the concentrations of 100, 200, and 400 mg/kg for five days also revealed its hepatoprotective effects in a dose-dependent manner. However, the dose of 400 mg/kg against hepatotoxicity induced by CCl4 was more effective than the other two doses.

The liver is the main site for the detoxification of drugs and toxicants. The metabolic process does not usually damage the liver itself; however, many toxic drugs or chemicals can cause liver injury (29). Additionally, CCl4 is the best-considered model used for hepatotoxicity induction in animals (30). The collected results suggested a significant greater in the serum amounts of ALT and AST in the CCl4 receiving mice. This finding can be due to major hepatic injury, because these two variable are main biomarkers of liver injury. They are typically limited in the cytosol and ultimately free into the blood flow after tissue injury (31). Previous studies also supported such findings that CCl4 causes hepatotoxicity along with increasing ALT and AST amounts (32). The present findings also demonstrated that AST and ALT amounts significantly decreased in the groups treated with the different doses of Iranian caper leaves extract. These results suggested that Iranian caper leaves extract could protect mice liver against CCl4-induced injury. Furthermore, CCl4 significantly reduced the GSH levels, as the main non-enzymatic antioxidant, as well as CAT as an enzymatic antioxidant in the mice’s liver. This indicates that CCl4 produced oxidative stress in the liver. The decrease of GSH levels in the present study was in line with previous findings (33, 34). The measurement of the GSH levels as the non-enzymatic antioxidant, and CAT as the enzymatic antioxidant defense were thus considered as appropriate indicators of the amount of antioxidant defense in the liver tissue. It can be supposed that antioxidant defense was reduced by CCl4 in the mice’s liver (35). The reduction of GSH levels might also reflect increased ROS generation stimulated by CCl4 (36).
A possible reason for the reduction of GSH levels could be that the metabolites of CCl₄ probably reacted with glutathione. In this respect, previous studies demonstrated that trichloromethyl radical could react with the sulfhydryl groups of glutathione and other protein thiols and consequently change antioxidant/oxidant proportion (37, 38). Along with the declined activity of CAT might be due to the gathering of H₂O₂ because CAT could convert H₂O₂ into H₂O and oxygen, then neutralize it (39). The inhibition of antioxidant defense system in hepatocytes by the metabolites of CCl₄ was likely to cause the accumulation of H₂O₂ in the liver tissues and be aided by decreased CAT activity. Numerous studies have established that the removal of free radical via antioxidant agents might decrease oxidative damage induced by CCl₄, and as a result, protect the liver from further injuries (40, 41).

In our study, the levels of GSH and CAT activity were recovered in treatment with Iranian caper leaves extract and boosted defense beside free radicals. This data suggested that the defensive properties of Iranian caper leaves extract against oxidative stress induced by CCl₄ probably due to the present of phenolic compounds in high amounts (42, 43). The phenolic compounds can counteract the free radicals caused by CCl₄ metabolites in the mice liver. Consistent with the current study, prior research reported that Iranian caper leaves extract augmented the levels of GSH and CAT in hepatotoxicity induced by tert-butyl hydroperoxide in the mouse's liver (13).

Trichloromethyl, which is derived from CCl₄ metabolism, is a highly reactive metabolite that is a major factor in the production of ROS and lipid peroxidation. This can be the main mechanism in the pathogenesis of hepatotoxicity induced by CCl₄ (Figure 5) (44). Therefore, reduced oxidative stress biomarkers like ROS and MDA, and increased antioxidant system can exceptionally protect the liver from oxidative stress caused by CCl₄ (45). The group treated by CCl₄ alone demonstrated high levels of lipid peroxidation and ROS; however, Iranian caper leaves extract could protect the samples against CCl₄ via suppressing lipid peroxidation and decreasing free radical generation evidenced by improving MDA and ROS levels.

The results of the histological analysis showed that CCl₄ treatment augmented the infiltration of inflammatory cells in mice liver. White blood cells respond nonspecifically to stimulants in the immune system and quickly migrate to the sites of damage and initiate inflammatory responses as liver injury happen following excessive ROS generation. Therefore, the infiltration of inflammatory cells can be reflected as an indicator of the inflammatory response (46). However, in this study, Iranian caper leaves extract inhibited inflammation caused by CCl₄ and could relieve hepatotoxicity induced by CCl₄ by overpowering the inflammatory reaction in the mice’s liver.

In this study, the liver in the CCl₄-treated mice demonstrated characteristic morphological findings, such as severe degenerative hepatocytes, widened sinusoids, deteriorating cells, the necrosis and inflammatory cells. Histopathological changes have led to cellular damages, like deterioration in membrane integrity. Furthermore, the CCl₄ administration facilitated the lipid peroxidation of lipid structures in the liver tissue, resulting in sub-cellular damages as observed in histopathological examinations. However, Iranian caper leaves extract attenuated morphological alterations caused by CCl₄. Previous studies similar to our study have reported necrosis and inflammation caused by CCl₄ in the mice liver (47, 48).

**Conclusion**

The present study evaluated the protective role of Iranian caper leaves extracts at different levels. Biochemical, molecular, and pathological findings suggested that Iranian caper leaves extract ameliorated hepatotoxicity induced by CCl₄ in the studied mice (Figure 5). The obtained results supported the pharmacological use of Iranian caper leaves for liver injuries induced by drugs and chemicals. However, more studies are required to analyze the biochemical contents of Iranian caper leaves extract (Khuzestan Province).

**Figure 5** The effects of Iranian caper leaves extract and CCl₄ on the mouse's liver. CCl₄ in the liver metabolizes to trichloromethyl free radical (CCl₃*) by CYP₃A₁₁, then trichloromethyl radical reacts with oxygen and forms a highly toxic reactive trichloromethyl peroxy radical (CCl₃OO•), decreases the levels of GSH and CAT, and consequently increases the levels of MDA that result in a rise in AST, ALT, amounts and inflammation. Iranian caper leaves extract administration also modifies all examined endpoints induced by CCl₄.

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**Authors' contributions**

Hebatullah Kalantari: Participated in research design, Anis Alijani, Parvin Kheradmand, and Maedeh Goodarzian: Conducted experiments, Leila Zeidooni: Performed data
analysis, Hebatullah Kalantari, Leila Zeidooni, and Anis Alijani: Contributed to the writing of the manuscript.

Conflicts of interests
The authors declare no competing interests.

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