



Hepatoprotective Activity of Caspian Saffron (*Crocus caspius* Fisch and Mey) Flowers against CCl₄- Induced Acute Liver Injury in Mice

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Abstract

Background and objectives: Free radicals and other oxidants have important roles in liver cell toxicity. Some natural products are potent scavengers of oxidative agents. *Crocus caspius* is an endemic species of Caspian forest. Flowers of *Crocus* genus contain phenolic compound and carotenoid as antioxidant agent. This study investigated the antioxidant and hepatoprotective activity of *Crocus caspius* Fisch and Mey flowers (Caspian saffron) hydro-alcoholic extract (CCFE) against acute oxidative hepatotoxicity induced by CCl₄ in mice. **Methods:** The antioxidant activity of the extract was evaluated by 2, 2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging assay. *Crocus caspius* flowers were investigated for presence of certain phytochemicals and total phenol and flavonoid contents were determined. For evaluation of hepatoprotective activity, the BALB/c mice were pre-treated with 50, 100, 200, and 500 mg/kg, of the extract intraperitoneally for 5 days and then received CCl₄ (0.5 mL/kg, in olive oil). Liver injury was determined by serum biochemical markers such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), glutathione content of liver tissue and histopathological studies. **Results:** The phytochemical screening in Caspian saffron flowers indicated the presence of carotenoids, saponins, sterols, flavonoids, and tannins. The extract exhibited antioxidant activity in DPPH radical scavenging assay (IC₅₀ 171.5 µg/mL). Pre-treatment groups with the extract demonstrated significant decrease in serum levels of ALT, AST, and ALP (p<0.05) and concomitant increase in GSH content (p<0.05). Histopathological observation determined hepatocellular protective effects of the extract. **Conclusion:** The results represented the protective activity of the extract against oxidative hepatotoxicity.

Keywords: antioxidant; carbon tetrachloride; *Crocus caspius*; hepatoprotective

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Introduction

Liver possess many vital roles in human body such as metabolism, detoxification of drugs and toxins, storage of nutrients, excretion of some waste metabolites, involvement in digestion and

immune response [1]. Many factors such as viral hepatitis B&C, alcohol consumption, food habits, some drugs (cyclophosphamide, acetaminophen, etc.) and toxins (aflatoxin B1, pyrrolizidine

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alkaloids, etc.) are the main reasons of hepatotoxicity and hepatocellular carcinoma. One of the mechanisms of liver damage is related to oxidative stress, under this condition free radicals attack cell macromolecules (lipid, protein and DNA) so causing cellular and tissue damage [2-5] Hepatic toxicity caused by carbon tetrachloride (CCl₄) is a current experimental model for simulation of liver injury in animal model. CCl₄ affected by hepatic enzymes produces free radicals (CCl₃[•]), then react with molecular oxygen and forms toxic trichloromethyl free radical (CCl₃[•]) [6]

Except for some procedures such as vaccination for viral hepatitis, there are few important drugs for the treatment of liver diseases in modern medicine. In most cases liver transplantation might be the only effective way.

Nowadays medicinal herbs are the most important therapeutic agents in hepatotoxicity; several of them play protective roles against certain hepatotoxins. Also some are antiviral, antioxidants and liver cell proliferation stimulants [7].

Crocus genus includes 150 species distributed in Mediterranean region, west and middle of Asia. *Crocus caspius* Fisch and Mey is a perennial, bulbous flowering plant from Iridaceae family which grows in south of Caspian Sea (as endemic species to Iran). Its common name is Caspian crocus or Caspian saffron [8].

Saffron (*C. sativa*) a well-known species of crocus genus is a valuable species and its stigma are used as flavor, colorant, and medicine. Crocin and safranal, the active compounds of saffron, have shown antioxidant and anticancer properties. Saffron flowers have exhibited hepatoprotective effect in CCl₄ hepatotoxicity model. Other studies have reported that alcoholic and hydroalcoholic extracts of corm and aerial parts of *C. caspius* is effective antioxidants [9,10]

In this study we have investigated the protective activity of hydro-alcoholic extract of *C. caspius* flowers against CCl₄-induced hepatotoxicity in male mice.

Material and Methods

Ethical considerations

This study was approved by the Ethical Committee of Mazandaran University of Medical Sciences (ID: IR. MAZUMS. REC. 1394. 1448). The animal experiments were carried out following the principles of working

with laboratory animal protocols of Animal Research Institute, Mazandaran University of Medical Sciences, Mazandaran, Iran.

Plant materials and extraction

Crocus caspius flowers were collected from Hezarjarib, Mazandaran province, Iran during November 2015. They were identified by plant taxonomists. A voucher specimen was deposited at the Herbarium of Faculty of Pharmacy, Mazandaran University of Medical Science, Iran (voucher No: E₂-2-512). The collected material was dried under shade for 6 days and ground to coarse powder then macerated with ethanol 70 % for 4 days. Finally, the fluid extract was concentrated under reduced pressure and dried with freeze dryer. [11]

Chemicals

Carbon tetrachloride, 5, 5-dithiobis-(2-nitrobenzoate) (DTNB), glutathione (GSH), and ascorbic acid were obtained from Merck (Germany); 1, 1-diphenyl-2-picryl hydrazyl (DPPH) was purchased from sigma, (St Louis, MO, USA). ALT, AST, and ALP kits were obtained from Biolabo, France. All the other chemicals used were of analytical grade.

Preliminary phytochemical screening

The hydro-alcoholic extract of *C. caspius* was screened for the presence of various phytochemicals including carotenoids, alkaloids, sterols and triterpenoids, saponins, anthraquinone glycosides, tannins and flavonoids [12].

Determination of phenolics content

The amount of total phenolics of hydro-alcoholic extract of *C. caspius* was determined by Folin-Ciocalteu method. Sample solution (0.1 mL) was mixed with 0.25 mL, Folin reagent 1 N. After 5 min, 1.25 mL 20% sodium carbonate solution was added and shaken vigorously. The absorbance of samples was measured at 725 nm after 40 min incubation at room temperature with a double beam Perkin Elmer UV/Visible spectrophotometer. Calibration curve was obtained by standard concentrations of tannic acid. The total phenolics content was expressed as equivalents of tannic acid [13].

Determination of flavonoids content

The total flavonoid content was determined by aluminum chloride method. Methanolic Sample solution (0.5 mL) was mixed with 1.5 mL

methanol, 0.1 mL 10% anhydrous aluminum chloride in methanol, 0.1 mL 1 M potassium acetate and 2.8 mL distilled water. After 30 min incubation at room temperature, the absorbance of samples was measured at 415 nm. Calibration curve was prepared by standard concentrations of methanolic solution of quercetin. The total flavonoid content was expressed as equivalents of quercetin [14].

DPPH-free radical scavenging activity

DPPH (2, 2-diphenyl-1-picrylhydrazyl) which is a stable radical was used for determination of free radical scavenging activity of the extract. Each dose of the extract and standard sample was mixed with ethanolic solution of DPPH then mixtures were incubated at room temperature in dark for 30 min and the absorbance was read at 517 nm. Ascorbic acid was used as the standard and methanolic solution of DPPH as the negative control. Radical scavenging activity was calculated by the following formula:

$$[(A_c - A_s)/A_c] \times 100.$$
 Where A_c and A_s were absorbances of control and sample, respectively. IC_{50} , the concentration of sample which was required for scavenging 50% of DPPH free radicals was finally calculated [15].

Animals

The animals were obtained from institute of experimental animal research center, Mazandaran, Iran. Eight to ten weeks old mice weighing 20-25 g were used. All mice were maintained under standard conditions (12 h light/dark cycles at room temperature) and fed with the rodent pellet and tap water.

Induction of liver injury

Male mice were divided into 8 groups, 6 animals each. Liver injury was induced in mice by intraperitoneal (i.p.) injection of CCl_4 (0.5 mL/kg) dissolved in equal volume of olive oil [16]

The animals were grouped as follows: Group I, without any treatment were the normal group; Group II, treated only with CCl_4 (i.p.); Group III, IV, V, VI, treated with hydro-alcoholic extract of *C. caspius* suspended in normal saline at doses of 50, 100, 200, 500 mg/kg daily for 5 days followed by CCl_4 on day 5, respectively; Group VII and VIII treated with olive oil and vitamin C (500mg/kg) as negative and positive groups, respectively (i.p.) daily for 5 days followed by CCl_4 on day 5.

Measurement of glutathione content

Hepatic glutathione (GSH) content was determined with 5, 5-dithiobis-(2-nitrobenzoate) (DTNB) at 412 nm. After scarification, the liver was immediately removed; 100 mg of liver tissue was minced with scissors and homogenized in 1 mL of 0.02 M EDTA on ice. Then the homogenized liver was centrifuged at 5000 rpm for 15 min. Next, 2.5 mL of 0.4 M tris buffer (pH 8.9) and 0.5 mL DTNB was added to 1 mL supernatant. The solution was vortexed and the absorbance was measured. The GSH solutions were used for plotting the standard curve. GSH concentration was expressed as micromoles per gram of wet tissue weight [17].

Serum biochemical assay

The animals were anesthetized 24 h after receiving the acute dose of CCl_4 . The blood was collected from the carotid artery, and then serum was separated by centrifugation (3000 rpm at 4 °C for 10 min). The serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were measured using assay kit (Biolabo, France) [18]

Histopathological analysis

The liver tissue was fixed in buffered formalin and embedded in paraffin; then, cut into thin sections of 4 μ m thickness. Afterwards, the sections were deparafinized and stained with hematoxylin and eosin (H&E) for microscopical examination [19]

Statistical analysis

The results were expressed as Mean \pm SEM. Differences among groups were assessed with one-way analysis of variance (ANOVA) and TUKEY test with SPSS16 software. $P < 0.05$ was regarded as significant.

Results and Discussion

Phytochemical screening of Caspian saffron flowers indicated the presence of carotenoids, flavonoids, saponins, sterols, and tannins in *C. caspius* flower extract.

Total phenolics and total flavonoids content of hydro-alcoholic extract of *C. caspius* flowers were reported as tannic acid and quercetin, respectively by reference to standard curve. The hydro-alcoholic extract exhibited high phenol 239.9 mg tannic acid/g and flavonoid 82.04 mg quercetin/g of extract.

The extract showed DPPH radical scavenging activity with IC_{50} of (171.5 $\mu\text{g/mL}$). IC_{50} value of vitamin C as the positive control group was determined to be 10.1 $\mu\text{g/mL}$.

After intraperitoneal injection of CCl_4 to the mice (group II) levels of ALT, AST, and ALP were significantly elevated in serum compared with normal group that received normal saline ($p < 0.05$). Furthermore, the hepatic GSH level was significantly decreased ($p < 0.05$) compared to the normal control animals (table 1.).

The photomicrographs of the liver in all groups have been exhibited in figure 1. Normal structure of the liver was observed in control group. The slides of the liver in CCl_4 toxicity group revealed focal necrosis and degeneration, periportal leucocyte infiltration, sinusoidal dilation, and granulomatous formation. However, *C. caspius* extract administration with different doses (50, 100, 200, 500 mg/kg. i.p.) in CCl_4 treated animals mitigated the pathological alterations compared to CCl_4 group. After 5 days, liver enzymes

decreased dose dependently and cellular glutathione contents increased compared with the CCl_4 group; so that, the effective dose (500 mg/kg) exhibited the results almost similar to the normal group (table 1). Histopathological study in treatment groups (groups III-VI) exhibited that liver lesions improved and in high doses of the extract in the liver sections, tissue architecture was preserved and less necrosis could be observed. Reactive oxygen species (ROS) are considered an important factor in the pathogenesis of liver damage. Cytochrome P450 enzymes in liver cells convert CCl_4 to very toxic free radicals that cause lipid peroxidation leading to hepatic cell necrosis, hence CCl_4 is used as a laboratory model for screening hepatoprotective activity of drugs. Cell disruption due to oxidant agents leads to increasing hepatic enzymes. The hepatic enzymes such as ALP, ALT, and AST in serum are used as biochemical markers for evaluation of liver function [20].

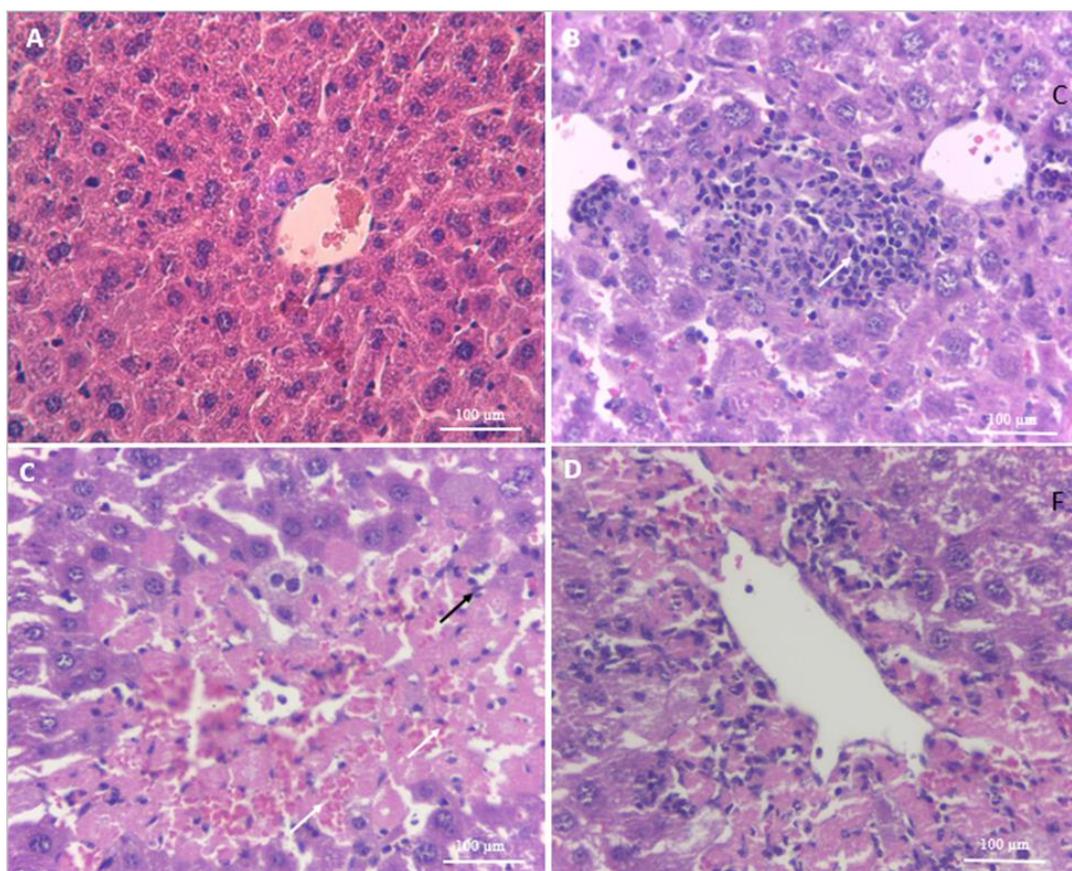


Figure 1. Micrograph of liver from all groups: (A) normal architecture of liver in physiological saline-treated group, (B) Liver tissue of CCl_4 group with hepatocyte cells degeneration (arrow) and cytoplasmic vacuolization and granulomatous formation (arrow), (C) Liver tissues of CCl_4 + CCFE (50, 100 and 200 mg/kg) with eosinophilic cytoplasmic cells (White arrow) and mild inflammatory cell infiltration (Black arrow) and reduced necrosis compared to the ccl_4 group, (D) CCl_4 + CCFE (500 mg/kg) with slight leucocyte infiltration compared to the ccl_4 group. (H & E staining, Mag: $\times 40$, Scale bar = 100 μm)

Table 1. Effect of *Crocus caspius* flowers hydro-alcoholic extract on CCl₄- induced liver damage in mice. Values present Mean±SD for six mice in each group.

Groups	Treatment	GSH(U/L)	AST(U/L)	ALT(U/L)	ALP(U/L)
I	Normal saline	140.66±3.05	117.00±2.00	61.00±1.00	146.00±3.6
II	CCl ₄ (25mg/kg. i.p)	73.00±4.00 ^a	151.33±1.52 ^a	78.33±4.04 ^a	200.00±2.5 ^a
III	<i>Crocus caspius</i> (50 mg/kg i.p.)	83.00±5.29	152.00±1.00	77.66±3.51	195.66±4
IV	<i>Crocus caspius</i> (100 mg/kg i.p.)	97.00±4.00*	146.66±1.52	75.66±3.05	188.66±2.5*
V	<i>Crocus caspius</i> (200 mg/kg i.p.)	117.33±4.5**	138.33±3.78*	70.00±2.00*	178.33±3.00*
VI	<i>Crocus caspius</i> (500 mg/kg i.p)	132.00±4.35**	93.00±6.49**	67.33±3.51**	160.66±3.5**
VII	Olive oil (vehicle)	114.00±2.56*	153.00±3	85.00±4.58	153.00±4.5**
VIII	Vitamin C	136.25±7.27**	126.40±3.91*	70.17±6.99*	195.25±5.73*

P values: ^a ≤0.05 vs. normal saline, *≤0.05; **≤0.01 vs. CCl₄ treated group

GSH is an endogenous radical scavenging factor which has a protective role against oxidative agents in the body and prevents cell necrosis. Therefore, decrease in liver GSH levels leads to oxidant stress status [6].

In this study we used different doses of the extract for evaluation of the hepatoprotective effect against CCl₄ toxicity. The serum levels of ALT, AST, ALP and hepatic GSH contents were employed as diagnostic markers of hepatocellular injury. In treatment groups with 50, 100, 200, and 500 mg/kg of the extract, the serum levels of ALT, AST, and ALP decreased dose dependently compared with the CCl₄ group. Upon treatment with 500 mg/kg of the extract these enzymes decreased similar to normal group.

Also, after administration of CCl₄, histopathological examination showed periportal leucocyte infiltration, granulomatous formation, periportal leucocyte infiltration, focal necrosis and degeneration and inflammation in the liver tissue structure. Liver tissues from extract-treated animals showed significant improvement in histopathology.

The extract significantly raised the hepatic GSH content.

The results exhibited that parenteral administration of the extract resulted in significant hepatoprotective effects against free radicals. Moreover, histopathological study confirmed the liver function improvement in treated groups. *Crocus* genus has several species with phytochemicals such as carotenoids and phenolic compounds [21,22].

Recently, the hepatoprotective effect of *Crocus sativus* L. flower extract was evaluated in CCl₄ experimental model. It showed significant protective effect on hepatic injury [23]. The hepatoprotective effect of saffron extract and crocin has been proven with acetaminophen and

acrylamide-induced liver injury model, respectively. The main mechanisms of acetaminophen and acrylamide liver toxicity is oxidative damage and GSH content depletion [24,25]. According to previous researches carotenoids isolated from *C. sativus* L. have antioxidant properties [26,27]. Other phytochemicals such as phenolic compounds in *C. sativus* L. are natural antioxidant which are able to scavenge reactive oxygen species (ROS) and to inhibit lipid peroxidation [28].

Ebrahimzadeh and coworkers reported the significant antioxidant effect of *C. caspius* aerial parts by several in vitro methods such as DPPH assay, metal chelating activity, reducing power, hydrogen peroxide, and nitric oxide-scavenging activity [10] which are supporting the antioxidant activity evaluated by DPPH test in our study.

For the first time we reported high quantities of phenolic compounds such as flavonoids and carotenoids in *C. caspius* flowers which are probably responsible for hepatoprotective activity. In some previous reports flavone and flavonol glycosides have been isolated from the flower extracts of different species of *Crocus*, that are likely to be present in the Caspian saffron showed liver protective function [29]

Different chemicals in natural sources play effective roles as hepatoprotectives and have now become a promising therapy for liver diseases. Plants such as turmeric (*Curcuma longa*), milk thistle (*Silybum marianum*), artichoke (*Cynara scolymus*) and many others are used in the treatment of liver disease [30]. Silymarin, the flavonolignan from *Silybum marianum* is a unique hepatoprotective compound. Beside the antioxidant activity of hepatoprotective agents, these compounds act in different ways, including anti-inflammatory activities, as cell permeability regulators and membrane stabilizers, for

stimulation of liver regeneration and inhibition of deposition in collagen fibers [31].

In conclusion, the results of the present study demonstrated that *C. caspius* extract could significantly prevent the oxidative hepatic damage induced by CCl₄ in mice. The extract exhibited significant in vitro and in vivo antioxidant effect. The hepatoprotective effect of the hydroalcoholic extract may be due to antioxidant activities of some phytochemicals such as phenolic compounds and carotenoids. More detailed investigations on the isolation and characterization of bioactive compounds that may be responsible for liver protection are in progress.

Author contributions

Emran Habibi and Mohammad Shokrzadeh designed and supervised the experiments. Parisa Habibpour and Ali Ziar were involved in the experiments. Fereshteh Talebpour Amiri interpreted the pathological result. Hossein Bakhshi Jouybari performed phytochemical evaluation and wrote the manuscript.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

DPPH: 2, 2-diphenyl-1-picryl hydrazyl, ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase, GSH: glutathione, IC50: half maximal inhibitory concentration, ROS: reactive oxygen species, i.p.: interaperitoneal injection