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Journal homepage: www.tiprpress.com E-mail: chm@tiprpress.com**Original article****Protective Effect of Chlorogenic Acid against Carbon Tetrachloride-induced Acute Liver Damage in Rats**Zong-xi Sun^{1, 2†}, Song Liu^{3†}, Zhi-quan Zhao^{1, 2}, Rui-qiang Su^{1, 2*}

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ABSTRACT

Objective To evaluate the protective effect of chlorogenic acid (CGA) on carbon tetrachloride (CCl₄)-induced liver injury of rats. **Methods** The anti-oxidative activity of CGA was investigated with several established *in vitro* systems. The hepatoprotective activity of CGA against CCl₄-induced acute liver injury in rats was studied. The levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and total bilirubin (TB) were measured. The histopathological examination was carried out to supplement the biochemical results. **Results** CGA possessed strong anti-oxidative ability *in vitro*. The CCl₄-induced liver toxicity experiment showed that the rats pretreated with CGA (300 or 500 mg/kg) had lower levels of ALT, AST, ALP, and TB than those of the CCl₄-treated group. These data were supplemented with histopathological examination of rat liver sections. CGA did not show any mortality at the dose up to 5000 mg/kg. **Conclusion** CGA could protect the liver against CCl₄-induced oxidative damage in rats, and the possible mechanism of the activity may be due to its free radical-scavenging and anti-oxidative activity.

Key words

acute liver damage; anti-oxidation; carbon tetrachloride; chlorogenic acid; hepatoprotection

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1. Introduction

Phenolic compounds are secondary metabolites commonly found in plants. Recently, these compounds have attracted considerable attention due to their free radical-scavenging and anti-oxidative activities with the potential beneficial effects in human health (Chirinos et al, 2010). Plant phenolics could basically be categorized into several classes based on the number of phenol rings they contained and on

the structural elements that bound these rings to one another (Manach et al, 2004). Among these, the phenolic acids, flavonoids, and tannins are regarded as the main phenolic compounds (Balasundram et al, 2006). A major class of phenolic acids are hydroxycinnamic acids, and chlorogenic acid (3-*O*-caffeoylquinic acid, CGA) is a major analogues of hydroxycinnamic acids (Sato et al, 2011).

Previous studies reported that CGA had excellent anti-oxidative activities which could explain some biological

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properties (Xiang and Ning, 2008; Karthikesan et al, 2010; Sasaki et al, 2010). Anti-oxidants from plant extracts play an important role in liver protection (Morisco et al, 2008; Srivastava and Shivanandappa, 2009; Zeashan et al, 2009; Akanitapichat et al, 2010; Huang et al, 2012). However, less work has been conducted to study the roles of CGA on the hepatoprotective activity. Therefore, the protective effect of CGA was evaluated using the animal model of hepatotoxicity induced by CCl_4 .

2. Materials and methods

2.1 Materials

Chlorogenic acid was purchased from Chengdu Biopurify Phytochemicals Ltd. (> 98%, batch No.12082009, China). Ascorbic acid (vitamin C, VC) and butylated hydroxytoluene (BHT) was purchased from National Institute for Food and Drug Control (Beijing, China). α, α -Diphenyl- β -picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and silymarin were purchased from Sigma-Aldrich Co. (USA). Linoleic acid and β -carotene were purchased from Fluka Co. (USA). All other chemicals used for analysis were obtained from China Medicine (Group) Shanghai Chemical Reagent Corporation (China).

2.2 Test animals

Male Sprague-Dawley rats weighing 200–250 g were obtained from the New Drug Evaluation Center, Lunan Pharmaceutical Group Co., Ltd. (Linyi, China). Rats were kept in a continuously ventilated room at a mean temperature of $(22 \pm 2)^\circ\text{C}$ with a relative humidity (40%–70%) under 12 h light and dark cycles. Throughout this study, the animals had free access to a standard pellet diet and water. Animals were allowed to acclimatize to the housing conditions for 3 d before experimentation. The study received approval from the Institutional Animal Ethical Committee (IAEC) of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Lunan Pharmaceutical Group Co., Ltd. (Linyi, China).

2.3 In vitro anti-oxidative activity

The free radical-scavenging activity on DPPH was assessed according to a previously published method (Shimada et al, 1992). An aliquot (2.7 mL) of 0.2 mmol/L DPPH solution in ethanol was added to test tubes containing 0.3 mL CGA at various concentration. The mixture was then shaken vigorously and incubated in dark for 1 h. The absorbance (A) was subsequently determined at 517 nm. The free radical-scavenging activity was calculated by the formula as follows:

$$\text{Free radical-scavenging activity} = [(A_s - A_i) / A_s] \times 100$$

where A_s is the A value of DPPH alone and A_i is the A value of DPPH in the presence of various samples

The concentration of BHT and VC identical to the

experimental samples was used as reference.

The ability to scavenge the ABTS radical cation was measured using an improved procedure (Jaitak et al, 2010). ABTS radical cation solution was prepared by the reaction of 7 mmol/L ABTS and 2.45 mmol/L potassium persulfate at room temperature in the dark for 12–16 h. The solution was then diluted with 80% ethanol to obtain the A value of (0.700 ± 0.005) at 734 nm. The test substances (0.3 mL) at various concentration were mixed with ABTS radical cation solution (2.7 mL). The mixture was left to stand for 30 min, and the A value at 734 nm was recorded. BHT and VC with the same concentration were used as references. The value of free radical scavenging was calculated using the above equation for DPPH.

The anti-oxidative activity of CGA was measured using the slightly modified β -carotene bleaching method of Siddhurajub and Becker (2007). Briefly, 4 mL of β -carotene solution (0.3 mg/mL chloroform) was added into a round-bottomed 500-mL flask containing 80 mg of linoleic acid and 800 mg of Tween 80. The mixture was then evaporated at 40°C using a rotary evaporator to remove chloroform. Immediately, 200 mL of distilled water was added slowly to the mixture with vigorous agitation to form a stable emulsion. Then, 3 mL of the emulsion was transferred into different test tubes containing 0.2 mL of the samples at 400 $\mu\text{g}/\text{mL}$ and was incubated in a water bath at 50°C for 2 h. BHT and VC were used as positive control. As soon as the emulsion was added to each tube, the initial A value was determined at 470 nm. The lipid peroxidation inhibition (LPO_t) was calculated using the formula as follows:

$$\text{LPO}_t = 1 - (A_0 - A_t) / (A_0^0 - A_t^0)$$

where $(A_0$ and A_0^0) were the A values measured at the initial incubation for the samples and control, respectively, and A_t and A_t^0 were the A values measured in the samples and control at $t = 2$ h, respectively

The ferrothiocyanate (FTC) method was adapted from Tyug et al (2010) with modifications. CGA (400 μg) in ethanol (4 mL), 2.5% linolenic acid (4 mL) in ethanol, phosphate buffer (8 mL, 50 mmol/L, pH 7.0), and distilled water (4 mL) were mixed in a vial with a screw cap. The mixed solution was placed in a dark oven at 40°C . Exactly 0.1 mL of the solution was added into 75% ethanol (9.7 mL) and 30% ammonium thiocyanate (0.1 mL). After 3 min, ferrous chloride (0.1 mL, 20 mmol/L, 3.5% hydrochloric acid) was added into the reaction mixture. The A value of the mixture was measured at 500 nm at 24-h intervals until a constant highest value was reached. A mixture without the sample was used as negative control, while BHT and VC were used as standard compounds.

The A value was evaluated using the thiobarbituric acid (TBA) method reported by Kikuzaki and Nakatani (1993). A total of 20% trichloroacetic acid (2 mL) and 0.67% 2-thiobarbituric acid (2 mL) were mixed with 1 mL of sample solution prepared as described in the FTC section above. The mixture was put in a boiling water bath for 10 min. After cooling, it was centrifuged at 3000 r/min for 20 min. The A value of the supernatant was recorded at 552 nm.

2.4 Acute oral toxicity

The single-dose acute oral toxicity was evaluated following the *Organization for Economic Cooperation and Development (OECD) Guidelines for the Testing of Chemicals* (No. 420) (OECD, 2001). After acclimatization, the rats were administered with CGA (5000 mg/kg). All the animals were observed individually for clinical symptoms of toxicity and mortality for 14 d after administration.

2.5 CCl₄-induced hepatotoxicity

Rats were randomly divided into six groups with six rats in each group. The rats in Group I (vehicle control) were ig administered with distilled water with 0.3% sodium carboxymethylcellulose (CMC-Na, 1 mL/kg) for 5 d, and ip administered with olive oil (1 mL/kg) on days 2 and 3. The rats in Group II (CCl₄ control) were ig administered with 0.3% CMC-Na (1 mL/kg) for 5 d, and ip administered with CCl₄-olive oil (1:1, 2 mL/kg) on days 2 and 3. The rats in Group III were ig administered with the standard drug silymarin (100 mg/kg) for 5 d, and ip administered with CCl₄-olive oil (1:1, 2 mL/kg) on days 2 and 3, after 30 min of silymarin administration. The rats in Groups IV–VI (test groups) were ig administered with a dose of body weight of CGA (100, 300, and 500 mg/kg) for 5 d. Additionally, after 30 min of CGA administration, they were also ip administered with CCl₄-olive oil (1:1, 2 mL/kg) on days 2 and 3. At the end of the experiment (on day 6), the animals were sacrificed by an overdose of pentobarbital and exsanguinated from the carotid. The serum was separated via 10 min centrifugation at 2000 g and at 4 °C for the biochemical parameter assessment. The livers were dissected and cleaned of extraneous tissue. Part of the liver tissue was immediately transferred into 10% formalin for the histopathological investigation.

2.6 Biochemical assays

Alamine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and total bilirubin (TB) were measured with an Automatic Biochemistry Analyzer (Mindray BS-200) using commercial assay kits (Mindray, China) according to the instructions of manufacturer.

2.7 Histopathological studies

For light microscopic examination, liver tissues were fixed in 10% formalin for 48 h and embedded in paraffin. The paraffin blocks were cut to 5 μm slice using a rotary microtome. Then, the sections were stained with hematoxylin-eosin and observed under a microscope (BX53, Olympus Corporation, Japan) to detect the histopathological changes in the liver.

2.8 Statistical analysis

All the experiments were done in triplicate and the

results were expressed as $\bar{x} \pm s$. Data were analyzed by One-way ANOVA, and the statistically significant effects were further analyzed using Duncan's multiple range test. Statistical significance was determined at $P < 0.05$.

3. Results and discussion

The anti-oxidative activity of CGA was investigated by the free radical-scavenging capacity on DPPH and ABTS which were stable radicals used as tools to estimate the anti-oxidative activities of components. For the DPPH assay, the concentration required to scavenge 50% of radicals (IC₅₀) of CGA, BHT, and VC was (51.12 ± 2.86), (48.92 ± 2.78), and (36.50 ± 1.43) μg/mL, respectively. In the ABTS assay, the IC₅₀ values of CGA, BHT, and VC were (9.77 ± 0.36), (6.81 ± 0.25), and (2.95 ± 0.04) μg/mL, respectively.

Linoleic acid, which liberates a peroxy free radical through the removal of hydrogen atoms from diallylic methylene groups, oxidized the β-carotene to lose its chromophore and characteristic orange color (Moyo et al, 2010). The reaction process could be inhibited by anti-oxidative compounds because of the reaction with free radicals. Consequently, the degradation rate of β-carotene relies on the anti-oxidative capacity of the substances. The inhibitory rates of CGA on LPO, VC, and BHT were (65.57 ± 2.01)%, (86.62 ± 1.04)%, and (95.08 ± 1.92)%, respectively. While CGA had good anti-oxidative activity, it was weaker than that in the references.

The FTC test was conducted to measure the amount of peroxide in the initial stage of LPO. Peroxides resulting from linoleic acid peroxidation could oxidize Fe²⁺ to Fe³⁺. Fe³⁺ ions formed a thiocyanate complex with SCN⁻, which had a maximum A value at 500 nm (Liu and Yao, 2007). Thus, high A value was a reflection of a high concentration of peroxides formed during the emulsion incubation. As shown in Figure 1, the A value of the control increased steadily and reached a maximum on day 8. The A value of BHT was nearly invariable at 0.2, indicating the strongest ability to inhibit linoleic acid peroxide. The A value of CGA was higher than that of BHT but lower than that of VC. Therefore, CGA had a moderate anti-oxidative capacity, but was less effective than BHT.

After acid and heat treatment, the lipid peroxides were decomposed to MDA, which could also be produced during the oxidation process. TBA reacted with MDA to form a red

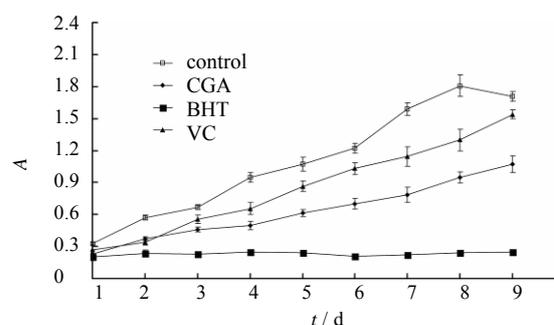


Figure 1 Anti-oxidative activity assayed by FTC method

complex that could be determined by spectrophotometry at 532 nm (Guillén-Sans and Guzmán-Chozas, 1998). The *A* values obtained for BHT, CGA, and VC were (1.036 ± 0.107) , (1.253 ± 0.183) , and (1.708 ± 0.115) , respectively, compared with the control (1.905 ± 0.136) . The anti-oxidative activity of CGA was more efficient than that of VC, but inferior to that of BHT.

Previous studies also indicated that CGA exhibited an effective scavenging capacity for DPPH, hydroxyl, and superoxide anion radicals. It was also found to possess a reducing ability and to inhibit the peroxidation of linoleic acid (Wu, 2007; Sato et al, 2011). Additionally, the ABTS radical-scavenging ability and β -carotene bleaching property were confirmed.

In the acute oral toxicity study, CGA (5000 mg/kg) showed no treatment-related symptoms of toxicity or mortality in any animals during the 14 d study. In addition, no weight loss was observed and all the internal organs

examined at necropsy were free from any gross pathological changes. Therefore, the oral toxicity of CGA could be classified in the category “unclassified” (the acute lethal toxicity is greater than 5000 mg/kg) according to the Globally Harmonized Classification System of OECD (OECD, 2001).

The hepatoprotective effects of CGA on serum biochemical parameters in CCl₄-treated rats are presented in Table 1. The activities of ALT, AST, ALP, and TB were significantly higher in the CCl₄ group than those in the control group ($P < 0.01$). The rats treated with CGA (100 mg/kg) had significantly lower AST, ALP, and TB ($P < 0.05$), while the level of ALT was comparable to that of the CCl₄ group ($P > 0.05$). The rats treated with CGA (300 or 500 mg/kg) and the standard treatment silymarin (100 mg/kg) showed a significant decrease in the parameters that were elevated in the CCl₄-treated group ($P < 0.05$). The results observed after the administration of 300 mg/kg CGA were comparable to those of silymarin at 100 mg/kg ($P > 0.05$).

Table 1 Effect of CGA on biochemical parameters of CCl₄-treated rats ($\bar{x} \pm s$, $n = 6$)

Groups	Doses / (mg·kg ⁻¹)	ALT / (IU·L ⁻¹)	AST / (IU·L ⁻¹)	ALP / (IU·L ⁻¹)	TB / (mg·dL ⁻¹)
control		34.50 ± 4.04	117.67 ± 11.41	202.20 ± 21.23	1.38 ± 0.26
CCl ₄		51.83 ± 12.09**	178.67 ± 14.81**	337.00 ± 50.35**	2.33 ± 0.36**
silymarin	100	37.83 ± 6.11 ^Δ	118.67 ± 14.40 ^{ΔΔ}	214.50 ± 37.03 ^{ΔΔ}	1.50 ± 0.18 ^{ΔΔ}
CGA	100	46.31 ± 7.82	129.89 ± 29.00 ^{ΔΔ}	248.67 ± 33.76 ^{ΔΔ}	1.93 ± 0.31 ^Δ
	300	39.85 ± 8.64 ^Δ	116.85 ± 13.59 ^{ΔΔ}	227.46 ± 24.17 ^{ΔΔ}	1.61 ± 0.24 ^{ΔΔ}
	500	37.27 ± 5.89 ^Δ	113.96 ± 14.05 ^{ΔΔ}	213.95 ± 29.11 ^{ΔΔ}	1.55 ± 0.29 ^{ΔΔ}

** $P < 0.01$ vs control group; ^Δ $P < 0.05$ ^{ΔΔ} $P < 0.01$ vs CCl₄-treated group

The CCl₄-induced hepatotoxicity model was being widely used for the evaluation of the hepatoprotective effects of plant extracts (Awaad et al, 2012). CCl₄ was activated by the cytochrome P450 system to give the trichloromethyl radical (CCl₃) and further combined with oxygen to form the trichloromethyl peroxy radical CCl₃O₂ (Bhattacharjee and Sil, 2007; Zhou et al, 2010). Both of these products bound covalently to the cellular macromolecules and produced degradation of the lipid membranes of adipose tissue. This led to the formation of lipid peroxides. Moreover, LPO was one of the principal mechanisms of the hepatotoxicity of CCl₄ (Souza et al, 1997). Therefore, free radical scavenging provided the important means to inhibit the chain reaction of LPO by CCl₄. In this assay, the free radical-scavenging activity of CGA was determined using DPPH and ABTS radicals. Our results showed that CGA had potent scavenging activity *in vitro*, which suggested that it was also an effective anti-oxidant.

CCl₄ could lead to hepatic injury that histologically resembled viral hepatitis. Loss of the metabolic enzymes that located in intracellular structure resulted from changes in the endoplasmic reticulum (Recknagel, 1983). Therefore, the hepatoprotective effect was assessed by biochemical and histopathological examination. In this present study, the pretreatment with CGA prior to the administration of CCl₄ showed a significant decrease of ALT and AST levels in serum compared to the rats treated with CCl₄ alone. This was

the evidence that both stabilization of plasma membrane and repair of CCl₄-induced hepatic tissue damage. The injection of CCl₄ led to the elevation of ALP and TB levels, reflecting a pathological alteration in biliary flow. CGA treatment reduced the levels of both, indicating that could stabilize biliary dysfunction caused by CCl₄ in the rat liver. Histopathological findings of liver tissue had directly supported the results from the serum assays. To a great extent, the detrimental effects exerted by CCl₄ toxicity were effectively reversed or improved.

Histopathological observations of liver sections showed that CCl₄, as compared with the control (Figure 2A), exhibited the most severe damage of the groups with massive fatty steatosis (Figure 2B). The treatment of silymarin is shown in Figure 2C. Hepatic lesions caused by CCl₄ were alleviated somewhat with the treatment of CGA. Different doses of CGA (Figures 2D–2F) showed the different protective effects against the acute liver damage. The liver sections of CGA at 500 mg/kg were almost comparable to the control.

4. Conclusion

This present study illustrates that CGA possesses strong anti-oxidative activity *in vitro* and exerts a potent protective effect against CCl₄-induced acute liver injury. The *in vivo* hepatoprotective activity of CGA might result from its free radical-scavenging and anti-oxidative activity. However, more

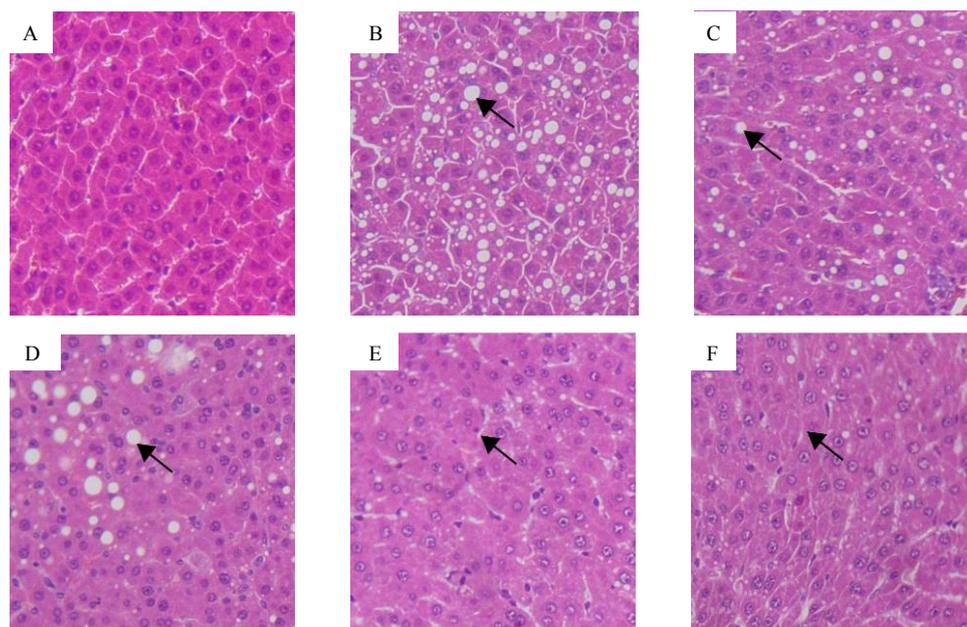


Figure 2 Effects of silymarin and CGA on liver histopathology of CCl₄-treated rats

A: vehicle control B: CCl₄ control C: CCl₄ + silymarin 100 mg·kg⁻¹ D: CCl₄ + CGA 100 mg·kg⁻¹
E: CCl₄ + CGA 300 mg·kg⁻¹ F: CCl₄ + CGA 500 mg·kg⁻¹

researches are still needed to confirm the protective effect of CGA using larger sample sizes of animals to explore its possible mechanisms of the action.

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Introduction of Cover Picture



Chelidonium majus Linn. is a herbaceous perennial plant, the only species in the genus *Chelidonium* L. It is native to Europe and western Asia and introduced widely in North America.

C. majus has an erect habit, and reaches 30 to 120 cm high. The leaves are lobed and wavy-edged, 30 cm long. When injured, the plant exudes a yellow to orange latex. The flowers consist of four yellow petals, each about 1 cm long, with two sepals. A double-flowered variety occurs naturally. The flowers appear from late spring to summer in umbelliform cymes of about four flowers. The seeds are small and black, borne in a long capsule. Each has an elaiosome, which attracts ants to disperse the seeds.

The whole plant is toxic in moderate doses as it contains a range of isoquinoline alkaloids but there are numerous therapeutic uses when used at the correct dosage. The effect of the fresh herb is of a mild analgesic, cholagogic, antimicrobial, oncostatic, and central nervous system sedative. In *in vitro* experiments on animal cells, celandine is shown to be cytostatic. An immune stimulating effect has also been noted. The latex could be employed for cauterizing small open wounds. The characteristic latex also contains proteolytic enzymes and the phytocystatin chelidostatin, a cysteine protease inhibitor. These co-constituents could explain the topical use of greater celandine against warts and moles.

(Photo by Qi-fang Lei)