Hepatoprotective effects of Coptidis Rhizoma aqueous extract on carbon tetrachloride-induced acute liver hepatotoxicity in rats

Xinshen Ye[a], Yibin Feng[a*], Yao Tong[a], Kwan-Ming Ng[b], SaiWah Tsao[c], George KK Lau[d], Chowing Sze[a], Yanbo Zhang[a], Jun Tang[a], Jiangang Shen[a], Seiichi Kobayashi[e]

a School of Chinese Medicine, The University of Hong Kong, 10 Sassoon Road, Pokfulam, Hong Kong.

b Department of Chemistry and Open Laboratory of Chemical Biology of the Institute of Molecular Technology for Drug Discovery and Synthesis, Faculty of Science, The University of Hong Kong, Pokfulam Road, Hong Kong.

c Department of Anatomy, Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Pokfulam, Hong Kong.

d Department of Medicine, The University of Hong Kong, 21 Sassoon Road, Pokfulam, Hong Kong.

e Faculty of Healthy Science, Hokkaido University, Kita 15, Nishi 7 Kita-ku, Sapporo, Japan.

* Corresponding author

Tel: (852) 2589 0482

Fax: (852) 2872 5476

E-mail address: yfeng@hku.hk

Mail address: *School of Chinese Medicine, The University of Hong Kong, 10 Sassoon Road, Pokfulam, Hong Kong.*

Abstract

Ethnopharmacological relevance: Coptidis Rhizoma (CR, Chinese name is *Huanglian*) has been used in treating infectious and inflammatory diseases for two thousand years in Traditional Chinese Medicine (TCM). Its related pharmacological basis for the therapeutics has been studied intensively, but CR can be also used for vomiting of "dampness-heat type or acid regurgitation" due to "liver-fire attacking stomach" in TCM, which symptoms seem to link the hepatic and biliary disorders, yet details in the therapies of liver diseases and underlying mechanism(s) remain unclear. *Aim of the Study:* in the present study, hepatoprotective effect of Coptidis Rhizoma aqueous extract (CRAE) and its possible mechanism were studied in rats intoxicated with carbon tetrachloride (CCl_4). *Materials and Methods:* Sprague-Dawley (SD) rats aged 7 weeks old were intraperitoneally injected with CCl_4 at a dose of 1.0 ml/kg as a 50% olive oil solution. The rats were orally given the CRAE at doses of 400, 600, 800 mg/kg and 120 mg/kg berberine body weight (BW) after 6 hours of CCl_4 treatment. At 24 hours after CCl_4 injection, samples of blood and liver were collected and then biochemical parameters and histological studies were carried out. *Results:* the results showed that CRAE and berberine inhibited significantly the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and increased the activity of superoxide dismutase (SOD). Observation on the hepatoprotective effect of berberine was consistent to that of CRAE. *Conclusion:* the study is the first time to demonstrate that CRAE has hepatoprotective effect on acute liver injuries induced by CCl_4, and the results suggest that the effect of CRAE against CCl_4-induced liver damage is related to antioxidant property.

Keywords: Coptidis Rhizoma aqueous extract; Carbon tetrachloride; Aspartate aminotransferase; Alanine aminotransferase; Superoxide dismutase; Liver histopathology.

Abbreviations: CR, Coptidis Rhizoma; CRAE, Coptidis Rhizoma aqueous extract; CCl_4, Carbon tetrachloride; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; SOD, Superoxide dismutase; SD rats, Sprague-Dawley rats; H & E staining, Hematoxylin and eosin staining.

Article Outline

1. Introduction

Coptidis Rhizoma (CR, Chinese name is *Huanglian*) is a Chinese herbal medicine used as a clearing heat and detoxifying agent, and has been used to treat syndromes incurred by *damp-heat, fire* or *toxicity* in Traditional Chinese Medicine (TCM) for two thousand years, which can be conceived as inflammatory diseases. Extensive studies exhibited that CR has many pharmacological actions with strong clinical implications, including antibacterial, antiviral, antiinflammatory, antineoplastic, antihypertensive, antioxidative, antihyperglycemic and cholesterol-lowering effects (Chang and But, 2004; Choi, et al., 2007; Kim, et al., 2008; Fukuda, et al., 1998; Li, et al., 2000; Sanae, et al., 2001; Yokozawa, et al., 2003, 2004). Traditionally, CR can be used for vomiting with "dampness-heat type" or "acid regurgitation due to liver-fire attacking stomach" in TCM, whose symptoms seem to be linked with the hepatic and biliary disorders, yet its therapeutic potential remains unexplored. In TCM clinical practice, CR is a key component in many TCM formulae. Typically, *Huanglian Jiedu* decoction (or Oren- gedoku-to in Japanese or JT-15, including Coptidis Rhizoma, Radix Scutellariae, Cortex Phellodendri, and Frucuts Gardeniae) has been used for the therapies of hepatitis and liver dysfunction in addition to gastric ulcers, dermatitis, dementia, and cerebrovascular diseases in Japan (Itoh, 2001) and has intensively studied for scientific basis of hepatitis and liver dysfunction in Japan (Ohta, et al., 1997, 1998, 2004), but whether CR has liver protection or not is unknown. Berberine, due to the phytochemical analysis, was the main ingredient in CR (Xu et al., 2004). It was reported that herbs with high content of berberine exhibit preventive effect or curative effect on liver damage (Nadkarni, 1976, Gilani and Janbaz, 1995; Janbaz and Gilani, 2000), indicating that CR is potential for the treatment of liver injury and hepatitis. According to the history of CR use, clinical indications of

CR-containing composite formulae, and clinical indications of berberine-containing plant species in other traditional medicine, we have used CR to prescribe for various liver diseases in TCM clinical practice in China (Feng et al., 2008). It is interesting to explore the exact effect of CR on liver damage and underlying mechanisms.

It was reported that the changes associated with CCl_4-induced liver damage are similar to that of acute viral hepatitis (Rubinstein, 1962), drug/chemicals-induced hepatopathy and oxidative stress (Recknagel et al., 1989; Kadiiska et al., 2000), so CCl_4-induced hepatotoxicity model is frequently used for the investigation of hepatoprotective effects of drugs and plant extracts. *CRAE* has shown to ameliorate renal oxidative injury in vivo and in vitro (Yokozawa et al 1999, 2004, 2005). Previous study revealed that preventive effect of huanglian-jie-du-tang extract on progression of CCl_4-induced acute liver injury in rats is related to its antioxidant properties (Ohta et al, 1997, 1998, 2001). Whether CRAE contributes to its antioxidant effects on CCl_4-induced acute liver injury in rats is not known yet.

In the present study, CCl4 was therefore introduced to induce liver damage on experimental animal model, and the curative effect of CRAE was examined via determining serum Alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum and tissue superoxide dismutase (SOD) level as well as hitopathological study. Phytochemistry of CRAE was also analyzed via High performance liquid chromatogram (HPLC) in this study. The experimental results demonstrated the potential effects of CRAE in protecting liver function, reducing oxidative stress, and improving histopathological structures in the rat model of CCl_4–induced liver damage. The study not only provides helpful information for the application of CRAE in liver disease, but also promotes the understanding of the pharmacological mechanisms of CRAE in the acute toxic liver injury.

2. Materials and methods

2.1. Plant materials and extraction procedures

Crude herb, Coptidis Rhizoma (*Coptis chinensis* Franch.) was collected from Sichuan province, China and authenticated under the guidance of The Pharmacopeia of China (2005). Plant materials were dried under shade and cut into small pieces before extraction. For the preparation of CRAE, one gram of crude CR was boiled in 10ml of distilled water at 100˚C for 1 hour. Total was 500g of crude CR. The solution was percolated through filter paper (Whatman, pleated filter grade 597 1/2, 4–7 μm) and then sterilized by filtration through a 0.2 μm pore filter (Minisart®-plus, Sartorius), while the residue was further extracted under the same condition once. The filtrates collected from the extraction were combined and evaporated to dryness by vacuum at temperature. The dry extract powder obtained (40g) was stored in –20 ºC freezer and used in following experiments.

The chemical profile of CRAE was recorded by high performance liquid chromatography (HPLC) with photodiode array (PDA) detection. CRAE powder (26.6 mg) was accurately weighed and dissolved in 9 ml acidified methanol (a mixture of fuming hydrochloric acid and methanol in 1:100 proportion). The solution was heated in a 60 ^{o}C water bath for 15 min, followed by ultrasonication for 30 min. The solution was then filtered by using a 0.45 μm Millex Syringe filter unit and subjected to HPLC analysis. The HPLC analytical system is composed of a Waters 600s solvent delivery system coupled with a 717 plus autosampler (with injection volume at 10 μL) and a 996 photodiode array detector. A reverse-phase C_{18} column (Alltech Alltima HP C18, 250 mm × 4.6 mm, 5 μm)eluting with a mobile phase (acetonitrile:25 mM potassium dihydrogen phosphate in H_2O (25: 75)) in an isocratic manner and at a flow rate of 1 ml/min was employed. The eluate was monitored at the wavelength of

345 nm. The column temperature was kept at 24 °C. Berberine is used as the reference standard for identifying and quantifying the major component in CRAE. The whole analysis was duplicated for confirmation.

Atomic absorption spectroscopic (AAS) analysis of five toxic heavy metals, including arsenic, cadmium, chromium, lead and mercury, was preformed on PerkinElmer Analyst 800 atomic absorption spectrometer with autosampler.

2.2. Drugs and chemical test agents

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) test kits were purchased from Stanbio, USA. SOD assay kit was from Biovision, USA. Carbon tetrachloride (CCl_4), olive oil and berberine, were purchased from Sigma, USA. Standards of arsenic, cadmium, chromium, lead and mercury were purchased from the Sigma, USA.

2.3. Test Animals

Adult male SD rats 7 weeks weighing 250 ± 20 g were obtained from animal centre of The University of Hong Kong. The animals left for 2 days for acclimatization to animal room conditions were maintained on standard pellet diet and water *ad libitum* at a temperature of 20–25 °C under a 12 h light/dark cycle throughout the experiment. The food was withdrawn on the day before the experiment, but free access of water was allowed. The rats were randomly assigned. A minimum of 8 animals were used in each group. All animals received human care and study protocols complied with the guidelines of the animal centre of the University of Hong Kong. Throughout the experiments, animals were processed according to the suggested international ethical guidelines for the care of laboratory animals.

2.4. CCl4-induced acute liver damage model in rats and CRAE treatment

The animals were randomly divided into five groups, that is, normal, CCl_4 alone, and 400, 600, 800 mg/kg BW CRAE and 120 mg/kg berberine BW treatment on CCl_4 groups, containing eight rats in each. Rats were intraperitoneally (*i.p.*) injected with CCl_4 at a dose of 1.0 ml/kg as a 50 % olive oil solution according to the previously reported methods with some modification (Moghaddam, et al. 1998; Feng, et al. 2000) and control ones with the same dose of olive oil. Our previous clinical practice showed the therapeutic effect of CRAE on liver injury, infectious hepatitis and even cancer (Feng, et al., 2008), the dosage of raw herb we use in clinic was about 30~50 gram in single use or 9-18 gram in formulation. Excellent effect and no toxicity were observed in our clinical practice. The result and dosage in present study fit our clinical study, that is, if we use conversion table to compute the clinical dosage from the animal dosage, 400~800 mg/kg, it will be 25~50 gram, near our clinical study. On the other hand, lower than 400mg/kg may have no effect in our dose screening in rats. Hence, the CRAE was dissolved in distilled water at a concentration of 400, 600 or 800 mg/kg BW orally administered to rats injected with or without CCl_4 treatment at 6 hours after CCl_4 exposure. The control rats were orally given the same volume of distilled water. These animals were fasted with free access to water throughout the experiment.

Twenty-four hours after the CCl_4 administration, blood samples were withdrawn by cardiac puncture when the animals had been anaesthetized with ketamine /xylazine mixture (ketamine 67mg/kg, xylazine 6mg/kg, i.p). The animals were sacrificed by an overdose of pentobarbitone (Phenobarbital 200mg/kg, i.p) or diethyl ether immediately after blood collection. Blood samples collected in heparinized tubes were centrifuged at 3000 × g for 10 min to obtain serum. Serum samples were used to determine SOD as well as to test ALT and

AST activities. On the other hand, the liver of each rat was promptly removed and used to determine the tissue level SOD and for further histopathological study.

2.5. Serum ALT and AST analysis

Biocon standard kits and an auto-analyzer (Hitachi 736-60, Tokyo, Japan) (UV-Rate) were used to measure serum ALT and AST activities in serum samples according to the method published before (Wilkinson et al., 1972). Values are derived based on the "absorptivity micromolar extinction coefficient" of NADH at 340 nm. ALT and AST activities expressed in terms of units per liter (U/L) are the amount of enzyme oxidizing one μmol/L of NADH per minute.

2.6. SOD levels in serum and liver tissues

The sensitive SOD assay kit (SOD Assay Kit-WST) utilizes mitochondrial activity that produces a water-soluble formazan dye upon reduction with superoxide anion. The rate of the reduction with a superoxide anion is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD. Therefore, the inhibition activity of SOD determined by a colorimetric method was used for the determination of the serum and liver SOD level in this study.

Serum and liver homogenate collected from test animals were for measurement of SOD in blood and liver tissues. Procedure was carried out by according to SOD Assay Kit-WST Technical Manual (Dojindo Laboratories, Kumamoto, Japan).

2.7. Histopathological studies

For the histopathological study, the livers of eight animals in each group were immediately removed after autopsy and the tissues were fixed in 10% buffered formaldehyde solution for a period of at least 24 h. The paraffin sections were then prepared (Automatic Tissue Processor, Lipshaw) and cut into 5 μm thick sections by a Leica RM 2016 rotary microtome (Leica Instruments Ltd., Shanghai, China). The sections were stained with hematoxylin and eosin staining (H & E staining) and then mounted with Canada balsam (Sigma, USA). The degree of liver damage was examined under the microscope (Leica Microsystems Digital Imaging, Germany). The images were taken using Leica DFC 280 CCD camera at original magnification of 10×10. Through grading the liver sections numerically to assess their histological features, acute liver injury was evaluated by three independent researchers. Vacuolation, nuclei, hepatocyte necrosis, inflammatory cell infiltration and central vein and portal triad were used as criteria, and a combined score of histological features was given for each liver section. The parameters were graded from score 0 to 6, with 0 indicating no abnormality, 1–2 indicating mild injury, 3–4 indicating moderate injury and 5–6 with severe liver injury (Wills and Ahsa, 2006; Wang et.al., 2008).

2.8. Statistical analysis

The data obtained were analyzed by one-way of variance (ANOVA) and Student–Newman–Kelus post hoc tests for the significant interrelation between the groups. Data were expressed as mean ± standard error of the mean and were analyzed with SPSS, version 11.5 software. Differences between group means were calculated by a one-way analysis of variance. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Quality control of CRAE

The crude herb of *huanglian* was identified by morphological characteristics of CR (*Coptis chinensis Franch.*) according to The Pharmacopeia of China (2005) and the voucher specimens (no. CR20060616) was deposited in the Herbarium of School of Chinese Medicine, The University of Hong Kong. The plant and crude herb were shown in Figure 1A and B.

The HPLC chemical profile of CRAE is shown in Fig. 1 C. Nine characteristic chromatographic peaks were recorded at 345 nm. The chemical identity of the peak at 14.98 min was confirmed as berberine by chromatographic peak matching of berberine reference standard at 15.02 min (Fig. 1 D) and the similar UV-Visible spectra with λ_{max} at 345 nm. A calibration curve of berberine reference standard showing a good linearity over the concentration range from 10 to 160 μg/ml with a regression coefficient at $r^2 = 0.9999$ (Fig. 1 E) was obtained. The content of berberine in CRAE powder was determined to be 20.3 mg in 100 mg of the powder (i.e., 20.3 % weight by weight) with deviation less than 1% in duplicated analysis. Among other eight peaks, five (including the peaks 4, 5, 6, 7 and 8) showed the similar UV spectra of berberine with λ_{max} at 345 nm.

As shown in Table 1, the contents of five heavy metals (Arsenic, Cadmium, Chromium, Lead, and Mercury) in *CRAE* determined from the AAS analysis fell in the range less than the maximum limit (20 ppm or μg/g) as regulated in China Pharmacopeia (2005) and the World Health Organization (WHO). The amount of the five harmful elements in CRAE is in the safe range for herbal test use.

3.2. Liver protective effects of CRAE and berberine on acute liver damage in rats

Compared with the normal group, the ALT activities in serum of the control treated with CCl_4 at an *i.p.* dose of 1.0 ml/kg was significantly elevated (P<0.01) after 24 h. While treatment with 400 mg/kg BW, CRAE decreased remarkably the levels of serum ALT in rats treated with CCl_4 though higher than the normal rats. The orally administered CRAE at a higher dose of 600 mg/kg BW and 800 mg/kg BW could significantly reduce the serum ALT level and restored them to normal levels when compared with rats treated with CCl_4 control (P<0.01). This result showed that the oral treatment with 800, 600 and 400 mg/kg BW of CRAE could inhibit the elevated ALT activities in rats intoxicated with CCl_4 in a dose-dependent manner (Table. 2).

Compared with the normal group, serum AST activities were significantly elevated (P<0.01) by 6 h after the CCl_4 treatment. Post-administration of 600 and 800 mg/kg BW CRAE significantly decreased the AST activities in serum in contrast to CCl_4-treated rats (P<0.05, P<0.01), but 400 mg/kg BW CRAE did not show effect on the AST levels in the CCl_4-treated rats (Table.2).

Significantly reduced serum ALT and AST levels in liver damage rats treated with berberine 120 mg/kg were also observed (Table. 2).

3.3. Effect of CRAE and berberine on the levels of serum SOD and liver homogenate SOD activities in acute liver damage of rats

The inhibition rate of serum SOD activities in control rats treated with CCl_4 alone was remarkably decreased after 24 h (P<0.01) compared with the normal ones, which showed the injured liver functions by CCl_4. While treated with 800, 600, 400 mg/kg BW CRAE and 120 mg/kg BW berberine, the inhibition rates of SOD were significantly elevated (P<0.01) compared with the control rats, especially the high dose at 800 mg/kg BW could restore the value to the normal level (Table 2). Liver homogenate SOD activities were similar to serum expression (Table 2).

3.4. Effect of CRAE and berberine on histopathological changes of liver in acute liver damage of rats

The histological changes associated with the hepatoprotective activity in three dosages of CRAE and berberine basically supported the measuring of the serum enzyme activities. There was no abnormal appearance or histological changes in the liver of normal control rats, which received olive oil only (Fig. 2 A). CCl_4 administration caused classical damage in the rat liver at 24 h, as demonstrated by severe hepatocyte necrosis, inflammatory cells infiltration, fatty degeneration, hemorrhage, and hydropic degeneration (Fig. 2 B), vacuole generation and microvascular steatosis were frequently observed. The administration of BW at dose of 800, 600 and 400 mg/kg could largely rescue the severity of CCl_4-induced liver intoxication, in which the high dose at 800 mg/kg was most effective (Fig. 2 C-E). The histological patterns showed dose dependant improvements for fatty change, necrosis and lymphocyte infiltration in contrast to treatment with CCl_4 showing an obvious formation of necrosis. Improvement results were observed in liver section from animals treated with 120 mg/kg berberine (Fig 2 F). The scoring of histological damage was displayed in Table 2.

4. Discussion and conclusions

Three clues led us to study CR against liver diseases: traditional use and our TCM daily practices for liver diseases (Feng et al., 2008), liver protective effects of

Hunaglian-containing composite formula (Huanglian je du decoction) (Ohta et al., 1997, 1998, 2004), and berberis aristata (major compound is berberine which is the same as CR) is used for liver disease in South Asian areas, only showed preventive, but not curative effects on liver damage in rats treated with CCl_4 (Gilani and Janbaz, 1995; Janbaz and Gilani, 2000). We assume that CR is a main effective herb in Huanglian je du decoction for liver protection and CR should possess curative effects on liver damage. The aim of the present study was to investigate the potential hepatoprotective effects of Coptidis Rhizoma aqueous extract (CRAE) on the free radical damage of liver caused by CCl_4 in rats.

Firstly, quality control was conducted for the raw herb of CR and CRAE to guarantee the reliability of our experimental results. The plant materials of Coptidis rhizhoma, Chinese name is "*Haunglian*", have three species. Rhizoma of Coptis Chinensis Franch, one of the species, was chosen for our study due to its plentiful cultivation in China and most popular in TCM practice. Miao et al. (1997) have shown that when *Coptidis rhizoma*, *Scutellariae radix*, and *Phellodendri cortex* are extracted with 50% methanol, the *Scutellariae radix* extract has much higher O_2^--scavenging activity than the *Phellodendri cortex* extract, while the *Coptidis rhizoma* extract has little O_2^--scavenging activity. Yokozawa et al. (1997) have reported that although the boiled water extract of *Coptidis rhizoma*, *Gardeniae frucuts*, *Scutellariae radix* or *Phellodendri cortex* inhibits lipid peroxidation induced by H_2O_2 in rat liver homogenates, the *Coptidis rhizoma* extract has the highest inhibitory activity, followed in the order of strength by the *Gardeniae frucut* extract > the *Scutellariae radix* extract > the *Phellodendri cortex* extract. We assume that water extract of CR should possess better bioactivities according to the above two reports. In addition, the water extract of CR is clinically applied form, and thus being used in our study. The yield amount of CRAE obtained from raw herb was similar to previous reports (Li, et al., 2000; Yokozawa, et al., 2004). The consistency of chemical composition in the CRAE is important in safeguarding the reliability of the research results. The chemical profile of CRAE was recorded by the RP-HPLC/PDA analysis. The HPLC chemical profile could be delineated by the measurement of relative retention times of major characteristic peaks using berberine as a marker. The resulting chromatogram was used as a standard for assessment of all extracts used in the current study. The HPLC chemical profile of the CRAE was similar to the previous report (Li, et al., 2000). As toxic heavy metals may induce toxicity including liver damage (Duffus, 2002; Wang, et al., 2007) and traditional Chinese medicines have drawn attention by its heavy metals (Cooper K, et al., 2007), five commonly found toxic metals, including aresenic, cadimum, chromium, lead and mercury were analyzed and their contents in CRAE were in safe range set by the Pharmacopeia of China (2005) and WHO.

Many drugs or chemical substances are known to cause hepatic injuries, such as acetaminophen, $CC1_4$, D-galactosamine (GalN), aflatoxins and dimethylnitrosamine (DMN), among which, liver injury induced by CCl_4 is the best-characterized system of the xenobiotic-induced hepatotoxicity and a commonly used model for screening the drugs with anti-hepatotoxicity and/or hepatoprotective activity (Brattin et al., 1985). CCl_4-induced liver damage, a free radical damage model, results from oxidative stress that could directly injure hepatocellular membrane by lipid peroxidation, followed by a series of cascades of cellular events such as the massive release of inflammatory mediators or cytokines, which eventually lead to liver injuries (Pessayre, 1995; Dizdaroglu, et al., 2002; Higuchi and Gores, 2003). Superoxide dismutase (SOD) is one of the most important antioxidative enzymes, whose activities decrease after CCl_4 injection. Therefore we selected SOD as parameter for the antioxidative effects of CR. The present study showed that CCl_4 administration caused severe acute liver damage in rats, which was demonstrated by significant elevation of serum AST, ALT levels, decreased SOD activities (Table 2), and classic histopathological changes

(Fig.2), indicating that CCl4-induced liver damage in animal model can be used to evaluate the curative effect of CRAE.

All data in our study consistently demonstrated that CRAE treatment at a dose of 800, 600 and 400 mg/kg BW had a potent protective effect against oxidative stress and acute liver damage induced by CCl_4 in rats, as revealed by remarkable elevation of SOD activities in the liver and serum (Table 2). Additionally, CRAE could ameliorate acute liver damage to a high degree, as demonstrated by reduction of serum ALT and AST levels and the improvement of the histopathological changes (Table 2, 3 and Fig.2). Apart from mild hydropic degeneration of hepatocytes, the liver had a nearly normal appearance in CCl_4-treated rats simultaneously treated with CRAE at a dose of 800 mg/kg BW (Fig.2 and Table 3). The results also indicated a remarkable elevation of SOD activities in the liver and serum of rats for CRAE treatment at 24 h after CCl_4 administration. It was previously reported that CRAE effectively scavenged the NO radical in-vitro (Yokozawa et al 2000, 2004), so the action mechanisms underlying hepatoprotection of CRAE may be related to both its radical scavenging properties and indirect effects as a regulator of antioxidative systems in which more details should be figured out in the future.

Phytochemical analysis indicated that berberine is also a major compound in CRAE (see Fig. 1 C and 1 D). Previous studies have demonstrated that berberine showed hepatoprotection possibly through inhibitory action on hepatic drug metabolizing enzymes, cytochrome P450s *(Gilani and Janbaz, 1995; Janbaz and Gilani 2000)*, but no evident curative effect of berberine against CCl_4-induced acute liver damage is investigated before. Our present study shows that 120 mg/kg of berberine can significantly decrease the elevation of serum AST and SLT level induced by CCl_4 treatment in animal model, and the recovery of SOD activity both in serum and tissue indicates berberine as an antioxidant agent in liver protection. This convinces our observation on CRAE's action on liver damage and further the knowledge that berberine may be the major active component in CRAE when it was used for liver diseases. Berberine 120 mg/kg BW which is the equal of CRAE 600 mg/kg BW (calculated by about 20% of berberine in CRAE according to Fig.1) displays good liver protective effect (effect close to CRAE 800 mg/kg BW). Detailed dose-effect relationship and bioavailability of berberine on hepatoprotective effect need to be further studied.

In conclusion, the study is the first time to demonstrate that CRAE has an impressive hepatoprotective effect on acute liver injuries induced by CCl_4, which might be considered to be therapeutic effect in clinical situations. Berberine may be the major active component in CRAE for hepatoprotective effect. As a possible mechanism, CRAE could alleviate liver injury through antioxidative effects. On the other hand, the model of CCl_4-induced hepatic injury in the rat is similar to many features of acute hepatitis induced by toxicants and virus, hence our results could partially explicate therapeutic principle for CRAE in TCM clinical application, suggesting that CRAE could be used as a potential new drug for acute liver injury.

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References

Brattin, W.J., Glende, E.A., Recknagel, R.O., 1985. Pathological mechanisms in carbon tetrachloride hepatotoxicity. Free Radical Biology and Medicine 1:27–28.

Chang, H.M., Paul, P.H But, 2001. Pharmacology and applications of Chinese Materia Medica. World Scientific Publishing, Hong Kong, 1061-1077.

Choi, U.K., Kim, M.H., Lee, N.H., 2007. Optimization of antibacterial activity by Gold-Thread (Coptidis Rhizoma Franch) against Streptococcus mutans using evolutionary operation-factorial design technique. Journal of microbiology and biotechnology, 17:1880-4.

Dizdaroglu, M., Jaruga, P., Birincioglu, M., Rodrriguez, H., 2002. Free radical-induced damage to DNA: mechanisms and measurement. Free Radical Biology and Medicine 32:1102–1115.

Feng, Y., Nagamatsu, T., Suzuki, Y., Kawada, T., Feng, Y.G., Kobayashi, S., Koike, T., 2000. Pharmacological studies on the diuretic action of Chinese-Japanese formulations: the application and evaluation of its pharmacological screening. Journal of Traditional Medicine 17, 122-130.

Feng, Y., Luo, W.Q., Zhu, S.Q., 2008. Explore new clinical application of Huanglian and corresponding compound prescriptions from their traditional use. China Journal of Chinese Materia Medica, 33, 1221-1225.

Fukutake, M., Yokota, S., Kawamura, H., Iizuka, A., Amagaya, S., Fukuda, K., Komatsu, Y.,1998. Inhibitory effect of Coptidis Rhizoma and Scutellariae Radix on azoxymethaneinduced aberrant crypt foci formation in rat colon. Biological & pharmaceutical bulletin. 21:814-817.

Gilani, A.H., Janbaz, K.H., 1995. Preventive and curative effects of Berberis aristata Fruit extract on paracetamol- and CCl4-induced hepatotoxicity. Phytotherapy Research 9:489 -494.

Higuchi, H., Gores, G.J., 2003. Mechanisms of liver injury: an overview. Current Molecular Medicine 3:483–490.

Janbaz, K.H., Gilani, A.H., 2000. Studies on preventive and curative effects of berberine on Chemical-induced hepatotoxicity in rodents. Fitoterapia 71, 25-33.

Kadiiska, M.B., Gladen, B.C., Baird, D.D., Dikalova, A.E., Sohal, R.S., Hatch, G.E., Jones, D.P., Mason, R.P., Barrett, J.C., 2000. Biomarkers of oxidative stress study: are plasma antioxidants markers of CCl4 poisoning? Free Radical Biology and Medicine 28:838-845.

Kim, H.Y., Shin, H.S., Park, H., Kim, Y.C., Yun, Y.G., Park, S., Shin, H.J., Kim, K., 2008. In vitro inhibition of coronavirus replications by the traditionally used medicinal herbal extracts, Cimicifuga rhizoma, Meliae cortex, Coptidis rhizoma, and Phellodendron cortex. Journal of clinical virology, 41:122-128.

Li, X.K., Motwani, M., Tong, W., Bornmann, W., Schwartz, G.K., 2000. Huanglian, A Chinese herbal extract, inhibits cell growth by suppressing the expression of cyclin B1 and inhibiting CDC2 kinase activity in human cancer cells. Molecular Pharmacology 58:1287-1293.

Miao, Z., Kayahara, H. and Tadasa, K., 1997. Superoxide-scavenging and tyrosinase-inhibitory activities of the extracts of some Chinese medicines. Bioscience, Biotechnology, and Biochemistry 61, 2106–2108.

Moghaddam, A.P., Eggers J.S., Calabrese E.J., 1998. Evaluation of sex difference in tissue repair following acute carbon tetrachloride toxicity in male and female Sprague-Dawley rats. Toxicology 130, 95-105.

Nadkarni, A.K., 1976. In: Indian materia medica, Vol. I Popular Prakashan Private Ltd, Bombay, 187–189.

Ohta, Y., Kongo-Nishimura, M., Hayashi, T., Kishikawa, T., 2004. Effect of Oren-gedoku-to (*Huanglian*-Jie-Du-Tang) extract on disruption of hepatic antioxidant defense systems in rats treated with D-galactosamine. Journal of Ethnopharmacology 94, 323-329.

Ohta, Y., Sasaki E., Nishida K., Kongo M., Hayashi T., Nagata M., Ishiguro I., 1998. Inhibitory effect of Oren-gedoku-tu (Huanglian-Jie-Du-Tang) extract on hepatic triglyceride accumulation with the progression of carbon tetrachloride-induced acute liver injury in rats. Journal of Ethnopharmacology 6, 75-80.

Ohta,Y., Sasaki, E., Nishida, K., Hayashi, T., Nagata, M., Ishiguro, I., 1997. Preventive effect of oren-gedoku-to (huanglian-jie-du-tang) extract on progression of carbon tetrachloride- induced acute liver injury in rats. American Journal of Chinese Medicine, 25, 57-68.

Pessayre, D., 1995. Role of reactive metabolites in drug-induced hepatitis, Journal of Hepatology, 23:16–24.

P.J. Wills, V.V. Asha, 2006. Protective effect of *Lygodium flexuosum* (L.) Sw. extract against carbon tetrachloride-induced acute liver injury in rats. Journal of Ethnopharmacology, 108: 320–326

Rubinstein, D., 1962. Epinephrine release and liver glycogen levels after carbon tetrachloride administration. American Journal of Physiology 203:1033-1037.

Recknagel, R.O., Glende, E.A.Jr., Dolak, J.A., Waller, R.L., 1989. Mechanisms of carbon tetrachloride toxicity. Pharmacology & therapeutics 43:139-154.

Sanae, F., Komatsu, Y., Chisaki, K., Kido, T., Ishige, A., Hayashi, H. 2001. Effects of San'o-shashin-to and the constituent herbal medicines on theophylline-induced increase in arterial blood pressure of rats. Biological & pharmaceutical bulletin. 24:1137-1141.

Tao Wang, Ning-Ling Sun, Wei-Dong Zhang, Hui-Liang Li, Guo-Cai Lua, Bo-Jun Yuan, Hua Jiang, Jia-Hong She, Chuan Zhang, 2008, Protective effects of dehydrocavidine on carbon tetrachloride-induced acute hepatotoxicity in rats. Journal of Ethnopharmacology. 117:300–308;

Xu, J.T., Wang, L.Q., Xu, B., 2004. Research development of *Coptis chinensis*. Acta Academia Medicines Sonica 26:704-707.

Yasukawa, K., Takido, M., Ikekawa, T., Shimada, F., Takeuchi, M., Nakagawa, S., 1991. Relative inhibitory activity of berberine-type alkaloids against 2-O-tetradecanoylphorbol-13-acetate-induced inflammation in mice. Chemical & pharmaceutical bulletin (Tokyo). 39:1462-1465.

Yokozawa, T., Dong, E., Liu, Z.W. and Oura, H., 1997. Antiperoxidation activity of traditional Chinese prescriptions and their main crude drugs in vitro. Natural Medicines 51, 92–97.

Yokozawa, T., Satoh, A., Cho, E.J., Kashiwada, Y., Ikeshiro, Y., 2005. Protective role of Coptidis Rhizoma alkaloids against peroxynitrite-induced damage to renal tubular epithelial cells. Journal of Pharmacy and Pharmacology. 57:367-74.

Yokozawa, T., Ishida, A., Kashiwada, Y., Cho, E.J., Kim, H.Y., Ikeshiro, Y., 2004. Coptidis Rhizoma: protective effects against peroxynitrite-induced oxidative damage and elucidation of its active components. Journal of Pharmacy and Pharmacology. 56:547-56.

Yokozawa, T., Chen, C.P., Tanaka, T., 2000. Direct scavenging of nitric oxide by traditional crude drugs. Phytomedicine. 6: 453-463.

Yokozawa T, Ishida A, Cho EJ, Nakagawa T. 2003. The effects of Coptidis Rhizoma extract on a hypercholesterolemic animal model. Phytomedicine. 10:17-22.

Table 1. Content (ppm or µg/g) of five selected toxic metals in CRAE

Heavy metals	Mean ± RSD (%)	Calibration correlation cofactor (%)
Aresenic	-0.26±32.54%	99.95
Cadimum	0.48±18.90%	99.91
Chromium	0.01±8.67%	99.82
Lead	-0.04±8.84%	99.90
Mercury	0.14±39.69%	99.40

Table 2 Effect of CRAE and berberine on CCl4-induced liver damage in rat (Mean±S.D., N=8)

Group	ALT(U, in serum)	AST(U, in serum)	SOD (%, in serum)	SOD (%, in tissue)
Normal	19.877±7.34	61.759±30.62	77.26±0.11	72.42±5.89
Control	133.27±32.11##	342.11±55.27##	10.80±0.21##	9.32±5.31##
CRAE 400 mg/kg	66.26±11.24**##	366.22±52.16##	26.70±0.23*#	21.61±4.97*#
CRAE 600 mg/kg	41.17±10.66**	155.39±30.24*#	40.30±0.15*#	46.22±4.46*#
CRAE 800 mg/kg	23.29±12.76**	20.78±10.77**	73.70±0.20**	70.63±6.54**
Berberine 120 mg/kg	26.06±7.48**	23.48±5.91**	75.21±1.79**	68.42±8.13**

#<p<0.05 compared with normal group

##p<0.01 compared with normal group

*p<0.05 compared with CCl_4 control group

**p<0.01 compared with CCl_4 control group

Table 3 Microscopic observation on CRAE and berberine against CCl4-induced acute liver damage (Mean ± S.D., n=8)

Group	**Vacuolation**	**nuclei**	**hepatocyte necrosis**	**inflammatory cell infiltration**	**central vein and portal triad**	**combined score**
Normal	0.4±0.3	1.0±0.7	0.5±0.2	0.6±0.3	1.1±0.6	0.5±0.3
Control	5.0±0.6##	0.7±0.2	4.2±0.8##	3.9±1.6##	0.5±0.3#	4.9±0.5##
CRAE 400 mg/kg	4.2±1.1*	0.8±0.6	2.2±1.7*	2.8±1.3*	1.3±0.8*	3.6±1.4*
CRAE 600 mg/kg	3.0±1.0**	1.1±0.3	2.1±1.5**	2.1±1.3**	1.1±0.6*	2.7±0.9**
CRAE 800 mg/kg	1.9±0.7**	1.2±0.6	1.5±1.2**	1.3±0.9**	0.9±0.5	1.7±0.6**
Berberine 120 mg/kg	1.7±1.3**	1.8±0.2	1.4±0.4**	1.2±0.5**	1.1±0.4*	1.6±0.8**

#<p<0.05 compared with normal group

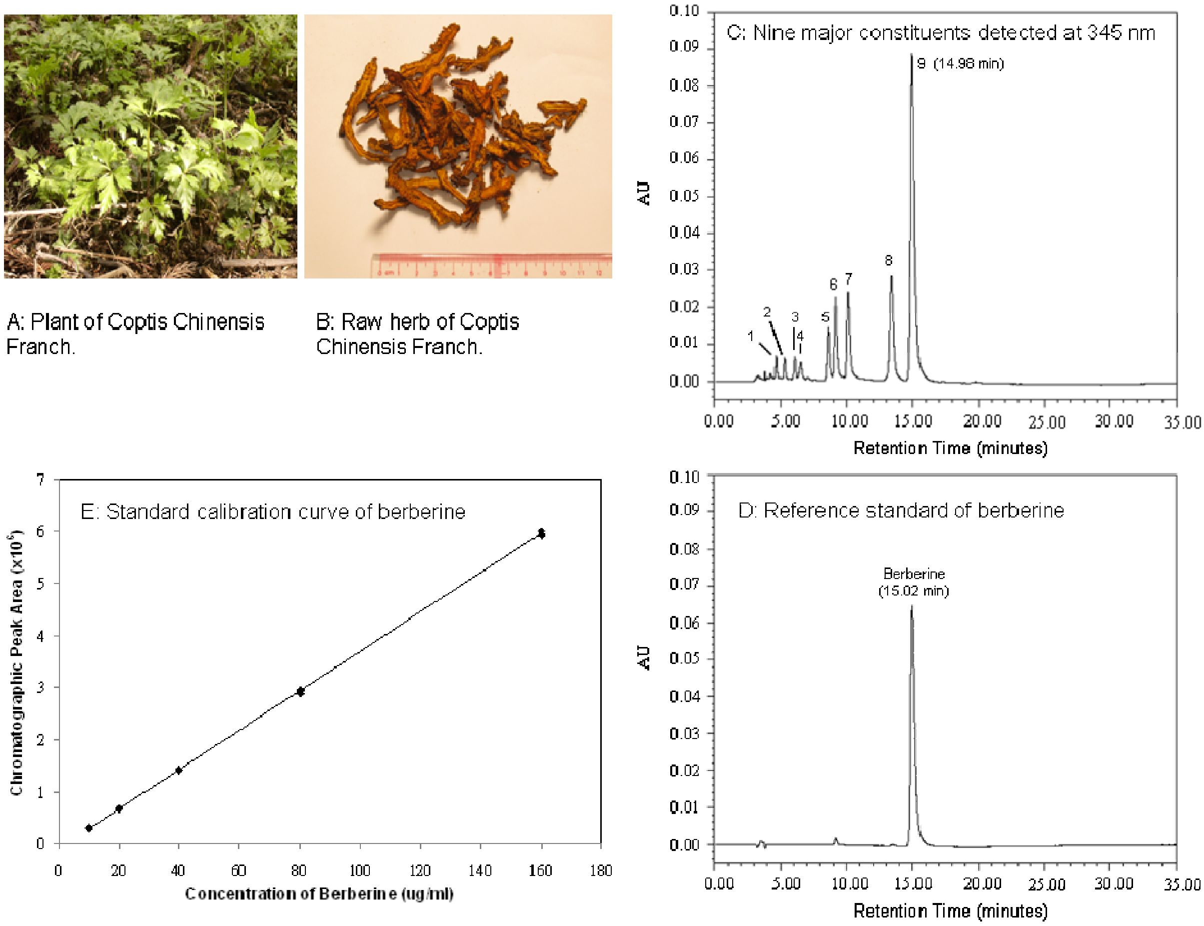
##p<0.01 compared with normal group

*p<0.05 compared with CCl_4 control group

**p<0.01 compared with CCl_4 control group

Ye, et al. Fig.1

Figure 1.



Ye, et al. Fig.2

Figure 2.

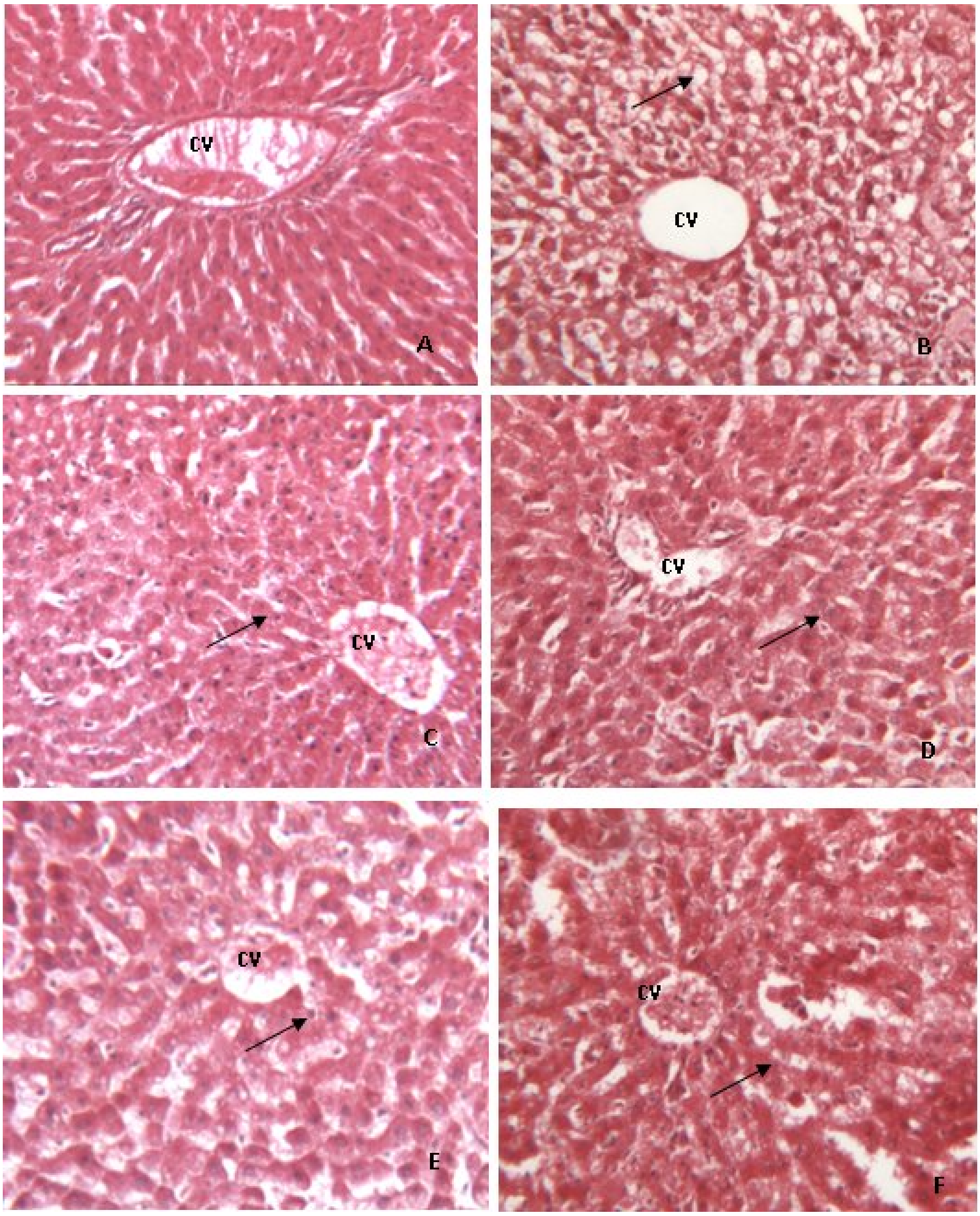


Figure captions

Figure 1. Huanglian identification and HPLC chemical profile of CRAE. A. The species of CR plant is *Coptis Chinensis* Franch. B. Raw herb of CR. C. The HPLC chromatogram of CRAE D. The HPLC chromatogram of berberine reference standard. E. Standard calibration curve of berberine.

Figure 2. The photomicrography of liver sections from rats treated with CCl_4, the post-doses of CRAE at 400, 600 and 800 mg/kg BW, and olive oil vehicle. A. liver section of normal rat; B. liver section of the control rat treated with CCl_4; C. liver section of the CCl_4-treated rat post-dosed by CRAE at 800 mg/kg BW; D. liver section of the CCl_4-treated rat post-dosed by CRAE at 600 mg/kg BW; E. liver section of the CCl_4-treated rat post-dosed by CRAE at 400 mg/kg BW; F. liver section of the CCl_4-treated rat post-dosed by berberine at 120 mg/kg BW.