# Hepatoprotective Effect of *Crossostephium chinensis* (L.) Makino in Rats

Tien-Ning Chang,* Yu-Ling Ho,§ Guan-Jhong Huang,*
Shyh-Shyun Huang,* Chao-Jung Chen,†,‡,// Po-Chow Hsieh,*
Ying-Chen Chiang* and Yuan-Shiun Chang*,¶

*School of Chinese Pharmaceutical Sciences and
Chinese Medicine Resources, College of Pharmacy

†Graduate Institute of Integrated Medicine

‡Proteomics Core Laboratory
Office of Research and Development
China Medical University, Taichung 40402, Taiwan

§Department of Nursing, Hungkuang University
Taichung 433, Taiwan

¶Chinese Crude Drug Pharmacy

//Department of Medicine Research
China Medical University Hospital
Taichung 40402, Taiwan

Abstract: The hepatoprotective potential of *Crossostephium chinensis* (L.) Makino water extract (CCW) on carbon tetrachloride ($CCl_4$) induced liver damage was evaluated in preventive and curative rat models. Not only were indicators of hepatic damage including GPT, GOT, lipid peroxides and TBARS were examined, the activities of antioxidant enzymes (SOD, CAT, GPx) and GSH were examined as well. The results showed that CCW (0.1, 0.5 and 1.0 g/kg) significantly reduced the elevated levels of GPT and GOT by $CCl_4$ administration ($p < 0.05$). TBARS level was dramatically reduced, and SOD, CAT, GPx and GSH activities were significantly increased. In addition, CCW decreased NO production and TNF-$\alpha$ activation in $CCl_4$-treated rats. Therefore, we speculate that CCW protects against acute liver damage through its radical scavenging ability. CCW inhibited the expression of MMP-9 protein, indicating that MMP-9 played an important role in the development of $CCl_4$-induced chronic liver damage in rats. In LC-MS-MS analysis, the chromatograms of CCW with good hepatoprotective activities were established. Scopoletin may be an important bioactive compound in CCW.

Correspondence to: Dr. Yuan-Shiun Chang, School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, College of Pharmacy, China Medical University, 91, Hsueh Shih Road, Taichung 40402, Taiwan. Tel: (+886) 4-2203-0380, Fax: (+886) 4-2208-3362, E-mail: yschang@mail.cmu.edu.tw
Dr. Guan-Jhong Huang, Tel: (+886) 4-2205-3366 (ext. 5508), Fax: (+886) 4-2208-3362, E-mail: gjhuang@mail.cmu.edu.tw

## Introduction

A number of medicinal plants and formulations are used to treat hepatic diseases in traditional medicines around the world (Pineiro-Carrero and Pineiro, 2004). Similarly, herbal medicines also play a major role in the treatment of hepatic disorders in Taiwan. Some pteridophyte plants used by tribal and folk medicine practitioners are unexplored by modern science, and are promising sources of effective hepatoprotective agents. *Crossostephium chinensis* (L.) Makino, a member of the Compositae family, is a low lying bush distributed throughout coastal regions in Taiwan. There are many applications for *Crossostephium chinensis* (L.) Makino. For example, the rhizome, root and leaf are ethnomedicinally useful in the treatment of rheumatoid arthritis. Treating hepatic disorders with *Crossostephium chinensis* is also practiced by some TCM doctors in Southern Taiwan. In our previous study, *Crossostephium chinensis* showed both good antioxidant and antiproliferative activities in comparison to other antioxidative chemical compounds such as glutathione (GSH), butylated hydroxytoluene and rutin *in vitro* (Chang *et al.*, 2009).

Reactive oxygen species (ROS) are causative factors of degenerative diseases, including some hepatopathies (Poli, 1993). The production of free radicals and oxidative stress can be aggravated by a variety of factors, such as ionizing radiation and exposure to drugs or xenobiotics (e.g. $CCl_4$). $CCl_4$, an analog of human hepatotoxin, has been used extensively in animal models to induce liver damage. Liver damage caused by $CCl_4$ is characterized by inflammation in the early stage. In damaged hepatocytes, $CCl_4$ is reductively bioactivated by cytochrome P450 2E1 into a trichloromethyl radical ($\cdot CCl_3$), which subsequently converts into a peroxyl radical ($\cdot OOCCl_3$) in the presence of oxygen. These reactive free radical metabolites can covalently bind to macromolecules and thereby initiate lipid peroxidation (Goeptar *et al.*, 1995). Protective effects of various natural products on $CCl_4$ induced hepatotoxicity have been reported (Halim *et al.*, 1997; Wang *et al.*, 2010), and antioxidation is one of the key processes.

After lipid peroxidation and hepatocellular membrane damage due to $CCl_4$, a release of inflammatory mediators from activated hepatic macrophages follows. This sequential process is thought to potentiate $CCl_4$-induced hepatic damage (Badger *et al.*, 1996). Macrophages release a number of inflammatory mediators with cytotoxic potentials (Laskin and Pendino, 1995). Two mediators of interest are tumor necrosis factor-$\alpha$ (TNF-$\alpha$) and nitric oxide (NO). TNF-$\alpha$ is unique among cytokines in that it can also induce cytotoxicity directly and has been implicated in apoptosis (Wang *et al.*, 2010). Nitric oxide is a highly reactive oxidant, produced by parenchymal and nonparenchymal liver cells from L-arginine via the action of inducible nitric oxide synthase (NOS II) (Geller *et al.*, 1993). When released by macrophages against infectious agents, nitric oxide has been shown to inhibit mitochondrial respiration and DNA synthesis (Nathan, 1992). Macrophages and inflammatory mediators, including TNF-$\alpha$ and nitric oxide, have been implicated in liver damage induced by a number of different toxicants (Laskin and Pendino, 1995). Recently it

has been shown that $CCl_4$ can also directly act on stellate cells (independent of macro-phages); necrotic hepatocytes release large amounts of IL-1, which directly stimulate stellate cells by binding to IL-1 receptors (Yao *et al.*, 2004).

MMPs, a family of zinc- and calcium-dependent proteinases, participate in the degra-dation of extracellular matrix (Visse and Nagase, 2003). Endothelial cells are an important source of MMPs (Singh *et al.*, 2010), and various cell types such as neutrophils (Pugin *et al.*, 1999), macrophages and hepatic stellate cells have been demonstrated to produce MMPs around hepatocyte damaged sites (Knittel *et al.*, 1999). In recent years, increasing discoveries of activities involving MMP-2 and -9 in the liver have been reported in animal hepatitis models (Wielockx *et al.*, 2001). IL-1 receptor knockout mice have been demon-strated to be resistant to lethality in a MMP-9 dependent manner, indicating a causative relationship. Although many hepatic cells are capable of producing MMP-9 *in vitro*, hepatic stellate cells have been demonstrated as the major source in hepatic injury (Yan *et al.*, 2008). The objective of this study was to better understand the efficacy of CCW in treating liver damage. In addition, we examined the expression of MMP-9 in $CCl_4$-induced liver damage and the effect of CCW on MMP-9 expression.

## Materials and Methods

### Chemicals

$CCl_4$, silymarin, olive oil, and thiobarbituric acid (TBA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Glutathione peroxidase (GPx), superoxide dismutase (SOD), and glutathione (GSH) were purchased from Randox Laboratory Ltd. TNF-$\alpha$ concentration was quantified using a commercial ELISA kit (Biosource International Inc., Camarillo, CA, USA). Scopoletin was purchased from Sigma Chemicals Co., #S2500.

### Preparation of Plant Extract

Plants were collected from their natural habitat in September of 2007 and authenticated. A voucher specimen was deposited in the institute. Dried whole herbs (around 45 g each) were boiled with 1000 ml distilled water for 1 h. Filtration and collection of the extracts were done three times. The resulting decoction (about 1000 ml) was evaporated to 10 ml and then dried under vacuum at 50°C. The dried extract was dissolved in distilled water (stock 4 mg/ml), concentrated in a freeze dryer into powder form, and stored at −20°C. This powder was named CCW. The yield was calculated based on the dry weight of whole herbs used and the quantity of dry mass obtained after the extraction. The yield was 3.13%.

### Analyses of Scopoletin and CCW by LC-MS-MS

CCW was weighed and dissolved in water and then filtered through a 0.22 $\mu$m filter. LC-MS-MS was performed on a Bruker Daltonics (Billerica, MA, USA) HCTultra PTM Discovery quadrupole ion trap mass spectrometer. The ion of mass spectrum was acquired

in MS/MS mode. The ion of 193.04 m/z (MH$^+$ of scopoletin) was selected as the precursor ion, and the product ion spectrum was scanned from 50 to 300 m/z. The trap mass spectrometer was coupled with a Dionex Ultimate 3000. There was a great electrospray source in between the LC system (Sunnyvale, CA, USA) and the mass spectrometer (Bruker Daltonics). The LC system was equipped with a RP$_{18}$ column (XTerra, 5 $\mu$m, 4.6 × 150 mm, Waters, Milford, MA), and the liquid flow was pumped from the loading pump without splitting. For each LC-MS-MS run, 5 $\mu$L of sample solution was injected. Buffer A (0.1% FA-H$_2$O) and buffer B (0.1% FA-ACN) were used for LC separation. The LC gradient was conducted as follows: 5% B to 20% B in 5 min, 20% B to 50% B in 5 min, 50% B to 80% B in 5 min, 50% B to 80% B in 5 min, and 80% B to 90% B in 5 min. The column was finally re-equilibrated with 5% B for 5 min before the next run. The flow rate was set to 400 $\mu$l/min. The peaks of CCW were identified by comparing to the standard, scopoletin.

### Animals

Male Sprague-Dawley rats, aged six to eight weeks and weighing 180–200 g, were obtained from BioLASCO Taiwan Co. Ltd. They were housed in a controlled temperature of 25–28 °C with 12 h light/12 h dark cycles, and fed with a standard diet and water *ad libitum*. Animal studies were conducted according to regulations of the Institute Animal Ethics Committee, and the protocol was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals. Rats were divided into six groups of eight animals each. Animals in the normal control and CCl$_4$ control (VEH, vehicle group) were orally administered with distilled water. The positive control rats were administered with silymarin (25 mg/kg in 1% carboxymethyl cellulose) once daily for seven days. In the three experimental groups, rats were pre-treated orally with CCW (0.1, 0.5 and 1.0 g/kg) once daily for seven days, respectively. One hour after the last treatment, all the rats, except for those in the normal control, were treated with CCl$_4$ (1.5 ml/kg in olive oil, 20%, ip). 24 h after the CCl$_4$ treatment, animals were anesthetized with ethyl ether, and blood samples were collected through their carotid arteries.

    Thereafter, the whole liver tissue was rinsed in ice-cold normal saline, and immediately placed in same volume of cold normal saline and homogenized at 4 °C. Then the homogenate was centrifuged at 13,000 g for 20 min. The supernatant was stored in the refrigerator at −20 °C for the antioxidant enzymes (CAT, SOD, GPx and GSH) activity assays, MDA and MMP-9 assays. Also, blood was withdrawn for GOP, GPT, NO, TNF-$\alpha$ assay and antioxidant capacity.

### Effect of CCW on Liver Functions in CCl$_4$-Treated Rats

The blood was centrifuged at 3000× g (BACKMAN, German) at 4 °C for 15 min to separate the serum. The levels of serum glutamate-oxalate-transaminase (sGOT) and serum glutamate pyruvate transaminase (sGPT) were assayed using clinical test kits (Roche, Germany) spectrophotometrically (Cobas Mira Plus, Germany).

*Antioxidant Enzyme Activities*

The following biochemical parameters were analyzed to detect hepatoprotective activities of CCW. Catalase (CAT) activity was measured by the method of Aebi (1984). A reaction mixture (2 ml) comprised of 0.01 ml of liver supernatant, 0.09 ml of 1% Triton X-100 and 1.9 ml of 20 mM phosphate buffer was added to a crystal cuvette containing 1 ml of 0.03 M $H_2O_2$. The change in absorbance at 240 nm was read after 1 min. Using the reaction time interval ($\Delta t$) between absorbance $A1$ and $A2$, the rate constant (K) was calculated with the equation $K = (2.3/\Delta t) \log (A1/A2)$. The specific activity of the enzyme was expressed as K/mg protein.

Superoxide dismutase (SOD) activity was measured according to the method of Beauchamp and Fridovich (1971). 150 $\mu$l of liver supernatant was mixed with the reaction mixture, which was comprised of 0.1 mM EDTA, 25 mM NBT, 0.1 mM xanthine and 50 mM sodium carbonate buffer (pH 10.2). Distilled water was added to a final volume of 3 ml. The reaction was initiated by adding 2 mU/ml of xanthine oxidase and maintained under two 40 W lamps at 25°C. After 15 min, the inhibition rate of NBT reduction was spectrophotometrically determined at 560 nm. One unit of SOD was defined as the amount of enzyme required for inhibition of the reduction of NBT by 50% (Lee and Yu, 1990).

Glutathione peroxidase (GPx) activity was measured according to the method of Paglia and Valentine (1967). The 150 $\mu$l liver supernatant was added to the reaction mixture comprised of 1 mM EDTA, 1 unit of glutathione reductase, 1 mM glutathione, 0.25 mM $H_2O_2$ and 1 mM sodium azide in 50 mM phosphate buffer (pH 7.0). The reaction was initiated by the addition of 0.2 mM NADPH, and GPx activity was defined as the amount of enzyme required to oxidize 1 $\mu$M of NADPH in 1 min. The results were expressed as U/mg protein.

*Determination of GSH*

Hepatic GSH level was determined according to the method of Ellman *et al.* (1959) with slight modifications. Briefly, 720 $\mu$l of liver supernatant in 200 mM Tris buffer (pH 7.2) was diluted to 1440 $\mu$l with the same buffer. 5% TCA (160 $\mu$l) was added and mixed thoroughly. The samples were then centrifuged at $10,000 \times$ g for 5 min at 4°C. Ellman's reagent (DTNB solution) (660 $\mu$l) was added to the liver supernatant (330 $\mu$l). Finally the absorbance was recorded at 405 nm.

*Determination of Hepatic Lipid Peroxidation*

The malondialdehyde (MDA) content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid-reactive substances (TBARS) (Uchiyama *et al.*, 1978). Briefly, the liver supernatant was filtered through four folded-gauze. 0.5 ml liver supernatant was mixed with 3 ml of H3PO4 1% (v/v) and 1 ml of TBA 0.6% (w/v), and then heated to and maintained at 100°C for 45 min. The samples were allowed to cool down to room temperature before 3 ml of *n*-butanol was added. After shaking vigorously, the butanolic phase

was obtained by centrifugation at $4000\times$ g for 10 min to determinate the concentration at 535 nm. The standard was 1, 1, 1, 3-tetraethoxypropane.

## Determination of Nitric Oxide

NO production was assessed indirectly by measuring the plasma nitrite level with a calorimetric method based on the Griess reaction (Green *et al.*, 1982). Plasma samples were diluted four times with distilled water and deproteinized by adding 1/20 volume of zinc sulfate (300 g/L) to a final concentration of 15 g/L. After centrifugation at $10,000\times$ g for 5 min at room temperature, 100 $\mu$l of the supernatant was applied to a microliter plate well, followed by 100 $\mu$l of Griess reagent (1% sulfanilamide and 0.1% $N$−1-naphthy-lethylenediamine dihydro-chloride in 2.5% polyphosphoric acid). After 10 min of color development at room temperature, the absorbance was measured at 540 nm with a Micro Reader (Hyperion, Inc., FL, USA). Nitrite was quantified by using sodium nitrate as the standard.

## Measurement of Plasma Antioxidant Capacity

Blood samples were collected in heparinized tubes and centrifuged for 20 min (2000 g). The plasma was stored at $-80°C$ until processed.

Plasma antioxidant capacity was determined by the randox total antioxidant status method. This assay is based on 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) incubated with a peroxidase (metmyoglobin) and hydrogen peroxide ($H_2O_2$) to produce the radical cation $ABTS^+$. The ABTS solution has a relatively stable blue-green color that can be measured at 600 nm. Antioxidants in the added sample cause suppression of this color production to a degree propotional to their concentration. The Trolox equivalent anti-oxidant capacity of plasma from an adult reference population has been measured, and the method has been optimized and validated (Miller *et al.*, 1993). We used this automated method to investigate the total plasma antioxidant capacity of CCW.

## Measurement of Serum TNF-$\alpha$

The serum level of TNF-$\alpha$ was determined using a commercially available enzyme linked immunosorbent assay (ELISA) kit (Biosource International Inc., Camarillo, CA, USA) according to the manufacturer's instructions. TNF-$\alpha$ was determined from a standard curve. The concentrations were expressed in pg/ml.

## Histopathology

Small pieces of liver were fixed in 10% buffered formalin and embedded in paraffin. Sections of 5–6 $\mu$m were cut and stained with hematoxylin and eosin before they were examined for histopathological changes under the microscope (Nikon, ECLIPSE, TS100,

Japan). Images were taken with a digital camera (NIS-Elements D 2.30, SP4, Build 387) at original magnification of $\times 200$.

Liver sections were graded numerically to assess the degree of histological features of acute hepatic injury. Centrilobular necrosis or zonal necrosis, characterized by the presence of fatty infiltration, ballooning and damaged liver cells around the central vein, was prominent in histological findings.

### Determination of MMP-9 Activity by Zymography

The activity of MMP-9 was measured by gelatin zymography protease assay (Yang *et al.*, 2005). Briefly, the liver supernatant was prepared with SDS sample buffer without boiling or reduction, and subjected to 0.1% gelatin-8% SDS-PAGE electrophoresis. After electrophoresis, the gel was washed with 2.5% Triton X-100 and incubated in a reaction buffer (40 mM Tris-HCl, pH 8.0; 10 mM $CaCl_2$ and 0.01% $NaN_3$) at $37\,^\circ$C for 12 h before staining with Coomassie brilliant blue R-250.

### Statistical Analysis

Results were expressed as mean $\pm$ S.E.M., and all statistical comparisons were made by means of one-way ANOVA test followed by Tukey post-hoc analysis. A *p*-value less than or equal to 0.05 was considered significant.

## Results

### Analyses of Scopoletin and CCW by LC-MS-MS

In LC-MS-MS analyses, chromatograms of CCW with good hepatoprotective activities were established. Scopoletin was found to be present, as shown in Fig. 1.

### Effect of CCW on Liver Functions in $CCl_4$-Treated Rats

The hepatoprotective activity of CCW was evaluated by measuring the activities of serum markers. Significant increases in sGOT and sGPT were observed in the $CCl_4$ control group. On the other hand, administration of CCW significantly reduced the levels of sGOT and sGPT in the experimental groups. Figure 2 shows that 1.0 g/kg of CCW significantly lowered the levels of sGOT and sGPT.

### Antioxidant Enzyme Activities

Antioxidant enzyme (SOD, CAT and GPx) activities in the rat livers are shown in Fig. 3. SOD, CAT, and GPx activities were lower in $CCl_4$-treated rats as compared to the normal control. Interestingly, pretreatments with silymarin and CCW significantly increased SOD, CAT and GPx activities.
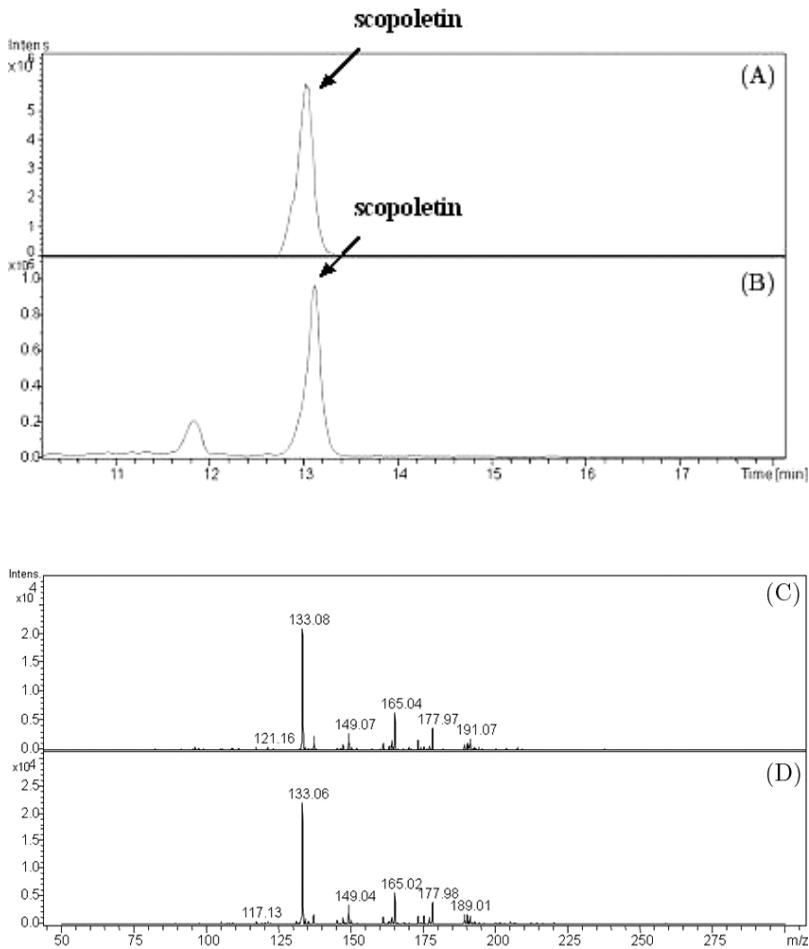
Figure 1. The chemical profile of CCW was analyzed by LC-MS-MS; product ion spectrum was part of the extracted ion chromatogram (m/z 133.0) (precursor ion expressed as m/z 193.0). Chromatographic pattern from the LC-MS-MS analysis of scopoletin (A); chromatographic pattern of CCW (B); product ion spectrum of scopoletin (precursor ion expressed as m/z 193.04) (C); and product ion spectrum of major peak at 13.1 min (D).

### Determination of GSH

As shown in Fig. 4, GSH levels in $CCl_4$-treated rats were obviously decreased. Pre-treatment with silymarin or CCW (0.5 or 1.0 g/kg) significantly increased the level of GSH. GSH level was 11.35 U/mg protein in the normal control and the level was decreased to about 5.80 U/mg protein after $CCl_4$ treatment; however pretreatment with 0.1, 0.5 and 1.0 g/kg of CCW increased the levels to 6.63, 6.64 and 6.86 U/mg protein respectively. Pre-treatment with silymarin also increased the GSH level to 6.06 U/mg protein.
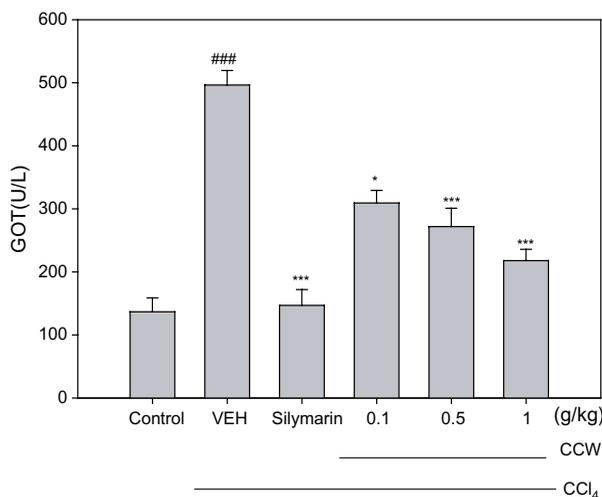
(A)
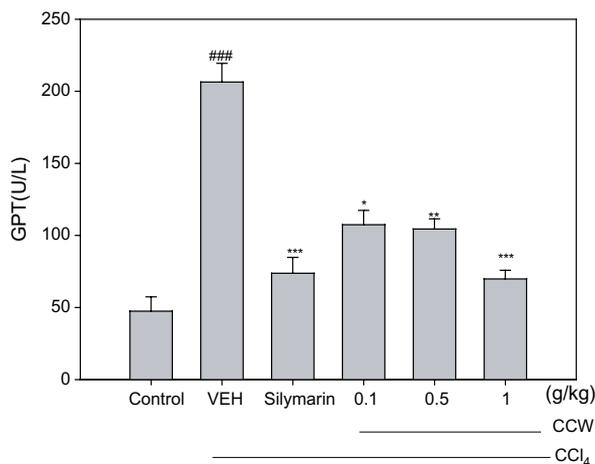


(B)

Figure 2.  Effect of the aqueous extract of *Crossostephium chinensis* (L.) Makino on serum GOT (A) and GPT (B) in CCl$_4$-treated rats after 24 h. The rats were pretreated with CCW (0.1, 0.5 and 1 g/kg) once daily for seven days. Three hours after the final administration, the rats were treated with CCl$_4$ (1.5 ml/kg, ip). The rats were killed 24 h after the CCl$_4$ treatment. Hepatotoxicity was determined by quantifying the serum activities of GOT and GPT. Each value was the mean ± S.E.M. from eight rats in triplicates. ###compared to the control group. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to the CCl$_4$ control.

### *Determination of Hepatic Lipid Peroxidation*

TBARS level in the liver was assessed as an indicator of tissue lipid peroxidation. CCl$_4$ treatment significantly increased the level of TBARS in the liver. However, pretreatment with CCW (0.5 or 1.0 g/kg) significantly decreased the level of TBARS. As shown in
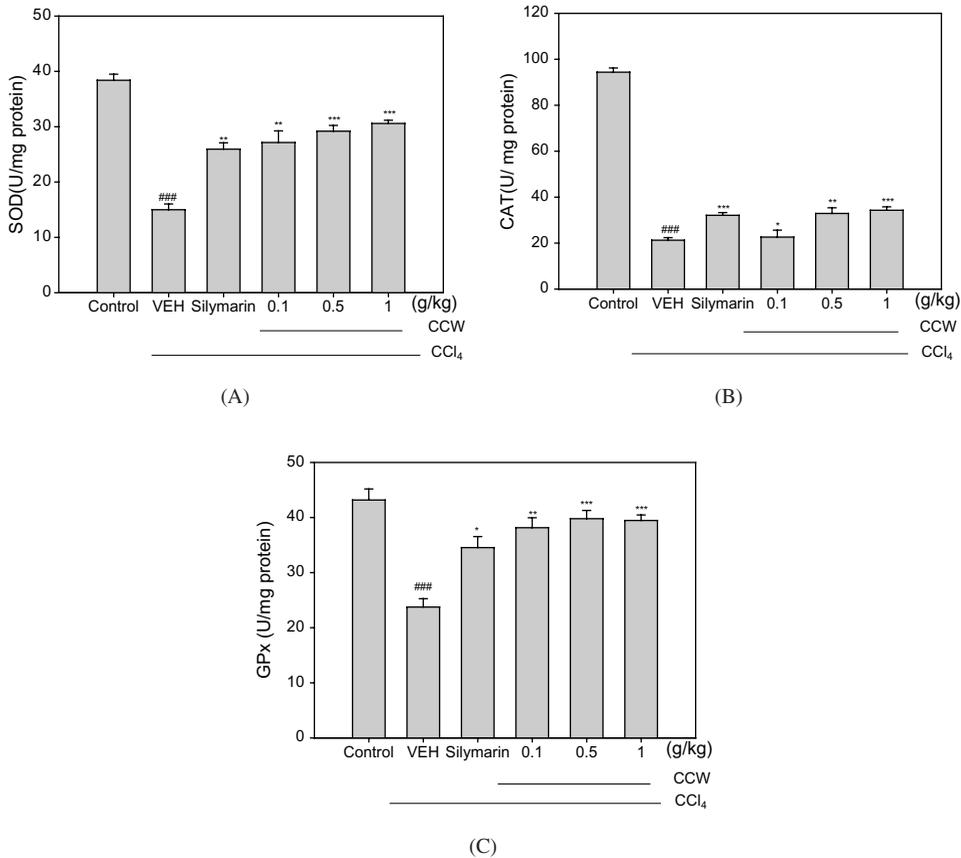
Figure 3. Effect of CCW on antioxidant enzymes in CCl$_4$-treated rats. Activities of superoxide dismutase (SOD) (A), catalase (CAT) (B) and glutathione peroxidase (GPx) (C). The values are means $\pm$ SEM from eight rats done in triplicates. ###compared to the control group. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to the CCl$_4$ control.

Fig. 5, TBARS level was 0.20 $\mu$M/mg protein in the normal control group and 1.35 $\mu$M/mg protein in the CCl$_4$ control group. TBARS levels dropped greatly to 0.35, 0.29 and 0.22 $\mu$M/mg protein with pretreatments of 0.1, 0.5 and 1.0 g/kg of CCW respectively.

*Determination of Nitric Oxide*

As shown in Fig. 6A, production of NO in the plasma was increased to 15.50 $\mu$M/mg in rats treated with CCl$_4$ only as compared to 4.36 $\mu$M/mg in normal control rats. However, silymarin and CCW pretreatments showed efficacies in decreasing such elevation. Pretreatment with silymarin potently decreased NO level to 5.50 $\mu$M/mg protein. NO productions in the experimental groups were significantly decreased to 10.78 and 5.39 $\mu$M/mg protein at 0.5 and 1.0 g/kg of CCW pretreatments, respectively.
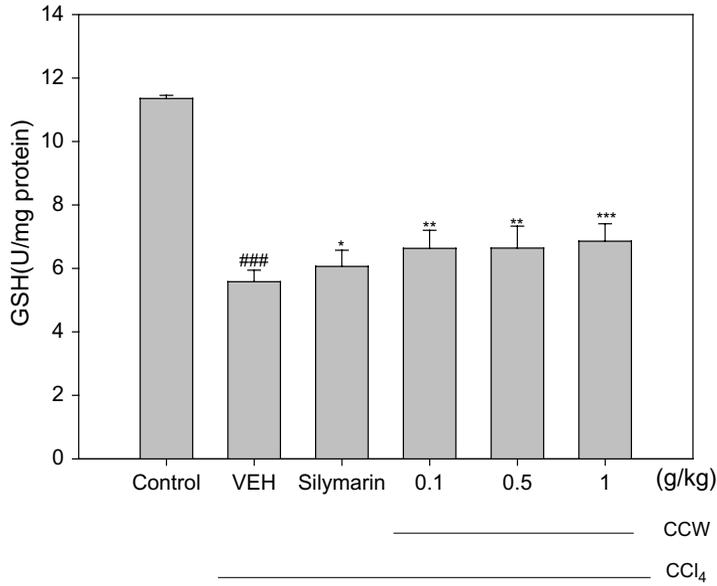
Figure 4. Effect of CCW on glutathione contents in $CCl_4$-treated rats. The values are mean $\pm$ SEM done in triplicates. ###compared with to the control group. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to the $CCl_4$ control.
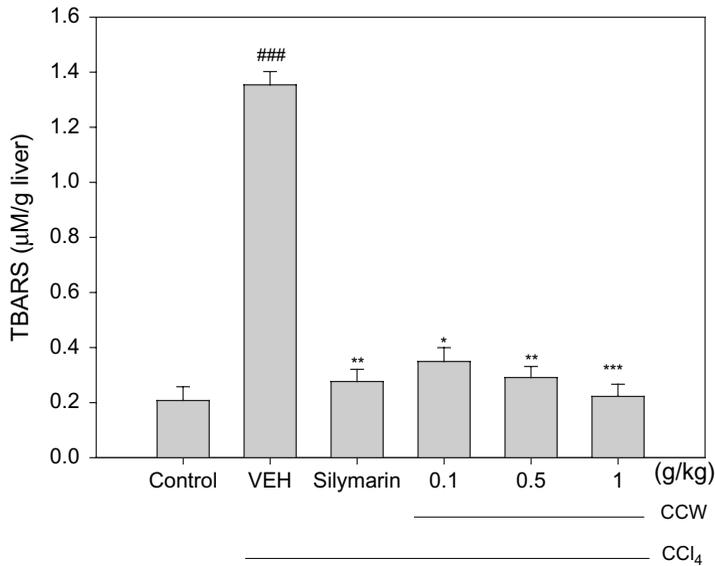


Figure 5. Effect of CCW on TBARS formation in $CCl_4$-treated rats. The values are mean $\pm$ S.E.M done in triplicates. ###compared to the control group. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to the $CCl_4$ control.
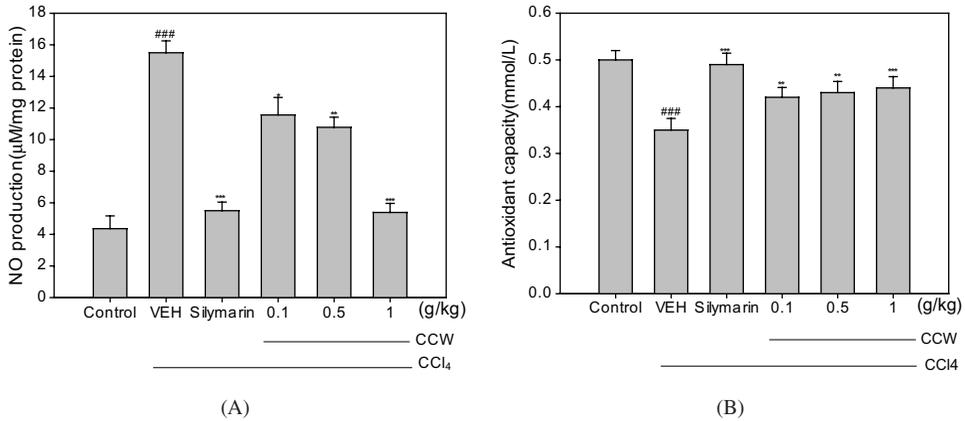
Figure 6. Effect of CCW on NO production (A) and antioxidant capacity (B) in $CCl_4$-treated rats. The values are mean $\pm$ S.E.M in triplicates. ###compared to the control group. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ compared to the $CCl_4$ control.

*Measuring Plasma Antioxidant Capacity*

As shown in Fig. 6B, antioxidant capacity in the plasma of $CCl_4$ treated rats was significantly reduced as compared to normal control rats. The antioxidant capacity in the normal control was 0.50 mM; while it was only 0.35 mM in the $CCl_4$ control. However, pre-treatment of silymarin increased the plasma antioxidant level to 0.49 mM. Similarly, the antioxidant capacities were also significantly augmented to 0.43 mM and 0.44 mM for the experimental groups pretreated with 0.5 g/kg and 1.0 g/kg of CCW.

*Measurement of Serum TNF-$\alpha$*

As shown in Fig. 7, TNF-$\alpha$ concentration in normal control rats was 17.26 pg/ml, while in the rats treated with $CCl_4$, the concentration was dramatically elevated to 173.24 pg/ml. However silymarin significantly decreased TNF-$\alpha$ concentration to 126.89 pg/ml. As for the rats pretreated with 0.5 g/kg and 1.0 g/kg of CCW, their TNF-$\alpha$ concentrations were dropped to 133.66 pg/ml and 125.91 pg/ml, respectively. These values were also significantly lower compared to rats in the $CCl_4$ control group.

*Histopathological Change*

The rats treated with $CCl_4$ only had a high degree of fibrosis in their livers. The three experimental groups, on the other hand, had markedly lower fibrosis and significant histological improvements. Histopathological changes in the normal control, silymarin, $CCl_4$ control and CCW experimental groups are shown in Fig. 8.
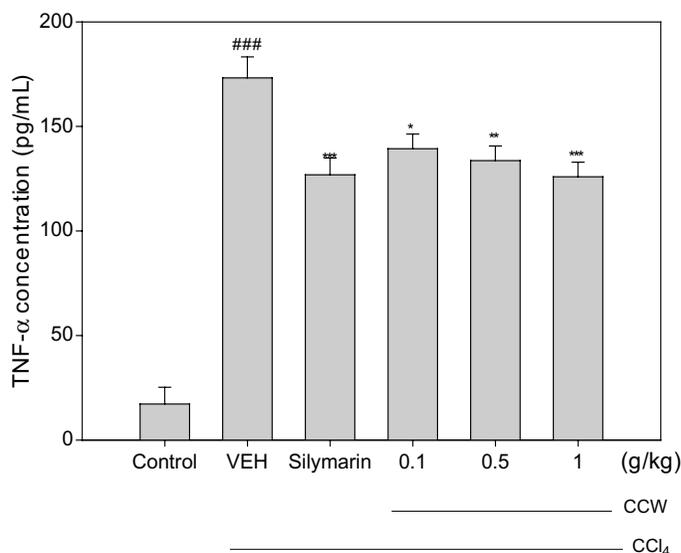
Figure 7. Effect of CCW on TNF-$\alpha$ concentration in CCl$_4$-treated rats. The values are mean $\pm$ S.E.M done in triplicates. ###compared to the control group. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to the CCl$_4$ control.

## Determination of MMP-9 Activity by Zymography

In the zymography assay, it was found that CCW effectively reduced MMP-9 expressions in the sera of animals with CCl$_4$-induced hepatic damage in a dose-dependent manner (Fig. 9).
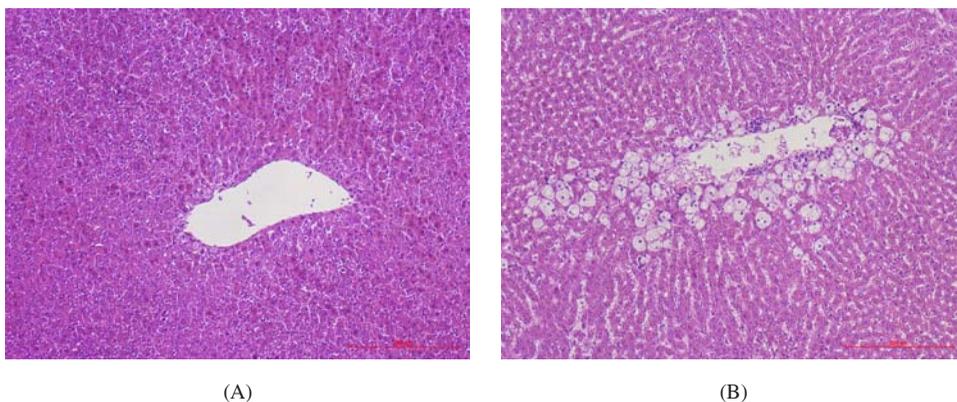


(A)                                        (B)

Figure 8. Effect of CCW on CCl$_4$-induced liver damage. The rats were pre-treated with CCW (0.1, 0.5, 1 g/kg) once daily for seven days. Three hours after the final treatment, the rats were treated with CCl$_4$ (0.5 ml/kg, ip). Rats were killed 24 h after the CCl$_4$ administration and their livers were removed, fixed and embedded in paraffin. Sections were stained with hematoxylin-eosin (200×). Normal control (A); CCl$_4$ (1.5 ml/kg) (B); silymarin (200 mg/kg) + CCl$_4$ (1.5 ml/kg) (C); CCW (0.1 g/kg) + CCl$_4$ (1.5 ml/kg) (D); CCW (0.5 g/kg) + CCl$_4$ (1.5 ml/kg) (E); CCW (1 g/kg) + CCl$_4$ (1.5 ml/kg) (200×) (F).
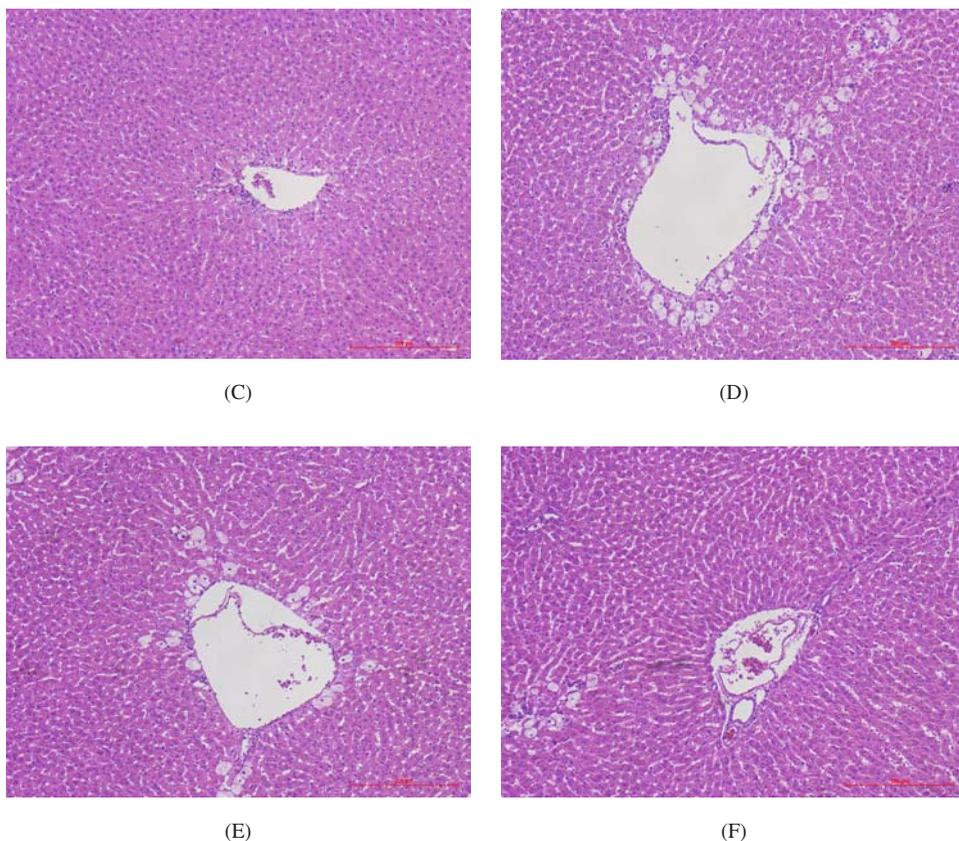
(C)

(D)

(E)

(F)

Figure 8. (*Continued*)

## Discussion

It has been reported that liver injury induced by $CCl_4$ is the best characterized system of xenobiotic-induced hepatotoxicity and a commonly used model for the screening of anti-hepatotoxic and/or hepatoprotective activities of drugs (Recknagel *et al.*, 1989). The principle causes of $CCl_4$-induced hepatic damage are lipid peroxidation, decreased activity of antioxidant enzymes, and generation of free radicals (Poli, 1993). For therapeutic strategies of liver damage, we postulate that it is important to find antioxidant compounds, which are able to reduce liver injuries due to trichloromethyl free radicals, generated by $CCl_4$. In all the antioxidant models of this study, CCW showed its ability to scavenge free radicals in a dose-dependent manner.

Scopoletin possesses antioxidant (Khan *et al.*, 2009; Chang *et al.*, 2009) and hepato-protective activities (Kang *et al.*, 1998). The presence of this compound in *Crossostephium chinensis* (L.) Makino may explain why this plant has been used to treat liver damage in folk medicine (Wu *et al.*, 2009).
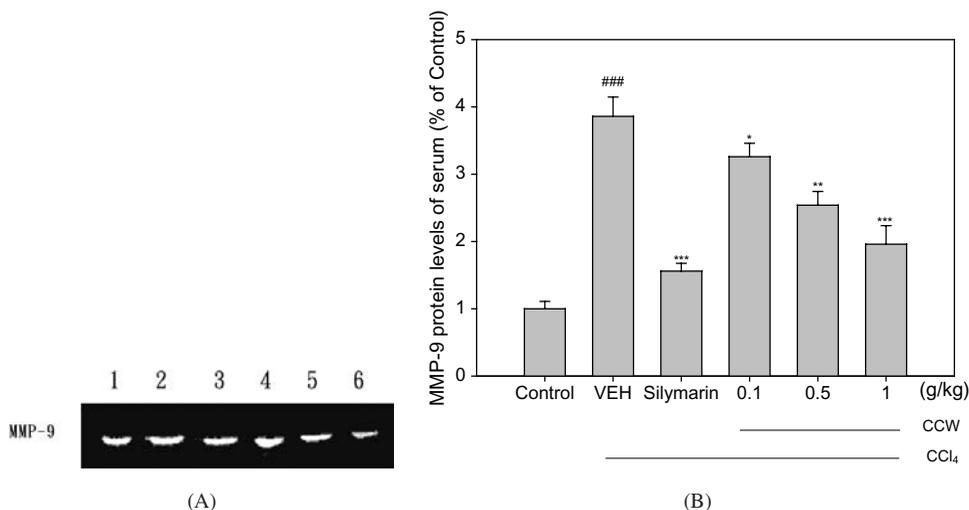
(A)                                    (B)

Figure 9. Effect of CCW on the activity of MMP-9 in SDS-PAGE zymography. Rat liver supernatant were subjected to gelatin zymography for the analysis of MMP-9 activity: (1) normal control; (2) $CCl_4$ 40% (1.5 ml/kg); (3) $CCl_4$ 40% (1.5 ml/kg) + silymarin (200 mg/kg); (4) $CCl_4$ 40% (1.5 ml/kg) + CCW (0.1 g/kg); (5) $CCl_4$ 40% (1.5 ml/kg) + CCW (0.5 g/kg); (6) $CCl_4$ 40% (1.5 ml/kg) + CCW (1.0 g/kg); (A) The levels of MMP-9 protein in the zymography of liver supernatant (B). Each value was the mean ± S.E.M. ###compared to the control group. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to the $CCl_4$ control.

The results of the present study demonstrated that oral administration of CCW effectively protected rats against $CCl_4$-induced acute liver damage. Although $CCl_4$-induced histopathological lesion was not obviously present, biological and hepatic damages due to $CCl_4$ administration were clearly evident from severe body weight loss and liver swelling (Table 1). $CCl_4$ is known to cause hepatic damage with marked elevations of serum GPT and GOT, and such elevations were observed in the present study. However, CCW ameliorated the body weight loss and liver swelling induced by $CCl_4$ (Table 1).

Moreover, antioxidant enzyme (SOD, CAT and GPx) activities were increased by CCW treatment (Fig. 3); GSH activity was also increased (Fig. 4). One possible mechanism to explain why SOD, CAT, and GPx activities dropped in the beginning was because these

**Table 1. Effect of CCW on Body and Liver Weights in $CCl_4$-Treated Rats**

| Groups | Body Weight (g) | Liver Weight (g) |
|---|---|---|
| Normal Control | 220.85 ± 0.21 | 11.76 ± 0.12 |
| $CCl_4$ + Silymarin (200 mg/kg) | 218 ± 0.16 | 11.61 ± 0.79 |
| $CCl_4$ 40% (1.5 ml/kg) | 210 ± 0.49 | 13.50 ± 0.37** |
| $CCl_4$ + CCW (0.1 g/kg) | 212 ± 0.40 | 12.51 ± 0.32 |
| $CCl_4$ + CCW (0.5 g/kg) | 214 ± 0.05* | 12.08 ± 0.23* |
| $CCl_4$ + CCW (1.0 g/kg) | 217 ± 0.18* | 11.95 ± 0.17* |

*Note*: The values are means ± S.E.M. done in triplicates. *$p < 0.05$ as compared to the negative control. **$p < 0.05$ as compared to the $CCl_4$ control.

compounds were degraded or saturated to block $CCl_4$-induced massive free radical production, however a latent regeneration likely induced by CCW administration would follow (Taniguchi *et al.*, 2004). It has also been reported that antioxidant enzyme expression can be altered by hydrogen peroxide (Rohrdanz and Kahl, 1998). Since it has been indicated in the present study that CCW augmented SOD, CAT, and GPx activities and blocked massive ROS production in the presence of $CCl_4$, it can probably also scavenge small quantities of ROS inevitably generated due to incomplete reduction of $O_2$ in the electron transfer of normal aerobic metabolism. The radical scavenging capacity of CCW may be attributed to synergic activities of stimulated SOD, CAT and GPx enzymes and the antioxidative activity of CCW. Therefore, we speculate that CCW played an important role particularly in the early stage of $CCl_4$-induced liver damage. Additionally, CCW also showed the ability to prevent $CCl_4$-induced increment of hepatic TBARS level (Fig. 5), suggesting that CCW was able to inhibit lipid peroxidation and its progression in the liver. The present study demonstrated that oral administration of CCW played a role in reducing oxidative stress associated with hepatotoxicity. After one week of treatment, parameters such as lipid peroxidation (Fig. 5), SOD, CAT and GPx in $CCl_4$ treated rats almost all improved to values close to those of the normal control.

NO level in the plasma was increased significantly with the administration of $CCl_4$; however, CCW was able to block such increase. Previous studies have also demonstrated that NO, a prominent macrophage-derived inflammatory mediator, plays a role in the pathogenesis of $CCl_4$-induced hepatotoxicity (Morio *et al.*, 2001).

TNF-$\alpha$ is produced in response to inflammatory stimuli at sites of tissue injury and may contribute to hepatotoxicity. TNF-$\alpha$ not only exerts toxicity directly, it may also contribute to injury by potentiating $CCl_4$-induced oxidative stress and lipid peroxidation. This is supported by the finding that oxidative stress is increased in hepatocytes treated with TNF-$\alpha$ (Adamson and Billings, 1992). TNF-$\alpha$ has also been shown to increase the release of reactive oxygen intermediates and augment lipid peroxidation in cultured rat hepatocytes (Kim *et al.*, 1997). Although fatty changes in the liver are generally considered reversible (Teli *et al.*, 1995), recent studies have suggested that these might mask apoptotic cell death (Rashid *et al.*, 1999). At the present time we cannot exclude the possibility that apoptosis is increased in rats deficient in TNF-$\alpha$ signaling. In this regard, apoptosis has been described in hepatocytes after $CCl_4$ administration to rats (Cabre *et al.*, 1999). However, it should be noted that both pro- and anti-apoptotic proteins are expressed in the livers of wild-type and TNF receptor p55/p75 double knockout rats following $CCl_4$ administration (Horn *et al.*, 2000), suggesting only a limited role of TNF-$\alpha$ mediated apoptosis in $CCl_4$-induced hepatotoxicity.

MMPs have been shown to be involved in animal hepatitis models (Wielockx *et al.*, 2001). It is known that MMPs can cleave the extracellular matrix, leading to the disintegration of tissue integrity and infiltration of neutrophils and macrophages (Wielockx *et al.*, 2001). Additionally, MMP-9 has been suggested to participate in the paracetamol-induced hepatotoxicity mediated by sinusoidal endothelial cell injury, which results in the impairment of microcirculation in chronic hepatitis (Ito *et al.*, 2005). In the present study, the level of MMP-9 in the liver, as well as the liver supernatant level of MDA, began to

increase 24 h after $CCl_4$ treatment, suggesting that MMP-9 was involved in the development of $CCl_4$-induced liver damage. However, CCW inhibited the expression of MMP-9, as well as reduced $CCl_4$-induced liver inflammation and cell damage. This study suggested that the inhibitory effect of CCW on MMP-9 production in $CCl_4$-induced liver damage might be beneficial to both acute and chronic hepatitis. It is worthy to progress further experiments on CCW related to chronic liver damage and liver fibrosis.

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