1 Running title: Hepatoprotective effect of *Flemingia macrophylla* in rats

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3 # Hepatoprotective Effect of the Aqueous Extract of *Flemingia*
4 # *macrophylla* on Carbon Tetrachloride-Induced Acute Hepatotoxicity
5 # in Rats through Anti-oxidative Activities

6

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1 **Abstract**

2 The study investigated the protective effect of the aqueous extract of *Flemingia*
3 *macrophylla* (AFM) against hepatic injury induced by CCl_4. Alanine aminotransferase
4 (ALT) and aspartate aminotransferase (AST) were detected as biomarkers in the
5 blood to indicate hepatic injury. Product of lipid peroxidation (MDA), superoxide
6 dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and reduced
7 glutathione (GSH) contents were evaluated for oxidative stress in hepatic injury.
8 Moreover, histopathological observation was assayed for the degree of hepatic injury.
9 After oral administration of AFM, 0.5 g/kg and 1.0 g/kg doses significantly decreased
10 ALT and AST, attenuated the histopathology of hepatic injury, ameliorated oxidative
11 stress in hepatic tissue, and increased the activities of CAT, SOD and GSH-Px.
12 Observation on the hepatoprotective effect of daidzein and genistein were consistent
13 to that of AFM. This study demonstrated for the first time that AFM has
14 hepatoprotective effect on acute liver injuries induced by CCl_4, and the results
15 suggested that the effect of AFM against CCl_4-induced liver damage was related to
16 antioxidant property.

17

18 *Keywords: Flemingia macrophylla*; Carbon Tetrachloride; Hepatotoxicity; Nitric
19 Oxide; Tumor Necrosis Factor-α

1 **Introduction**

2

3 Reactive oxygen species (ROS) including oxygen free radicals are causative
4 factors of degenerative diseases, including some hepatopathies (Poli, 1993). The
5 enhanced production of free radicals and oxidative stress can be induced by a variety
6 of factors, such as ionizing radiation, and exposure to drugs or xenobiotics (e.g.,
7 carbon tetrachloride). CCl_4, an analogue of human hepatotoxin, has been used
8 extensively in animal models to induce liver damage. Liver damage caused by CCl_4 is
9 characterized by inflammation in the early stage. In damaged hepatocytes, CCl_4 is
10 reductively bioactivated by cytochrome P450 2E1 into a trichloromethyl radical (·
11 CCl_3), which is subsequently converted into a peroxyl radical (·$OOCCl_3$) in the
12 presence of oxygen. These reactive free radical metabolites can covalently bind to
13 macromolecules and also initiate lipid peroxidation (Goeptar *et al.*, 1995).
14 Antioxidant action plays an important role by which various natural products protect
15 against CCl_4-induced liver damage (Halim *et al.*, 1997).

16 CCl_4 toxicity is thought to be mediated by at least two sequential processes. The
17 first involves cytochrome P450-mediated metabolism of CCl_4 to a highly reactive
18 trichloromethyl radical, which initiates lipid peroxidation and leads to hepatocellular
19 membrane damage (Sipes *et al.*, 1974). This is followed by the release of

1 inflammatory mediators from activated hepatic macrophages, which are thought to
2 potentiate CCl_4-induced hepatic damage (Badger *et al*., 1996). Macrophages release a
3 number of inflammatory mediators with cytotoxic potentials. Two mediators of
4 interest are tumor necrosis factor-α (TNF-α) and nitric oxide (NO). TNF-α is unique
5 among cytokines in that it can also induce cytotoxicity directly and has been
6 implicated in apoptosis (Wang *et al*., 1995). NO is a highly reactive oxidant, produced
7 by parenchymal and nonparenchymal liver cells from L-arginine via the action of
8 inducible nitric oxide synthase (NOS II). When released by macrophages against
9 infectious agents, NO has been shown to inhibit mitochondrial respiration and DNA
10 synthesis (Nathan, 1992). Macrophages and inflammatory mediators, including
11 TNF-α and nitric oxide, have been implicated in liver damage induced by a number of
12 different toxicants (Laskin and Pendino, 1995).

13 The *Flemingia* genus, known as 'I-Tiao-Gung' in Chinese, is distributed in
14 tropical areas. The traditional usages of the roots of *Flemingia* species have been for
15 the treatment of rheumatism, arthropathy, leucorrhea, menalgia, menopausal
16 syndrome, chronic nephritis, and improvement of bone mineral density (Li *et al*.,
17 2008). Only few studies have confirmed the pharmacological activity of members in
18 the *Flemingia* genus. For example, it was reported that the root extract of *F.*
19 *philippinensis* (*F. prostrata* (FP)) exhibited anti-oxidative, anti-inflammatory,

estrogenic, and anti-estrogenic activities (Li *et al*., 2008). The stem of *F. macrophylla* has been used in traditional medicine as an antirheumatic and anti-inflammatory agent and for improving blood circulation. Furthermore its flavonoids have inhibitory effects on Aβ -induced neurotoxicity (Shiao *et al*., 2005). In our previous studies, FM had both antioxidant and antidiabetic activities (Hsieh *et al*., 2010). The objective of this study was to better understand the hepatoprotective effect of FM by investigating the protective effect of AFM in CCl_4-induced rat liver damage.

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 (GSH-Px), superoxide dismutase (SOD), and glutathione (GSH) were purchased from Randox Laboratory Ltd. TNF−α and IL-1β concentrations were quantified using a commercial ELISA kit (Biosource International Inc., Camarillo, CA). Daidzin, daidzein, genistin, and genistein were purchased from Sigma Chemicals Co.

Plant Material

Plant materials were collected from Taichung county, Taiwan. They were identified and authenticated by Dr. Chao-Lin Kuo, Associate Professor and Chairman, Department of Chinese Medicine Recourses, China Medical University, Taichung, Taiwan.

Preparing the Aqueous Extract

Dried herb roots were boiled with one liter distilled water for 1 hour. Filtrate and collection of the extracts were done three times. The filtrate was concentrated to powder by a freeze dryer (Christ Alpha, Germany) and stored at -20℃.

Compositional Analysis of AFM by HPLC

HPLC was performed with a Hitachi Liquid Chromatography (Hitachi Ltd., Tokyo, Japan), consisting of two L-7100 model pumps, and one L-7455 model photodiode array detector (254 nm). AFM extracts were filtered through No. 1 filer-paper. The filtrate was diluted to 100 μL with 70% methanol. This solution was then passed through a 0.45μ m PVDF-filter and the filtrate was injected into an HPLC (10 μL) equipped with a Mightysil RP-18 GP column (5 μm, 250 × 4.6 mm I.D.). The method involved the use of a binary gradient with mobile phases containing: (A) phosphoric acid in water (0.1%, v/v) and (B) H_2O/CH_3CN：20/80 (v/v). The solvent

gradient elution program was as follows: 0-15 min, 100-95% A, 0-5% B; 15-20 min, 95-85% A, 5-15% B; 20-30 min, 85-45% A, 15-55% B; 30-45 min, 45-25% A, 55-75% B; 45-50 min, 25-0% A, 75-100% B; and finally 50-60 min, 0% A, 100% B. The flow-rate was kept constant at 0.8 mL/min. A precolumn of μ-Bondapak^{TM}C_{18} (Millipore, Milford, MA, USA) was attached to protect the analytical column.

Animals

Male SD rats, aged six to eight weeks and weighing 180-200 g, were selected for the study. They were maintained at a controlled temperature of 25-28℃ with 12h light/dark cycles and fed a standard diet and water *ad libitum*. Animal studies were conducted according to the 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 (n=8). Rats in the normal control and negative control were orally administered with distilled water. The positive control was orally administered with silymarin (25 mg/kg in 1% carboxymethyl cellulose) once daily for 7 days. In the three experimental groups, the rats were pretreated orally with AFM (0.1g/kg, 0.5g/kg, and 1.0 g/kg) once daily for seven consecutive days. 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). 24h

after the CCl_4 treatment, animals were anesthetized with ethyl ether, and blood samples were collected through their carotid arteries. The mortality rate and body weight were recorded daily.

Histopathology

Small pieces of liver, fixed in 10 % buffered formalin were processed for embedment in paraffin. Sections of 5-6 μm 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 ×200.

Antioxidant Enzyme Activities

The following biochemical parameters were analyzed to detect the hepatoprotective activity of AFM. SOD activity was determined by monitoring the inhibition of cytochrome *c* reduction at 550 nm using xanthine and a xanthine oxidase system. One SOD unit was defined as the amount of enzyme required to inhibit cytochrome *c* reduction by 50 % (Lee and Yu, 1990).

Catalase activity was measured by the method of Aebi (1984). A reaction mixture (2 mL), which contained 0.01 mL of liver homogenate, 0.09 mL of 1 % Triton X-100

1 and 1.9 mL of 20 mM phosphate buffer, was added to a crystal cuvette containing 1

2 mL of 0.03 M H_2O_2. The change in absorbance after 1 min was read at 240 nm. Using

3 the reaction time interval (Δt) of absorbance ($A1$ and $A2$), the rate constant (K) was

4 calculated using the equation $K = (2.3/\Delta t) \log (A1/A2)$. The specific activity of the

5 enzyme was expressed as K/mg protein.

6 GSH-Px activity was measured according to the method of Paglia and Valentine

7 (1967). The liver supernatant was added to the reaction mixture comprised of 1 mM

8 EDTA, 1 unit of glutathione reductase, 1 mM glutathione, 0.25 mM H_2O_2 and 1 mM

9 sodium azide in 50 mM phosphate buffer (pH 7.0). The reaction was initiated by the

10 addition of 0.2 mM NADPH, and GSH-Px activity was defined as the amount

11 required to oxidize 1 μM of NADPH in one min. Resulting values of GSH-Px activity

12 were expressed as U/mg protein in each supernatant.

13

14 *Determination of GSH*

15 Hepatic GSH level was determined as described previously (Ellman *et al.*, 1959)

16 with slight modifications. Briefly, 720 μL of liver homogenate in 200 mM Tris buffer,

17 pH 7.2, was diluted to 1440 μL with the same buffer. Five percent TCA (160 μL) was

18 added and mixed thoroughly. The samples were then centrifuged at 10,000 × *g* for 5

19 min at 4℃. Ellman's reagent (DTNB solution) (660 μL) was added to the supernatant

1 (330 μL). Finally the absorbance was taken at 405 nm.

2

3 *Determination of Hepatic Lipid Peroxidation*

4 The malondialdehyde (MDA) content, a measure of lipid peroxidation, was

5 assayed in the form of thiobarbituric acid-reactive substances (TBARS) as previously

6 described (Uchiyama *et al*., 1978). Briefly, 1 g of liver was homogenized in 10 mL of

7 KCl 1.15 % (w/v), and the homogenate was filtered through four folded-gauze. 0.5

8 mL of liver homogenate was mixed with 3 mL of H_3PO_4 1 % (v/v) and 1 mL of TBA

9 0.6 % (w/v), and then heated to and maintained at 100℃ for 45 min. The samples

10 were allowed to cool down to room temperature and 3 mL of *n*-butanol was added.

11 After shaking vigorously with the vortex, the butanolic phase was obtained by

12 centrifugation at 4,000 × *g* for 10 min to determine the absorbance at 535 nm. The

13 standard was 1, 1, 1, 3-tetraethoxypropane.

14

15 *Determination of Nitric Oxide (NO)*

16 The production of NO was assessed indirectly by measuring the nitrite levels in

17 the plasma by a calorimetric method based on the Griess reaction (Green *et al*., 1982).

18 Plasma samples were diluted four times with distilled water and deproteinized by

19 adding 1/20 volume of zinc sulfate (300 g/L) to a final concentration of 15 g/L. After

1 centrifugation at 10,000 × g for 5 min at room temperature, 100 μL supernatant was
2 applied to a microliter plate well, followed by 100 μL of Greiss reagent (1 %
3 sulfanilamide and 0.1 % *N*-1-naphthylethylenediamine dihydro-chloride in 2.5 %
4 polyphosphoric acid). After 10 min of color development at room temperature, the
5 absorbance was measured at 540 nm with a Micro Reader (Hyperion, Inc., FL, USA).
6 Nitrite was quantified by using sodium nitrate as the standard curve.
7
8 *Measurement of Serum TNF-α and* IL-1β
9 The serum level of TNF-α and IL-1β were determined using a commercially
10 available enzyme linked immunosorbent assay (ELISA) kit (Biosource International
11 Inc., Camarillo, CA) according to the manufacturer's instructions. TNF-α and IL-1β
12 were determined from a standard curve. The concentrations were expressed in pg/mL.
13
14 *Statistical Analysis*
15 Results were expressed as mean ± S.E.M. and all statistical comparisons were
16 made by means of one-way ANOVA test followed by Tukey post-hoc analysis. A
17 p-value less than or equal to 0.05 was considered significant.
18
19

Results

Compositional Analysis of AFM by HPLC

Four marker compounds were selected for HPLC chromatographic fingerprint analysis of AFM as shown in Fig. 1, with the markers identified as daidzin (retention time, 27.3 min), daidzein (43.5 min), genistin (35.8 min) and genistein (48.7 min) (Butyl *p*-hydroxybenzoate is internal standard (IS)). Based on the plots of the peak-area (y) vs. concentration (x, μg/mL), the regression equations of the three phenolic constituents and their correlation coefficients (r^2) were as follows: daidzin, $y = 0.0509x + 0.2852$ ($r^2 = 0.9981$); daidzein, $y = 0.0276x + 0.0241$ ($r^2 = 0.9988$); genistin, $y = 0.0619x + 0.3888$ ($r^2 = 0.9954$); genistein, $y = 0.0424x + 0.0659$ ($r^2 = 0.9984$). The relative amount of the four compounds found in AFM was in the order: genistein (3.7 mg/g) > genistin (2.6 mg/g) > daidzein (0.3 mg/g) > daidzin (0.1 mg/g).

Activities of ALT and AST in Serum

The serum activities of ALT and AST were used as biochemical markers for the early acute hepatic damage. The effect of oral administration of AFM on serum AST and ALT levels is as shown in Fig. 2. AFM at the dose of 1.0 g/kg significantly reduced serum AST and ALT levels in liver damaged rats ($p < 0.001$). The standard

1 drug, silymarin at dose of 10 mg/kg, also had similar effects. It was confirmed that
2 AFM could ameliorate hepatic function in CCl_4 induced liver injury.
3
4 *Effect of AFM on Liver Histology*
5 The histological features of the livers from the control and experimental groups
6 are as shown in figure 3. Fig. 3A shows the hepatic cell structure of the control and is
7 a representation of normal liver lobular architecture, which has no pathological
8 changes and with central vein and radiating hepatic cords. Fig. 3B shows multiple and
9 extensive areas of portal inflammation and hepatocellular necrosis randomly
10 distributed throughout the parenchyma, as well as a moderate increase in
11 inflammatory cell infiltration. Changes were improved in AFM and silymarin
12 pretreated rats, which exhibited areas of normal liver architecture and patches of
13 necrotic hepatocytes (Fig. 3 C–F).
14
15 *Effect on CAT, SOD, and GSH-Px Activities in CCl_4-Induced Hepatic Injury*
16 CAT is a key component of the antioxidant defense system. Inhibition of these
17 protective mechanisms results in enhanced sensitivity to free radical-induced cellular
18 damage. Excessive production of free radicals may result in alterations in the
19 biological activity of cellular macromolecules. CAT activities in total liver

homogenates were shown in Fig. 4A. CAT activity of liver homogenate of the CCl_4 group (3.46 ± 0.16 U/mg protein) was conspicuously lower than that of the control group (5.18 ± 0.21 U/mg protein). CAT activities of liver homogenates from the 0.5 g/kg (3.70 ± 0.17 U/mg protein, $p < 0.05$) and 1.0 g/kg (4.36 ± 0.29 U/mg protein, $P < 0.01$) AFM groups were significantly higher than that of the CCl_4 group. In this study, CAT was increased by the administration of AFM, suggesting that it could restore CAT enzymes.

SOD plays an important role in the elimination of ROS derived from the peroxidative process of xenobiotics in hepatic tissues. SOD activity of the liver homogenate in the CCl_4 group (9.97 ± 0.46 U/mg protein) was lower than that of the control group (14.83 ± 0.63 U/mg protein). The SOD activities of liver homogenates of the groups treated with 0.1 g/kg (11.40 ± 0.51 U/mg protein, $p < 0.05$), 0.5 g/kg (12.48 ± 0.83 U/mg protein, $p < 0.01$), and 1.0 g/kg (13.47 ± 0.87 U/mg protein, $p < 0.001$) of AFM were significantly higher than that of the CCl_4 group (Fig. 4B). The observed increase in SOD activity suggested that AFM had an efficient protective mechanism in response to ROS.

Fig. 4C shows the GSH-Px activity of the liver homogenate of CCl_4 treated rats after continual administration with different doses of AFM and 10 mg/kg silymarin. GSH-Px activity of liver homogenate from the CCl_4 group (3.02 ± 0.20 U/mg protein)

1 was lower than that of the control group (4.78 ± 0.31 U/mg protein). GSH-Px
2 activities of liver homogenates from the experimental groups pretreated with 0.1 g/kg
3 (3.86 ± 0.17 U/mg protein, $P < 0.01$), 0.5 g/kg (4.43 ± 0.21 U/mg protein, $P < 0.001$),
4 and 1.0 g/kg (4.76 ± 0.29 U/mg protein, $P < 0.001$) AFM were significantly higher
5 than that of the CCl_4 group.
6
7 *Effect on the GSH Levels in CCl_4 Treated Rats*
8 GSH is an intracellular reductant and plays major roles in catalysis, metabolism
9 and transport. It protects cells against free radicals, peroxides and other toxic
10 compounds. It is widely known that a deficiency of GSH within living organisms can
11 lead to tissue disorder and injury. Significant depletion of GSH was detected in CCl_4
12 treated rats (10.85 ± 0.63 U/mg protein in the control vs. 5.60 ± 0.21 U/mg protein in
13 the CCl_4 group). Furthermore, 0.5 g/kg and 1.0 g/kg of AFM significantly ameliorated
14 CCl_4-induced depletion of GSH in the hepatic tissue (7.52 ± 0.49 U/mg protein in the
15 0.5 g/kg AFM group and 8.31 ± 0.91 U/mg protein in the 1.0 g/kg AFM group, with P
16 < 0.05 and $P < 0.01$ when compared to the CCl_4 group) (Fig. 5).
17
18 *Effect on Hepatic TBARS Levels*
19 CCl_4 caused a marked lipid peroxidation in hepatic tissue. The localization of

1 radical formation resulting in lipid peroxidation, measured as MDA in rat liver
2 homogenate, is as shown in Fig. 6. MDA content in the liver total homogenate was
3 dramatically increased in the CCl_4 (2.33 ± 0.16) group as compared to the control
4 group (0.69 ± 0.08). MDA level was significantly inhibited in 0.1 g/kg (2.04 ± 0.11, p
5 < 0.05), 0.5 g/kg (1.34 ± 0.18, $p < 0.01$) and 1.0 g/kg (0.85 ± 0.07, $p < 0.001$) of AFM
6 treated groups.
7
8 *Effect on Serum NO, TNF-α, and IL-1β Levels*
9 CCl_4 induced hepatotoxicity was associated with marked increase in the level of
10 NO, TNF-α, and IL-1β. As shown in Fig. 7A, the production of NO in the plasma was
11 significantly increased in CCl_4-treated rats as compared to the normal control group
12 (4.04 ± 0.35 μM vs. 2.45 ± 0.14 μM). However, pretreatment of silymarin and AFM
13 decreased NO production in CCl_4-treated rats. NO level was significantly inhibited in
14 the groups pretreated with 0.5 g/kg (3.27 ± 0.23 μM, $p < 0.01$) and 1.0 g/kg (2.64 ±
15 0.33 μM, $p < 0.001$) of AFM.
16 The production of TNF-α and IL-1β in the serum was significantly increased in
17 CCl_4-treated rats (132.47 ± 1.46 pg/mL and 377.79 ± 34.80 pg/mL) as compared to
18 the normal control group (82.23 ± 11.20 pg/mL and 145.02 ± 18.93 pg/mL). At the
19 dose of 0.1 g/kg, AFM produced significant decreases in TNF-α and IL-1β levels, as

1 shown in Fig. 7(B) and 7(C). Furthermore, treatment with AFM (0.5 and 1.0 g/kg)

2 over 7 days, similar to silymarin, produced more pronounced ($p < 0.001$)

3 dose-dependent decreases in the level of TNF-α and IL-1β.

4

5 **Discussion**

6

7 Liver injury induced by CCl_4 is a classical system of xenobiotic-induced

8 hepatotoxicity and has been used extensively for decades for the screening of

9 antihepatotoxic/hepatoprotective activities of different drugs (Alqasoumi, 2010).

10 Oxidative stress has been postulated as a major molecular mechanism involved in

11 experimental animal models. In the present study, we have evaluated the

12 hepatoprotective effect of AFM against CCl_4 induced acute hepatotoxicity in rats. The

13 consistency of chemical composition in AFM is important in safe guarding the

14 reliability of the research results. The chemical profile of AFM was recorded by

15 HPLC analysis. The HPLC chemical profile could be delineated by the measurement

16 of relative retention times of major characteristic peaks using genistein and daidzein

17 as markers. The resulting chromatogram was used as the standard for the assessment

18 of all extracts used in the present study. Genistein has anti-inflammatory effects on

19 experimental liver damage caused by CCl_4; it reduces liver damage by preventing

1 lipid peroxidation and strengthening antioxidatrichoromethyl and trichloromethyl
2 peroxy radicals (Shen *et al*., 2009).

3 It is well known that CCl_4 is activated by the cytochrome P450 system. The
4 initial metabolite is the trichloromethyl free radical, which is believed to stimulate the
5 biochemical events that ultimately culminate in liver cell necrosis (Lin *et al*., 2000).
6 In response to hepatocellular injury initiated by the biotransformation of CCl_4 into
7 reactive radicals, “activated” Kupffer cells respond by releasing increased amount of
8 active oxygen species and other bioactive agents (Yam *et al*., 2007). CCl_4-induced
9 generation of peroxy and superoxide radicals result in the inactivation of CAT and
10 SOD. Our results also showed that CCl_4 significantly decreased the activities of CAT,
11 SOD and GSH-Px in the liver. Cells have a number of self-protecting mechanisms
12 against toxic effects of ROS, including free radical scavengers and chain reaction
13 terminators such as SOD, CAT, and GPx systems. SOD removes superoxide radicals
14 by converting them into H_2O_2, which in turn, can be rapidly converted into water by
15 CAT and GPx. However, such protective effect of AFM against NO has not been
16 elucidated and may be related to its antioxidant properties. Cellular injury occurs
17 when ROS generation exceeds the cellular capacity of removal (Tang *et al*., 2010).

18 Oxidative stress causes depletion of intracellular GSH, leading to serious
19 consequences (Ha *et al*., 2005). AFM administration inhibited lipid peroxidation at

1 higher levels after CCl_4 treatment. Interestingly, 0.5 g/kg and 1.0g/kg of AFM were
2 capable of increasing the activity of endogenous antioxidant enzymes (SOD, CAT,
3 and GSH-Px) and the level of GSH in hepatic tissue. AFM pretreatment was
4 demonstrated to inhibit MDA from producing reactive oxygen radicals.
5 Anti-inflammatory effect has also been shown in the hepatoprotective agent,
6 silymarin.

7 The pro-inflammatory cytokine, TNF-α, has been reported to play a key role in
8 the pathogenesis of various liver diseases. Following its release from activated
9 Kupffer cells, TNF-α aggravates both oxidative stress and inflammatory responses in
10 the liver (Nagata *et al*, 2007). The key role of TNF-α in CCl_4 induced liver damage
11 has also been substantiated in an earlier study where treatment with soluble TNF-α
12 receptors prevented liver injury and decreased mortality in rats. TNF-α has also been
13 shown to increase the release of reactive oxygen intermediates and to augment lipid
14 peroxidation in cultured rat hepatocytes (Roome *et al.*, 2008). AFM not only
15 inhibited the release of inflammatory mediators NO, TNF-α, and IL-1β, based on the
16 findings of our study, the hepatoprotective effect of AFM could also be attributed to
17 its anti-inflammatory properties.

18 These results have provided evidence for the pharmacological effect of AFM in

1 CCl_4-induced hepatotoxicity. Overall, AFM not only provided maximum conjugation
2 with injurious free radicals and diminished their toxic properties, but also suppressed
3 the inflammatory responses in CCl_4-induced liver injury. Further studies will be
4 required to fully understand the association between CCl_4 induced oxidative stress and
5 inflammatory responses in the liver with the hepatoprotective effect of AFM.
6

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13 **Figure Legend**

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15 Figure 1. HPLC chromatogram of AFM. The peaks indicate the following 1. daidzin;
16 2. genistin; 3. daidzein; 4. genistein. IS: butyl *p*-hydroxybenzoate.

17

18 Figure 2. Effect of AFM on the activities of serum AST (A) and ALT (B) in
19 CCl_4-treated rats after 24 h of treatment. The rats were pretreated with AFM (0.1, 0.5,

1 and 1.0 g/kg) once daily for seven consecutive days. Three hours after the final

2 treatment, the rats were treated with CCl_4 (1.5 mL/kg, ip) and then killed 24 h later.

3 Hepatotoxicity was determined 24 h later by quantifying the serum activities of AST

4 and ALT. Each value is the mean ± S.E.M. $^{\#}p < 0.05$, compared with the control

5 group. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ compared with the CCl_4 group.

6

7 Figure 3. The effect of *Flemingia macrophylla* on CCl_4-induced liver damage. The

8 rats were pretreated with AFM (0.1, 0.5, and 1.0 g/kg) once daily for seven

9 consecutive days. Three hours after the final treatment, the rats were treated with CCl_4

10 (0.5 mL/kg, ip) and then killed 24 h later. In turn, their livers were removed, fixed and

11 embedded in paraffin. Sections were stained with hematoxylin-eosin (x 100). (A)

12 normal control; (B) received CCl_4 （1.5 mL/kg）; (C) silymarin (200 mg/kg) + CCl_4

13 (1.5 mL/kg); (D) AFM (0.1 g/kg) + CCl_4 (1.5 mL/kg); (E) AFM (0.5 g/kg) + CCl_4

14 （1.5 mL/kg）; (F) AFM (1 g/kg) + CCl_4 (1.5 mL/kg) (200×).

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16 Figure 4. Effect of AFM on antioxidant enzyme activities of CCl_4-treated rats.

17 Activities of SOD, superoxide dismutase, CAT, catalase and GPx, glutathione

18 peroxidase are shown in the figure. The values are mean ± S.E.M. done in triplicates.

19 $^{\#}p < 0.05$, compared with the control group. $*p < 0.05$, $**p < 0.01$ and $***p <$

1 *0.001* compared with the CCl_4 group.

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3 Fig. 5. Effect of AFM on glutathione contents in CCl_4-treated rats. The values are

4 mean ± SEM done in triplicates. $^{\#}p < 0.05$, compared with the control group. $^{*}p <$

5 0.05, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the CCl_4 group.

6

7 Figure 6. Effect of AFM on TBARS formation in CCl_4-treated rats. The values are

8 mean ± S.E.M done in triplicates. $^{\#}p < 0.05$, compared with the normal control

9 group. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the negative group.

10

11 Figure 7. Effect of AFM on (A) NO production (B) TNF-α and (C) IL-1β

12 concentrations in CCl_4-treated rats. The values are mean ± S.E.M in triplicates. $^{\#}p <$

13 0.05, compared with the normal control group. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p <$

14 *0.001* compared with the negative group.

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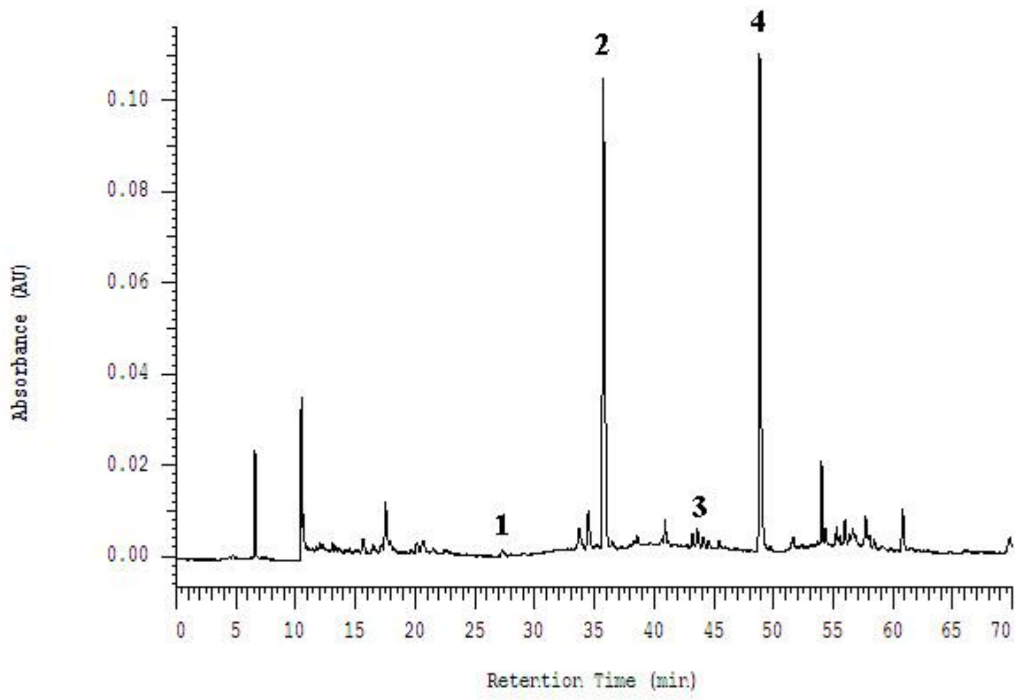
25 Figure 1.



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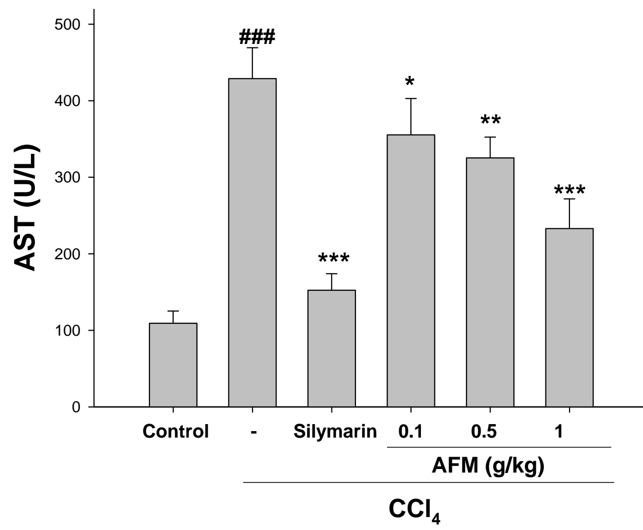
1 (A)



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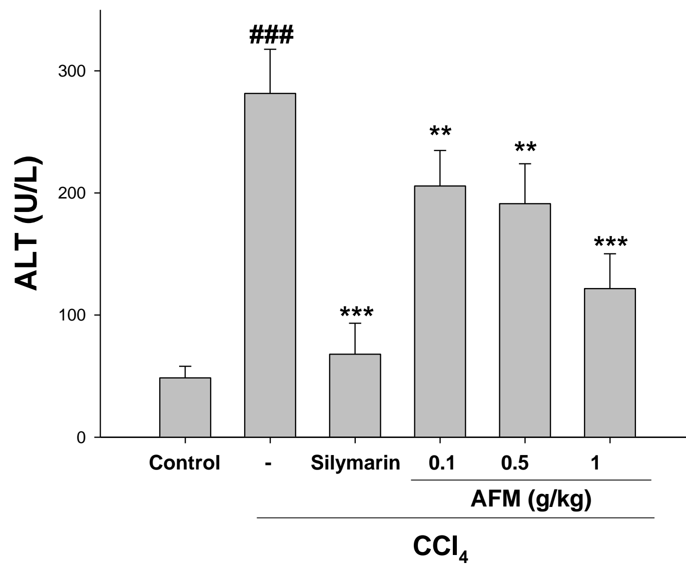
3 (B)



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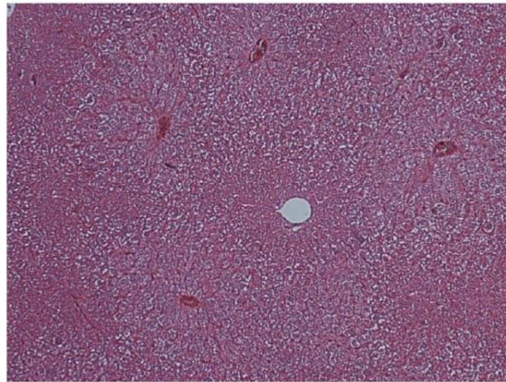
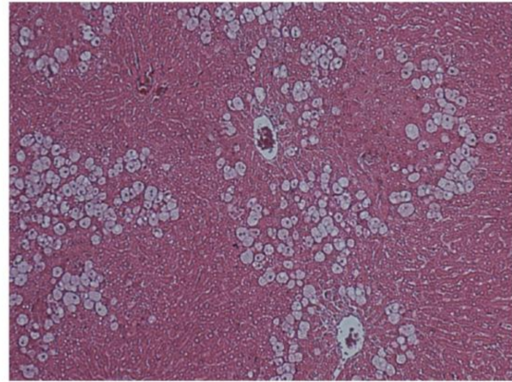
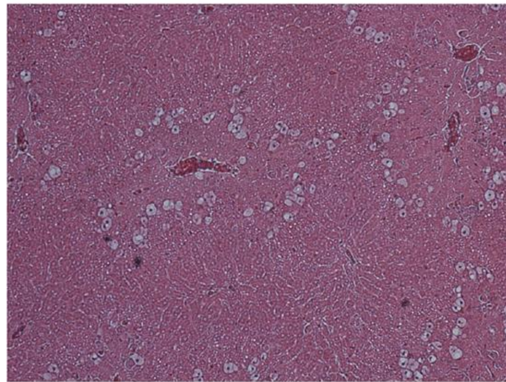


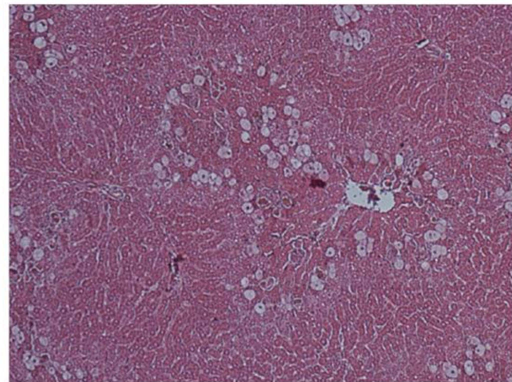
A. Control group



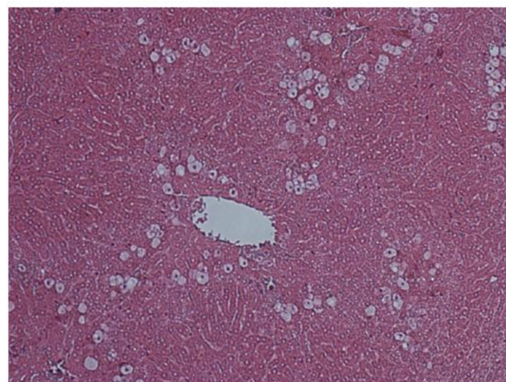
B. CCl_4 (1.5 mL/kg)



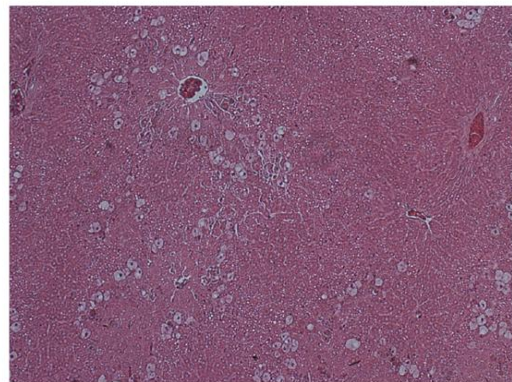
C. Silymarin (200 mg/kg) + CCl_4 (1.5 mL/kg)



D. AFM (0.1 g/kg) + CCl_4 (1.5 mL/kg)



E. AFM (0.5 g/kg) + CCl_4 (1.5 mL/kg)



F. AFM (1.0 g/kg) + CCl_4 (1.5 mL/kg)

Figure 4.

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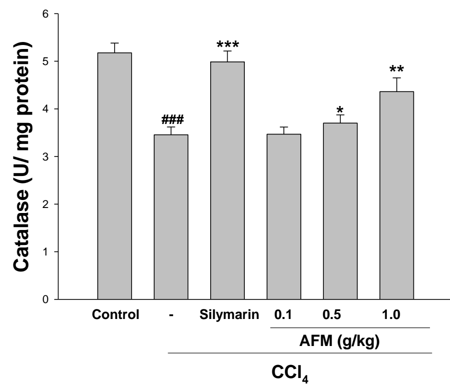

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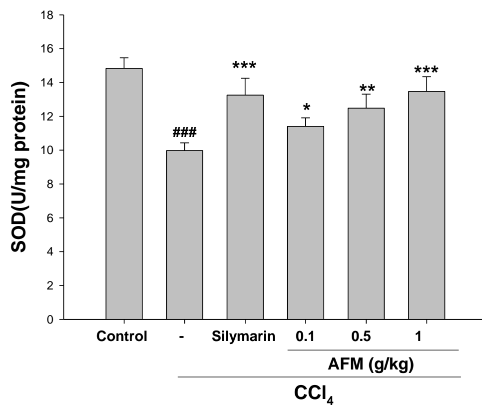

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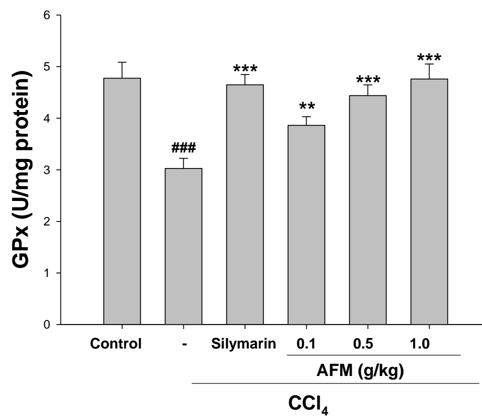

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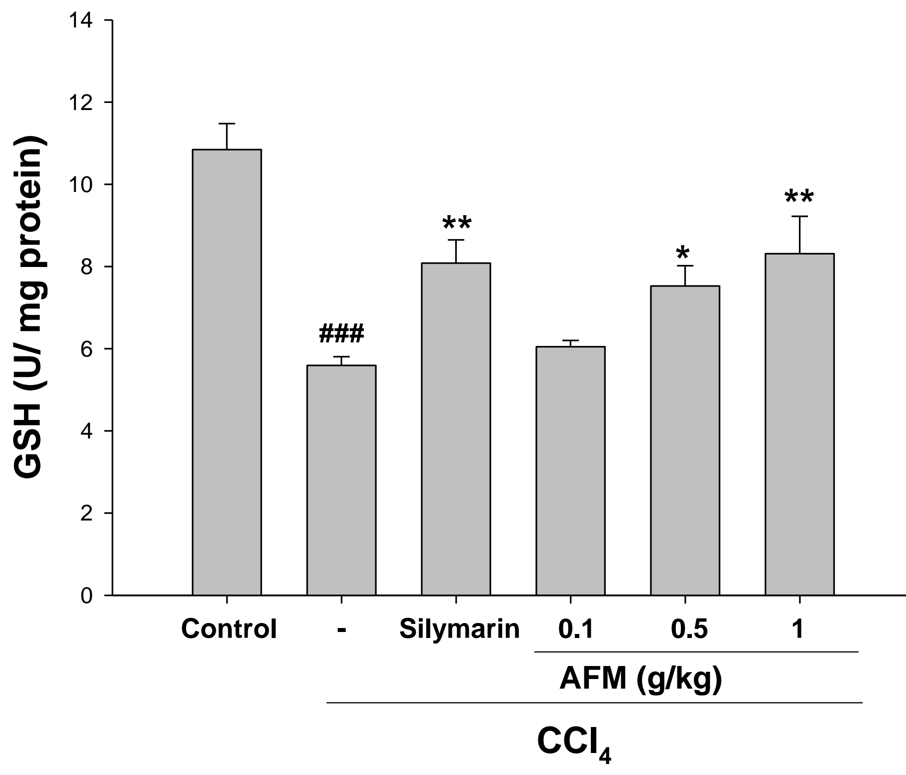
9 Figure 5.



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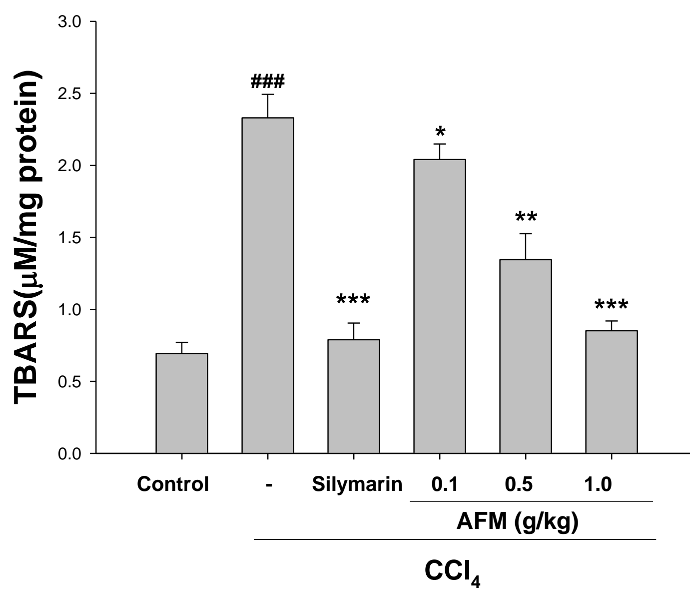

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8 Figure 7.

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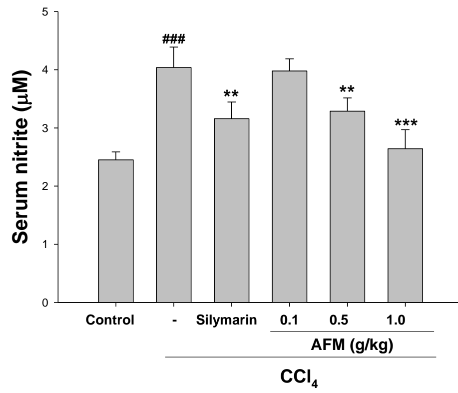

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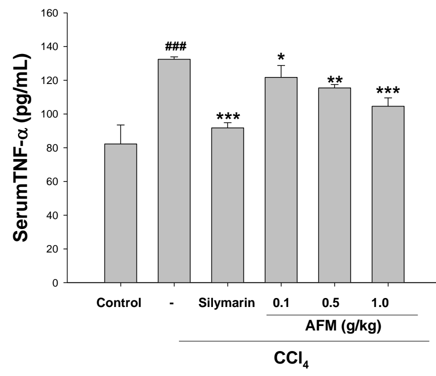

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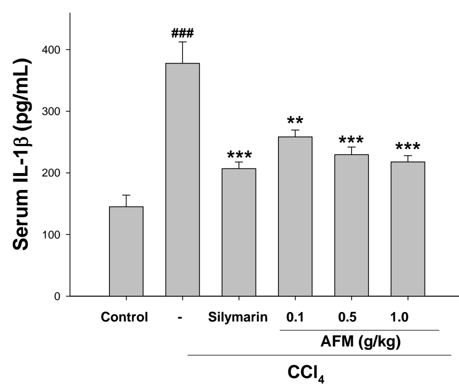

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