Running title: Hepatoprotective effect of *Vitis thunbergii* in rats

Hepatoprotective effect of the ethanol extract of *Vitis thunbergii* on carbon tetrachloride-induced acute hepatotoxicity in rats through anti-oxidative activities

Jeng-Shyan Deng[a], Yi-Chih Chang[b], Chi-Luan Wen[c], Jung-Chun Liao[d], Wen-Chi Hou[e], Sakae Amagaya[f], Shyh-Shyun Huang[d], Guan-Jhong Huang [g,*]

[a]Department of Health and Nutrition Biotechnology, Asia University, Taichung 413, Taiwan
[b]Department of Medical Laboratory Science and Biotechnology, China Medical University, Taiwan
[c]Taiwan Seed Improvement and propagation Station, Council of Agriculture, Propagation Technology Section, Taichung, Taiwan
[d]School of Pharmacy, China Medical University, Taichung 404, Taiwan
[e]*Graduate Institute of Pharmacognosy, Taipei Medical University, Taipei 250, Taiwan*
[f]Department of kampo Pharmaceutical Sciences, Nihon Pharmaceutical University, Saitama 362-0806, Japan
[g]School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, China Medical University, Taichung 404, Taiwan

*** Corresponding author:**

Dr. Shyh-Shyun Huang

School of Pharmacy, College of Pharmacy, China Medical University, Taichung 404, Taiwan; E-mail address: wenhsin.press@msa.hinet.net

Dr. Guan-Jhong Huang

School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, College of Pharmacy, China Medical University, Taichung 404, Taiwan; Tel: +886- 4-2205-3366. Ext: 5508. Fax: +886- 4-2208-3362, E-mail address: gjhuang@mail.cmu.edu.tw

1. Introduction

The oxidative damage caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS) may generate various diseases in the human body, such as aging, arthritis, cancer, inflammation, heart diseases and other human diseases (Poli, 1993). The enhanced production of oxidative stress can be induced by a variety of factors, such as ionizing radiation, and exposure to drugs or xenobiotics (e.g., carbon tetrachloride). CCl_4, an analogue 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, a highly reactive species that triggers lipid peroxidation and leads ultimately to hepatotoxicity (Goeptar et al., 1995). Antioxidant action plays an important role by which various natural products protect against CCl_4-induced liver damage (Halim et al., 1997).

Vitis thunbergii Sieb. & Zucc. var. *taiwaniana* Lu (Vitaceae) an endemic plant, an original medicinal plant in Taiwan, which has long been used as folk medicines for treatments of hepatitis, jaundice, diarrhea, and arthritis (Lin et al, 2003). The active components from *Vitis thunbergii* were reported to be resveratrol derivatives (Huang et al, 2005) and polyphenols compounds (Dou et al., 2003). *Vitis thunbergii* has been shown to have anti-inflammatory (Wang et al, 2010), antihypertensive, (Huang et al.,

2010) and neuroprotective activities (Chung et al., 2011).

The objective of this study was to better understand EVT antioxidant effects *in vitro* and *in vivo*. The hepatoprotective activity of its plant extracts has been associated with its antioxidant activity.

2. Materials and methods

2.1. 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−α and IL-1β concentrations were quantified using a commercial ELISA kit (Biosource International Inc., Camarillo, CA). The antibody against iNOS, COX-2, and β-actin were purchased from Cell Signaling Technology (Beverly, MA). Gallic acid, protocatechuic acid, catechin, vanillic acid, caffeic acid, and syringic acid were purchased from Sigma Chemicals Co.

2.2. 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. A voucher specimen had been deposited in the school of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, China Medical University, Taichung, Taiwan.

2.3. Preparation of the extracts of plant materials

Dried sample of the aerial part of *Vitis thunbergii* (1 kg) was macerated with 3 L ethanol for 24 h at room temperature. Filtration and collection of the extract were done three times. The filtrates were collected, concentrated with a vacuum evaporator until the volume was below 10 mL and then freeze-dried. The yield obtained was 6.3% (*w*/*w*).

2.4. Compositional analysis of EVT by HPLC

HPLC was performed with a Hitachi Liquid Chromatography (Hitachi Ltd., Tokyo, Japan), consisting of two model L-5000 pumps, and one model L-7455 photodiode array detector (254 nm). Samples (10 mg/mL) were filtered through a 0.45 µm PVDF-filter and injected into the HPLC column. The injection volume was 10 µL and the separation temperature was 40°C. The column was a Mightysil RP-18 GP (5 µm,

250 mm × 4.6 mm I.D.). The method involved the use of a binary gradient with mobile phases containing: (A) phosphoric acid in water (0.6‰, *v/v*) and (B) MeOH (*v/v*). The solvent gradient elution program was as follows: from 88% A to 78% A in 60 min, from 78% A to 68% A in 15 min. The flow-rate was kept constant at 1.0 mL/min. A precolumn of μ-Bondapak™ C_{18} (Millipore, Milford, MA, USA) was attached to protect the analytical column. For photodiode array detection, the wavelengths of phenolic compounds at their respective maximum absorbance wavelength can monitored at the same time. Identification is based on retention times and on-line spectral data in comparison with authentic standards. Quantification is performed by establishing calibration curves for each compound determined, using the standards. The crude extract was partitioned five times with 20 mL ethyl acetate. The ethyl ether portions were combined, filtered and then concentrated by a rotary evaporator and the residue dissolved in 1 mL of LC-grade methanol and filtered through ultra membrane filter (pore size 0.45 μm: Millipore) before HPLC analysis.

2.5. In vitro antioxidant activities of the crude extracts

2.5.1. Determination of antioxidant activity by ABTS$^{\cdot+}$ scavenging ability

The ABTS$^{\cdot+}$ scavenging ability was determined according to the method of Huang et al., (2007). Aqueous solution of ABTS (7 mM) was oxidized with potassium

peroxodisulfate (2.45 mM) for 16 hours in the dark at room temperature. The $ABTS^{·+}$ solution was diluted with 95% ethanol to an absorbance of 0.75 ± 0.05 at 734 nm (Beckman UV-Vis spectrophotometer, Model DU640B). An aliquot (20 μL) of each sample (125 μg/mL) was mixed with 180 μL $ABTS^{·+}$ solution and the absorbance was read at 734 nm after 1 min. Trolox was used as a reference standard.

2.5.2. Determination of antioxidant activity by DPPH radical scavenging ability

The effects of crude extracts and positive controls (GSH and BHT) on DPPH radicals were estimated according to the method of Huang et al., (2007). Aliquot (20 μL) of crude extracts at various concentrations were each mixed with 100 mM Tris-HCl buffer (80 μL, pH 7.4) and then with 100 μL of DPPH in ethanol to a final concentration of 250 μM. The mixture was shaken vigorously and left to stand at room temperature for 20 min in the dark. The absorbance of the reaction solution was measured spectrophotometrically at 517 nm. The percentages of DPPH decolorization of the samples were calculated according to the equation: % decolorization = $[1-(ABS_{sample}/ABS_{control})] \times 100$. EC_{50} value was the effective concentration at which DPPH radicals were scavenged by 50% and was obtained by interpolation from linear regression analysis.

2.5.3. Determination of total polyphenol content

The total polyphenol contents of the crude extracts were determined according to the method of Huang (2007). 20 μL of each extract (125 μg/mL) was added to 200 μL distilled water and 40 μL of Folin-Ciocalteu reagent. The mixture was allowed to stand at room temperature for 5 min and then 40 μL of 20 % sodium carbonate was added to the mixture. The resulting blue complex was then measured at 680 nm. Catechin was used as a standard for the calibration curve. The polyphenol content was calibrated using the linear equation based on the calibration curve. The total polyphenol content was expressed as mg catechin equivalence (CE)/g dry weight.

2.5.4. Determination of total flavonoid content

The total flavonoid content of the crude extracts was determined according to the method of Lamaison and Carnet (1990). Aliquots of 1.5 mL of extracts were added to equal volumes of a solution of 2 % $AlCl_3 \cdot 6H_2O$. The mixture was vigorously shaken, and the absorbance at 430 nm was read after 10 min of incubation. Rutin was used as a standard for the calibration curve. The total flavonoid content was calibrated using the linear equation based on the calibration curve. The total flavonoid content was expressed as mg rutin equivalent/g dry weight. The dry weight indicated was the sample dry weight.

2.6. 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°C 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 each (n=8). Rats in the normal control and negative control were orally administered with distilled water. The positive control was orally administered with silymarin (200 mg/kg in 1% carboxymethyl cellulose) once daily for 7 days. In the three experimental groups, the rats were pretreated orally with EVT (100, 200, and 400 mg/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%, *i.p.*). 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.

2.7. Evaluation of Acute Toxicity

Different doses of EVT were orally administered to 5 groups of 10 mice in order to estimate acute toxicity. Signs of toxicity during the first hour were observed and recorded. Ten control animals were given a vehicle of 0.5% CMC. After the acute phase, animals were observed for 14 days, keeping a record of the toxicity and mortality (Rivera et al., 2004). Food and water were provided throughout the experiment. If the mice died, they would be checked for autopsy and biochemical profiles.

2.8. 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.

2.9. Antioxidant enzyme activities

The following biochemical parameters were analyzed to check the hepatoprotective activity of EVT by the methods given below. Total superoxide dismutase (SOD) activity was determined by the inhibition of cytochrome *c* reduction

(Flohe and Otting 1984). The reduction of cytochrome *c* was mediated by superoxide anions generated by the xanthine/xanthine oxidase system and monitored at 550 nm. One unit of SOD was defined as the amount of enzyme required to inhibit the rate of cytochrome *c* reduction by 50%. Total catalase (CAT) activity was estimated as described elsewhere (Aebi 1984). In brief, the reduction of 10 mM H_2O_2 in 20 mM of phosphate buffer (pH 7) was monitored by measuring the absorbance at 240 nm. The activity was calculated by using a molar absorption coefficient, and the enzyme activity was defined as nanomoles of dissipating hydrogen peroxide per milligram protein per minute. Total GPx activity was determined as previously reported (Paglia and Valentine, 1967). Briefly, the enzyme solution was added to a mixture containing hydrogen peroxide and glutathione in 0.1 mM Tris buffer (pH 7.2) and the absorbance at 340 nm was measured. Activity was evaluated from a calibration curve, and the enzyme activity was defined as nanomoles of NADPH oxidized per milligram protein per minute.

2.9.1. Determination of GSH

Hepatic GSH level was determined as described previously (Ellman, 1959) with slight modifications. Briefly, 720 μL of liver homogenate in 200 mM Tris buffer, pH 7.2, was diluted to 1440 μL with the same buffer. Five percent TCA (160 μL) was

added to it and mixed thoroughly. The samples were then centrifuged at 10,000 × *g* for 5 min at 4℃. Ellman's reagent (DTNB solution) (660 μL) was added to the supernatant (330 μL). Finally the absorbance was taken at 405 nm.

2.9.2. 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) as previously described (Uchiyama et al., 1978). Briefly, 1 g of liver was homogenized in 10 mL of KCl 1.15 % (*w/v*). 0.5 mL of liver homogenate was mixed with 3 mL of H_3PO_4 1 % (*v/v*) and 1 mL of TBA 0.6 % *(w/v*), and then heated to and maintained at 100℃ for 45 min. The samples were allowed to cool down to room temperature and 3 mL of *n*-butanol was added. After shaking vigorously with the vortex, the butanolic phase was obtained by centrifugation at 4,000 × *g* for 10 min to determine the absorbance at 535 nm. The standard was 1, 1, 1, 3-tetraethoxypropane.

2.9.3. Determination of nitric oxide (NO)

The production of NO was assessed indirectly by measuring the nitrite levels in the plasma by 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 × *g* for 5 min at room temperature, 100 μL supernatant was applied to a microliter plate well, followed by 100 μL of Greiss reagent (1 % sulfanilamide and 0.1 % *N*-1-naphthylethylenediamine 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 curve.

2.9.4. Measurement of serum TNF-α and IL-1β

The serum level of TNF-α and IL-1β were determined using a commercially available enzyme linked immunosorbent assay (ELISA) kit (Biosource International Inc., Camarillo, CA) according to the manufacturer's instructions. TNF-α and IL-1*β* were determined from a standard curve. The concentrations were expressed in pg/mL.

2.9.5. Protein Lysate Preparation and Western blot Analysis of iNOS and COX-2.

Liver tissues were homogenized in lysis buffer (0.6% NP-40, 150 mM NaCl, 10 mM HEPES (pH 7.9), 1 mM EDTA, and 0.5 mM PMSF) at 4℃. We used BSA (bovine serum albumin) as a protein standard to calculate equal total cellular protein amounts. Protein samples (30 μg) were resolved by denaturing sodium dodecyl

sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using standard methods, and then were transferred to PVDF membranes by electroblotting and blocking with 1% BSA. The membranes were probed with the primary antibodies (iNOS, COX-2, and β-actin) at 4℃ overnight, washed three times with PBST, and incubated for 1 h at 37 ℃ with horseradish peroxidase conjugated secondary antibodies. The membranes were washed three times and the immunoreactive proteins were detected by enhanced chemiluminescence (ECL) using hyperfilm and ECL reagent (Amersham International plc., Buckinghamshire, U.K.). The results of Western blot analysis were quantified by measuring the relative intensity compared to the control using Kodak Molecular Imaging Software and represented in the relative intensities.

2.10. Statistical analysis

All experiments were done in triplicate and results were reported as mean ± S.D. Data were analyzed by one way ANOVA. Statistically significant effects were further analyzed and means were compared using Duncan's multiple range test. Statistical significance was determined at $p < 0.05$.

3. Results

3.1. Fingerprint Analysis by HPLC

To establish the fingerprint chromatogram for the quality control of EVT. Gallic acid, protocatechuic acid, catechin, vanillic acid, caffeic acid, and syringic acid were used as markers. An optimized HPLC-PAD (photodiode array detector) technique was employed. Meanwhile, HPLC chromatograms showed five marker components present in EVT. As shown in Fig. 1, these phenolic components have been identified as gallic acid, protocatechuic acid, catechin, vanillic acid, caffeic acid, and syringic acid by their retention time and UV absorbance of purified standards. According to the plot of peak-area ratio (y) vs. concentration (x, μg/mL), the regression equations of the five constituents and their correlation coefficients (r) were determined as follows: gallic acid, $y = 1.111x + 8.101$ ($r^2 = 0.999$); protocatechuic acid, $y = 0.622x + 4.301$ ($r^2 = 0.999$); catechin, $y = 0.443x + 3.104$ ($r^2 = 0.999$); vanillic acid, $y = 0.836x + 5.686$ ($r^2 = 0.999$); caffeic acid, $y = 1.643x + 11.65$ ($r^2 = 0.999$), syringic acid, $y = 1.410x + 10.19$ ($r^2 = 0.999$). The relative amounts of the six phenolic compounds found in EVT were in the order of catechin (65.93 mg/g) > vanillic acid (12.08 mg/g) >gallic acid (4.54 mg/g) > protocatechuic acid (4.11 mg/g) > syringic acid (3.65 mg/g) > caffeic acid (2.47 mg/g), respectively.

3.2. *In vitro* assays

3.2.1. ABTS radical scavenging activity. ABTS assay are often used in evaluating

total antioxidant power of single compounds and complex mixtures of various plants. ABTS assay was expressed as trolox equivalent antioxidant activity (TEAC) values. Higher TEAC value represented that the sample had a stronger antioxidant activity. Our results showed that EVT (109.35± 0.12 μg/mg extracts) had higher antioxidant potentials (Table 1).

3.2.1. DPPH radical scavenging activity.

DPPH radical was scavenged by antioxidants through the donation of a proton forming the reduced DPPH. The color changed from purple to yellow after the reduction, which could be quantified by its decrease of the absorbance at wavelength 517 nm. Radical scavenging activity increased with increasing percentage of the free radical inhibition. In DPPH radical scavenging activity assay, our results indicated that an EC50 value of EVT was 192.69 ± 2.56 μg/mL while BHT had a value of 42.46 ± 1.08 μg/mL, respectively (Table 1).

3.3. Total phenolic and flavonoid content.

Most antioxidant activities from plant sources are correlated with phenolic-type compounds. Total phenol content was estimated as 82.13± 0.58 μg catechin equivalent/mg dry weight. Total flavonoid content was 45.45 ± 0.21 μg rutin

equivalent/ mg dry weight.

3.4. Evaluation of Acute Toxicity in Mice.

When EVT was studied by oral administration in mice, no signs of toxicity were observed. In the LD_{50} experiment, no mice died under the dose of 5 g/kg.

3.5. 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 the oral administration of EVT on the serum AST and ALT levels of hepatic-damaged rats is shown in Table 2. EVT (400 mg/kg) reduced serum AST and ALT levels of the rats with hepatic damage, significantly ($p<0.001$). Silymarin (200 mg/kg) used as the standard drug indicated the similar effect. It is confirmed that EVT could ameliorate hepatic function in CCl_4 induced liver injury.

3.6. Effect on serum NO, TNF-α, and IL-1β levels

CCl_4 induced hepatotoxicity was associated with marked increase in the levels of NO, TNF-α, and IL-1β. As shown in Table 2, the production of NO in the plasma was significantly increased in CCl_4-treated rats as compared to the normal control group

(12.26 ± 0.68 μM in control vs. 20.19 ± 1.75 μM in only CCl_4 treatment). However, pretreatments of EVT decreased NO production in CCl_4-treated rats. NO level was significantly inhibited in the groups pretreated with 100 mg /kg (18.56±1.88 μM, $p <$ 0.05), 200 mg/kg (17.43 ± 0.85 μM, $p < 0.01$) and 400 mg/kg (15.63 ± 1.11 μM, $p <$ 0.001) of EVT.

The production of TNF-α and IL-1β in the serum was significantly increased in CCl_4-treated rats (152.47±1.46 pg/mL and 363.46 ± 21.89 pg/mL) as compared to the normal control group (81.07 ± 2.23 pg/mL and 152.47±1.46 pg/mL, respectively). At the dose of 100 and 200 mg/kg, EVT produce any change in the TNF-α and IL-1β level while significant decreases in the TNF-α and IL-1β level were observed ($p<0.001$ or $p<0.001$). Like silymarin, treatment with EVT (400 mg/kg) over 7 days produced a significant ($p < 0.001$) dose-dependent decrease in the levels of TNF-α and IL-1β (Table 1).

3.7. Effect of EVT on liver histology

The histological features of the livers from the control and experimental groups are as shown in Fig. 2. Figure 2A shows the cell structure of livers of the control animals and it is a representation of normal liver lobular architecture, which has no pathological changes and with central vein and radiating hepatic cords (Fig. 2A).

Increased fatty degeneration, cytoplasmic vacuolization and necrosis provided histopathological evidence of tissue injury in the CCl_4-treated group (Fig. 2B). Changes were improved in EVT and silymarin pretreated rats, which exhibited areas of normal liver architecture and patches of necrotic hepatocytes (Fig. 2. C–F).

3.8. Effect on CAT, SOD, and GPx activities in CCl_4-induced hepatic injury.

CAT activities in the total liver homogenate were shown in Table 3. CAT activity of liver homogenate in CCl_4 group (3.21 ± 0.06 U/mg protein) was conspicuously lower than that of the control group (5.32 ± 0.15 U/mg protein). CAT activities of liver homogenates from the 200 mg/kg (3.59 ± 0.28 U/mg protein, $p < 0.05$) and 400 mg/kg (4.23 ± 0.29 U/mg protein, $P < 0.01$) of EVT groups were significantly higher than those in the CCl_4 group.

The effect of EVT on SOD activity in the liver was shown in Table 3. SOD activity of the liver homogenate in CCl_4 group (9.68 ± 0.23 U/mg protein) was lower than that of the control group (14.56 ± 0.57 U/mg protein). The SOD activities of liver homogenates of groups treated with 100 mg/kg (11.57 ± 1.04 U/mg protein, $p < 0.05$), 200 mg/kg and 400 mg/kg (13.33 ± 0.93 U/mg protein; 13.69 ± 1.01 U/mg protein, $p < 0.01$) were significantly higher than those in the CCl_4 group.

Table 3 shows the GPx activity of the liver homogenate on CCl_4 treated rat after

administration with EVT. GPx activity of liver homogenate in the CCl_4 group (3.23±0.15 U/mg protein) was lower than that of the control group (4.56 ± 0.21 U/mg protein). The GPx activities of liver homogenates of the experimental groups pretreated with 100 mg/kg (3.67 ± 0.28 U/mg protein, $P < 0.05$), 200 mg/kg (4.14 ± 0.24 U/mg protein, $P < 0.01$), and 400 mg/kg (4.43 ± 0.14 U/mg protein, $P < 0.001$) EVT were significantly higher than those of the CCl_4 group. The observed increase of antioxidant activities suggested that EVT had an efficient protective mechanism in response to ROS.

3.9. Effect on the GSH levels in CCl_4 treated rats

GSH protects cells against free radicals, peroxides and other toxic compounds. Tissue levels of GSH often decrease upon elevation of local oxidative stress. Deficiency of GSH within living organisms can lead to tissue disorder and injury. Oxidative stress caused by CCl_4 significantly reduced liver GSH level (5.31 ± 0.14 U/mg protein in only CCl_4 treatment), however, CCl_4-treated rats administrated with 200 and 400 mg/kg EVT (7.67 ± 0.81 U/mg protein, $P < 0.01$; 8.61± 0.26 U/mg protein, $P < 0.001$) increased the GSH levels, as compared to the rats treated with CCl_4, significantly (Table 3). The results indicating that EVT (200-400 mg/kg) treatment significantly protected against liver GSH reduction.

3.10. Effect on hepatic TBARS levels

The localization of radical formation resulting in lipid peroxidation, measured as MDA in rat liver homogenate, is as shown in Table 3. MDA contents in the liver total homogenate were dramatically increased in CCl_4-treated (1.59 ± 0.12 nmole/mg protein) compared to the control group 0.48 ± 0.05 nmole/mg protein). MDA level was significantly inhibited in 100 mg/kg (1.03 ± 0.17 nmole/mg protein, $p < 0.05$), 200 mg/kg (0.82 ± 0.16 nmole/mg protein, $p < 0.01$) and 400 mg/kg (0.68 ± 0.09 nmole/mg protein, $p < 0.001$) of EVT treated groups.

3.11. Analysis of iNOS/COX-2 expression following EVT treatment in rats with CCl_4-induced liver injury

We investigated the changes of the activation of iNOS and COX-2 by EVT in CCl_4-treated rats (Fig. 3). The results showed that CCl_4 treatment stimulates to increase activation of iNOS and COX-2. However, the treatment of EVT decreased the iNOS and COX-2 expression in CCl_4-induced rats. Namely, the relative intensities of bands about iNOS and COX-2 expressions were reduced by 74.5 % and 71.1 % at 400 mg/kg of EVT, respectively, compared to CCl_4 treatment alone.

4. Discussion

Oxidative stress plays a crucial role in the development of CCl_4-induced hepatotoxicity and a connection between oxidative stress and lipid peroxidation has been reported (Somasundaram et al., 2010). Studies have noted that CCl_4 is widely used to induce liver damage because it is metabolized in hepatocytes by cytochrome P450, generating a highly reactive carbon-centered trichloromethy radical, leading to initiating a chain of lipid peroxidation and thereby causing liver fibrosis (Fang et al., 2008). In the present study, we evaluated the hepatoprotective effect of EVT against CCl_4 induced acute hepatotoxicity in rats. The consistency of chemical composition in EVT is important in safe guarding the reliability of the research results. The chemical profile of EVT was recorded by HPLC analysis. The HPLC chemical profile could be delineated by the measurement of relative retention times of major characteristic peaks using gallic acid, protocatechuic acid, catechin, vanillic acid, caffeic acid and syringic acid as markers. The resulting chromatogram was used as a standard for the assessment of all extracts used in the present study.

HPLC was used to quantify the components of EVT. The fingerprint chromatograms demonstrated gallic acid, protocatechuic acid, catechin, vanillic acid, caffeic acid, and syringic acid as its ingredients. Our results demonstrated that 1 g of dry weight of EVT contains polyphenol amounts equal to 82.13± 0.58 mg CE/g,

respectively. This result confirms that total polyphenol contents of natural products are regular indices of their antioxidant activity.

The DPPH or ABTS have been popular radical scavenging tests for natural products. Free radicals could induce biological damage and pathological events, such as inflammation, aging, and carcinogenesis (Halliwell, 1999). In this study, EVT showed significant antioxidant activities. The HPLC chromatogram of EVT demonstrated six phenolic components identified as gallic acid, protocatechuic acid, catechin, vanillic acid, caffeic acid, and syringic acid had higher good antioxidant activities (Table 1). The higher radical scavenging activity of EVT seems to be closely correlated with its polyphenolic constituents through other active components. Apparently, these marker compounds in EVT could contribute to its antioxidant effects. They could account for the high antioxidative of EVT. In general, free radical scavenging and antioxidant activities of phenolics (e.g., phenolic acids, flavonoids) depend on the number and position of hydrogen-donating hydroxyl groups on the aromatic ring of the phenolic molecules, and are also affected by other factors, such as glycosylation of aglycones, other H-donating groups (–NH, –SH), etc., In addition, many scientific papers have reported that gallic acid was evaluated for its hepatoprotective activity against CCl_4-induced physiological and biochemical alterations in the liver (Anand et al., 1997). The oral administration with

protocatechuic acid for 28 consecutive days significantly decreases the intensity of hepatic damage induced by CCl_4 in rats (Hung et al., 2006). Catechin can be considered as a chemotherapeutic against hepatopathies (Abdel-Hamid et al., 2007). The administration of syringic acid and vanillic acid suppress hepatic fibrosis in chronic liver injury (Itoh et al., 2010). Caffeic acid exhibits hepatoprotective activity against paracetamol and CCl_4-induced hepatic damage (Janbaz et al., 2004). Therefore, the *in vitro* and *in vivo* antioxidant activities of EVT may be associated with the phenolic compounds in the extracts.

Hepatic cells contain higher concentrations of AST and ALT in the cytoplasm, and AST, particularly exists in the mitochondria. Due to damage caused to hepatic cells, the leakage of cytosol will cause increased levels of hepatospecific enzymes in the serum. The elevated serum enzyme levels such as AST and ALT are indicative of cellular leakage and functional integrity of cell membrane in the liver (Zeashan et al., 2009). The measurement of serum AST and ALT levels serve as a mean for the indirect assessment of the condition of the liver. In the present study, the capability of EVT in controlling CCl_4 induced toxicity was demonstrated in that EVT pre-treated animals which had decreased AST and ALT levels when compared with the CCl_4 group, significantly.

Because antioxidants are reported to exert anti-inflammatory activity (Huang, 2011), we also evaluated the *in vivo* anti-inflammatory activity of EVT by measuring release of TNF-α, a pro-inflammatory cytokine that becomes elevated in acute and chronic diseases. Some phytochemicals have been shown to inhibit inflammation by blocking inflammatory pathways downstream of cytokine release, and also by reducing macrophage production of proinflammatory factors (Tripathi et al., 2007). The pro-inflammatory factors, TNF-α has been reported to play a key role in the pathogenesis of various liver diseases. Following its release from activated Kupffer cells, TNF-α aggravates both oxidative stress and inflammatory responses in the liver (Nagata et al, 2007). The key role of TNF-α in CCl_4 induced liver damage has been substantiated in an earlier study in which treatment with soluble TNF-α receptors prevented liver injury and decreased mortality in rats (Hsieh, et al., 2011). TNF-α also stimulates the release of cytokines from macrophages and induces phagocyte oxidative metabolism and NO production. Although several studies have showed that NO protects against CCl_4-induced liver injury using a NOS inhibitor, certain evidence indicates that excessive NO production by iNOS can lead to hepatic injury (Zhu and Fung, 2000). Recent reports also demonstrated that iNOS overproduction occurs in the liver of rats with CCl_4-induced acute liver injury, which suggested that iNOS may act as a mediator in the pathogenesis of hepatotoxicity in rats (Zhu

and Fung, 2000). Increased expression of COX-2, a known inflammatory mediator has been observed in the present study. Increased expression of COX-2 and iNOS indicate that there is a rise in the inflammation in CCl_4 treated rats. In this study, we evaluated that EVT not only inhibited the release of inflammatory mediators NO, TNF-α, and IL-1β, the hepatoprotective effect of EVT also could be attributed to its anti-inflammatory properties.

CCl_4-induced lipid peroxidation is highly dependent on the bioactivation of the trichoromethyl and trichloromethyl peroxy radicals (Shen et al., 2009). MDA, a product of lipid peroxidation, was increased in rat liver by CCl_4 induction. However, we showed that EVT significantly reduced MDA formation. In other words, the mechanism of the inhibitory effects, by which the EVT protects against lipid peroxidation, may involve radical-scavenging capability. CCl_4 not only initiates lipid peroxidation but also reduces tissue GPx, GR, CAT, and SOD activities, and this depletion may result from oxidative modification of these proteins (Augustyniak et al., 2005). Our results also showed that CCl_4 challenge significantly decreased the activities of CAT, SOD and GPx in the liver. Cells have a number of mechanisms to defend themselves from the toxic effect of ROS including free radical scavengers and chain reaction terminators such as SOD, CAT, and GPx systems. SOD removes superoxide radicals by converting them into H_2O_2 which can be rapidly converted into

water by CAT and GPx. Cellular injury occurs when ROS generation exceeds the cellular capacity of removal (Wu et al., 2009).

EVT administration effectively protected against the loss of these antioxidant activities after CCl_4 administration, and it also significantly reduced the loss of hepatic GSH. GSH, an endogenous reductant, is well known to serve diverse biological functions, including protection of cells from oxidative damage by ROS and free radicals (Gabele et al., 2009). Some phytochemicals have also been shown to stimulate syntheses of antioxidant enzymes and detoxification systems at the transcriptional level, through antioxidant response elements (Masella et al., 2005).

Overproduction of NO in the liver has been implicated as an important event in endotoxin shock and in other models of hepatic inflammation and injury. NO is known to react with superoxide radical, forming peroxynitrite, an even more potent oxidizing agent. Therefore, this endotoxin shock may alter the balance existing between NO production and its target proteins and enzymes, leading to GSH depletion, free radical generation and up-regulation of iNOS (Foresti, et al., 1997). Previous report demonstrated that CCl_4 administration increased NO level in the blood plasma or CCl_4-treated animals (Muriel, 1998). Similarly, under acute CCl_4 intoxication we revealed considerable NO level in blood plasma (Table 2), and increased expression of iNOS and COX-2 (Fig. 3A). EVT prevented the accumulation of the plasma NO

level in CCl_4-treated rats. The treatment with EVT leads to a reduction in the expression level of both iNOS and COX-2. This may in part be the result of the regulatory activity and expression of NF-κB, and need to be proved.

These results have provided the evidence for the pharmacological effect of EVT in CCl_4-induced hepatotoxicity. Overall, EVT not only provided maximum conjugation with injurious free radicals and diminished their toxic properties, but also suppressed the inflammatory responses in CCl_4-induced liver injuries. The possible mechanisms could be suggested that EVT is able to protect the liver against cellular oxidative damage and maintenance of intracellular level of antioxidant enzymes as well as to act immunoregulatory. However, further studies on the active compounds and their biochemical mechanisms responsible for the hepatoprotective effect of *Vitis thunbergii* will be necessary.

Acknowledgement

The authors want to thank the financial supports from the National Science Council (NSC100-2313-B-039-004- and NSC 100-2320-B-039-033-), China Medical University (CMU) (CMU100-ASIA-15 and CMU100-TC-11) and Taiwan Department of Heath Clinical Trial and Research Center of Excellence (DOH101-TD-B-111-004).

References

Abdel –Hamid, N.M., Faddah, L.M., Al-Rehany, M.A., Ali, A.H., Bakeet, A.A., 2007. New role of antinutritional factors, phytic acid and catechin in the treatment of CCl_4 intoxication. Annals of hepatology 6, 262-266.

Aebi, H., 1984. Catalase *in vitro*. Methods in Enzymology 105, 121-126.

Anand, K.K., Singh B., Saxena, A.K., Chandan, B.K., Gupta, V.N., Bhardwaj, V., 1997. 3, 4, 5-Trihydroxy benzoic acid (gallic acid), the hepatoprotective principle in the fruits of *Terminalia belerica*-bioassay guided activity. Pharmacological Research 36, 315-321.

Augustyniak, A., Wazkilwicz, E., Skrzydlewaka, 2005. Preventive action of green tea from changes in the liver antioxidant abilities of different aged rats intoxicated with ethanol. Nutrition 21, 925-932.

Chung, I.M., Yeo, M.A., Kim, S.J., Moon, H.I., 2011. Neuroprotective effects of resveratrol derivatives from the roots of *Vitis thunbergii* var. sinuate against glutamate-induced neurotoxicity in primary cultured rat cortical cells. Human and Experimental Toxicology 30, 1404-1408.

Dou, D.Q., Cooper, J.R.M., He, Y.H., Pei, Y.P., Takaya, Y., Niwa, M., Chen ,Y.J., Yao, X.S., Zhou, R.P., 2003. Polyphenols from *Vitis thunbergii* Sieb. et Zucc. Journal of Chinese Pharmaceutical Sciences 12, 57-59.

Ellman, G.L., 1959. Tissue sulphydryl group. Archives of Biochemistry and Biophysics 82, 70-77.

Fang, H.L. Lai, J.T., Lin, W.C., 2008. Inhibitory effect of olive oil on fibrosis induced by carbon tetrachloride in rat liver. Clinical Nutrition 27, 900-907.

Flohe L, Otting F. 1984. Superoxide dismutase assays. Methods in Enzymology 105, 93–104.

Foresti, R., Clark, J. E., Green, C. J., Motterlini, R., 1997. Thiol compounds interact with nitric oxide in regulating heme oxygenase- 1 induction in endothelial cells: involvement of superoxide and peroxynitrite anions. The Journal of Biological Chemistry 29, 18411-18417.

Gabele, E., Fron, M., Aeteel, G.E., Uesugi, T., Hellerbrand, C., Scholmerich, J., 2009. TNF-alpha is required for cholestasis-induced liver fibrosis in the mouse. Biochemical and Biophysical Research Communications 378, 348-353.

Goeptar, A.R., Scheerens, H., Vermeulen, N.P., 1995. Oxygen and xenobiotic reductase activities of cytochrome P450. Critical Reviews in Toxicology 25, 25-65.

Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 1982. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. Analytical Biochemistry 126, 131-138.

Halim, A.B., el-Ahmady, O., Hassab-Allah, S., Abdel-Galil, F., Hafez, Y., Darwish, A., 1997. Biochemical effect of antioxidants on lipids and liver function in experimentally-induced liver damage. Annals of Clinical Biochemistry 34, 656-663.

Halliwell, B., 1999. Antioxidant defense mechanisms: from the beginning to the end. Free Radical Research 31, 261-272.

Huang, Y.L., Tsai, W.J., Shen, C.C., Chen, C.C., 2005. Resveratrol derivatives from the roots of *Vitis thunbergii*. Journal of Natural Products 68, 217-220.

Huang, G.J., Chen, H.J., Chang, Y.S, Sheu, M.J., Lin, Y.H., 2007. Recombinant Sporamin and its synthesized peptides with Antioxidant Activities *in vitro*. Botanical studies 48, 133-140.

Huang, C.Y., Wen, C.L., Lu, Y.L., Lin, Y.S., Chen, L.G., Hou, W.C., 2010. Antihypertensive activities of extracts from tissue cultures of *Vitis thunbergii* var. *taiwaniana*. Botanical Studies 51, 317-324.

Huang, M.H., Huang, S.S., Wang, B.S., Wu, C.H., Sheu, M.J., Hou, W.C., Lin, S.S., Huang, G.J., 2011. Antioxidant and anti-inflammatory properties of *Cardiospermum halicacabum* and its reference compounds *ex vivo* and *in vivo*. Journal of Ethnopharmacology 133, 743-750.

Hung, M.Y., Fu, T.Y., Shih, P.H., Lee, C.P., Yen, G.C., 2006. Du-Zhong (*Eucommia*

ulmoides Oliv.) leaves inhibits CCl4-induced hepatic damage in rats. Food and Chemical Toxicology 44, 1424-1431.

Hsieh, P.C., Ho, Y.L., Huang, G.J., Huang, M.H., Chiang, Y.C., Huang, S.S., Hou, W.C., Chang, Y.S. 2011. Hepatoprotective effect of the aqueous extract of *Flemingia macrophylla* on carbon tetrachloride-induced acute hepatotoxicity in rats through anti-oxidative activities. American Journal of Chinese Medicine 39, 349-365.

Itoh ,A., Isoda, K., Kondoh, M., Kawase, M., Watari, A., Kobayashi, M., Tamesada, M., Yagi, K., 2010. Hepatoprotective effect of syringic acid and vanillic acid on CCl_4-induced liver injury. Biological & Pharmaceutical Bulletin 33, 983-987.

Janbaz, K.H., Saeed, S.A., Gilani, A.H., 2004. Studies on the protective effects of caffeic acid and quercetin on chemical-induced hepatotoxicity in rodents. Phytomedicine 11, 424-430.

Lamaison, J. L. C., Carnet, A., 1990. Teneurs en principaux flavonoids des fleurs de Crataegeus monogyna Jacq et de *Crataegeus laevigata* (Poiret D. C) en fonction de la vegetation. Pharmaceutica Acta Helvetiae 65, 315-320.

Lin, I. H. 2003. The Catalogue of Medicinal Plant Resources in Taiwan, Committee on Chinese Medicine and Pharmacy, Department of Health, Taipei, Taiwan.

Masella, R. Benedetto, R. Di Vari, R. Filesi, C., Giovannini, C., 2005. Novel

mechanisms of natural antioxidant compounds in biological systems: Involvement of glutathione and glutathione-related enzymes. The Journal of Nutritional Biochemistry 16, 577-586.

Muriel, P., 1998. Nitric oxide protection of rat liver from lipid peroxidation, collagen accumulation, and liver damage induced by carbon tetrachloride. Biochemical Pharmacology 56, 773-779.

Nagata, K., Suzuki, H., Sakaguchi, S., 2007. Common pathogenic mechanism in development progression of liver injury caused by non-alcoholic or alcoholic steatohepatitis. The Journal of Toxicological Sciences 32, 453-468.

Paglia, D.E., Valentine, W.N., 1967. Studies on the quantitive and qualitative characterization of erythrocyte glutathione peroxidase. Journal of Laboratory and Clinical Medicine 70, 158-169.

Poli, G., 1993. Liver damage due to free radicals. British Medical Bulletin 49, 604-620.

Rivera, F., Gervaz, E., Sere, C., Dajas, F., 2004. Toxicological studies of the aqueous extract from *Achyrocline satureioides* (Lam.) DC (Marcela). Journal of Ethnopharmacology 95, 359-362.

Shen, X., Tang, Y., Yang, R., Yu, L., Fang, T., Duan, J., 2009. The protective effect of Zizyphus jujube fruit on carbon tetrachloride-induced hepatic injury in mice by

anti-oxidative activities. Journal of Ethnopharmacology 122, 555-560.

Somasundaram, A., Karthikeyan, R., Velmurugan, V., Dhandapani, B., Raja, Muthu., 2010. Evaluation of hepatoprotective activity of *Kyllinga nemoralis* (Hutch & Dalz) rhizomes. Journal of Ethnopharmacology 127, 555–557.

Tripathi, S., Maier, K.G., Bruch, D., Kittur, D.S., 2007. Effect of 6-gingerol on pro-inflammatorycytokine production and costimulatory molecule expression in murine peritoneal macrophages. The Journal of Surgical Research 138, 209-213.

Uchiyama, M., Mihara, M., 1978. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Analytical Biochemistry 86, 271-278.

Wang, C.K., Chen, L.G., Wen, C.L., Hou, W.C., Hung, L.F., Yen, S.J., Shen, Y.J., Lin, S.Y., Liang, Y.C., 2010. Neuroprotective activity of *Vitis thunbergii* var. *taiwaniana* extracts *in vitro* and *in vivo*. Journal of Medicinal Food 13, 170-178.

Wu, Y.H., Zhang, X.M., Hu, M.H., Wu, X.M., Zhao, Y., 2009. Effect of *Laggera alata* on hepatocyte damage induced by carbon tetrachloride *in vitro* and *in vivo*. Journal of Ethnopharmacology 126, 50–56.

Zeashan, H., Amresh, G., Singh, S., Rao, C.V., 2009. Hepatoprotective and antioxidant activity of *Amaranthus spinosus* against CCl_4 induced toxicity. Journal of Ethnopharmacology 125, 364–366.

Zhu, W., Fung, P. C., 2000. The roles played by crucial free radicals like lipid free radicals, nitric oxide, and enzymes NOS and NADPH in CCl(4)-induced acute liver injury of mice. Free Radical Biology and Medicine, 29, 870-880.