Protective effect of *Acacia confusa* bark extract and its active compound gallic acid against carbon tetrachloride-induced chronic liver injury in rats

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ARTICLE INFO

ABSTRACT

Acacia confusa Merr. (Leguminosae), a species native to Taiwan, is widely distributed on the hills and low-lands of Taiwan, and has been traditionally used as a medicine. The hepatoprotective effects of *A. confusa* bark extract (ACBE) and its active constituent gallic acid were evaluated against carbon tetrachloride (CCl_4)-induced hepatotoxicity in rats. CCl_4-induced hepatic pathological damage and significantly increased the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and malondial-dehyde (MDA) in plasma, and cytochrome P4502E1 (CYP2E1) protein expression in hepatic samples, and decreased the activities of superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) in erythrocytes. Treatment with ACBE, gallic acid or silymarin could decrease significantly the AST, ALT, and MDA levels in plasma, and CYP2E1 expression in liver tissues, and increase the activities of SOD and GPX in erythrocyte when compared with CCl_4-treated group. Liver histopathology also showed that ACBE, gallic acid or silymarin could significantly reduce the incidence of liver lesions induced by CCl_4. These results suggested that the ACBE and gallic acid exhibit potent hepatoprotection against CCl_4-induced liver damages in rats, and the hepatoprotective effects of ACBE and gallic acid may be due to the modulation of antioxidant enzymes activities and inhibition of lipid peroxidation and CYP2E1 activation.

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1. Introduction

According to the statistics tabulated by the Department of Health, Taiwan in 2007, chronic liver disease and cirrhosis consti-tute 7th leading cause of death in Taiwan. Unfortunately, there are certain limitations in the treatment of hepatitis. A number of researchers pointed out that in addition to hepatic problems, car-bon tetrachloride (CCl_4) also causes acute and chronic hepatic damage due to free radical generations (Liao et al., 2007; Jain et al., 2008; Hung et al., 2006; Wills and Asha, 2006). CCl_4 is metab-olized by cytochrome P4502E1 (CYP2E1) to become trichloro-methyl radical ($\cdot CCl_3$) and proxy trichloromethyl radical ($\cdot OOCCl_3$), which are assumed to initiate free radical-mediated lipid peroxidation leading to the accumulation of lipid-derived oxida-tion products that cause liver injury (Liao et al., 2007; Poli et al., 1987; Recknagel et al., 1989; Shyur et al., 2008). Silymarin, a mix-ture of flavonolignanes from milk thistle (*Silybum marianum* L.), is a hepatoprotective herbal medicine worth $180 million dollars of business annually in Germany alone. Silymarin could effectively prevent CCl_4-induced liver injury, indicating that oxidative stress is responsible for CCl_4-induced hepatic disorder (Liao et al., 2007). A number of studies showed that various herbal extracts could protect liver against CCl_4-induced oxidative stress by altering the levels of increased lipid peroxidation, and enhancing the de-creased activities of antioxidant enzymes such as superoxide dis-mutase (SOD), catalase (CAT) and glutathione-S-transferase (GST) as well as the decreased level of the hepatic-reduced glutathione (GSH) (Lu and Liu, 1992; Jain et al., 2008; Hsiao et al., 2003; Hung et al., 2006; Wills and Asha, 2006).

Acacia confusa Merr. (Leguminosae), a native species to Taiwan, is widely distributed on the hills and lowlands of Taiwan, and has been traditionally used as a medicine (Wu et al., 2005). An aqueous extract of *A. confusa* leaves was used in Taiwan for wound healing and anti-blood-stasis (Kan, 1978). The crude extracts of *A. confusa* bark contain a wide variety of phenolic compounds (Chang et al., 2001; Tung et al., 2007, 2009). Recent studies have shown that some phenolic compounds can prevent CCl_4-induced hepatic dam-age, and the ability of phenolic compounds might be related to their antioxidant properties (Hung et al., 2006; Jadon et al., 2007). Thus, the objective of this study was performed to evaluate

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the potential protective effects of ethanolic crude extract and its active compound, gallic acid, from *A. confusa* bark in CCl₄-induced chronic liver injury in Sprague Dawley rats.

2. Materials and methods

2.1. Preparation of A. confusa bark extract (ACBE)

The bark of *A. confusa* was sampled from the experimental forest of National Taiwan University in Nan-Tou County. The species was identified by Sheng-You Lu of the Taiwan Forestry Research Institute, and a voucher specimen (AC001) was deposited at the School of Forestry and Resource Conservation, National Taiwan University. The materials were air dried at room temperature (25 °C). The dried samples were cut into small pieces and soaked in 70% ethanol at room temperature for 7 days. The extract was filtered through Whatman No. 2 filter paper, and the filtrate was concentrated in a rotary evaporator and then lyophilized. Gallic acid (3,4,5-trihydroxybenzoic acid) content in crude extracts isolated and identified from the EtOAc fraction of *A. confusa* bark ranged from 31.6 to 34.7 µg per gram of crude extract.

2.2. Treatment of animals

A total of 42 male Sprague Dawley rats (body weight 220-230 g) were obtained from the Laboratory Animal Center of National Taiwan University of College and Medicine. The rats were housed in individual cages with free access to water and standard powdered diet (Lab5001 powder from USA PMI) at a temperature- and humidity-regulated environment (22 ± 2 °C, $65 \pm 5\%$ RH) with 12 h dark/light cycle. Experiments were started after the animals were allowed to adapt to the individual stainless-steel cages for one week. Regarding to the dosage used in the experiment, it is well-known that protocatechuic acid (3,4-dihydroxyl benzoic acid), the analog of gallic acid, exhibited an excellent protection activity against CCl₄-induced hepatic damage in rats at a dose of 100 mg/kg of bw (Hung et al., 2006). However, gallic acid has better antioxidant activity than protocatechuic acid by various *in vitro* assays (Tung et al., 2007, 2009). Thus, in this study, the major bioactive phytocompound of ethanolic extract from *A. confusa* bark, i.e. gallic acid, was tested at a relatively low dose of 50 mg/kg of bw. For the examination of both aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in the plasma, 42 rats were randomly assigned to seven groups ($n = 6$): olive oil (control group), CCl₄ injection (CCl₄ group), dietary supplementation with silymarin at a dose of 50 mg/kg plus CCl₄ injection (silymarin + CCl₄ group), dietary supplementation with ACBE at a relatively low dose of 50 mg/kg plus CCl₄ injection (L-ACBE + CCl₄ group), dietary supplementation with ACBE at a relatively high dose of 250 mg/kg plus CCl₄ injection (H-ACBE + CCl₄ group), dietary supplementation with ACBE at a relatively high dose of 250 mg/kg (H-ACBE group), dietary supplementation with gallic acid at a dose of 50 mg/kg plus CCl₄ injection (GA + CCl₄ group). Rats were fed powdered diet admixed with silymarin, ACBE or gallic acid combination for two weeks prior to CCl₄ injection and throughout the experiment. Silymarin has been used in the treatment and prevention of liver disease in clinic (Chang et al., 2009; Kuriakose and Kurup, 2008; Gowri Shankar et al., 2008). Thus, in this study, it was used as a positive control. During the 8-week experimental period, the amounts of silymarin, ACBE and gallic acid were administered according to weekly body weight. CCl₄-induced hepatotoxic rats were subcutaneously injected with 0.75 mL/kg bw of 40% CCl₄ dissolved in olive oil once a week, while rats in control group were injected with olive oil only.

Blood samples were collected from the tail vein into heparin-containing tubes and plasma samples were obtained by centrifuging at 1200g for 15 min. After removing plasma, erythrocytes were obtained by washing two times with ice-cold physiological saline. At the end of the experiment, each rat was to be anesthetized by ethyl ether, the liver was immediately perfused with ice-cold normal saline, then carefully removed, rinsed in ice-cold normal saline, blotted dry and weighed. All samples were stored at −80 °C for assay.

2.3. Pathological histology

Liver tissue was fixed in 10% buffered formaldehyde and processed for histological examination according to three kinds of histopathological stain including hematoxylin and eosin (H&E), Masson, and silver stains.

2.4. Western blot analysis of hepatic microsomal CYP2E1 protein

Western blot analysis of hepatic microsomal CYP2E1 protein was measured according to the method of Huang et al. (2005). Liver tissues were homogenized in 10 volumes of ice-cold buffer (0.25 mol/L sucrose, 10 mmol/L Tris–HCl, and 0.25 mmol/L phenylmethylsulfonyl fluoride, pH 7.4). The homogenates were centrifuged at 17,000g for 20 min at 4 °C. Then the supernatant was centrifuged at 105,000g for 60 min at 4 °C to separate the microsomes. The microsomal pellet was dissolved in 50 mmol/L potassium phosphate buffer containing 1 mmol/L EDTA and 1 mmol/L DTT (pH 7.4). Then, 30 µL of microsomal protein was separated by

10% SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes. Membranes were incubated in blocking solution (50 g/L non-fat milk in PBS with 1 mL/L Tween 20). These blocking steps were performed at room temperature for 2 h. Membranes were incubated with primary mouse anti-rat CYP2E1 monoclonal antibody (Cat. No.: PM32, Oxford Biomedica Inc., San Diego, CA) or mouse anti-actin monoclonal antibody (Cat. No.: MAB1501, Chemicon International Inc., Temecula, CA) for 2 h. After washing, membranes were incubated with a goat anti-mouse IgG peroxidase-conjugated secondary antibody directed against primary antibody. Membranes were developed by an enhanced chemiluminescence western blot detection system.

2.5. Preparation of liver homogenate and determination of protein content

First, 1 g of liver tissue was added with 4 mL of 4 °C buffer (0.25 mM sucrose, 10 mM Tris–HCl, and 0.25 mM phenylmethylsulfonyl fluoride, pH 7.4). The Polytron homogenizer (Glas-Col, Terre Haute, IN, USA) was utilized to homogenate the tissue. The homogenate solution was centrifuged at 10,000g for 20 min at 4 °C. The upper layer was collected and stored at −80 °C for assay. In order to express the antioxidant enzyme activities per gram of protein, total protein concentration of liver tissues was determined colorimetrically using a Bio-Rad DC protein assay kit (Cat. No.: 500-0116; Bio-Rad Laboratories, Hercules, CA, USA).

2.6. Determination of biochemical parameters of plasma

The enzymatic activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), triacylglycerol (TC), and total cholesterol (TC) in the plasma were measured colorimetrically with an automatic analyzer (Beckman-Coulter Synchron LX20 analyzer; Beckman, Fullerton, CA).

2.7. Assay of antioxidant enzymes activities in erythrocytes and liver tissues

2.7.1. Glutathione peroxidase (GPX) activity

GPX activity of erythrocytes and liver tissues was determined with a commercial kit (RS 504; Randox Laboratories, Antrim, UK). First, 20 µL of the diluted sample were added to 1 mL of mixed substrate (4 mmol/L GSH, 0.5 U/L GRD, and 0.34 mmol/L NADPH dissolved in 50 mmol/L phosphate buffer, pH 7.2, 4.3 mmol/L EDTA). Then, 40 µL of cumene hydroperoxide (diluted in deionized water) were added to the mixture. The reaction mixture was incubated at 37 °C, and the absorbance at 340 nm was determined every 1 min up to 3 min using the UV–vis spectrophotometer.

2.7.2. Glutathione reductase (GRD) activity

GRD activity of erythrocytes and liver tissues was measured with a commercial kit (Calbiochem 359962; Calbiochem-Novabiochem). First, 200 µL of the diluted sample and 400 µL of 2.4 mmol/L GSSG buffer (dissolved in 125 mmol/L potassium phosphate buffer, pH 7.5, 2.5 mmol/L EDTA) were added to 400 µL of 0.55 mmol/L NADPH (dissolved in deionized water). The absorbance was measured at 340 nm every 1 min up to 5 min using the UV–vis spectrophotometer.

2.7.3. Superoxide dismutase (SOD) activity

SOD activity of erythrocytes and liver tissues was measured with a commercial kit (SD 125; Randox Laboratories). First, 50 µL of the diluted sample and 1.7 mL of the mixed substrate (50 µmol/L xanthine and 25 µmol/L 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride, INT) were added to 250 µL of xanthin oxidase. The reaction mixture was incubated at 37 °C, and the absorbance was measured at 340 nm every 1 min up to 3 min using the UV–vis spectrophotometer.

2.7.4. Catalase (CAT) activity

CAT activity of erythrocytes and liver tissues was determined according to the method reported by Beers and Sizer (1952). First, 100 µL of the diluted sample and 1 mL of 59 mmol/L H₂O₂ (dissolved in 50 mmol/L potassium phosphate buffer, pH 7.0) were added to 1.9 mL of deionized water. The absorbance was measured at 240 nm every 1 min up to 3 min using the UV–vis spectrophotometer.

2.8. Determination of GSH/GSSG in erythrocytes and liver tissues

2.8.1. Total glutathione (GSH) concentration

The concentration of reduced GSH in erythrocytes and liver tissues was carried out according to the method of Tietze (1969), using GSH (0–100 µM) as the standard. The diluted sample solution or standard (10 µL) was mixed with 95 µL of the reagent (2 U/mL glutathione reductase, 200 µM NADPH, and 2 mM EDTA in 50 mM phosphate buffer, pH 7.2), followed by the addition of 100 µL of the reagent (10 mM DTNB in 50 mM phosphate buffer, pH 7.2). The reaction mixture was then incubated at room temperature, and the absorbance at 405 nm was determined every 1 min up to 5 min using the ELISA reader (Labsystem Multiskan RC, Finland). The concentration was expressed as GSH (µM) in erythrocytes and liver tissues.

2.8.2. Oxidized glutathione (GSSG) concentration

The concentration of reduced GSSG in erythrocytes and liver tissues was measured according to the method of Tietze (1969), using GSSG (0–100 μM) as the standard. The diluted sample solution or standard (70 μL) was mixed with 4 μL of 1-methyl-2-vinylpyridinium trifluoromethanesulfonate (M2VP). The mixture was kept for 1 h. The reaction mixture (10 μL) was mixed with 95 μL of the reagent (2 U/mL glutathione reductase, 200 μM NADPH, and 2 mM EDTA in 50 mM phosphate buffer, pH 7.2), followed by the addition of 100 μL of the reagent (10 mM DTNB in 50 mM phosphate buffer, pH 7.2). The reaction mixture was then incubated at room temperature, and the absorbance at 405 nm was determined every 1 min up to 5 min using the ELISA reader (Labsystem Multiskan RC, Finland). The concentration was expressed as GSSG (μM) in erythrocytes and liver tissues.

2.8.3. Ratio of GSH and GSSG

The GSH/GSSH is then calculated by dividing the difference between the total GSH and GSSG concentrations (reduced GSH). GSH/GSSG = (total GSH − 2GSSG)/ GSSG.

2.9. Measurement of lipid peroxidation in plasma and liver tissues

The quantitative measurement of lipid peroxidation was analyzed by measuring the concentration of thiobarbituric acid reactive substances (TBARS) in plasma and liver tissues using the method of Ohkawa et al. (1979) with minor modifications. 20 μL sample (plasma or liver homogenates) or various levels of TMP (1,1,3,3-tetra-methoxypropane; as a standard) were shaken with 800 μL of 0.22% H_2SO_4 in a 2 mL centrifuge tube. 100 μL of 10% Phosphotungstic acid and 200 μL of 0.67% TBA (in H_2O: galacical acetic acid = 1:1, v/v) were added to the mixture, shaken, and warmed for 60 min in a boiling water bath followed by rapid cooling. Then it was shaken into a 600 μL of n-butyl-alcohol layer in a separation tube and MDA content in the plasma or liver homogenates were determined fluorometrically (excitation and emission wavelengths: 531 nm vs. 590 nm) using a Wallac Victor-2 1420 Multilabel Counter (Perkin–Elmer). The level of lipid peroxides was expressed as nmols MDA per g tissue (wet wt).

2.10. Statistical analysis

All results are expressed as mean ± SEM (n = 6). The significance of difference was calculated by Scheffe's test, and results with $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. Pathological histology

Fig. 1 shows that CCl_4-induced liver injury causes fatty degeneration of hepatic cells and vacuole formation in the central vein. Table 1 summarizes the data of liver damage induced by CCl_4 in pathological histology. The level of vacuole formation and inflammation were significant after chronic CCl_4 treatment. Treatments using low dose of silymarin and gallic acid, and high dose of ACBE reduce the injury score of vacuole formation and inflammation. Histological examination showed a preventive effect of ACBE and gallic acid on CCl_4-induced hepatoxicity.

3.2. Effect of A. confusa bark extract on plasma biochemical parameters of liver function

Serum aminotransferase activities have long been considered effective indicators of hepatic injury. The protective effects of ACBE and gallic acid on CCl_4-induced reduction of plasma AST and ALT activities are presented in Table 2. The plasma AST and ALT activities of CCl_4 group were dramatically elevated to 210 and 214 U/L, while these values were 68 and 38 U/L, respectively in the control group. However, the groups treated with 50 mg/kg of silymarin showed significant decrease in elevation of plasma AST and ALT activities significantly ($p < 0.05$), with the values of 116 and 102 U/L, respectively. In addition, there were no significant differences among the groups treated with 50 mg/kg of silymarin, 50 mg/kg of ACBE, and 250 mg/kg of ACBE. Furthermore, treatment with 50 mg/kg of gallic acid, an active compound of A. confusa bark, decreased significantly the elevation of plasma AST and ALT activ-

ities compared with those administered with 50 mg/kg of silymarin ($p < 0.05$).

3.3. Effect of A. confusa bark extract on antioxidant enzymes activities in erythrocytes

The activities of SOD, GRD, GPX, and CAT in erythrocytes of CCl_4-induced rats are shown in Table 3. In erythrocytes, CCl_4 treatment caused reduction of SOD (19 U/mg), GPX (2.3 U/mg), and CAT (852 U/mg) activities when compared with those in the control group, showing values of 49, 3.0 and 1381 U/mg, respectively ($p < 0.05$). Enhancement of SOD activity was observed in the groups treated with 50 mg/kg of silymarin (68 U/mg), 50 mg/kg of ACBE (61 U/mg), 250 mg/kg of ACBE (54 U/mg), and 50 mg/kg of gallic acid (97 U/mg) compared with those administered with CCl_4 alone ($p < 0.05$). However, except for treatment with gallic acid, no significant differences in GRD activity of erythrocytes were observed among the groups. Additionally, the groups treated with 50 mg/kg of silymarin (3.1 U/mg), 250 mg/kg of ACBE (3.0 U/mg), and 50 mg/kg of gallic acid (4.9 U/mg) were found to have significant increase in GPX activity when compared with the CCl_4 group ($p < 0.05$). Enhancement of CAT activity was observed in the group treated with 50 mg/kg of gallic acid (1939 U/mg) compared with that of the CCl_4 group ($p < 0.05$). Furthermore, treatment with 50 mg/kg of gallic acid increased significantly SOD, GRD, and CAT activities in erythrocytes compared with treatment with 50 mg/ kg silymarin ($p < 0.05$). On the other hand, the silymarin treatment showed enhanced induction of SOD and GPX compared to the CCl_4 control. However, there was no statistically difference in CAT although data showed the elevated trend. These results are similar to those reported in the previous study by Rastogi et al. (2001) in alfatoxin B1 induced-hepatotoxic rats. These findings may also be related with the study of Sonnenbichler et al. (1976) who reported that transcription in the livers of rats and mice is accelerated under the influence of silymarin. Furthermore, Machicao and Sonnenbichler (1997) demonstrated that in vitro experiments with isolated cell nuclei and nucleoli the enzymatic activity of DNA-dependent RNA polymerase I is stimulated by silymarin leading to an increase in the rate of protein synthesis.

3.4. Effect of A. confusa bark extract on antioxidant enzymes activities in liver tissues

The activities of SOD, GRD, GPX, and CAT in liver tissues of CCl_4-induced rats are shown in Table 4. Rats in the CCl_4 group showed significant reduction in the hepatic GRD (0.49 U/g) and GPX (0.21 U/g) activities when compared with the control group, showing values of 0.53 and 0.33 U/g ($p < 0.05$), respectively. On the other hand, hepatic GRD and GPX activities of the groups treated with 50 mg/kg of ACBE, 250 mg/kg of ACBE, and 50 mg/kg of gallic acid, showing values of 0.58/0.28, 0.60/0.32 and 0.54/0.32 U/g, respectively, were significantly increased compared with those in the CCl_4 group ($p < 0.05$). However, the hepatic GPX activity of the silymarin group showed no significant difference when compared with the CCl_4 group. No significant differences in hepatic CAT activity were observed between the control group and the CCl_4 group. However, the groups treated with 250 mg/kg of ACBE and 50 mg/ kg of gallic acid showed significant increase in hepatic CAT activity when compared with those administered with CCl_4 only ($p < 0.05$).

3.5. Effect of A. confusa bark extract on GSH/GSSG

In states of oxidative stress, GSH is converted into GSSG and becomes depleted, leading to lipid peroxidation. Therefore, using GSH/GSSG as a marker for the evaluation of oxidative stress is an effective method (Reckengel et al., 1991). Tables 5 and 6 show
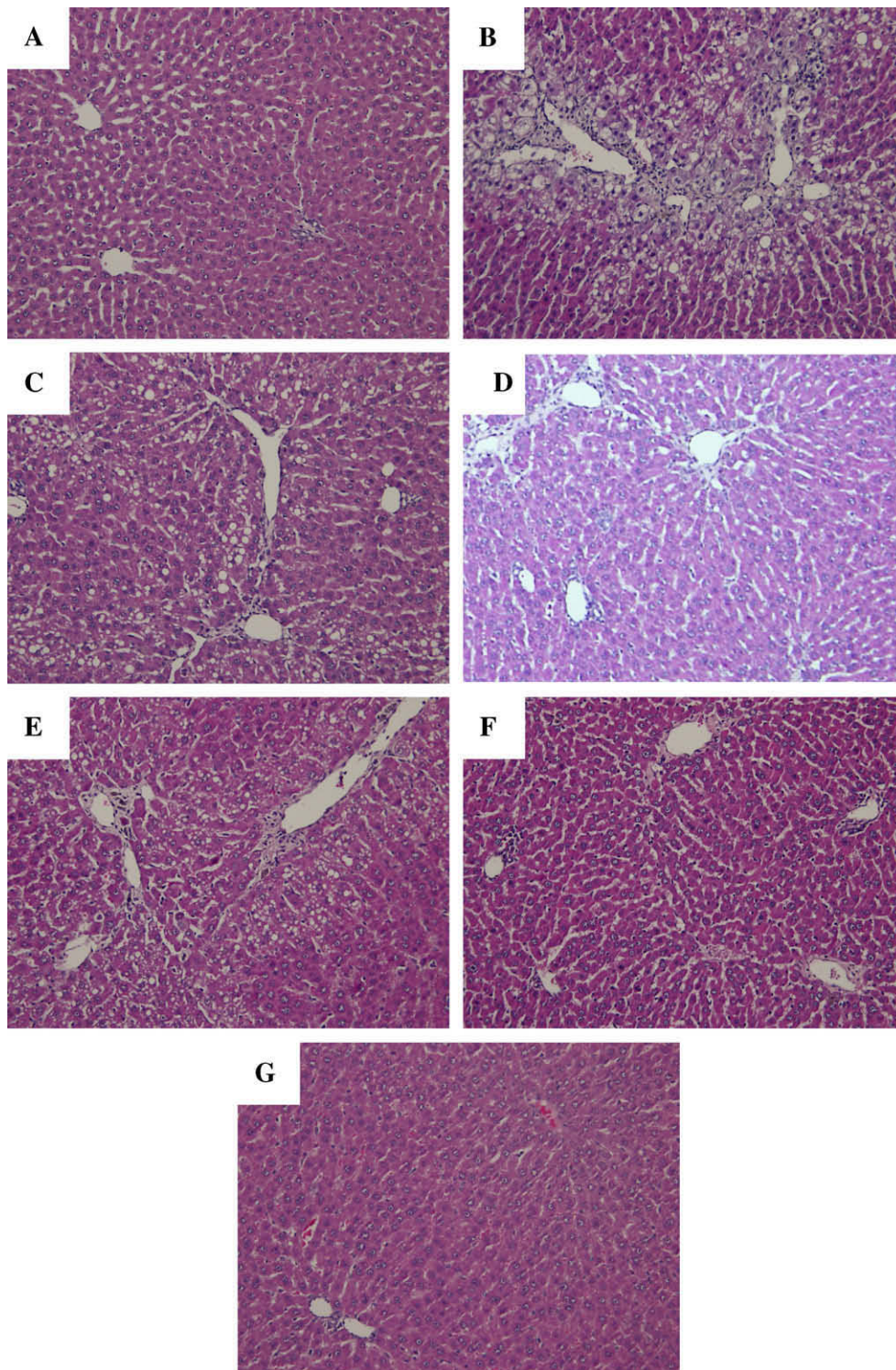
Fig. 1. Effects of *A. confusa* bark extract and gallic acid on CCl_4-induced hepatotoxicity in rats. (A) control group; (B) animals treated with 40% CCl_4 (0.75 mL/kg of bw) showed severe fatty degeneration and vacuole formation; (C) animals treated with silymarin (CCl_4 injection combined the supplementation of silymarin, 50 mg/kg of bw/day); (D) animals treated with low dose of crude extract (CCl_4 injection combined with supplementation of crude extract at a low dose, 50 mg/kg of bw/day); (E) animals treated with high dose of crude extract group (CCl_4 injection combined with the supplementation of crude extract at a high dose, 250 mg/kg of bw/day); (F) animals treated with gallic acid (CCl_4 injection combined the supplementation of gallic acid, 50 mg/kg of bw/day) and (G) animals only treated with crude extract (olive oil injection combined with the supplementation of crude extract at a high dose, 250 mg/kg of bw/day).

the effects of ACBE on GSH/GSSG of CCl_4-induced hepatotoxicity in rats. The administration of 250 mg/kg of ACBE and 50 mg/kg of gallic acid to rats resulted in an elevation of GSH/GSSG in erythrocytes, showing values of 82 and 107, respectively. In comparison with the CCl_4 group (27), erythrocyte GSH/GSSG was significantly increased in the high-dose group (82) but not in the low-dose group (35). Furthermore, GSH/GSSG in erythrocytes of the group treated with 50 mg/kg of gallic acid (107) increased significantly when compared with those treated with 50 mg/kg of silymarin (57) ($p < 0.05$). Interestingly, supplementation of gallic acid could

Table 1
Effect of A. confusa bark extract and gallic acid on hepatic histopathology scores of liver damage in rat treated with CCl_4.

Treatments[*]	Injury of scores[**]			
	Fatty change (Vacuole formation)		Inflammation	Necrosis
	Small	Big		
Normal control	0.00 ± 0.00^e	0.00 ± 0.00^b		
CCl_4 control	1.92 ± 0.08^a	0.75 ± 0.11^a	0.58 ± 0.08^a	0.08 ± 0.08^a
Silymarin + CCl_4	$0.83 \pm 0.21^{c,d}$	0.33 ± 0.17^b	$0.50 \pm 0.00^{a,b}$	0.17 ± 0.11^a
L-ACBE + CCl_4	$1.42 \pm 0.33^{a,b}$	0.00 ± 0.00^b	0.33 ± 0.11^b	0.08 ± 0.08^a
H-ACBE + CCl_4	$1.08 \pm 0.24^{b,c}$	0.00 ± 0.00^b	0.42 ± 0.08^b	0.00 ± 0.00^a
H-ACBE	$0.08 \pm 0.08^{d,e}$	0.00 ± 0.00^b	0.25 ± 0.11^b	0.00 ± 0.00^a
GA + CCl_4	0.42 ± 0.24^e	0.00 ± 0.00^b	0.25 ± 0.11^b	0.00 ± 0.00^a

[*] Seven groups were designed as follows: olive oil (control group), CCl_4 injection (CCl_4 group), dietary supplementation with silymarin at a dose of 50 mg/kg plus CCl_4 injection (silymarin group), dietary supplementation with A. confusa bark extract at a relatively low dose of 50 mg/kg plus CCl_4 injection (L-ACBE + CCl_4 group), dietary supplementation with A. confusa bark extract at a relatively high dose of 250 mg/kg plus CCl_4 injection (H-ACBE + CCl_4 group), dietary supplementation with A. confusa bark extract at a relatively high dose of 250 mg/kg (H-ACBE group), dietary supplementation with gallic acid at a dose of 50 mg/kg plus CCl_4 injection (GA + CCl_4 group). Each value represents the mean ± SEM of six rats.
[**] Different letters are significantly different at the level of $p < 0.05$ according to the Scheffe's test.

Table 2
Effect of A. confusa bark extract and gallic acid on plasma AST and ALT activities of CCl_4-induced hepatotoxicity in rats.

Treatment [*]	AST (U/L)[**]	ALT (U/L)
Normal control	68 ± 2^c	38 ± 1^c
CCl_4 control	210 ± 29^a	214 ± 29^a
Silymarin + CCl_4	116 ± 12^b	102 ± 11^b
L-ACBE + CCl_4	$84 \pm 8^{b,c}$	$71 \pm 9^{b,c}$
H-ACBE + CCl_4	$83 \pm 7^{b,c}$	$65 \pm 11^{b,c}$
H-ACBE	61 ± 2^c	62 ± 5^c
GA + CCl_4	77 ± 4^c	37 ± 1^c

[*] Each value represents the mean ± SEM of six rats.
[**] Different letters are significantly different at the level of $p < 0.05$ according to the Scheffe's test.

Table 3
Effect of A. confusa bark extract and gallic acid on antioxidant enzymes activities in the erythrocytes of CCl_4-induced hepatotoxicity in rats.

Treatment[*]	SOD (U/mg)[**]	GRD (U/g)	GPX (U/mg)	CAT (U/mg)
Normal control	49 ± 6^b	2.5 ± 0.2^b	3.0 ± 0.3^a	$1381 \pm 199^{b,c}$
CCl_4 control	19 ± 4^c	2.1 ± 0.1^b	2.3 ± 0.2^b	852 ± 16^d
Silymarin + CCl_4	68 ± 9^b	2.1 ± 0.2^b	3.1 ± 0.2^a	$1337 \pm 172^{b,c,d}$
L-ACBE + CCl_4	61 ± 4^b	2.6 ± 0.4^b	2.0 ± 0.2^b	$1100 \pm 120^{b,c,d}$
H-ACBE + CCl_4	54 ± 8^b	$3.1 \pm 0.4^{a,b}$	3.0 ± 0.3^a	$1224 \pm 63^{c,d}$
H-ACBE	46 ± 6^b	2.8 ± 0.2^b	3.2 ± 0.3^a	$1674 \pm 298^{a,b}$
GA + CCl_4	97 ± 5^a	5.1 ± 0.9^a	4.9 ± 0.9^a	1939 ± 222^a

[*] Each value represents the mean ± SEM of six rats.
[**] Different letters are significantly different at the level of $p < 0.05$ according to the Scheffe's test.

protect GSH/GSSG depletion induced by CCl_4 both in both erythrocytes and liver tissues.

3.6. Effect of A. confusa bark extract on CCl_4-induced lipid peroxidation levels

Tables 5 and 6 show the formation of TBARS in the plasma and liver tissues of rats treated with ACBE. Malondialdehyde (MDA) is the product of lipid peroxidation and is a common marker of lipid peroxidation. The content of TBARS in liver tissues showed no differences among groups (Table 6). However, the content of TBARS

Table 4
Effect of A. confusa bark extract and gallic acid on antioxidant enzymes activities in the liver tissues of CCl_4-induced hepatotoxicity in rats.

Treatment[*]	SOD (U/g)[**]	GRD (U/g)	GPX (U/g)	CAT (U/g)
Normal control	5.3 ± 0.3^b	$0.53 \pm 0.00^{b,c}$	0.33 ± 0.01^a	$55 \pm 11^{a,b}$
CCl_4 control	6.9 ± 0.3^a	0.49 ± 0.02^d	0.21 ± 0.00^d	48 ± 1^b
Silymarin + CCl_4	6.5 ± 0.2^a	$0.56 \pm 0.01^{a,b}$	$0.27 \pm 0.00^{c,d}$	57 ± 3^b
L-ACBE + CCl_4	6.9 ± 0.4^a	$0.58 \pm 0.01^{a,b}$	$0.28 \pm 0.01^{b,c}$	55 ± 2^b
H-ACBE + CCl_4	6.8 ± 0.4^a	0.60 ± 0.02^a	$0.32 \pm 0.01^{a,b}$	72 ± 11^a
H-ACBE	5.4 ± 0.1^b	$0.54 \pm 0.00^{b,c}$	$0.32 \pm 0.01^{a,b}$	53 ± 7^b
GA + CCl_4	5.6 ± 0.3^b	$0.54 \pm 0.02^{b,c}$	$0.32 \pm 0.01^{a,b}$	79 ± 12^a

[*] Each value represents the mean ± SEM of six rats.
[**] Different letters are significantly different at the level of $p < 0.05$ according to the Scheffe's test.

Table 5
Effect of A. confusa bark extract and gallic acid on TBARS concentration in the plasma and GSH:GSSG ratio in the erythrocytes of CCl_4-induced hepatotoxicity in rats.

Treatment[*]	TBARS (MDA, μM)[**]	GSH/GSSG
Normal control	4.5 ± 0.2^b	29 ± 4^c
CCl_4 control	6.2 ± 0.1^a	27 ± 3^c
Silymarin + CCl_4	$4.1 \pm 0.1^{b,c}$	$57 \pm 9^{b,c}$
L-ACBE + CCl_4	3.8 ± 0.1^c	$35 \pm 6^{b,c}$
H-ACBE + CCl_4	3.8 ± 0.1^c	$82 \pm 25^{a,b}$
H-ACBE	4.2 ± 0.1^b	$85 \pm 20^{a,b}$
GA + CCl_4	3.3 ± 0.2^d	107 ± 31^a

[*] Each value represents the mean ± SEM of six rats.
[**] Different letters are significantly different at the level of $p < 0.05$ according to the Scheffe's test.

Table 6
Effect of A. confusa bark extract and gallic acid on TBARS concentration and GSH:GSSG ratio in the liver tissues of CCl_4-induced hepatotoxicity in rats.

Treatment[*]	TBARS (MDA, nmol/g liver)[**]	GSH/GSSG
Normal control	$43 \pm 4^{a,b}$	20 ± 1^b
CCl_4 control	57 ± 5^a	15 ± 2^b
Silymarin + CCl_4	$42 \pm 5^{a,b}$	$41 \pm 14^{a,b}$
L-ACBE + CCl_4	$48 \pm 7^{a,b}$	$65 \pm 16^{a,b}$
H-ACBE + CCl_4	$51 \pm 8^{a,b}$	$65 \pm 11^{a,b}$
H-ACBE	39 ± 4^b	$61 \pm 1^{a,b}$
GA + CCl_4	39 ± 6^b	93 ± 43^a

[*] Each value represents the mean ± SEM of six rats.
[**] Different letters are significantly different at the level of $p < 0.05$ according to the Scheffe's test.

was significantly increased in the plasma of CCl_4-treated rats (6.2 μM) as compared with that of the control group (4.5 μM). Moreover, rats treated with 50 mg/kg of silymarin (4.1 μM), 50 mg/kg of ACBE (3.8 μM), 250 mg/kg of ACBE (3.8 μM), and 50 mg/kg of gallic acid (3.3 μM) showed significantly reduced content of TBARS in the plasma ($p < 0.05$) (Table 5). In addition, the concentrations of triacylglycerol and total cholesterol of plasma are shown in Fig. 2. The data show that there is no difference between vehicle control group and CCl_4-treated group. Therefore, we considered the changes of plasma lipid levels to be not influenced on the TBARS values by using the methods in our study. Consequently, these results suggest that oxidative stress induced by CCl_4 was blocked by the supplementation of ACBE and gallic acid.

3.7. Effect of A. confusa bark extract on hepatic CYP2E1 expression

Fig. 3 shows a representative western blot of CYP2E1 levels in liver tissues. Rats in the CCl_4 group with the value of 177%, showed significantly increased hepatic CYP2E1 levels relative to those observed in the control group (100%) ($p < 0.05$). However, the groups
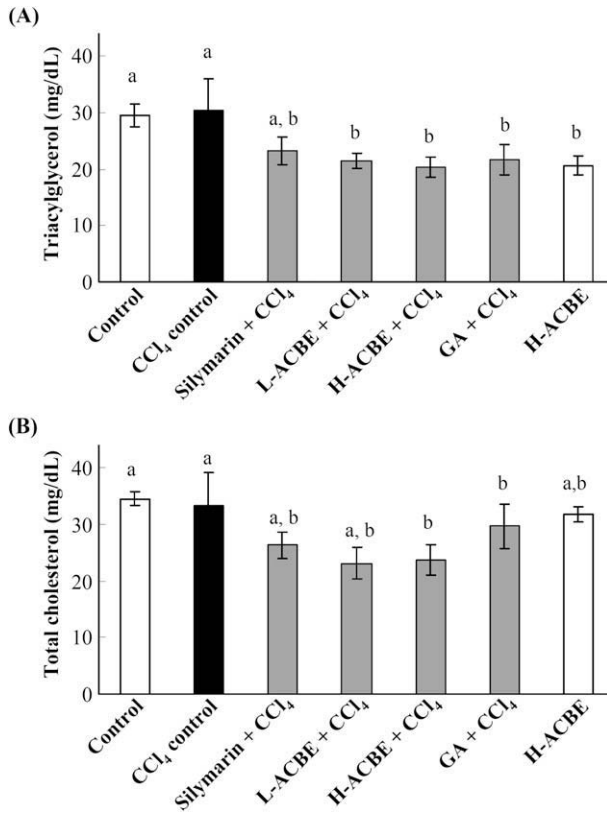
(A)

(B)

Fig. 2. Effects of *A. confusa* bark extract and gallic acid on triacylglycerol and total cholesterol concentrations of plasma in CCl$_4$-induced hepatotoxicity in rats. Results are mean ± SEM of six rats. The bars marked by different letters are significantly different at the level of $p < 0.05$ according to the Scheffe's test.

treated with 50 mg/kg of silymarin (43%), 50 mg/kg of ACBE (16%), 250 mg/kg of ACBE (26%), and 50 mg/kg of gallic acid (45%) had significantly decreased hepatic CYP2E1 levels relative to those observed in the CCl$_4$ group (177%) ($p < 0.05$).

4. Discussion

Earlier studies have shown that gallic acid, an active component of *A. confusa* bark, possesses potent antioxidant property, including DPPH free radical scavenging activity, ABTS free radical scavenging activity, superoxide free radical scavenging activity, and reducing power (Tung et al., 2007, 2009). The present study showed for the first time that ACBE and gallic acid possess chronic hepatoprotective effect as evidenced by the significant reduction in plasma AST and ALT activities and increase in antioxidant enzymes activities induced by CCl$_4$.

Carbon tetrachloride (CCl$_4$), a well-known model compound for producing chemical hepatic injury, requires biotransformation by hepatic microsomal CYP to produce toxic metabolites, namely trichloromethyl free radicals (Brautbar and Williams, 2002). CYP2E1 is the major isozyme involved in bioactivation of CCl$_4$ and subsequent production of free radicals (Sugiyama et al., 2006). ·CCl$_3$ initiates lipid peroxidation of the membrane of the endoplasmic reticulum and causes a chain reaction. In addition, injury to liver tissues alters their transport function and membrane permeability, leading to leakage of enzymes from the cells. Therefore, the marked release of AST and ALT into the circulation indicates severe damage to hepatic tissue membranes due to CCl$_4$ intoxication (Ahn et al., 2007). In the present study, general indicators of CCl$_4$-induced hepatotoxicity such as histopathological lesions and liver-specific enzyme activities (e.g. AST and ALT) in the plasma were evident. Wills and Asha (2006) reported that silymarin exhibited an excellent hepatoprotection activity at a dose of 50 mg/kg of
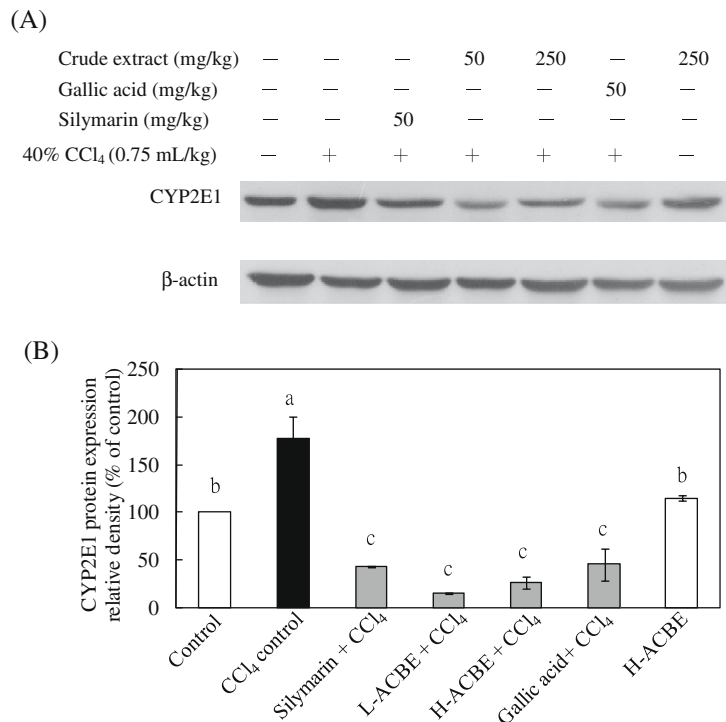
(A)

(B)

Fig. 3. Effects of *A. confusa* bark extract and gallic acid on Western blot of hepatic microsomal CYP2E1 in CCl$_4$-induced hepatotoxicity in rats. (A) The indicated apparent molecular masses of CYP2E1 and β-actin (internal control) were 54 and 43 kDa, respectively. (B) Results are mean ± SEM of six rats. The bars marked by different letters are significantly different at the level of $p < 0.05$ according to the Scheffe's test.

bw. In order to compare with silymarin, we select ACBE and gallic acid to test at a dose of 50 mg/kg of bw in this study. Additionally, the performance of hepatoprotection at a relative high dose of ACBE (250 mg/kg of bw) is also concerned. Accordingly, the results revealed that rats fed with 50 mg/kg of silymarin, 50 mg/kg of ACBE, 250 mg/kg of ACBE, and 50 mg/kg of gallic acid had significantly lowered plasma AST and ALT activities as compared to those administered with CCl_4 only ($p < 0.05$). The results are in agreement with those obtained by Jadon et al. (2007) who showed that gallic acid, at 50 mg/kg body weight, could decrease plasma AST and ALT activities elevated by acute hepatic damage. This evidenced that the administration of crude extract and gallic acid showed hepatoprotective effect under CCl_4-induced oxidative stress.

ACBE decreased lipid peroxides in the plasma, increased SOD activity, GPX activity and GSH/GSSG in erythrocytes, and increased GRD, GPX and CAT activities in liver tissues of CCl_4-induced rats ($p < 0.05$). Treatment with 250 mg/kg of ACBE increased significantly hepatic GPX and CAT activities compared with treatment with silymarin ($p < 0.05$). The histological changes induced by CCl_4 treatment were evidenced by centrilobular necrosis and bridging hepatic necrosis, and the groups treated with silymarin, ACBE and gallic acid had livers returned to normalcy. ACBE at a dose of 50 mg/kg, which was comparable with silymarin, showed excellent protection on CCl_4-induced liver damage. It was also found that ACBE at a dose of 250 mg/kg increased significantly GPX activities in erythrocytes and CAT activities in liver tissues when compared with ACBE at a dose of 50 mg/kg ($p < 0.05$). However, except for GPX activities in erythrocytes and CAT activities in liver tissues, there was no significant difference between doses of 50 and 250 mg/kg. Hence, in this present work, a dose-response study between doses of 50 and 250 mg/kg was unnecessary. On the other hand, in previous works, cinnamates and various derivatives of benzoic acid were identified from the EtOAc fraction of *A. confusa* (Tung et al., 2007, 2009). Phenolic compounds, which are widely distributed in plants, have been considered to play an important antioxidant role as dietary antioxidants for the prevention of oxidative damage in the living system (Hertog et al., 1993). Results revealed that administering ACBE decreased significantly CCl_4-induced hepatotoxicity in rats because of antioxidant activities of phenolic compounds.

Our previous study showed that phenolic acids were important antioxidant components in *A. confusa*, among which gallic acid had the highest antioxidant activities (Tung et al., 2007, 2009). Moreover, the antioxidant enzyme (SOD, GRD, GPX, and CAT) activities were increased by gallic acid in erythrocytes and liver tissues when compared with those administered with CCl_4 only ($p < 0.05$). The concentration of TBARS decreased significantly following gallic acid administration in the plasma and liver tissues ($p < 0.05$). These results clearly show that the antioxidant property of gallic acid reduces significantly the damage of CCl_4-induced liver injury and activates the biological defense system of the liver. Furthermore, gallic acid appears to scavenge free radicals (Tung et al., 2007), as shown by the inhibition of lipid peroxidation induced by CCl_4 *in vivo*. On the other hand, it has reported that CYP3A activity was reversibly inhibited by gallic acid in human hepatic microsomes *in vitro* (Stupans et al., 2002). Our results show that gallic acid significantly inhibited CCl_4-induced protein expression of CYP2E1 in rat livers, suggesting therapeutic potential for gallic acid in this experimental liver disorder. Further systematic studies on the inhibitory mechanisms of hepatic cytochrome P450 oxidases by gallic acid are warranted.

In conclusion, it is well-known that the hepatoprotective effect has a good correlation with the antioxidant activities (Hung et al., 2006; Jadon et al., 2007). In our previous researches, ACBE and gallic acid have been demonstrated to possess excellent antioxidant activities by various *in vitro* assays (Chang et al., 2001; Tung et al., 2007, 2009). In this current study, ACBE and gallic acid could reduce CCl_4-induced toxicity, particularly hepatotoxicity, by inhibiting lipid peroxidation and CYP2E1 activation, suppressing alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, and increasing antioxidant enzyme activity. Therefore, ACBE and gallic acid can be proposed to protect the liver against CCl_4-induced oxidative damage in rats, and the hepatoprotective effect might be correlated with its antioxidant and free radical scavenger effects.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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