*ORIGINAL ARTICLE*

**Basic Study**

# Caffeic acid phenethyl ester inhibits liver fibrosis in rats

Mei Li, Xiu-Fang Wang, Juan-Juan Shi, Ya-Ping Li, Ning Yang, Song Zhai, Shuang-Suo Dang

Mei Li, Xiu-Fang Wang, Juan-Juan Shi, Ya-Ping Li, Ning Yang, Song Zhai, Shuang-Suo Dang, Department of Infectious Diseases, the Second Affiliated Hospital of Medical School of Xi'an Jiaotong University, Xi'an 710004, Shannxi Province, China

Correspondence to: Shuang-Suo Dang, PhD, MD, Department of Infectious Diseases, the Second Affiliated Hospital of Medical School of Xi'an Jiaotong University, No. 157 Xi'wu Road, Xi'an 710004, Shaanxi Province, China. dang212@126.com
Telephone: +86-29-87679688
Fax: +86-29-87679688

## Abstract

**AIM:** To investigate the hepatoprotective effects and antioxidant activity of caffeic acid phenethyl ester (CAPE) in rats with liver fibrosis.

**METHODS:** A total of 75 male Sprague-Dawley rats were randomly assigned to seven experimental groups: a normal group ($n$ = 10), a vehicle group ($n$ = 10), a model group ($n$ = 15), a vitamin E group ($n$ = 10), and three CAPE groups (CAPE 3, 6 and 12 mg/kg, $n$ = 10, respectively). Liver fibrosis was induced in rats by injecting $CCl_4$ subcutaneously, feeding with high fat forage, and administering 30% alcohol orally for 10 wk. Concurrently, CAPE (3, 6 and 12 mg/kg) was intraperitoneally administered daily for 10 wk. After that, serum total bilirubin (TBil), aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured to assess hepatotoxicity. To investigate antioxidant activity of CAPE, malondialdehyde (MDA), glutathione (GSH) levels, catalase (CAT) and superoxide dismutase (SOD) activities in liver tissue were determined. Moreover, the effect of CAPE on α-smooth muscle actin (α-SMA), a characteristic hallmark of activated hepatic stellate cells (HSCs), and NF-E2-related factor 2 (Nrf2), a key transcription factor for antioxidant systems, was investigated by immunohistochemistry.

**RESULTS:** Compared to the model group, intraperitoneal administration of CAPE decreased TBil, ALT, and AST levels in liver fibrosis rats ($P$ < 0.05), while serum TBil was decreased by CAPE in a dose-dependent manner. In addition, the liver hydroxyproline contents in both the 6 and 12 mg/kg CAPE groups were markedly lower than that in the model group ($P$ < 0.05 and $P$ < 0.001, respectively). CAPE markedly decreased MDA levels and, in turn, increased GSH levels, as well as CAT and SOD activities in liver fibrosis rats compared to the model group ($P$ < 0.05). Moreover, CAPE effectively inhibited α-SMA expression while increasing Nrf2 expression compared to the model group ($P$ < 0.01).

**CONCLUSION:** The protective effects of CAPE against liver fibrosis may be due to its ability to suppress the activation of HSCs by inhibiting oxidative stress.

**Key words:** Caffeic acid phenethyl ester; Liver fibrosis; Oxidative stress; NF-E2-related factor 2; α-smooth

muscle actin

**Core tip:** It has been demonstrated that caffeic acid phenethyl ester (CAPE) has several biological and pharmacological properties. The aim of this study was to evaluate the hepatoprotective effects and antioxidant activity of CAPE in rats with liver fibrosis, as well as the underlying mechanism.

# INTRODUCTION

Caffeic acid phenethyl ester (CAPE), a flavonoid-like compound, is one of the major components of honeybee propolis. Honeybee propolis possesses a highly complex and variable chemical composition, and approximately 300 components have been identified[1,2]. CAPE has been widely accepted as a hepatoprotective folk medicine for many years. Its protective effects are attributed to its cytoprotective, anti-oxidant, anti-proliferative, and anti-inflammatory effects, and it has been shown to inhibit both lipoxygenase activity and lipid peroxidation[3-9]. CAPE can also inhibit phorbol ester-induced $H_2O_2$ production and tumor promotion[10,11], and it was reported that many CAPE activities were related to transcription factor inhibition of nuclear factor-kappa B (NF-κB)[10,12-14].

Liver fibrosis results from chronic damage to the liver in conjunction with the accumulation of extracellular matrix proteins, which is a characteristic of many chronic liver diseases[15]. Oxidative stress can cause excessive damage to hepatocytes through lipid peroxidation and protein alkylation. In addition, oxidative stress can liberate mediators and assist in the activation of hepatic stellate cells (HSCs), which are the predominant fibrogenic cell type producing collagen type I in the liver[16,17]. Therefore, oxidative stress has been recognized as a fundamental factor in the pathological process of liver fibrosis. NF-E2-related factor 2 (Nrf2) is a key transcription factor that regulates the induction of genes encoding antioxidant proteins and phase 2 detoxifying enzymes, which is involved in drug metabolism, detoxification and antioxidant defenses[18,19]. In nuclear protein extracts from normal and activated HSCs, Nrf1 and Nrf2 proteins were identified as part of the NAD(P)H quinine oxidoreductase 1 antioxidant response element (ARE) DNA/protein complex. Concomitant activation of the ARE defense and enhanced cell proliferation may confer protection against electrophile-mediated HSC toxicity during liver fibrosis[20]. All of these factors have led us to propose the following hypothesis: CAPE is more effective in inhibiting liver fibrosis and oxidative stress by activating Nrf2 expression compared to the effects of vitamin E (vit E), which is one of the well-known antioxidant agents.

# MATERIALS AND METHODS

## Chemicals

CAPE was obtained from Yuancheng Technology Co. (Wuhan, China; purity 98%). Vit E was obtained from Sigma Chemical Co. (St. Louis, MO). Serum total bilirubin (TBil), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were measured with an auto chemistry analyzer (Hitachi Co., Japan). Antibodies against α-smooth muscle actin (α-SMA) and Nrf2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and hydroxyproline detection kits, $CCl_4$, olive oil, and alcohol were also obtained from commercial sources.

## Animals and treatment

Seventy-five male Sprague-Dawley rats (254.50 ± 16.22 g) were obtained from Xi'an Jiaotong University Medical College. The rats were reared in a controlled environment at 21 ± 2 ℃ and 50% ± 5% relatively humidity under a 12-h dark/light cycle and were acclimatized for at least 1 wk prior to use. The experimental procedures were performed according to the animal care guidelines of the National Institutes of Health and performed under the Xi'an Jiaotong University Guidelines for Animal Experimentation.

The rats were randomly assigned to seven experimental groups as follows: a normal group that was given water and a standard chow diet ad libitum ($n = 10$); a vehicle group that was treated by intraperitoneal injection of 10% alcohol (vehicle for CAPE) and subcutaneous injection of olive oil (vehicle for $CCl_4$; $n = 10$); a model group (10% alcohol *via* the intraperitoneal route, once a day; $n = 15$); a vit E group (vit E dissolved in olive oil was administered intraperitoneally at 10 mg/kg, once daily; $n = 10$); and three CAPE groups (CAPE dissolved in 10% alcohol was administered intraperitoneally at 3, 6 or 12 mg/kg, once daily, respectively; $n = 10$). Except for the normal and vehicle groups, the other groups were injected with $CCl_4$ dissolved in 40% v/v olive oil at 1 mg/kg body weight (2 mg/kg for the first time) twice a week *via* the subcutaneous route, fed rich-fat forage, and given alcohol (30%) orally for 10 wk.

At the conclusion of the experiment, all rats were fasted for 12 h before sacrifice. Liver, kidneys and spleen were removed and weighed, and blood was collected for serum isolation. Liver tissue samples (0.5 g) were homogenized (1:10, w/v) in ice-cold normal

saline (NS). Homogenates were centrifuged at 5000 rpm for 10 min to remove debris. The supernatants, including the cytosolic fraction, were obtained and used for the analyses of select parameters. Protein concentrations of supernatants were measured by the Coomassie brilliant blue method[21].

### Determination of TBil, ALT and AST in serum
Serum TBil, ALT and AST levels were measured to assess hepatotoxicity. Briefly, blood samples were kept overnight in a 4 ℃ refrigerator before centrifugation at 3000 *g* for 10 min. Afterwards, serum samples were stored in a -80 ℃ freezer before analysis. TBil, ALT and AST in serum were evaluated by an autoanalyzer (Hitachi-7170, Hitachi, Japan).

### Determination of liver hydroxyproline
Liver samples were frozen at -20 ℃ before analysis. Hepatic collagen content was evaluated by hydro-xyproline (Hyp) quantification according to the method described and validated by Edwards and O'Brien[22] with some modifications. Briefly, liver tissues (75 to 100 mg) were homogenized in 1 mL of phosphate-buffered saline and hydrolyzed in 6 mol/L HCl overnight at 120 ℃. Next, 5 μL of hydrolysates was mixed with 5 μL of citrate acetate buffer, and then 100 μL of chloramine T solution was added. After 20 min of incubation at room temperature, 100 mL of Ehrlich's solution was added. The mixture was incubated for 15 min at 65 ℃, and absorbance was read at 550 nm. Recovery of known standard amounts was performed using similar liver samples to provide quantification. The hepatic hydroxyproline content is expressed as g/g wet liver weight.

### Determination of lipid peroxidation and GSH levels
Hepatic lipid peroxidation levels were determined by measuring MDA (a thiobarbituric acid reactive substance) levels[23]. Briefly, 10% hepatic homogenate samples were mixed with a thiobarbituric acid (TBA) reagent consisting of 0.375% TBA and 15% trichloroacetic acid in 0.25 mol/L hydrochloric acid. The reaction mixtures were placed in a boiling water bath for 40 min and then centrifuged at 4000 *g* for 5 min. Supernatant absorbance was measured at 535 nm. Results are expressed as nmol/mg protein. Hepatic GSH level was estimated by a colorimetric method using 5,5'-dithio bis-(2-nitrobenzoic acid) (DTNB)[24]. Samples were mixed with sodium phosphate buffer and DTNB. The mixture was incubated for 5 min at room temperature. Absorbance was measured at 405 nm. GSH content was determined from a standard curve generated with known concentrations of GSH. Results are expressed as μmol/mg protein.

### Determination of antioxidant enzymes
CAT and SOD assays were performed according to the manufacturer's instructions as previously described[25].

Briefly, CAT activity was defined as the enzyme concentration required to decompose 1 μmol $H_2O_2$ within 1 min. This reaction was initiated by addition of 1.0 mL of freshly prepared 20 mmol $H_2O_2$. The rate of $H_2O_2$ decomposition was measured spectrophotometrically at 240 nm for 1 min. To measure SOD activity, xanthine and xanthine oxidase were used to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyl tetrazolium chloride to form a red formazan dye. Absorbance was then measured at 550 nm. CAT and SOD activities are expressed as UI/mg protein.

### Histological examination
Fresh liver tissues that had been trimmed to a thickness of 3 mm were placed in plastic cassettes and immersed in neutral buffered formalin for 24 h. Fixed tissues were processed routinely, embedded in paraffin, sectioned, deparaffinized, and rehydrated using standard techniques. The extent of CCl4-induced necrosis was evaluated by assessing the morphological changes in liver sections stained with hematoxylin-eosin (HE) and van Gieson (VG). Fibrosis was scored semi-quantitatively as described by Zhang *et al*[26]. Accordingly, 0 was accepted as normal liver; 1, as thickened perivenular collagen and thin collagen septa; 2, as thin septa with incomplete bridging between portal regions; 3, as thin septa and extensive bridging; and 4, as thickened septa with complete bridging of portal regions and nodular appearance.

### Immunohistochemistry
Immunohistochemistry for α-SMA was performed to identify HSC activation. Nrf2 expression in liver tissue was confirmed by immunohistochemistry, and the density of positive cells was measured with Image-Pro plus 6.0 software. Integrated optical density of positive staining of α-SMA and Nrf2 in each photograph was measured, and the average of all photographs in each group was calculated[27].

Immunohistochemical staining was performed on formalin-fixed paraffin-embedded liver sections (thickness, 5 mm). Following dehydration through xylene and a graded series of alcohol, the sections were washed with phosphate buffered saline (PBS; pH 7.4) and treated with 0.3% $H_2O_2$ for 20 min to block endogenous peroxidase activity. Tissue antigen retrieval was then accomplished by microwave heating for 2 min. After three washes with PBS, 10% goat non-immune serum was applied to liver sections for 30 min to prevent non-specific antibody binding. The sections were incubated with a primary mouse antibody (α-SMA or Nrf2, 1:100) in a humidified chamber at 37 ℃ for 3 h. A biotinylated secondary antibody and streptavidin peroxidase complex (ZSGB-BIO, Histostain-Plus Kit, Cat No: 85-9043) were consecutively applied at 37 ℃ for 15 min each, and three PBS washes were performed between applications. Antibody binding
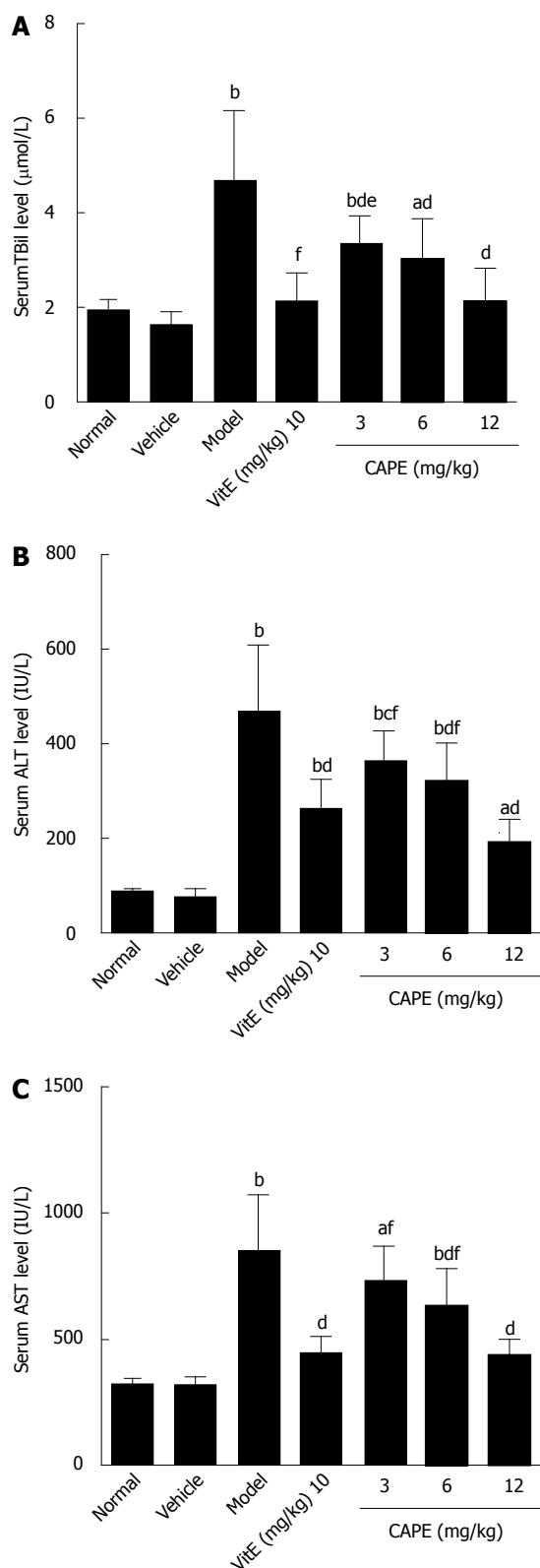
**A**



**B**



**C**



**Figure 1  Effects of caffeic acid phenethyl ester on serum TBil (A), ALT (B), and AST (C) levels in rats.** Bars and error bars represent mean ± SD, $t$ = 2.741-10.5, [a]$P$ < 0.05, [b]$P$ < 0.01 *vs* normal group, respectively; $t$ = 2.9-7.371, [c]$P$ < 0.05, [d]$P$ < 0.01 *vs* model group, respectively; $t$ = 3.225-5.229, [e]$P$ < 0.05, [f]$P$ < 0.01 *vs* CAPE (12 mg/kg) group, respectively. CAPE: Caffeic acid phenethyl ester; Vit E: Vitamin E.

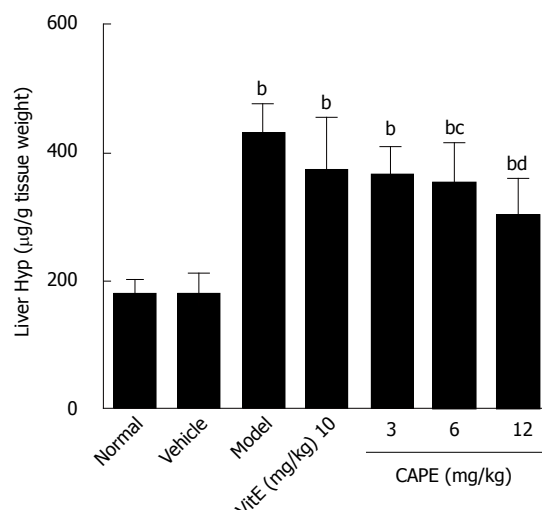was visualized by color development using 3, 3 diaminobenzidine/$H_2O_2$. Finally, the sections were



**Figure 2  Liver hydroxyproline contents in the groups.** Bars and error bars represent mean ± SD. $t$ = 4.959-10.38, [b]$P$ < 0.01 *vs* normal group; $t$ = 3.090-5.148, [c]$P$ < 0.05, [d]$P$ < 0.01 *vs* model group, respectively. CAPE: Caffeic acid phenethyl ester; Vit E: Vitamin E; Hyp: Hydroxyproline.

counter-stained with Mayer's hematoxylin and rinsed with tap water, and the slides were mounted and observed under a light microscope.

*Statistical analysis*
The GraphPad.prism version 5.0 software package was used for statistical analysis. Results of measurement data are expressed as mean ± SD and tested by analysis of variance followed by the Tukey-Kramer test. Results of ranked data were statistically analyzed by the nonparametric Kruskal-Wallis *H* test followed by the Wilcoxon test for paired comparisons. Differences were considered significant if the *P*-value was less than 0.05.

# RESULTS

*Effects of CAPE on experimental hepatotoxicity*
The biochemical results of analyses are presented in Figure 1. Serum TBil, ALT, and AST levels were significantly higher in the model group compared to the normal group ($P$ < 0.01). Intraperitoneal administration of CAPE significantly reduced ($P$ < 0.05) TBil, ALT, and AST levels in liver fibrosis rats when compared to the model group, and serum TBil was decreased by CAPE in a dose-dependent manner.

*Liver hydroxyproline*
As demonstrated in Figure 2, liver hydroxyproline content in the model group was 1.5 times higher than that in the normal group, and the liver hydroxyproline contents in both the 6 and 12 mg/kg CAPE groups were significantly lower than that in the model group ($P$ < 0.05 and $P$ < 0.001, respectively).

*Tissue levels of MDA and GSH*
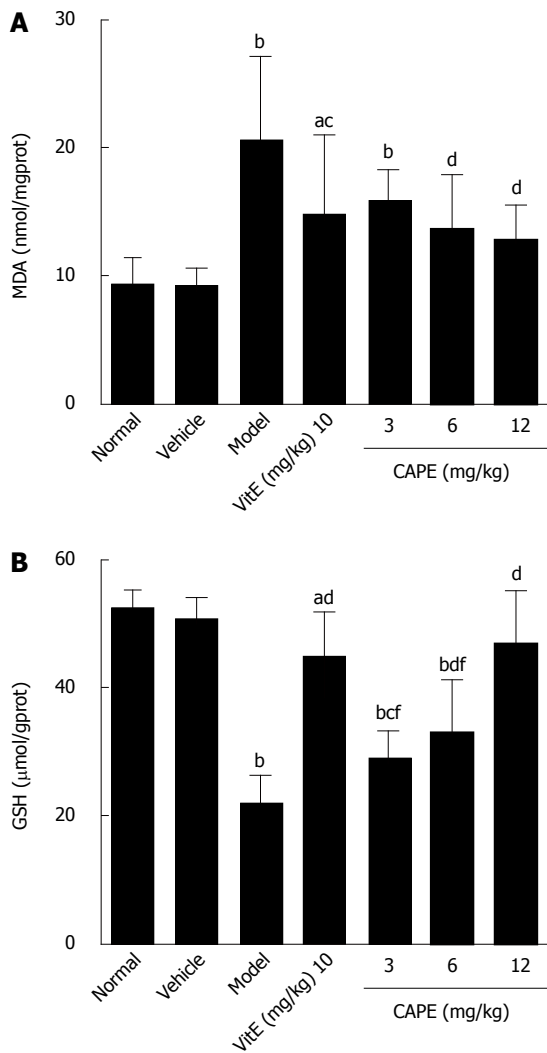To evaluate the effect of CAPE on liver lipid pero-

**Figure 3 Tissue levels of malondialdehyde (A) and glutathione (B) in the groups.** Bars and error bars represent mean ± SD. *t* = 2.767-9.292, [a]*P* < 0.05, [b]*P* < 0.01 *vs* normal group, respectively; *t* = 2.721-9.551, [c]*P* < 0.05, [d]*P* < 0.01 *vs* normal group, respectively; *t* = 5.154-6.903, [e]*P* < 0.05, [f]*P* < 0.01 *vs* CAPE (12 mg/kg) group, respectively. CAPE: Caffeic acid phenethyl ester; Vit E: Vitamin E; MDA: Malondialdehyde; GSH: Glutathione.

**Figure 4 Superoxide dismutase and catalase activities in liver tissue.** Bars and error bars mean ± SD. *t* = 4.552-11.06, [b]*P* < 0.01 *vs* normal group; *t* = 2.876-8.689, [c]*P* < 0.05, [d]*P* < 0.01 *vs* model group, respectively; *t* = 3.393-7.503, [f]*P* < 0.01 *vs* CAPE (12 mg/kg) group, respectively; *t* = 3.969-4.997, [g]*P* < 0.05, [h]*P* < 0.01 *vs* vit E group, respectively. CAPE: Caffeic acid phenethyl ester; Vit E: Vitamin E; SOD: Superoxide; CAT: Catalase.

xidation, MDA levels (an indicator of oxidative damage and one of the principal products of lipid peroxidation) were monitored. GSH is an important part of the non-enzymatic antioxidant system and is known to play an important role in liver injury; therefore, GSH levels were also determined in this study. The results of MDA and GSH are shown in Figure 3. In the CAPE (6 and 12 mg/kg) treated rats, the MDA levels were significantly lower, and the levels of GSH were significantly higher than those in the model group. Moreover, MDA and GSH concentrations in groups treated with 12 mg/kg CAPE were not significantly different from those in the normal group.

### Antioxidant enzymes activities

As shown in Figure 4, in a similar manner to GSH, CAT and SOD activities were significantly decreased in liver fibrosis rats induced with CCl4, alcohol and rich-fat
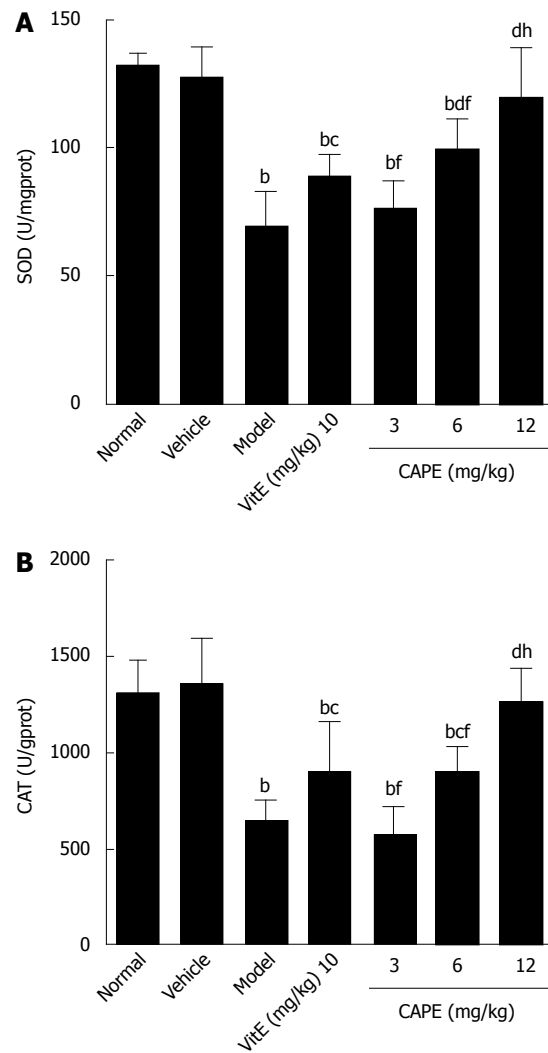
forage. CCl4, alcohol and rich-fat forage administration significantly depleted antioxidant enzyme (CAT and SOD) activities, whereas CAPE (6 and 12 mg/kg) treatment significantly protected against the depletion of CAT and SOD, and the effects of treatment with 12 mg/kg CAPE were more obvious compared to 10 mg/kg vit E.

### Pathological changes

Histopathological changes in all of the groups are shown in Figures 5 and 6 (HE and VG staining). In microscopic examinations of the samples, liver sections in the normal and vehicle groups showed normal parenchymal cells, portal system, and blood sinusoids (no histopathological changes). Administration of CCl4, alcohol and rich-fat forage induced severe alterations in liver histopathology. The model group liver sections showed peri-portal apoptosis, lymphocytic infiltration,
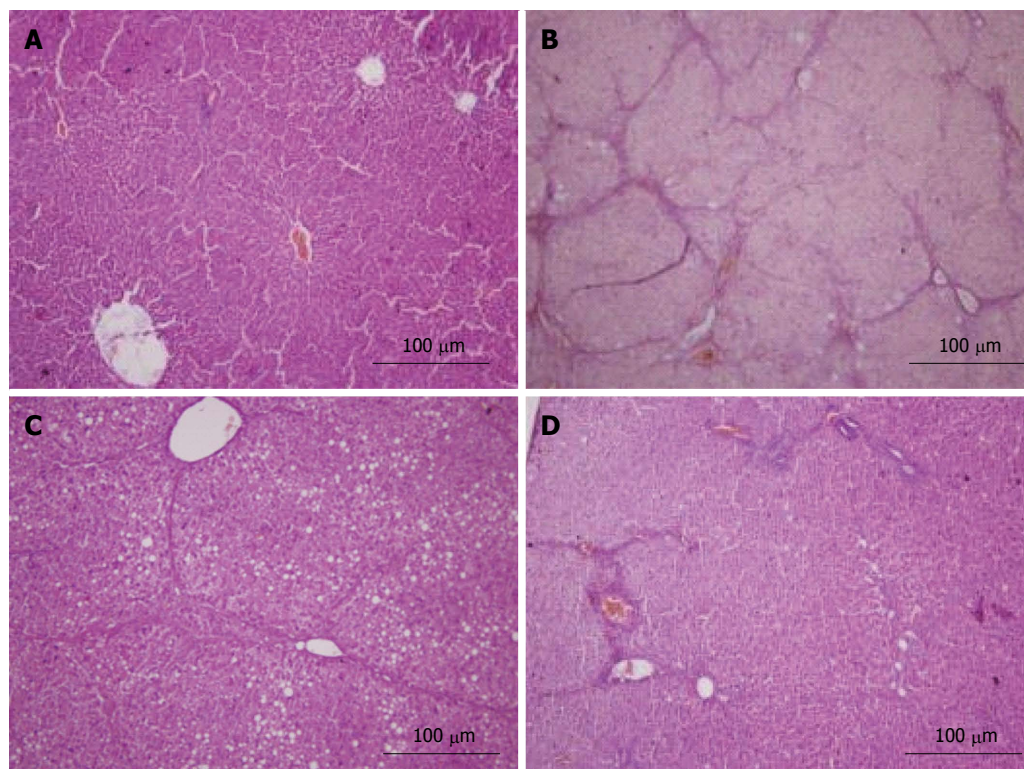
**Figure 5  HE staining.** All sections were stained with hematoxylin and eosin (× 100). A: Liver sections of the normal group did not show histopathological changes; B: Liver sections of the model group showed liver tissue structural disorder, apoptotic cells, lymphocytic infiltration and typical pseudo-lobule; C: Liver sections of the vitamin E  group showed steatosis, edema and swelling of hepatocytes, but no typical pseudo-lobule can be observed; D: Liver sections of the caffeic acid phenethyl ester (12 mg/kg) group showed spotty necrosis, steatosis, and blood sinusoids with mild congestion. The results of the other groups are not shown here.
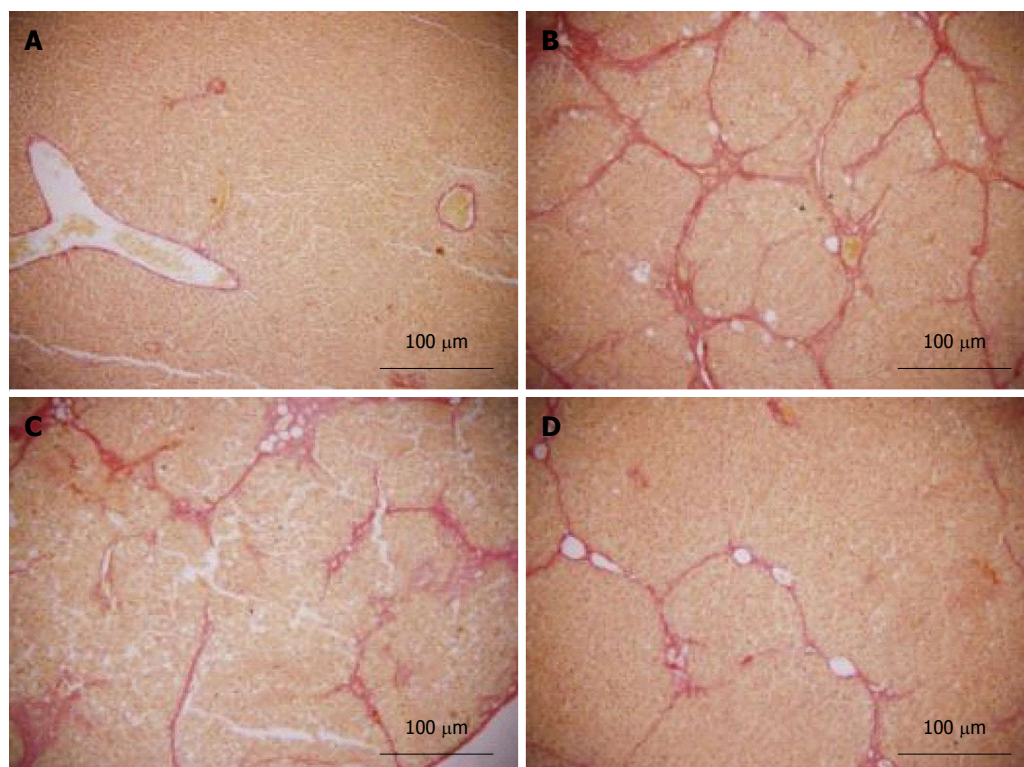


**Figure 6  Van Gieson staining.** All sections were stained with van Gieson (× 100). A: Liver sections of the normal group did not show fibroplasia; B: Liver sections of the model group showed thick bundles of proliferous collagenous fibers stained with eosin and typical pseudo-lobule; C: Liver sections of the vitamin E group showed a small amount of collagenous fibers; D: Liver sections of the caffeic acid phenethyl ester (12 mg/kg) group showed a small amount of fibroplasia, which was less than that in the vitamin E group. The results of other groups are not shown here.

**Table 1 Degree of liver fibrosis in the groups ($n$ = 6 for each group)**

| Group | 0 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| Normal | 6 | 0 | 0 | 0 | 0 |
| Vehicle | 6 | 0 | 0 | 0 | 0 |
| Model | 0 | 0 | 0 | 3 | 3 |
| Vit E | 0 | 4 | 2 | 0 | 0 |
| CAPE (mg/kg) | | | | | |
| 3 | 0 | 0 | 2 | 3 | 1 |
| 6 | 0 | 1 | 3 | 2 | 0 |
| 12 | 0 | 5 | 1 | 0 | 0 |

$H$ = 46.12. Model group vs normal group, $\chi^2$ = 9.778, $P$ < 0.05; Vitamin E (Vit E) group *vs* model group, $\chi^2$ = 8.899, $P$ < 0.05; Caffeic acid phenethyl ester (CAPE; 12 mg/kg) *vs* model group, $\chi^2$ = 9.209, $P$ < 0.05. The results were statistically analyzed by the nonparametric Kruskal-Wallis $H$ test followed by Wilcoxon test for paired comparisons.

dilated central veins, thick bundles of proliferous collagenous fibers stained with eosin and typical pseudo-lobule. The liver did not show significant improvement in either the 3 or 6 mg/kg CAPE group, whereas the 12 mg/kg group had similar recovery as in the vit E positive control group. However, steatosis, edema and swelling of hepatocytes (cobble stone appearance), spotty necrosis and a small amount of collagenous fibers still could be observed in the CAPE (12 mg/kg) and Vit E groups. The degree of liver fibrosis in the groups is shown in Table 1.

### $\alpha$-SMA expression

In the model group, immunohistochemistry localized the $\alpha$-SMA protein predominantly to the fibrous septa, inflamed area and portal area, and we observed a significantly higher ($P$ < 0.001) expression level than in the normal group (Figure 7). CAPE treatment could down-regulate the $\alpha$-SMA expression in liver tissue. The expression level of $\alpha$-SMA in the 12 mg/kg CAPE group was significantly lower than those in the model group and the vit E group (Figure 8).

### Nrf2 expression

As shown in Figures 9 and 10, it was observed that Nrf2-positive cells detected by immunohistochemistry in liver tissue were increased in the model group compared to the normal group, and the expression levels of Nrf2 were increased in the 6 and 12 mg/kg CAPE groups compared with the model group. In addition, expression level of Nrf2 in the 12 mg/kg dose group was highest ($P$ < 0.05) in all the groups.

## DISCUSSION

CCl$_4$ induced hepatotoxicity derives from the reductive dehalogenation of CCl$_4$ that is catalyzed by CYP450 in the endoplasmic reticulum of liver cells to generate unstable trichloromethyl ($CCl_3^+$) and trichloromethyl
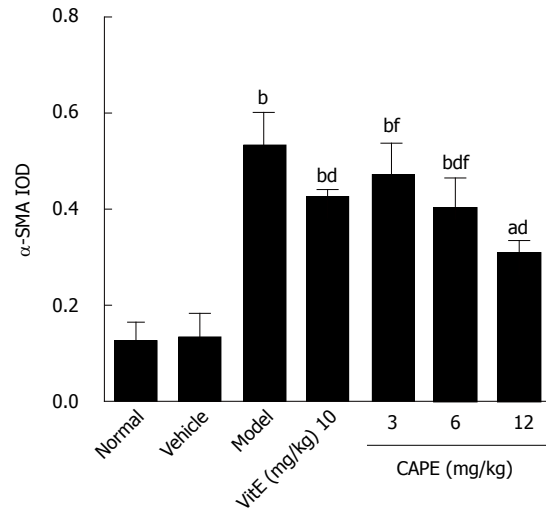


**Figure 7 $\alpha$-smooth muscle actin expression level in liver tissue.** Bars and error bars represent mean ± SD. $t$ = 6.557-14.487, $^aP$ < 0.05, $^bP$ < 0.01 *vs* normal group, respectively; $t$ = 3.784-7.913, $^cP$ < 0.05, $^dP$ < 0.01 *vs* model group, respectively; $t$ = 3.284-5.718, $^fP$ < 0.01 *vs* CAPE (12 mg/kg) group, respectively; $t$ = 4.129, $^gP$ < 0.05 *vs* vit E group, respectively. CAPE: Caffeic acid phenethyl ester; Vit E: Vitamin E; $\alpha$-SMA: $\alpha$-smooth muscle actin.

peroxyl ($CCl_3O_2^-$) radicals. Similarly, it was reported that alcohol-induced liver injury was associated with formation of the l-hydroxyl ethyl radical and lipid radicals[28]. Oxidative stress also plays an important role in high-fat forage induced liver injury. Free radicals attack and covalently bind to microsomal lipids and proteins, leading to lipid peroxidation and initiating secondary biochemical processes, which are the ultimate cause for unfolding the pathological consequences of CCl$_4$.

In the current study, CCl$_4$, alcohol and high-fat forage resulted in prominent hepatotoxicity as evidenced by the increases in serum TBil, ALT and AST levels and histopathology (HE and VG staining), and CAPE at all the three doses reduced TBil, ALT and AST levels in blood in a dose-dependent manner, indicating reduced hepatocellular damage. Histopathological observations also substantiated biochemical data of the study. CAPE treatment at 6 and 12 mg/kg resulted in the improvement in liver histoarchitecture and better recovery than that in the Vit E treated positive group.

Exogenous and endogenous protective agents with antioxidant properties demonstrated some protective effects against hepatotoxicity induced by various etiological factors. CAPE exhibits a number of beneficial effects against various types of degenerative diseases in humans, largely because the major components of CAPE have potent antioxidant activities[29,30]. GSH is a low molecular weight thiol antioxidant and a co-substrate for a variety of antioxidants and anti-xenobiotic enzymes of phase II pathway of the CYP450 cycle. It has been reported that the covalent binding of most toxicants to hepatic protein occurs only after
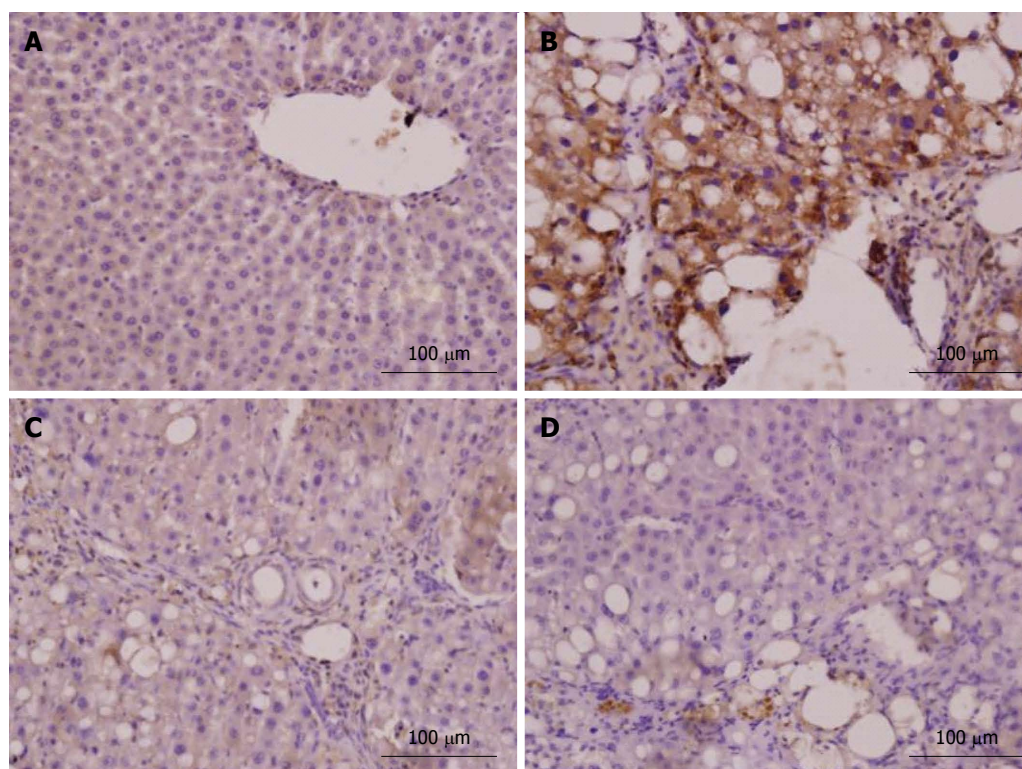
**Figure 8  α-smooth muscle actin expression in liver tissue (immunohistochemistry, × 400).** The α-smooth muscle actin (α-SMA) expression level in liver tissue was confirmed by immunohistochemistry (× 400). A: Liver sections of the normal group showed a small amount of α-SMA-positive cells only present in vascular wall; B: Liver sections of the model group showed that the α-SMA protein was predominantly present in the fibrous septa, inflamed area and portal area; C: Liver sections of the vitamin E group showed that α-SMA-positive cells were distributed in fibroplasia and inflamed area, but the number of positive cells was less than that in the model group; D: Liver sections of the CAPE (12 mg/kg) group showed that α-SMA-positive cells were sparse, located in inflamed area. The results of the other groups are not shown here.
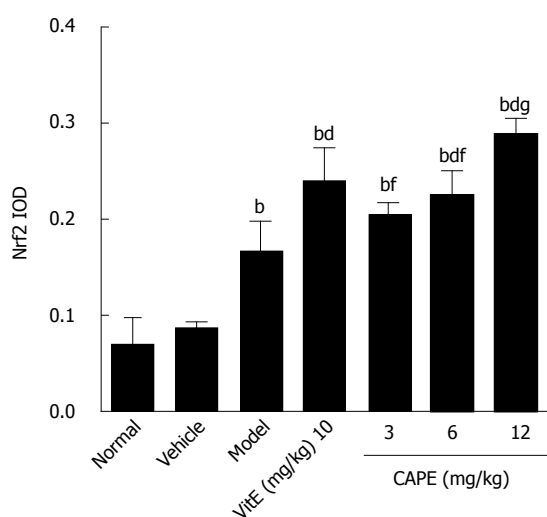


**Figure 9  NF-E2-related factor 2 expression level in liver tissue.** Bars and error bars represent mean ± SD. $t$ = 6.412-14.54, $^aP < 0.05$, $^bP < 0.01$ *vs* normal group, respectively; $t$ = 3.936-8.126, $^cP < 0.05$, $^dP < 0.01$ *vs* model group, respectively; $t$ = 4.190-5.627, $^eP < 0.05$, $^fP < 0.01$ *vs* CAPE (12 mg/kg) group, respectively; $t$ = 3.252, $^gP < 0.05$ *vs* vit E group, respectively. CAPE: Caffeic acid phenethyl ester; Vit E: Vitamin E; Nrf2: NF-E2-related factor 2.

depletion of GSH, and the severity of hepatic necrosis is related to the degree of covalent binding[31]. In addition, antioxidant enzymes such as CAT and SOD are easily inactivated by lipid peroxides or reactive oxygen species, which results in decreased activities of these enzymes in $CCl_4$ toxicity. Thus, antioxidant enzymes also play an important role in the detoxification of xenobiotics, catalyzing their conjugation with reduced GSH[32]. Finally, it has been reported that antioxidants and flavonoids can act as inhibitors of LPO by scavenging peroxy radicals derived from polyunsaturated fatty acids and interrupt the chain reactions[33]. Therefore, LPO was monitored by measuring MDA - an end product of LPO in membrane components of cells[34].

In the present study, the model group showed significantly decreased GSH levels, CAT and SOD activities, and increased MDA levels compared with the normal group, indicating increased oxidative stress due to free-radical induced liver damage. CAPE treatment significantly reduced the elevated MDA levels and also increased tissue GSH levels, CAT and SOD activities. These results suggest that suppression of oxidative stress by CAPE in rats is an important aspect of the hepatoprotective effect of CAPE against hepatocytoxicity induced with $CCl_4$, alcohol and rich-fat forage.

Transcription factor Nrf2 is activated in response to electrophiles and reactive oxygen species. During oxidative stress, Nrf2 is released from sequestration in the cytoplasm and translocates to the nucleus[35]. It
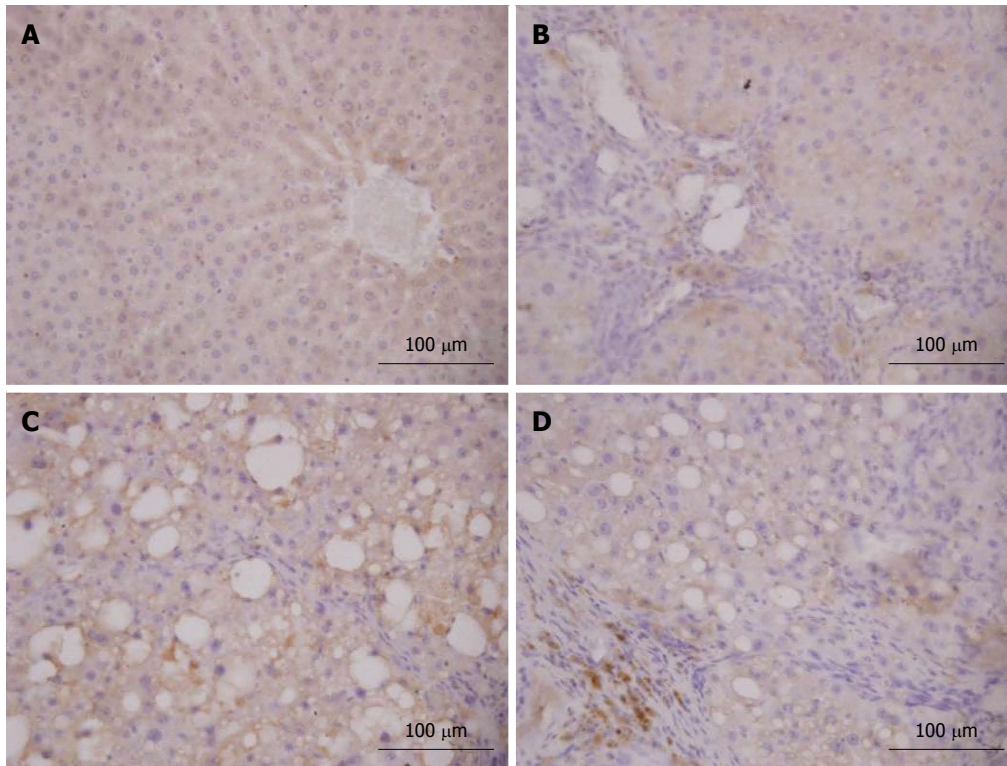
**Figure 10  NF-E2-related factor 2 expression in liver tissue (immunohistochemistry).** The NF-E2-related factor 2 (Nrf2) expression in liver tissue was confirmed by immunohistochemistry (× 400). A: Liver sections of the normal group showed a small amount of Nrf2-positive cells only present in the cytoplasm; B: Liver sections of the model group showed only a few Nrf2-positive cells present in the fibrous septa and inflamed area; C: Liver sections of the vitamin E group showed that Nrf2-positive cells were distributed in fibroplasia and inflamed area, but the number of positive cells was more than that in the model group; D: Liver sections of the caffeic acid phenethyl ester (12 mg/kg) group showed that Nrf2-positive cells were located mainly in fibrosis and inflamed area and were strongly stained. The results of the other groups are not shown here.

has been demonstrated that Nrf2 is a key transcription factor for both the inducible and constitutive expression of phase Ⅱ enzymes. Previous research has shown that Nrf2 knockout mice are more susceptible to acetaminophen-induced hepatocellular injury and that Nrf2 may also be important in protecting against liver fibrosis[36]. In liver fibrosis, HSCs serve as the major source of extracellular matrices in liver fibrosis. During the activation process, HSCs undergo phenotypic changes in cellular morphology to a myofibroblast-like cell type with expression of $\alpha$-SMA[12]. In the current study, immunohistochemistry results showed that treatment with CAPE at 6 mg/kg and 12 mg/kg significantly activated Nrf2, and inhibited $\alpha$-SMA. These data indicate that CAPE may be able to protect liver cells from oxidative stress induced damage and inhibit activation of HSCs through up-regulated Nrf2 expression.

It should be considered that the protective effect of CAPE against hepatotoxicity might not be solely dependent on the antioxidant effect of CAPE, which may exert its hepatoprotective effect by other means. For example, it was shown that CCl₄ induces liver cell apoptosis by cytochrome C release and caspase 3 activation, and causes hepatoxicity by increasing mRNA expression of NF-κB dependent cyclooxygenase-2 and iNOS. Alternatively, CAPE shows an anti-inflammatory action as lipoxygenase and cyclooxygenase inhibitor[37,38]. Furthermore, CAPE is a potent mitochondrial protective agent that blocks Ca$^{2+}$-induced cytochrome release and is a potent suppressor of pro-inflammatory cytokines by reducing caspase 1 activation and depressing NF-κB[39].

In conclusion, CAPE exhibits significant partial protection against hepatotoxicity as evidenced from the reversal of various biochemical indices and histopathology, which were altered due to CCl₄, alcohol and high-fat forage induced toxicity. The protective effects against liver damage could be due to the antioxidant effect of CAPE. However, the protective effect of CAPE may not always be associated with its antioxidant effects. Owing to the hepatoprotective potential, CAPE has clinical importance and could be used to develop a safe hepatoprotective medicine.

## COMMENTS

### Background

Liver fibrosis results from chronic damage to the liver in conjunction with the accumulation of extracellular matrix proteins. Oxidative stress has been recognized as a fundamental factor in the pathological processes of liver fibrosis. Caffeic acid phenethyl ester (CAPE) has been widely accepted as a hepatoprotective folk medicine for many years. However, the hepatoprotective

effects of CAPE on liver fibrosis and the underlying mechanism are unclear.

## Research frontiers

CAPE is one of the major components of honeybee propolis and has several biological and pharmacological properties, including cytoprotective, anti-oxidant, anti-proliferative, and anti-inflammatory effects. Moreover, it was reported that many CAPE activities were related to transcription factor inhibition of nuclear factor-kappa B.

## Innovations and breakthroughs

Oxidative stress can cause excessive damage to hepatocytes through lipid peroxidation and protein alkylation. NF-E2-related factor 2 (Nrf2) is a key transcription factor that regulates the induction of genes encoding antioxidant proteins and phase 2 detoxifying enzymes, which is involved in drug metabolism, detoxification and antioxidant defenses. CAPE may inhibit liver fibrosis and oxidative stress by activating Nrf2 expression.

## Applications

These findings show that the protective effects of CAPE against CCl₄, alcohol and high-fat forage induced liver fibrosis may be due to its ability to suppress activation of HSCs by inhibiting oxidative stress.

## Terminology

CAPE is a flavonoid-like compound and is one of the major components of honeybee propolis.

## Peer-review

This is an interesting study about CAPE in liver fibrosis. CAPE is a flavonoid-like compound and is one of the major components of honeybee propolis. In this study, the authors evaluated the hepatoprotective effects and antioxidant activity of CAPE in rats with liver fibrosis. The study design is good, and the results are interesting.

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