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Hepatoprotective activity of *Terminalia catappa* L. leaves and its two triterpenoids

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Abstract

The aim of this study was to evaluate the effect of the chloroform extract of *Terminalia catappa* L. leaves (TCCE) on carbon tetrachloride (CCl_4)-induced acute liver damage and D-galactosamine (D-GalN)-induced hepatocyte injury. Moreover, the effects of ursolic acid and asiatic acid, two isolated components of TCCE, on mitochondria and free radicals were investigated to determine the mechanism underlying the action of TCCE on hepatotoxicity. In the acute hepatic damage test, remarkable rises in the activity of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (5.7- and 2.0-fold) induced by CCl_4 were reversed and significant morphological changes were lessened with pre-treatment with 50 and 100 mg kg^{-1} TCCE. In the hepatocyte injury experiment, the increases in ALT and AST levels (1.9- and 2.1-fold) in the medium of primary cultured hepatocytes induced by D-GalN were blocked by pre-treatment with 0.05, 0.1, 0.5 g L^{-1} TCCE. In addition, Ca^{2+}-induced mitochondrial swelling was dose-dependently inhibited by 50–500 μM ursolic acid and asiatic acid. Both ursolic acid and asiatic acid, at concentrations ranging from 50 to 500 μM, showed dose-dependent superoxide anion and hydroxyl radical scavenging activity. It can be concluded that TCCE has hepatoprotective activity and the mechanism is related to protection of liver mitochondria and the scavenging action on free radicals.

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Introduction

Terminalia catappa L. is a combretaceous tree distributed in tropical and subtropical areas. The leaves, trunk bark and fruits have been used in folk medicine for the treatment of dermatitis, and for antipyretic and homeostatic purposes. The fallen leaves of this plant have been used to prevent hepatoma and for treating hepatitis in India, the Philippines and other areas. However, the effective components have not been well studied.

The effects of the water extract of *T. catappa* leaves and the tannins in this plant have been examined. It was reported that remarkable rises in the activity of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) induced by CCl_4 were blocked by pre-treatment with the water extract of *T. catappa* leaves. *T. catappa* also exerted antioxidant activity and superoxide radical scavenger effects (Lin et al 1997). Punicalagin and punicalin, two of the most abundant tannins, exhibited strong antioxidant and hepatoprotective activity (Lin et al 1998, 2001). However, the mechanisms remain unknown.

Our recent study revealed that triterpenoid was the most abundant component of the chloroform extract of *T. catappa* leaves (TCCE) and was effective as an anti-inflammatory (Fan et al 2004). Two triterpenoids, ursolic acid and asiatic acid, were isolated from TCCE in our laboratory for the first time (Fan et al 2004). It has been shown that triterpenoid has antioxidant, anti-cancer (Kim et al 2000) and hepatoprotective activities and can protect liver against a number of hepatotoxicants such as carbon tetrachloride (CCl_4), acetaminophen, D-galactosamine (D-GalN) and cadmium (Liu et al 1994; Miura et al 1999). Oleanolic acid and ursolic acid have been considered as the most effective anti-hepatotoxicant triterpenoids, and oleanolic acid has been widely used for treating hepatitis in China (Liu et al 1995). We speculated that triterpenoid may be one of the important hepatoprotective elements of *T. catappa*. In the present study, the relationship between the hepatoprotective effect of TCCE and the action of ursolic acid and asiatic

acid was addressed to search for the possible mechanisms underlying the hepatoprotective effect of TCCE.

First, the role of TCCE in prevention of hepatotoxicity was evaluated using CCl_4 and D-GalN induced liver injury models. Second, the effects of ursolic acid and asiatic acid against mitochondrial swelling were analysed. There is evidence that cell death is involved in liver injury and liver diseases. In fact, apoptosis and necrosis are the most crucial steps in the development of all types of liver injury, fibrosis, alcoholic liver disease and hepatitis. It has been recognized that the major function of mitochondria is not only to provide ATP by oxidative phosphorylation, but to play many other roles such as the modulation of intracellular Ca^{2+} homeostasis, pH control and induction of the apoptotic and excitotoxic cell death (Kaplowitz 2000). Indeed, mitochondrial dysfunction contributes to a great number of human and animal diseases (Wallace 1999). These findings suggest that protection of mitochondria may be an important strategy for the treatment of liver diseases. In fact, it has been found that some of the hepatoprotective drugs have significant protective effects on the liver mitochondria (Elimadia et al 2003). However, the protective effects of triterpenoid on liver mitochondria have not been investigated. It is also known that reactive oxygen species (ROS) mainly produced by mitochondrial respiration have a variety of pathological effects and play an important role in the inflammation process, especially in the process of all types of liver injury. We therefore investigated the actions of ursolic acid and asiatic acid on protecting liver mitochondria and scavenging free radicals to demonstrate the possible active components of *T. catappa* against hepatotoxicity.

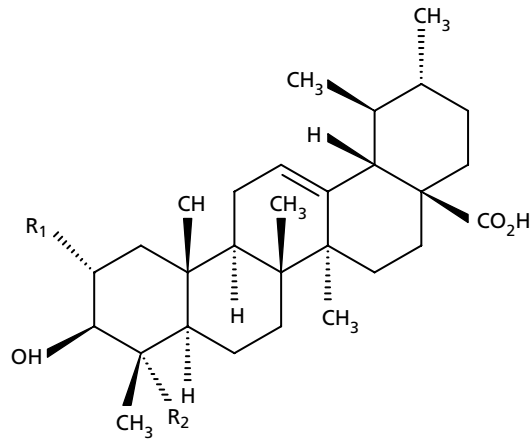
Materials and Methods

Chemicals

Succinate, rotenone, vitamin C, xanthine, xanthine oxidase, nitroblue tetrazolium, ciclosporin A and cytochrome C were purchased from Sigma Chemical Co. (St Louis, MO, USA). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) test kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other chemicals were of high purity from commercial sources.

Plant material

The leaves of plant *T. catappa* L. were collected in the south of China in 1998 and were identified by Gan Yao (Institute of Botany of Jiangsu Province, Chinese Academy of Sciences). Ursolic acid and asiatic acid were isolated from TCCE. Briefly, the dried cut leaves (1.1 kg) were extracted twice with ethanol by reflux for 1 h, evaporated and suspended in water, and followed by solvent partition with $CHCl_3$. Then, 32.0 g $CHCl_3$ soluble fraction (TCCE) was fractionated on a silica gel column (100–200 mesh, 30 mm × 500 mm), eluting with petrol/EtOAc (5:1–0:100) to afford some subfractions. One subfraction was recrystallized with petrol/EtOAc (1:2) to yield a white powder (20 mg, m.p. 222.0–226.9 °C, $[\alpha]_D$: +65, EtOH) identified as ursolic acid (Budzikiewicz et al 1963). Another fraction (yellow powder, 740 mg) was purified by silica gel column (200–300 mesh, 10 mm × 500 mm) chromatography, eluting with petrol/EtOAc (1:4–0:100) and EtOAc/EtOH (6:4–1:4) successively. The EtOAc/EtOH (6:4) fraction was recrystallized with EtOAc/EtOH (1:4) and yielded a white powder (70 mg, m.p. 289.3–296.8°C, $[\alpha]_D$:+56, $CHCl_3$), which was identified as asiatic acid (Kojima & Ogura 1986). Both of these compounds had >95% purity as determined by HPLC analysis (Figure 1).



	R_1	R_2
Ursolic acid	H	CH_3
Asiatic acid	OH	CH_2OH

Figure 1 Chemical structures of ursolic acid and asiatic acid.

Animals

Adult male ICR mice (Experimental Animal Center of Southeastern University, Grade II, Certificate No. 97003), weighing 18–22 g, were housed at a temperature of 20–25°C under a 12-h light/dark cycle with 50% relative humidity and kept in filtered, pathogen-free air. This study complied with current ethical regulations on animal research in our university and all mice used in the experiments were treated humanely.

CCl_4-induced hepatotoxicity in mice

Mice were divided into six groups of eight animals each. All animals except the normal group were administered 0.15% CCl_4 (in olive oil, 10 mL kg^{-1}, i.p.). The normal and CCl_4 control groups received olive oil and CCl_4, respectively, following 5 days of oral treatment with saline. The other groups received CCl_4 following treatment with TCCE (20, 50 and 100 mg kg^{-1}) or 200 mg kg^{-1} dimethyl diphenyl bicarboxylate (as a positive reference) in the same way. After 24 h, all mice were killed and blood was collected to determine serum ALT and AST activity using the test kits. After collecting blood, liver sections were taken and fixed in 4% neutral-buffered formalin and regularly prepared for examination under a photomicroscope.

D-GalN-induced hepatotoxicity in cultured hepatocytes

Fetal mouse hepatocytes were isolated by the method of Kaighn (1973). The isolated cells were suspended in Dulbecco's modified Eagle's medium containing 5% fetal calf serum, 20 U L^{-1} insulin and 0.5 mg L^{-1} dexamethasone and plated onto tissue culture dishes at 1×10^6 mL^{-1}. The viability of cells was measured by the trypan blue exclusion technique and the cells were used if the viability was greater than 95%. After 7 days of culture at 37°C in a 5% CO_2 atmosphere, hepatocytes were pre-treated with TCCE at various concentrations or 0.5 g L^{-1} dimethyl diphenyl bicarboxylate (as a positive control), and D-GalN (6.4 g L^{-1}) was added following 36 h of incubation. After 6 h, ALT and AST activity was measured using the test kits.

Measurement of mitochondrial swelling

Mitochondria were prepared from the livers of mice according to the method of Aprille et al (1977). Protein concentration was determined using Coomassie Brilliant Blue (Bradford 1976). Various concentrations of oleanolic acid, ursolic acid or asiatic acid were added to the assay mixture containing 1 g protein L^{-1} mitochondria, 125 mM sucrose, 50 mM KCl, 2 mM KH_2PO_4, 5 mM succinate, 5 μM rotenone and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid), pH 7.4. Swelling was induced when challenged with 50 μM $CaCl_2$ at 30°C (Uyemura et al 1997). Ciclosporin A (5 μM) was used as a positive reference. The extent of mitochondrial swelling was assayed by measuring the decrease in absorbance (A) at 540 nm and the inhibitory rate of mitochondrial swelling was calculated as follows: $(\Delta A_{control} - \Delta A_{drug})/\Delta A_{control} \times 100\%$, $\Delta A = A_{0\,min} - A$.

Assay of superoxide anions scavenging activity

Superoxide anions were generated in the xanthine/xanthine oxidase system and were measured by the nitroblue tetrazolium reduction method (Panteleon et al 2003). Xanthine oxidase (20 U L^{-1}) was added to the reaction mixture in 10 mM phosphate buffer (pH 7.4) containing 100 μM xanthine, 100 μM nitroblue tetrazolium in the absence or presence of oleanolic acid, ursolic acid or asiatic acid at various concentrations. Following incubation at 25°C for 5 min, absorbance was read at 550 nm. Superoxide dismutase (1×10^5 U L^{-1}) was used as a positive control. The percentage scavenging rate of superoxide anions was calculated as follows: $(\Delta A_{control} - \Delta A_{drug})/\Delta A_{control} \times 100\%$, $\Delta A = A_{0\,min} - A_{5\,min}$.

Assay of hydroxyl radicals scavenging activity

Hydroxyl radicals were generated in 3 mL of 0.15 mM sodium phosphate buffer (pH 7.4), which contained 100 μM vitamin C, 100 μM $CuSO_4$ and 100 μM cytochrome C, with or without oleanolic acid, ursolic acid or asiatic acid at various concentrations. The mixture was incubated at 25 °C for 90 min. The transmittance of colour change of cytochrome C was measured at 550 nm (Liu et al 1997). Vitamin E (60 μM) was used as a positive reference. The percentage scavenging rate was calculated as described above but $\Delta A = A_{0\,min} - A_{90\,min}$.

Statistical analysis

Differences among all groups were analysed by one-way analysis of variance followed by SNK *q*-test using SPSS 10 software (Guangzhou, China). A value of $P < 0.05$ was accepted as statistically significant.

Results

Effect of TCCE on CCl_4-induced injury in mice

Serum ALT and AST activities were remarkably increased (5.7-fold and 2.0-fold) after the injection of CCl_4 in mice (Figure 2). Treatment with various concentrations of TCCE (20, 50 and 100 mg kg^{-1}) significantly blocked these increases; the increase in ALT and AST activities was almost completely inhibited by 100 mg kg^{-1} TCCE.

The histological changes associated with the hepatoprotective activity of the three doses of TCCE basically supported the estimation of the serum enzyme activities. The liver sections of CCl_4-treated mice showed massive fatty changes, gross necrosis, broad infiltration of the lymphocytes and Kupffer cells around the central vein, and loss of cellular boundary (Figure 3B). The histological pattern of the livers of the mice treated with TCCE only showed a mild degree of fatty changes, necrosis and lymphocyte infiltration (Figure 3C–E).

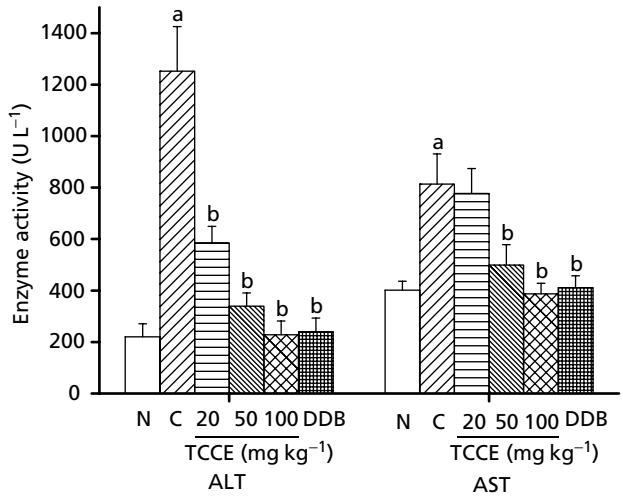


Figure 2 Inhibitory effect of the chloroform extract of *Terminalia catappa* L. leaves (TCCE) on the rise in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities induced by CCl_4 in mice. Mice were divided into six groups. The normal (N) and CCl_4 control (C) groups received olive oil and CCl_4 (10 mL kg^{-1}, i.p.), respectively, following 5 days of oral treatment of saline. Drug groups received CCl_4 following 5 days of oral treatment of TCCE or 200 mg kg^{-1} dimethyl diphenyl bicarboxylate (DDB; as a positive reference). After 24 h, all mice were killed and blood was collected to determine serum ALT and AST activities. Each column indicates the mean ± s.d., n = 8. [a] $P < 0.01$ vs normal; [b] $P < 0.01$ vs CCl_4 control group.

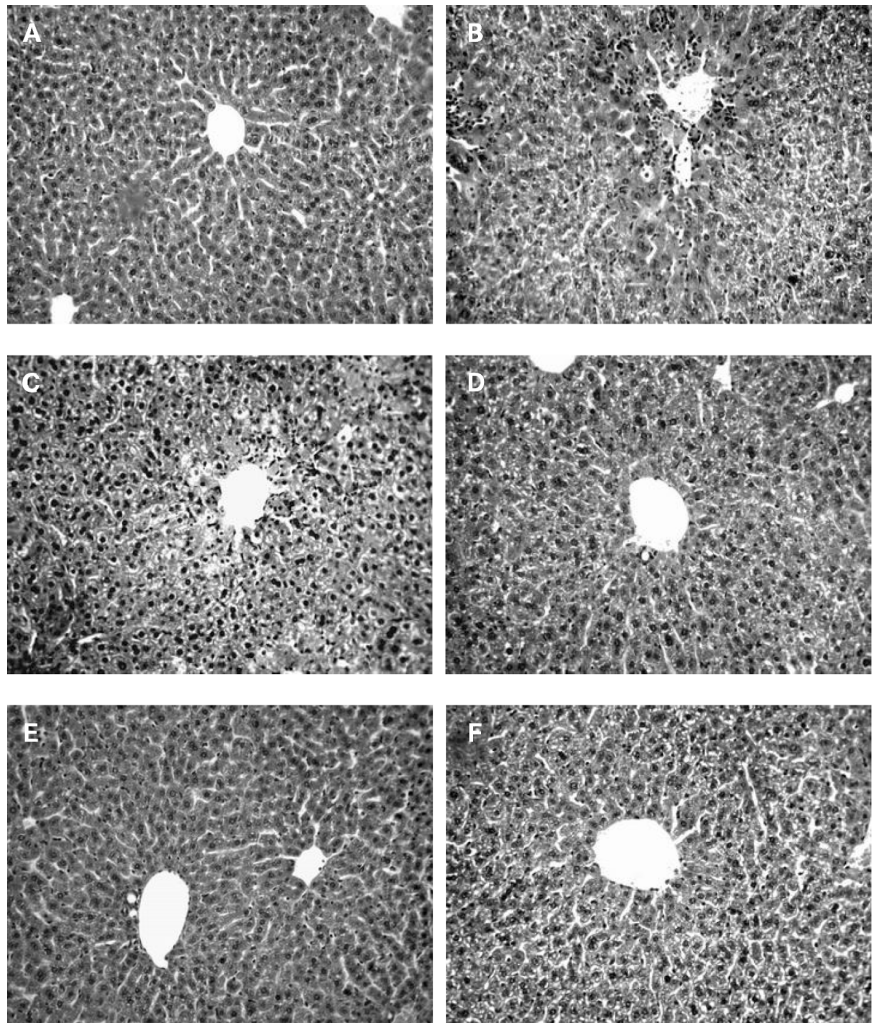


Figure 3 Effect of the chloroform extract of *Terminalia catappa* L. leaves (TCCE) on histopathological changes in the CCl_4-injured livers. A. Liver tissue structure from a normal mouse. B. Section of mouse liver from the CCl_4 control group 24 h after an intraperitoneal injection with 10 mL kg^{-1} CCl_4. C–E. Sections of livers from mice pre-treated with 20, 50, 100 mg kg^{-1} TCCE before CCl_4 injection, respectively. F. Section of liver from a mouse pre-treated with 200 mg kg^{-1} dimethyl diphenyl bicarboxylate before CCl_4 injection. HE stain, original magnification ×100.

Protective effect of TCCE on D-GalN-induced hepatocytes injury

Figure 4 shows the ALT and AST activities in culture medium of the hepatocytes in different groups. Administration of D-GalN resulted in a marked increase in ALT and AST activities (1.9-fold and 2.1-fold) compared with normal. However, the enhancement of supernatant enzyme activities induced by D-GalN was effectively inhibited by treatment of TCCE. Moreover, changes in ALT and AST activities were completely reversed in both 0.1 and 0.5 g L^{-1} TCCE groups.

Effects of ursolic acid and asiatic acid on Ca^{2+}-induced mitochondrial swelling

The swelling of liver mitochondria was induced by 50 μM Ca^{2+}, which could be attenuated by treatment with ursolic acid and asiatic acid (50, 150 and 500 μM). The inhibition rates of 500 μM ursolic acid and asiatic acid at 5 min were 73.2% and 76.1%, respectively, which were greater than that of oleanolic acid (50.5%) (Figure 5).

Scavenging action of ursolic acid and asiatic acid on free radicals

The scavenging activity of ursolic acid and asiatic acid against superoxide anions was evaluated in-vitro in the xanthine/xanthine oxidase system. As shown in Table 1, both ursolic acid and asiatic acid exerted superoxide anion scavenging activity in a dose-dependent manner. It was also found that 500 μM asiatic acid had the greatest scavenging rate (56.7%); the rates of oleanolic acid and ursolic acid were 39.3% and 46.2%, respectively, at the same concentration.

The two compounds also showed scavenging activity against hydroxyl radicals in a dose-dependent manner (Table 2). The scavenging rates of 500 μM ursolic acid and

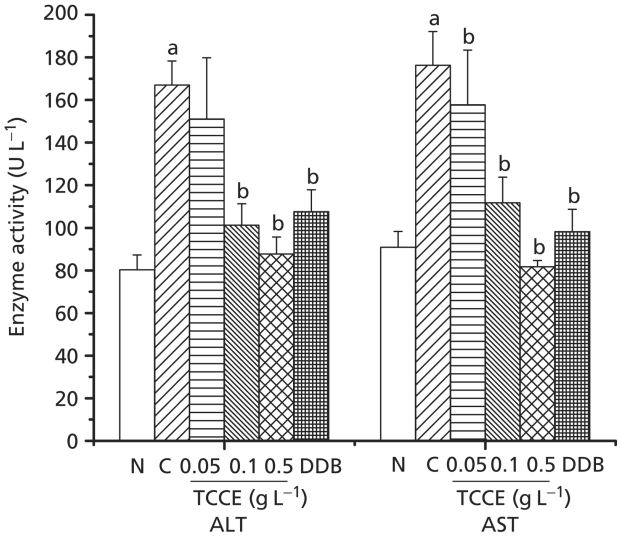


Figure 4 Effect of the chloroform extract of *Terminalia catappa* L. leaves (TCCE) on the rise in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in the medium of hepatocytes damaged by D-galactosamine (D-GalN). Cultured primary hepatocytes were divided into six groups. Hepatocytes were pre-treated with TCCE at various concentrations or 0.5 g L^{-1} dimethyl diphenyl bicarboxylate (DDB; as a positive control), and D-GalN (6.4 g L^{-1}) was added following 36 h of incubation. After 6 h, the activities of ALT and AST were measured. Each column indicates the mean ± s.d., n = 7. N, normal; C, D-GalN control. $^{a}P < 0.01$ vs normal; $^{b}P < 0.01$ vs D-GalN control group.

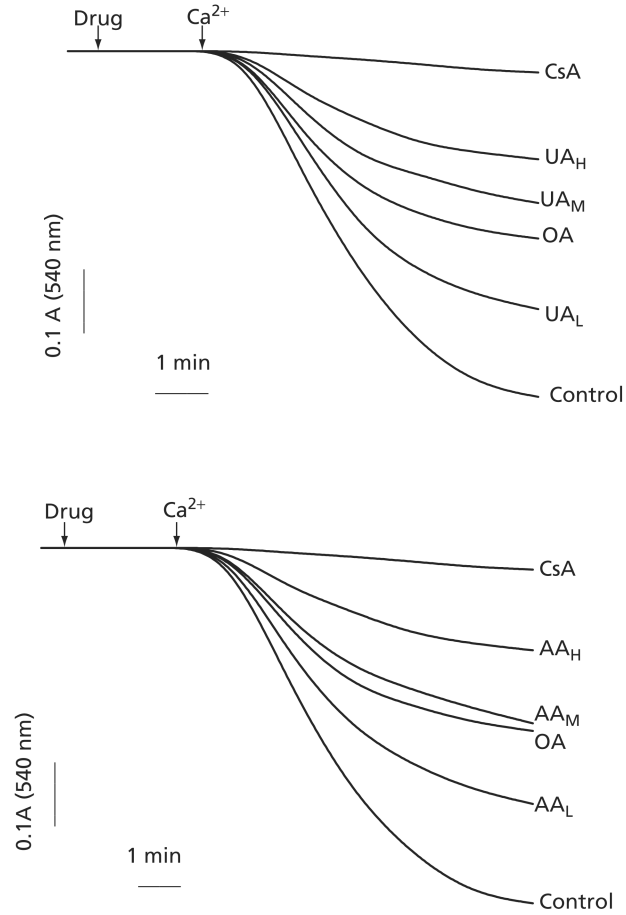


Figure 5 Inhibitory effects of ursolic acid (UA) and asiatic acid (AA) on Ca^{2+}-induced mitochondrial swelling. Mitochondrial swelling was assayed by measuring the decrease in absorbance at 540 nm. Swelling was induced by the addition of Ca^{2+} at 30°C. UA_L, UA_M and UA_H represented 50, 150 and 500 μM UA, respectively. AA_L, AA_M and AA_H represented 50, 150 and 500 μM AA, respectively. Ciclosporin A (CsA; 5 μM) was used as a common positive reference and oleanolic acid (OA) was a specific positive control with similar structure. The traces are representative of five experiments.

asiatic acid were 50.1% and 63.8%, respectively, which were greater than that of oleanolic acid (45.6%) at the same concentration.

Discussion

Liver damage induced by CCl_4 and D-GalN is commonly used to screen drugs with hepatoprotective activity (Itokazu et al 2000; Hewawasam et al 2003). CCl_4 is metabolized by the endoplasmic reticulum mixed-function oxidase system to its trichloromethyl or peroxytrichloromethyl free radical, which leads to auto-oxidation of the polyenoic fat present in the cytoplasmic membrane phospholipids. On the other hand, liver disease caused by the administration of D-GalN morphologically resembles virus-induced hepatitis in man. The hepatotoxicity of D-GalN is attributed to its metabolism in liver, which causes a decrease in several uracil nucleotides. As a result, it inhibits RNA and protein synthesis and disturbs the biosynthesis of glycoproteins, leading to deterioration of the cellular membranes. The above changes in hepatocytes induced by CCl_4 or D-GalN result in disturbance of Ca^{2+} homeostasis, inhibition of mitochondrial respiration and excessive generation of ROS. At the same time, the accumulation of ROS aggravates the damage in hepatocytes and mitochondria, resulting in membrane fragility, enzyme leakage and pathological degeneration (Lin et al 1999).

The results of the present study demonstrate that TCCE effectively protected mice against hepatotoxicity induced by both CCl_4 and D-GalN, as evidenced by the reversed serum ALT and AST activities and confirmed by morphological observations. ALT is thought to be one of the indices of the degree of cell membrane damage, while AST is an indicator for mitochondrial damage since mitochondria contain 80% of the enzyme (Daba & Abdel-Rahman 1998). So we can speculate that the protective effect of TCCE on liver tissue may be related to its protective effect on both hepatocytes and their mitochondria.

Ca^{2+}-induced liver mitochondrial swelling is a useful model for evaluating the effects of drugs or other substances on mitochondrial function (Elimadia et al 2003). Mitochondria isolated from the livers of normal mice were used to assess the direct effects of ursolic acid and asiatic acid on Ca^{2+}-induced liver mitochondrial swelling to determine the mechanism underlying the protection of TCCE on liver mitochondria. It was found that high-grade mitochondrial

Table 1 Effects of ursolic acid and asiatic acid on the generation of superoxide anions in the xanthine/xanthine oxidase system

Group	Dose	Decrease in absorbance$_{550}$	Scavenging rate (%)
Control		242.0 ± 16.9	
Oleanolic acid	500 μM	147.0 ± 18.9^{a}	39.3
Superoxide dismutase	1×10^5 U L^{-1}	132.1 ± 12.0^{a}	45.4
Ursolic acid	50 μM	177.9 ± 16.6^{a}	26.5
	100 μM	145.6 ± 10.9^{a}	39.9
	500 μM	130.1 ± 13.0^{a}	46.2
Asiatic acid	50 μM	172.1 ± 21.9^{a}	28.9
	100 μM	127.9 ± 18.6^{a}	47.2
	500 μM	120.3 ± 10.9^{abc}	56.7

Superoxide dismutase was used as a positive and oleanolic acid was a specific positive control. Data are mean $\pm$ s.d., n = 7. $^{a}P < 0.01$ vs the control group; $^{b}P < 0.01$ vs the oleanolic acid group; $^{c}P < 0.01$ vs the 500 μM ursolic acid group.

Table 2 Effects of ursolic acid and asiatic acid on the generation of hydroxyl radicals in the vitamin C/$CuSO_4$ system

Group	Dose (μM)	Decrease in absorbance$_{550}$	Scavenging rate (%)
Control		442.8 ± 23.4	
Oleanolic acid	500	241.3 ± 27.2^{a}	45.6
Vitamin E	60	213.0 ± 16.3^{a}	51.9
Ursolic acid	50	414.5 ± 30.1^{a}	6.4
	100	273.5 ± 21.2^{a}	38.2
	500	217.0 ± 17.1^{ab}	51.0
Asiatic acid	50	404.8 ± 31.7^{a}	8.6
	100	219.3 ± 18.5^{a}	50.5
	500	160.5 ± 30.4^{abc}	63.8

Vitamin E was used as a positive reference and oleanolic acid was a specific positive control. Data are mean $\pm$ s.d., n = 7. $^{a}P < 0.01$ vs the control group; $^{b}P < 0.01$ vs the oleanolic acid group, $^{c}P < 0.0\,1$ vs the 500 μM ursolic acid group.

swelling induced by Ca^{2+} could be inhibited by ursolic acid and asiatic acid in a dose-dependent manner, which supports the previous results indicating that TCCE had direct protective effects on mitochondria.

The scavenging effects of ursolic acid and asiatic acid on free radicals were further evaluated. Our results showed that both ursolic acid and asiatic acid exerted strong scavenging activity. The superoxide anions generated from the xanthine/xanthine oxidase system and hydroxyl radicals resulting from the vitamin C/$CuSO_4$ system were scavenged by ursolic acid and asiatic acid in a dose-dependent manner.

Excessive ROS can result in lipid peroxidation, protein oxidation, disturbance in calcium homeostasis and consequent loss of cell viability (Liu et al 1997; Panteleon et al 2003). Cellular accumulation of ROS could also induce the opening of mitochondrial permeability transition pores in mitochondrial membrane, which results in the inhibition of aerobic adenosine ATP synthesis, mitochondrial swelling and, finally, cell death (Youn et al 2002). From the above results, we might assume that the excess ROS in the mice treated with CCl_4 and D-GalN could be effectively scavenged by pre-treatment with TCCE. The decrease in the level of ROS could reduce the osmotic imbalance between the mitochondrial matrix and the intermembrane space, and protect mitochondria against swelling by inhibition of the excessive opening of permeability transition pores. Thus, the integrity of the mitochondrial membrane could be maintained and enzyme leakage decreased. Meanwhile, ATP synthesis could be recovered, and mitochondria and hepatocytes sustained.

It is interesting that although structurally similar to oleanolic acid, ursolic acid and asiatic acid had greater ROS scavenging rates and stronger inhibitory effects on mitochondrial swelling, suggesting that TCCE could be used clinically in the treatment of liver diseases. The reason for the differences in action among ursolic acid, asiatic acid and oleanolic acid may be owing to structural differences. The only structural difference between ursolic acid and oleanolic acid is in the conformation, while asiatic acid has two more hydroxyl radicals than oleanolic acid. Thus, it may be postulated that both the number of hydroxyl groups and conformation are related to the activity of these compounds.

Conclusion

The present study demonstrated that TCCE has hepatoprotective activity against both CCl_4-induced acute liver damage and D-GalN-induced hepatocyte injury, as evidenced by the inhibition of ALT and AST activities, and changes in liver morphology. The results suggest that two isolated triterpenoids, ursolic acid and asiatic acid, can scavenge superoxide anions and hydroxyl radicals, and protect liver mitochondria against Ca^{2+}-induced mitochondrial swelling in a dose-dependent manner. In general, the effective components of TCCE may include ursolic acid and asiatic acid, and the mechanisms underlying their protective effects may be associated with direct protection on mitochondria and hepatocytes, and the strong scavenging activities on ROS, which may benefit mitochondria indirectly.

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