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Mechanisms of Hepatoprotection of *Terminalia catappa* L. Extract on D-Galactosamine-Induced Liver Damage

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Abstract: The hepatoprotective effects of the extract of *Terminalia catappa* L. leaves (TCE) against D-Galactosamine (D-GalN)-induced liver injury and the mechanisms underlying its protection were studied. In acute hepatic injury test, it was found that serum ALT activity was remarkably increased (3.35-fold) after injection of D-GalN in mice. But with oral pretreatment of TCE (20, 50 and 100 mg/kg/d) for 7 days, change in serum ALT was notably reversed. In primary cultured hepatocytes from fetal mice, it was found that cell viability was decreased by 45.0% after addition of D-GalN, while incubation with TCE (0.1, 0.5 and 1.0 mg/ml) for 36 hours could prevent the decrease in a dose-dependent manner. Meanwhile, D-GalN-induced both the increase of AST level (1.9-fold) and the decrease of SOD activity (48.0%) in supernatant of primary cultured hepatocytes could also be inhibited by pretreatment with TCE. In order to study the possible mechanisms underlying its hepatoprotective effects, one effective component separated from TCE, 2α, 3β, 23-trihydroxyursane-12-en-28-oic acid (DHUA), was used to determine anti-mitochondrial swelling activity and superoxide radicals scavenging activity *in vitro*. It was found that at the concentration range of 50–500 μmol/L DHUA, Ca^{2+}-induced mitochondrial swelling was dose-dependently inhibited, and superoxide radicals scavenging activity was also shown in a dose-dependent manner. It was concluded that TCE has hepatoprotective activity and the mechanisms underlying its protective effects may be related to the direct mitochondrion protection and strong scavenging activity on reactive oxygen species (ROS).

Keywords: *Terminalia catappa* L.; 2α, 3β, 23-trihydroxyursane-12-en-28-oic acid (DHUA); Hepatoprotective Effects; D-Galactosamine; Mitochondria; Reactive Oxygen Species.

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Introduction

Terminalia catappa L. is a combretaceous plant broadly distributed in tropical and subtropical areas. The leaves of this plant have been used as a folk medicine for treating dermatitis and hepatitis in India, the Philippines, and some other areas. Previous studies show that *Terminalia Catappa* L. exerts antioxidative, anti-inflammatory and hepatoprotective activity (Lin *et al.*, 1997; Lin *et al.*, 1998; Lin *et al.*, 1999; Chyau *et al.*, 2002; Mau *et al.*, 2003) and can prevent carcinogenesis (Liu *et al.*, 1996, Chen *et al.*, 2000). Reports on the constituents of the leaves of this plant chiefly concern hydrolysable tannins (Tanaka *et al.*, 1986; Lin, 1992; Lin *et al.*, 1998) while other constituents were rarely investigated.

In this study, we evaluate the hepatoprotective effects of *Terminalia catappa* L. extract (TCE) by using one of hepatotoxins, D-GalN (D-galactosamine), which can induce acute hepatitis in mice closely resembling human viral hepatitis in morphological and functional changes (Keppler *et al.*, 1970; Sabesin and Ragland, 1978). Both primary cultured hepatocytes and liver injury models induced by D-GalN were used to study the protective effects of TCE by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and aspartate aminotransferase (AST), alanine aminotransferase (ALT) and superoxide dismutase (SOD) activities detection.

2α, 3β, 23-trihydroxyursane-12-en-28-oic acid (DHUA), a triterperoid, was isolated from TCE for the first time in our laboratory (Fan *et al.*, submitted). Triterperoids have various biological effects such as anti-oxidative, anti-inflammatory and hepatoprotective activity (Liu *et al.*, 1994; Liu *et al.*, 1995; Miura *et al.*, 1999). However, the biological effects of DHUA have not been investigated. In this study, we were interested in studying whether DHUA was a hepatoprotective component of TCE and its possible mechanisms of protective effects by using Ca^{2+}-induced mitochondrial swelling model and superoxide radicals scavenging test.

Materials and Methods

Materials

The plant *Terminalia catappa* L. was collected in southern China in 1998 and identified by Mr. Yao Gan (Institute of Botany of Jiangsu Province, Chinese Academy of Sciences). DHUA is isolated from TCE. Briefly, 1.1 kg dried cut leaves were extracted with ethanol by reflux for 1 hour and the resulting ethanol extract was subjected to evaporation to obtain 231.0 g (yield: 21.0% w/w) of crude extract (TCE). Then 100 g TCE was extracted with $CHCl_3$ and 13.85 g $CHCl_3$ soluble fraction was fractionated on silica gel columns, being eluted with petrol-EtOAc and EtOAc-EtOH, respectively, and recrystallized with EtOAc-EtOH to yield 30.3 mg white powder (yield: 0.03% w/w) which was identified as DHUA as follows.

TCE and DHUA were dissolved in 0.9% NaCl for oral treatment on mice or in 0.2% Tween-80 for addition to medium of hepatocytes or mitochondria. Dulbecco's Modified Eagle's Medium (DMEM) and trypsin were obtained from Gibco Life Technologies

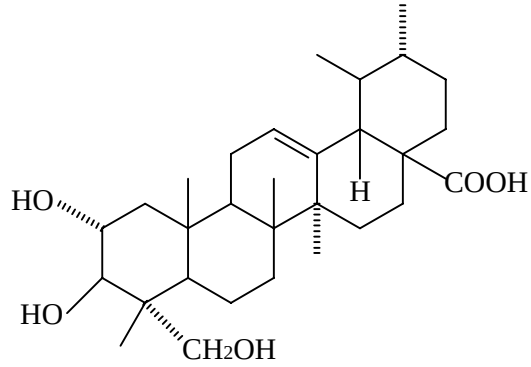

Figure 1. Structure of 2α, 3β, 23-trihydroxyursane-12-en-28-oic acid (DHUA).

(New York, USA). D-Galactosamine (D-GalN), MTT, succinate, rotenone, xanthine, xanthine oxidase, nitroblue tetrazolium (NBT), superoxide dismutase (SOD), were purchased from Sigma Chemical Co. (St Louis, MO, USA). Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and superoxide dismutase (SOD) testing kits were purchased from Nanjing Jiancheng Bioengineering Institutes (Nanjing, P. R. China). All other chemicals were of high purity from commercial sources.

Experimental Animals

Adult male ICR mice (18–22 g) and pregnant mice (with 17-day-old fetal mice for culture of primary hepatocytes) were purchased from the Experimental Animal Center of Southeastern University (Grade II, Certificate No. 97003). The mice were housed at a temperature of 20°C–25°C under a 12-hour light/dark cycle with 50% of relative humidity and kept in filtered, pathogen-free air. They were fed on commercial laboratory chow and give tap water.

Culture of Primary Hepatocytes

Hepatocytes were isolated from livers of 17-day-old mice embryos according to the method of Kaighn (Kaighn, 1973) with a little modification. Briefly, livers were minced, pooled and treated with trypsin (0.125%) for 10 minutes at 37°C and stopped with Hanks' Balanced salt solution. The homogenate was centrifuged at 600 g for 10 minutes, and the cell pellets were resuspended in DMEM containing 5% fetal calf serum, 20 U/L insulin and 0.5 mg/L dexamethasone and seeded onto 24-well (400 μl/dish) or 96-well (100 μl/dish) culture dishes at a density of 1×10^6 cells/ml. The viability of cells was measured by trypan blue exclusion technique and the cells were accepted if the viability was more than 95% and cultured at 37°C in 5% CO_2 atmosphere for 7 days. The culture medium was renewed every 3 days.

D-GalN-Induced Hepatotoxicity

In acute liver injury test, mice were divided into five groups as follows: control, D-GalN and D-GalN with 20, 50, 100 mg/kg TCE, respectively. The drug test groups were orally treated with various concentrations of TCE (ig) for 7 days before 800 mg/kg D-GalN was administrated intraperitoneal injection (ip). The mice in the control and D-GalN group only received saline (ig) for 7 days before ip saline or D-GalN, respectively. The blood samples were obtained from eyes for the detection of serum ALT activity 24 hours after saline or D-GalN injection.

In primary cultured hepatocytes model, hepatocytes were divided into five groups also: (1) Control, (2) D-GalN, (3) D-GalN + TCE (0.1 mg/ml), (4) D-GalN + TCE (0.5 mg/ml), and (5) D-GalN + TCE (1.0 mg/ml). The drug test groups were treated with various concentrations of TCE for 36 hours prior to 16.0 mg/ml D-GalN exposure. The control and D-GalN group received saline before addition of saline or D-GalN. After 6 hours of incubation, the AST and SOD activities and MTT assay were performed.

MTT Assay

A modified MTT assay (Hansen *et al.*, 1989), in which the yellow MTT is reduced to a purple formazan by mitochondrial dehydrogenase in living cells, was used to assess hepatocyte viability. Briefly, 25 μl MTT in PBS (0.1 mol/L, pH 7.2) was added to each well of 96-well plates (1.0 mg/ml final concentration). After 4 hours of incubation at 37°C, the reaction was stopped by adding 100 μl lysine buffer (20% sodium dodecyl sulfate in 50% dimethylformamide, pH 4.8). The amount of formazan product was determined 24 hours later by measuring absorbance at 570 nm at BIO-RAD Model 570 Microplate Reader.

Assay of AST, ALT and SOD Activities

In the liver injury test, 24 hours after D-GalN intoxication, the blood of experimental animals was collected from eyes. Blood samples were allowed to coagulate at room temperature for 30 minutes and the serum was separated by centrifugation at 4°C, 5000 g for 10 minutes. Serum ALT activity was measured according to the method described by Reitman (Reitman and Frankel, 1957). In the primary hepatocytes injury test, 6 hours after D-GalN incubation, the supernatant of cultured hepatocytes was collected, and AST and SOD activities were measured according to the method described by Reitman and Frankel (1957) and Sun *et al.* (1988), respectively.

Measurement of Mitochondrial Swelling

Mitochondria were prepared from the liver of mice according to the method of Apprille *et al.* (1977). The liver was removed and homogenized in ice-cold medium (225 mmol/L mannitol, 75 mmol/L sucrose, 50 μmol/L EDTA, and 10 mmol/L Tris-HCl, pH 7.5). The

homogenate was cleared by centrifugation at 500 g for 5 minutes and the supernatant was centrifuged at 8800 g for 10 minutes to form a mitochondria pellet. The pellet was washed three times, and suspended in homogenizing buffer. Protein concentration was determined using Coomassie Brilliant Blue (Bradford, 1976). The assay mixture containing mitochondria (1 mg protein/ml), 125 mmol/L sucrose, 50 mmol/L KCl, 2 mmol/L KH_2PO_4, 5 mmol/L succinate, 50 μmol/L $CaCl_2$, 5 μmol/L rotenone and 10 mmol/L Hepes, pH 7.4 with or without various concentrations of DHUA. OA (500 μmol/L) was used as a reference. Swelling was induced by the addition of Ca^{2+} at 30°C, and measured at 520 nm (Uyemura *et al.*, 1997).

Assay of Superoxide Scavenging Activity

Superoxide was generated by xanthine (100 μmol/L) and xanthine oxidase (0.02 U/ml) with or without various concentrations of DHUA in 3 ml of 10 mmol/L KH_2PO_4-KOH buffer, pH 7.4, and was detected using nitroblue tetrazolium (100 μmol/L) and quantified spectrophotometrically at 550 nm (Wu *et al.*, 2000). SOD (1×10^5 U/L) and OA (500 μmol/L) were used as reference inhibitors.

Statistical Analysis

Differences among all groups were analyzed by one-way analysis of variance (ANOVA), followed by SNK-q-test. $p < 0.01$ was accepted as statistically significance.

Results

Effects of TCE on D-GalN-Induced Hepatotoxicity

The hepatoprotective effects of TCE on D-GalN-induced acute liver injury are shown in Table 1. Serum ALT activities in the D-GalN-treated group were elevated by 3.35 fold from the control group at 24 hours after D-GalN administration ($p < 0.01$). While treatment of TCE at a dose of 20, 50 or 100 mg/kg completely blocked the increase in serum ALT caused by D-GalN ($p < 0.01$).

Effects of TCE on the viability of D-GalN-insulted primary hepatocytes were shown in Fig. 2. The cell viability was significantly decreased by 45.0% after addition of 16.0 mg/ml D-GalN for 6 hours. However, it was effectively inhibited with the pretreatment of various concentrations of TCE (0.1, 0.5 and 1.0 mg/ml) in a dose-dependent manner (the relative coefficient was 0.925). Among them, cell viability of 1.0 mg/ml TCE group was increased by 77.1% compared with D-GalN group, it almost totally blocked the change induced by D-GalN.

In the supernatant of primary cultured hepatocytes, as shown in Table 2, administration of D-GalN for 6 hours resulted in a marked increase in AST activity, which was 1.96-fold compared to the control, and a 48.0% decrease in SOD activity. However, D-GalN-induced

Table 1. Protective Effects of TCE on the D-GalN-Induced Hepatotoxicity in Mice

Group	Dose (mg/kg)	ALT Activity (IU/L)
Control		312.4 ± 31.6
D-GalN	800	1048.3 ± 399.8*
D-GalN +TCE	800 20	314.3 ± 65.1+
D-GalN +TCE	800 50	365.1 ± 106.0+
D-GalN +TCE	800 100	369.1 ± 91.5+

Each value represents mean ± SD of eight mice. *$p < 0.01$, significantly different from the control group; +$p < 0.01$, significantly different from the D-GalN group.

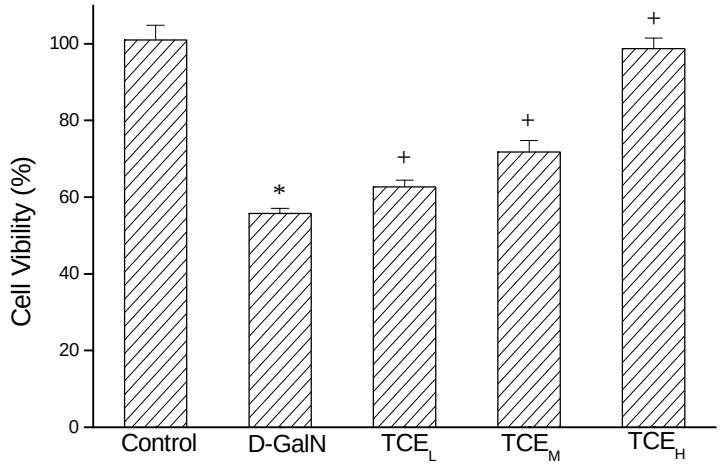

Figure 2. Effects of TCE on the survival rate of primary cultured hepatocytes insulted from D-GalN. TCE_L: 0.1 mg/ml TCE, TCE_M: 0.5 mg/ml TCE and TCE_H: 1.0 mg/ml TCE. Each value represents mean ± SD (n = 6). *$p < 0.01$, significantly different from the control group, +$p < 0.01$, significantly different from the D-GalN group (the relative coefficient was 0.925).

Table 2 Effects of TCE on AST and ALT Levels in the Supernatant of Primary Hepatocytes Insulted from D-GalN

Group	Dose (mg/ml)	AST Activity (IU/0.4 ml Cultured Medium)	SOD Activity (IU/0.1 ml Cultured Medium)
Control		9.43 ± 5.2	18.17 ± 15.9
D-GalN	16.0	18.52 ± 2.15*	9.59 ± 2.58*
D-GalN +TCE	16.0 0.1	10.12 ± 2.86+	17.08 ± 1.45+
D-GalN +TCE	16.0 0.5	9.20 ± 2.48+	18.09 ± 1.90+
D-GalN +TCE	16.0 1.0	9.62 ± 5.79+	18.88 ± 0.91+

Each value represents mean ± SD (n = 8). *$p < 0.01$, significantly different from the control group; +$p < 0.01$, significantly different from the D-GalN group.

damage was effectively inhibited by pretreatment of TCE at 0.1, 0.5 and 1.0 mg/ml for 36 hours. Moreover, the activities of AST and SOD in drug treatment groups were reversed to normal levels.

Protective Effects of DHUA against Ca^{2+}-Induced Mitochondrial Swelling

The swelling of liver mitochondria was induced when 50 μmol/L Ca^{2+} was added. DHUA (50, 150 and 500 μmol/L) would attenuate the swelling in a dose-dependent manner. The inhibitory rate of 150 μmol/L DHUA was similar to that of 500 μmol/L OA (33.93%), while 500 μmol/L DHUA had an inhibitory rate of 76.1%, which were greater than OA at the same concentration (Fig. 3).

Scavenging Activity of DHUA Against Superoxide Anions

The scavenging of superoxide anions generated from the xanthine/xanthine oxidase system *in vitro* was also evaluated. As shown in Fig. 4, treatment with DHUA showed superoxide scavenging activity in a dose-dependent manner (the relative coefficient was 0.970). And it was also found that the scavenging activity of DHUA was stronger than that of OA at the same concentration; 500 μmol/L DHUA scavenging rate (56.7%) was higher than that of OA (45.6%) at the same concentration, and even higher than that of SOD (45.4%).

Discussion

D-GalN is one of the most useful experimental hepatotoxins in screening and investigating hepatoprotective drugs. It is known that 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

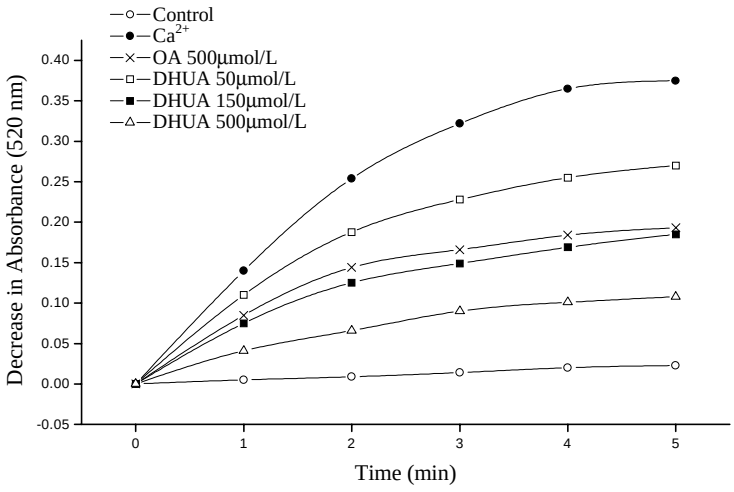

Figure 3. Protective effects of DHUA on Ca^{2+}-induced mitochondrial swelling. Mitochondrial swelling was assayed by measuring the decrease in absorbance at 520 nm. The assay mixture contained mitochondria (1 mg protein/mL), 125 mmol/L sucrose, 50 mmol/L KCl, 2 mmol/L KH_2PO_4, 5 mmol/L succinate, 50 μmol/L $CaCl_2$, 5 μmol/L rotenone and 10 mmol/L Hepes, pH 7.4 with or without various concentrations of DHUA. OA (500 μmol/L) was used as a reference. Swelling was induced by the addition of Ca^{2+}.

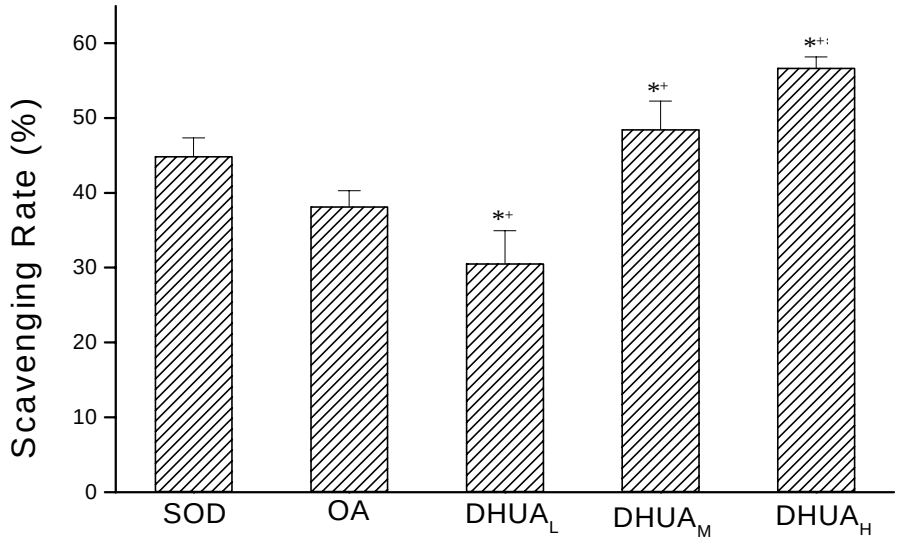

Figure 4. Scavenging activity of DHUA against superoxide anion. Superoxide was generated by xanthine (100 μmol/L) and xanthine oxidase (0.02 U/ml) with or without DHUA in 3 mL of 10 mmol/L KH_2PO_4-KOH buffer, pH 7.4, and was detected using nitroblue tetrazolium (100 μmol/L) and quantified spectrophotometrically at 550 nm. $DHUA_L$, $DHUA_M$ and $DHUA_H$ represents 50, 250 and 500 μmol/L DHUA, respectively. SOD (1×10^5 U/L) and 500 μmol/L OA were used as references. Each value represents mean ± SD (n = 6). $^*p < 0.01$, significantly different from the OA group. $^+p < 0.01$, significantly different from the SOD group.

to deterioration of the cellular membranes (Keppler *et al.*, 1970; Sabesin and Ragland, 1978) and resulting in disturbance in calcium homeostasis, inhibition of mitochondrial respiration (Miyahara *et al.*, 1982) and excessive generation of ROS. One of the most important endogenous antioxidants in hepatocytes (SOD) is exhausted and the SOD activity decreased. At the same time, the accumulation of ROS aggravates the damage in hepatocytes and mitochondria, which will result in the leak of AST and ALT (Elimadi *et al.*, 2001).

This study reveals the hepatoprotective effects of TCE against D-GalN injury in both *in vivo* and *in vitro* models. Our results indicate that the administration of TCE could effectively reverse the elevation of serum ALT activity in mice induced by D-GalN, which is in agreement with the studies of Lin *et al*. (1997 and 1998) on the protective effect of *Termimalia catappa* L. In primary cultured hepatocytes, it was found that after pretreatment of TCE, the decrease of MTT value and SOD activity, as well as the increase of AST activity induced by D-GalN were all prominently reversed to the same level as the control. The ALT enzyme is one of the indices for the degree of cell membrane damage, while AST is an index of mitochondrial damage, mitochondria contains 80% of the enzyme (Daba and Abdel-Rahman, 1998). MTT is known to represent the activity of mitochondrial succinate dehydrogenase (Slater *et al.*, 1963). Therefore, we could speculate from our results that TCE has a protective effect on both hepatocytes and their mitochondria.

Mitochondrial dysfunction has been shown to participate in the induction of apoptosis as well as in the excitotoxic cell death, which has been considered an important mechanism in liver injury (Pessayre *et al.*, 1999; Kaplowitz, 2000). In order to ascertain the mechanism of TCE's protection on mitochondria, a mitochondrial swelling test was performed. It was found that high-grade mitochondrial swelling induced by Ca^{2+} was effectively inhibited by different dosages of DHUA. This was in support of the previous result, i.e. TCE might have a direct protective effect on mitochondria.

On the other hand, ROS produced by metabolic processes have a variety of pathological effects and play an important role in the inflammation process (Liu *et al.*, 1997; Wu *et al.*, 2000). The increase in the level of ROS is known to induce the opening of mitochondrial permeability transition pores (PTP) in the mitochondrial membrane, which results in the inhibition of aerobic ATP synthesis, mitochondrial swelling and finally cell death (Elimadi *et al.*, 2001). A high ROS scavenging rate of DHUA also contributed to its strong protective effect on mitochondria.

With these results we can assume that, when mice were pretreated with TCE, D-GalN-induced excess of ROS such as superoxide anions were effectively scavenged. The decrease in ROS level may reduce the osmotic disbalance between mitochondrial matrix and the intermembrane space, and protect mitochondria against the excessive opening of PTP. Additionally, TCE can protect mitochondria against swelling induced by hepatotoxins. Thus, the integrity of the mitochondrial membrane was maintained and enzyme leakage was decreased. At the same time, ATP synthesis was recovered, and mitochondria and hepatocytes were finally sustained.

The results of this study suggest that TCE has strong hepatoprotective activity and the effective components may include DHUA. The mechanisms underlying its protective effects may be related to direct mitochondria protection and scavenging activity on ROS.

Acknowledgments

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