

Protective effect of *Lysimachia christinae* against acute alcohol-induced liver injury in mice

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Summary

Lysimachia christinae Hance (Primulaceae) is a medicinal plant. The present study was undertaken to investigate protection of *L. christinae* against acute alcohol-induced liver injury in mice, the related mechanism of oxidative stress and its hepatoprotective chemical compound for the first time. Mice were orally administered alcohol at 6 g/kg 2 h after a 75% ethanol extract of *L. christinae* (ET) (100, 200, 400 mg/kg), quercetin (2, 4, 8 mg/kg) isolated from *L. christinae*, or bifendate (150 mg/kg) for seven consecutive days by intragastric administration (*i.g.*) except the normal group. Serum and liver tissue samples were collected 6 h after alcohol administration and the amount of quercetin in ET was analyzed by high-performance liquid chromatography (HPLC) with a diode array detector (DAD). The results showed that alcohol-induced elevated serum alanine transferase (ALT) and aspartate transaminase (AST) activities were significantly reduced by ET (200, 400 mg/kg), quercetin (4, 8 mg/kg) and bifendate (150 mg/kg), respectively. Further analysis demonstrated that lipid peroxidation (LPO) levels significantly decreased, while glutathione amounts, glutathione-s transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) activities all increased in livers of ET-, quercetin-, and bifendate-treated mice. Besides, amount of quercetin in ET was 1.03%. Taken together, our results indicate that *L. christinae* can protect against acute alcohol-induced liver injury in mice, the potential mechanism can be related to inhibiting liver oxidative stress injury, and its main hepatoprotective compound is quercetin, for the first time.

Keywords: Ethanol extract, quercetin, hepatoprotective compound, oxidative stress

1. Introduction

Alcohol abuse is one of the main causes of liver disease worldwide and has become a social problem (1). Due to the increased frequency of drinking, incidence of alcoholic liver disease has increased in China, becoming another important risk factor for morbidity and mortality

in addition to viral hepatitis (2). However, there is no satisfactory therapy for alcoholic liver disease at present except for the combination of abstinence from alcohol and supportive care (3).

There is increasing evidence that oxidative stress plays a vital role in pathogenesis of alcoholic liver disease (4-7). During alcohol-induced oxidative stress, reactive oxygen species (ROS) are produced and it is extremely reactive. Such ROS may modify and inactivate lipids, proteins, DNA, and RNA, and thus induce cell dysfunction. To inhibit ROS-induced cell injury, the antioxidant system has been generated in the body. The system includes low-molecular-mass antioxidants such as glutathione, alpha-tocopherol, ascorbic acid and the main antioxidant enzymes such

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as, superoxide dismutase (SOD) and catalase (CAT) (8). Generally cellular antioxidants maintain a point of balance between oxidants and antioxidants, and thus prevent the organism from injury induced by ROS.

Lysimachia christinae Hance (Primulaceae), commonly known as "Jinqiancao", is distributed worldwide in temperate climates and is widely found in China (9,10). It is one of the herbs commonly used in traditional Chinese medicine for treatment of hepatitis virus, cholecystitis and cholagogic efficiency (11,12). A recent study has demonstrated that the water extract of *L. christinae* exhibits a marked anticholecystitis and cholagogic activity in animals (13). However, as for its hepatoprotective effect, there is no experimental report up until now. Furthermore, whether the potentially protective effect underlying *L. christinae* against alcohol-induced liver injury is related to the liver antioxidant system has not been known.

The present study was designed to observe the protective effect of *L. christinae* against acute alcohol-induced liver injury, the related mechanism on oxidative stress, and further explore its hepatoprotective chemical compound, for the first time.

2. Materials and Methods

2.1. Animals

Kunming (KM) male mice (18-22 g) were purchased from the Experimental Animal Center of Hebei Province (Hebei, China). Animals were given rodent laboratory chow and water *ad libitum*, and maintained under controlled conditions with a temperature of $22 \pm 1^\circ\text{C}$, relative humidity $60 \pm 10\%$ and a 12/12 h light/dark cycle (lights on at 7 am). All procedures were in strict accordance with china's legislation on using and caring for laboratory animals and with guidelines established by institute for experimental animals of Henan University of Traditional Chinese Medicine and were approved by the university committee for animal experiments.

2.2. Reagents

Bifendate (powdered pill suspended in 0.5% CMC-Na) was purchased from Zhejiang Medicine Co., Ltd., Xinchang Pharmaceutical Factory (Xinchang, China). Bradford protein assay kit was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Reduced glutathione (GSH), oxidized glutathione (GSSG), and NADPH were purchased from Roche Diagnostics GmbH (Mannheim, German).

2.3. Plant material and preparation of samples

L. christinae whole herbs were collected in Henan province and authenticated by Dr. Xiaolong Xie

(Pharmacognosy Department, Henan University of Traditional Chinese Medicine, Zhengzhou, China). A voucher specimen (JQC110207) was deposited in the herbarium of School of Pharmacy, Henan University of Traditional Chinese Medicine.

The dried herbs were crushed and preparation of the 75% ethanol extract of *L. christinae* (ET) and quercetin are described as follows.

L. christinae powders (about 1 kg) were soaked in 75% ethanol (w/v = 1:10) and incubated at room temperature for 120 min. The mixture was extracted three consecutive times at $85 \pm 5^\circ\text{C}$ with a rotary evaporator 180 min at a time. The combined extraction was centrifuged at $800 \times g$ for 10 min, and the supernatant was transferred to a glass container by decanting and concentrated under vacuum with a rotary evaporator under reduced pressure at $45 \pm 5^\circ\text{C}$ to about 86 g extracts. The yield of ET was 8.6% from *L. christinae* raw medicinal materials.

Quercetin was isolated from *L. christinae* herbs according to previous literature (14). After purification using a silica gel column and gel chromatography, the purity of quercetin was more than 98% as determined by high performance liquid chromatography (HPLC) with diode array detector (DAD).

2.4. Treatment protocol

Male mice were divided into several groups of 10 mice each. Mice of treated groups were administered orally with 6 g/kg alcohol 2 h after treatment with ET (100, 200, 400 mg/kg), quercetin (2, 4, 8 mg/kg), or the positive drug bifendate (150 mg/kg) everyday for seven consecutive days by intragastric administration (*i.g.*) except mice in the normal (non-alcohol treated) group. Mice in the normal and control (alcohol alone) groups both received daily oral administration (*p.o.*) of 0.5% CMC-Na (0.2 mL/10 g). Peripheral blood samples of groups were collected 6 h after alcohol administration for determination of serum biomarkers for the protective effect against acute alcohol-induced liver injury, and liver tissues for assay mechanism.

2.5. Assay for detecting serum biomarkers for liver injury

The blood samples were obtained from mice of all groups (10 mice per group) for the determination of serum biomarkers for liver injury. The serum alanine amino transferase (ALT) and aspartate amino transferase (AST) were assayed according to the reported methods (15).

2.6. Assay for detecting liver lipid peroxide (LPO) level

Liver tissues were homogenized in cold phosphoric acid (PBS). LPO was determined by the previous reported

method (16). Malondialdehyde (MDA) was formed as an end product of LPO and served as an index of the intensity of LPO. MDA reacts with total bile acids (TBA) to generate a pink colored product, which has an absorbance at 532 nm. The standard curve for MDA was constructed over the concentration range of 0-40 μ M. The level of lipid peroxides was expressed as micromoles of MDA per milligram of protein based on tissue protein concentration measured by the Bradford protein assay kit.

2.7. Assay for detecting liver glutathione amounts

Glutathione amount was measured immediately as described in the previous study (17). The reaction mixture contained samples, 150 μ L of a working solution (0.53 U/mL of glutathione reductase, 40.7 μ g/mL of DTNB, 1 mM EDTA in 100 mM sodium phosphate buffer, pH 7.0) and 50 μ L of 0.16 mg/mL NADPH solution. The change in absorbance was determined at 412 nm against the reagent blank after standing at room temperature for 30 min and glutathione amounts were determined in comparison with a standard curve. The glutathione amounts of mice livers were calculated based on tissue protein concentration measured by the Bradford protein assay kit.

2.8. Enzymatic assays

Tissues were homogenized in cold PBS, and centrifuged at $5,000 \times g$ for 5 min and the supernatant was transferred to new tubes for assay. The liver tissue activities of SOD, CAT, GPx, and GST were determined by previous literature method (18-21), respectively, and the results were calculated based on tissue protein concentrations measured by the Bradford protein assay kit.

2.9. Assay for detecting the amount of quercetin in ET

Amount of quercetin in ET was measured by HPLC-DAD. The chromatography conditions were used as follows.

Analysis was performed on a Prominence HPLC instrument (Agilent 1200 series) equipped with quaternary pump, DAD, on-line degasser, autosampler, and a column heater compartment. The sample was separated on a Welch Materials XDB-C18 column (4.6×200 mm, 5 μ m). The mobile phase consisted of methanol and water containing 0.4% (v/v) phosphoric acid with isocratic elution (v/v = 52:48). The flow rate was 0.8 mL/min, and column temperature was set at 25°C. The DAD detector was monitored in the range 200-400 nm, and the on-line UV spectra were recorded at 360 nm.

To obtain a calibration curve of quercetin, purified quercetin (6.13 mg, weighed accurately) was dissolved

in 80% methanol in a 10 mL low actinic volumetric flask. This solution was diluted with 80% methanol to obtain standard solutions for the calibration curve in a range of 1.838-29.408 μ g/mL on the column, and a 10 μ L aliquot was injected. ET was dissolved in 80% methanol and an aliquot (10 μ L) was injected into the above HPLC-DAD system. The amount of quercetin in ET was calculated using the calibration curve of quercetin.

2.10. Statistical analysis

Data is presented as mean \pm standard error of mean (S.E.M.). The differences among experimental groups were compared by one-way ANOVA (analysis of variance) followed by Student Newman Keuls (SNK) ($p < 0.05$) using the SPSS (Statistics Package for Social Science) program Version 11.5.

3. Results

3.1. Effects of ET and quercetin on serum biomarkers for alcohol-induced liver injury

Serum ALT and AST activities are liver injury biomarkers and their significant elevation often reflects liver injury (15). In the present study, ALT and AST were both found to be elevated significantly in mice treated with alcohol alone (Figures 1A and 1B), demonstrating that acute alcohol-induced liver injury was copied successfully in this study. After ig treatment with ET (200, 400 mg/kg), quercetin (4, 8 mg/kg) and bifendate (150 mg/kg), respectively, for seven consecutive days, such an excessive increase was significantly inhibited (Figures 1B and 1C). These results demonstrate that ET, quercetin isolated from *L. christinae* and bifendate all protect against acute alcohol-induced liver injury.

3.2. Effects of ET and quercetin on liver LPO level

MDA is one of the main end products of LPO (16). As shown in Figure 2A, MDA amounts increased in livers of mice treated with alcohol alone while ET (200, 400 mg/kg), quercetin (4, 8 mg/kg) and bifendate (150 mg/kg) all inhibited such an excessive increase (Figure 2A), which demonstrated that ET, quercetin, and bifendate could protect against alcohol-induced LPO injury in mice.

3.3. Effects of ET and quercetin on liver glutathione amounts

Glutathione is an antioxidant which helps protect cells against ROS such as free radicals and peroxides (17). Its excessive exhaustion can induce oxidative stress injury. In the present study, glutathione amounts decreased conspicuously ($p < 0.05$) in alcohol-treated

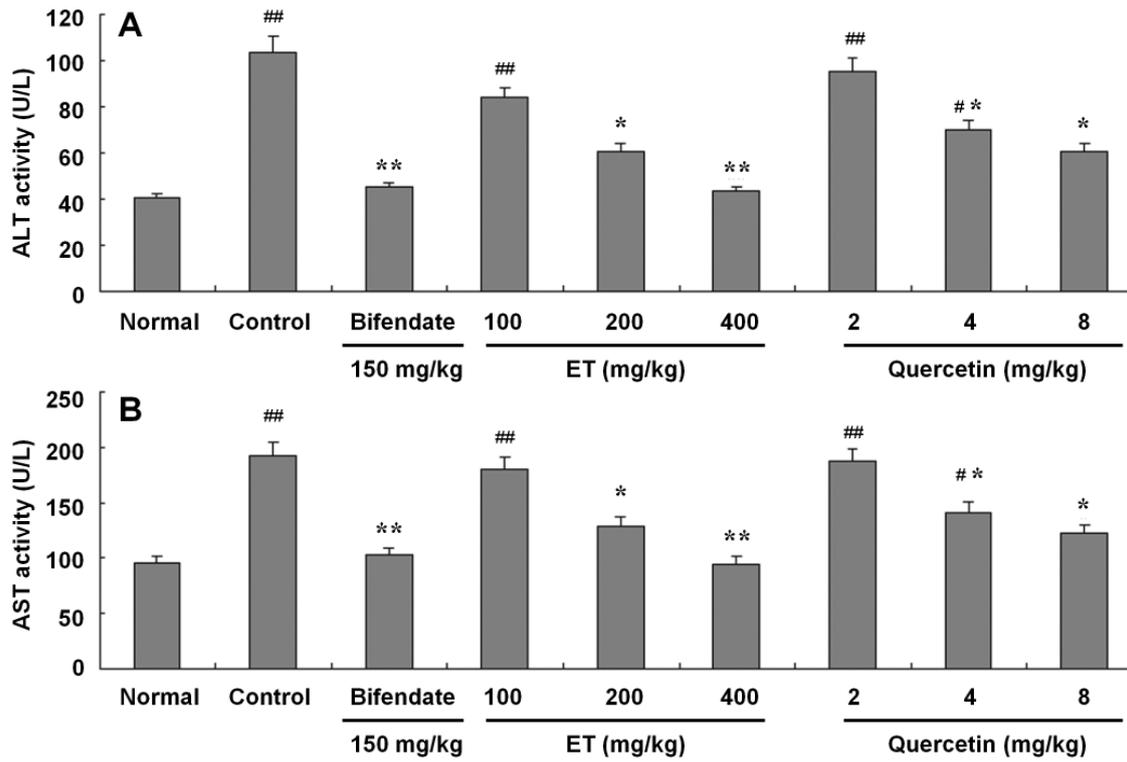


Figure 1. Effects of ET and quercetin isolated from *L. christinae* on the activities of serum ALT and AST in mice. Data are presented as mean ± S.E.M. (n = 10). Significant differences compared with normal (non-alcohol treated) group were designated as [#]p < 0.05 and ^{##}p < 0.01 and with control (alcohol alone) as ^{*}p < 0.05 and ^{**}p < 0.01.

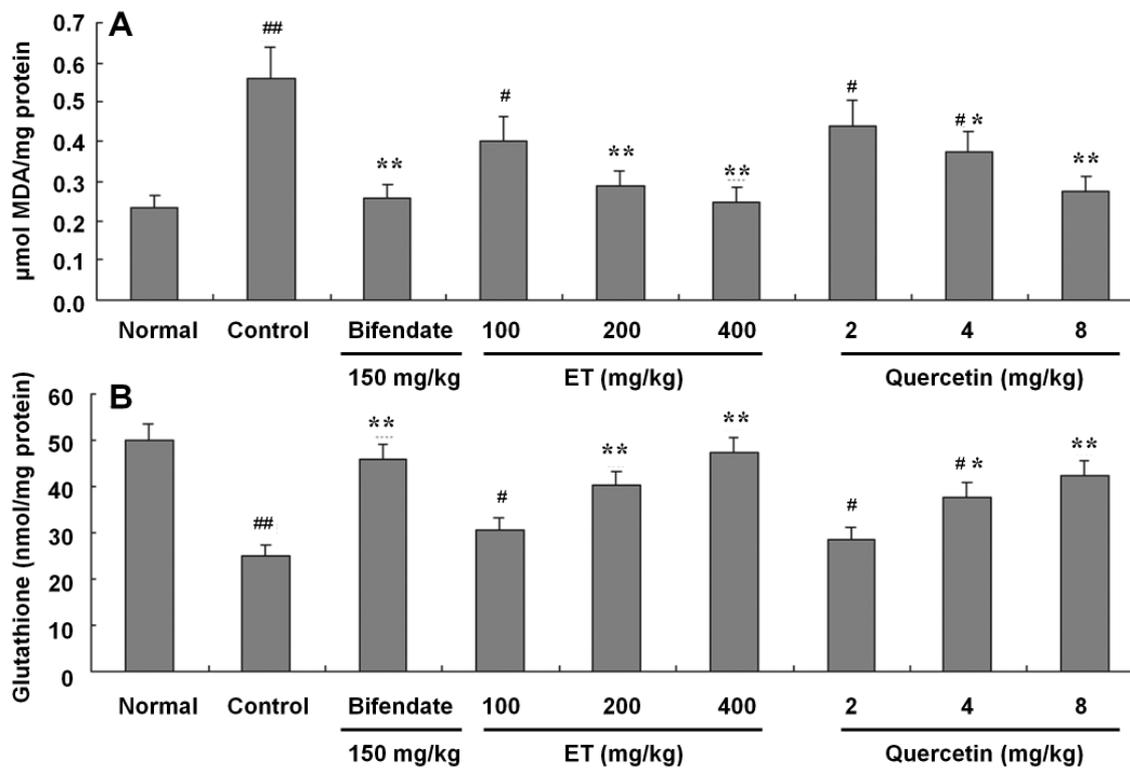


Figure 2. Effects of ET and quercetin isolated from *L. christinae* on the MDA level and glutathione amounts in mice liver tissues. Data are presented as mean ± S.E.M. (n = 10). Significant differences compared with normal (non-alcohol treated) group were designated as [#]p < 0.05 and ^{##}p < 0.01 and with control (alcohol alone) as ^{*}p < 0.05 and ^{**}p < 0.01.

mice liver, while ET (200, 400 mg/kg), quercetin (4, 8 mg/kg) and bifendate (150 mg/kg) all significantly prevented such a decrease (Figure 2B). The results suggested that ET, quercetin, and bifendate can protect against alcohol destroying the balance between cellular oxidants and antioxidants through inhibiting exhaustion of glutathione amounts and thus can likely protect against liver oxidative stress injury.

3.4. Effects of ET and quercetin on liver glutathione-related enzymes activities

GST and GPx are both intracellular glutathione-related enzymes, working with glutathione in participation in the process of oxidative stress injury (20,21). Our results showed that alcohol decreased the activities of GST and GPx in mice livers while ET (200, 400 mg/kg), quercetin (8 mg/kg) and bifendate (150 mg/kg) all inhibited such an obvious decrease (Figures 3A and 3B), demonstrating that the glutathione-related enzymes could participate in the protection of ET, quercetin, and bifendate against alcohol-induced oxidative stress injury. Our results further confirmed the balance between cellular oxidants and antioxidants was destroyed by alcohol, while ET, quercetin and bifendate could prevent such damage of this balance.

3.5. Effects of ET and quercetin on liver main antioxidant enzyme activities

SOD and CAT are both intracellular main antioxidant

enzymes, participating in the process of oxidative stress (18,19). Our results showed that alcohol decreased the activities of SOD and CAT in livers of mice while ET (200, 400 mg/kg), quercetin (8 mg/kg) and bifendate (150 mg/kg) all inhibited such an obvious decrease (Figures 4A and 4B). These results indicate that the main antioxidant enzymes might play a key role in the protection of ET, quercetin, and bifendate against the oxidative stress injury induced by alcohol.

3.6. Analysis of whether quercetin is the main hepatoprotective compound in ET

The amount of quercetin in ET was 1.03% as shown by HPLC-DAD analysis. The chemical structure of quercetin is shown in Figure 5A. The standard curve equation of quercetin was $y = 14.634x - 0.3958$ (y : Area; x : concentration of quercetin, $\mu\text{g/mL}$) and the correlation coefficient was $R^2 = 1$. HPLC chromatograms of quercetin and ET are shown in Figures 5B and 5C, respectively.

Further to confirm that quercetin was the main hepatoprotective compound in ET, we converted the hepatoprotective doses of ET into quercetin, and then compared these converted doses with actual ones of quercetin. According to the above amount of quercetin in ET, the effective doses of ET, 200 and 400 mg/kg, are equivalent to the ones of quercetin, 2.06 and 4.12 mg/kg (4 mg/kg as the actually effective dose of quercetin from this study).

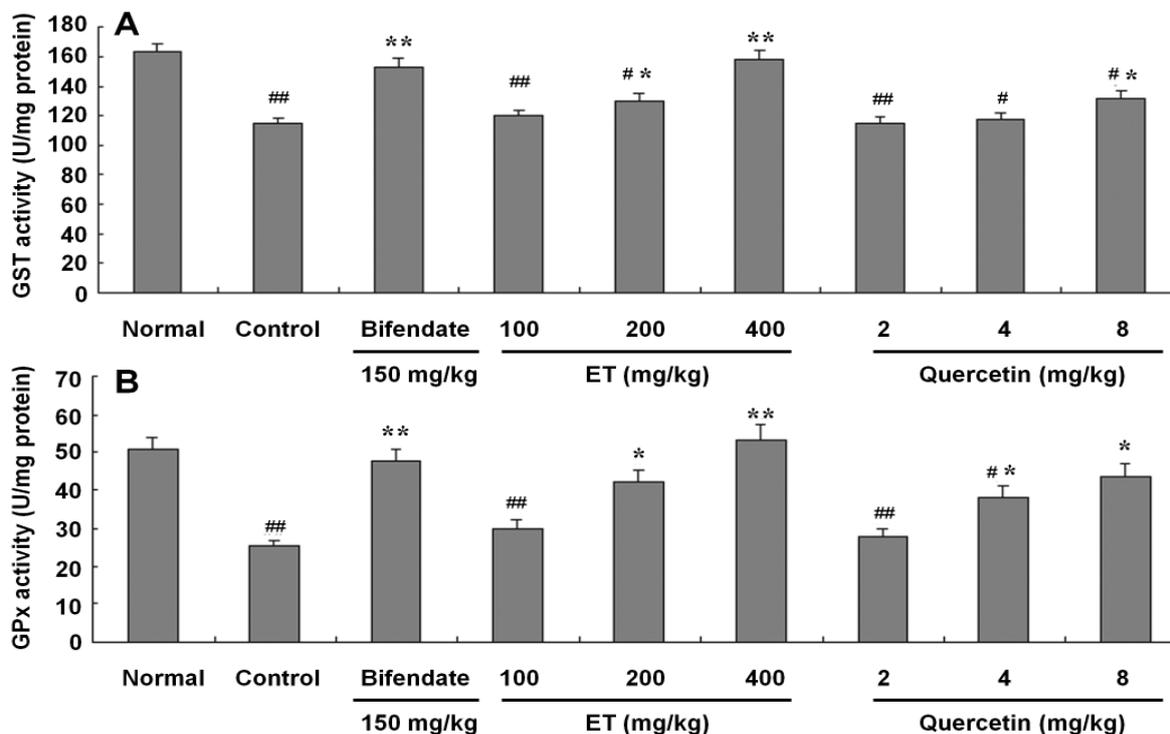


Figure 3. Effects of ET and quercetin isolated from *L. christinae* on liver GST and GPx activities. Data are presented as mean \pm S.E.M. ($n = 10$). Significant differences compared with normal (non-alcohol treated) group were designated as $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ and with control (alcohol alone) as $^*p < 0.05$ and $^{**}p < 0.01$.

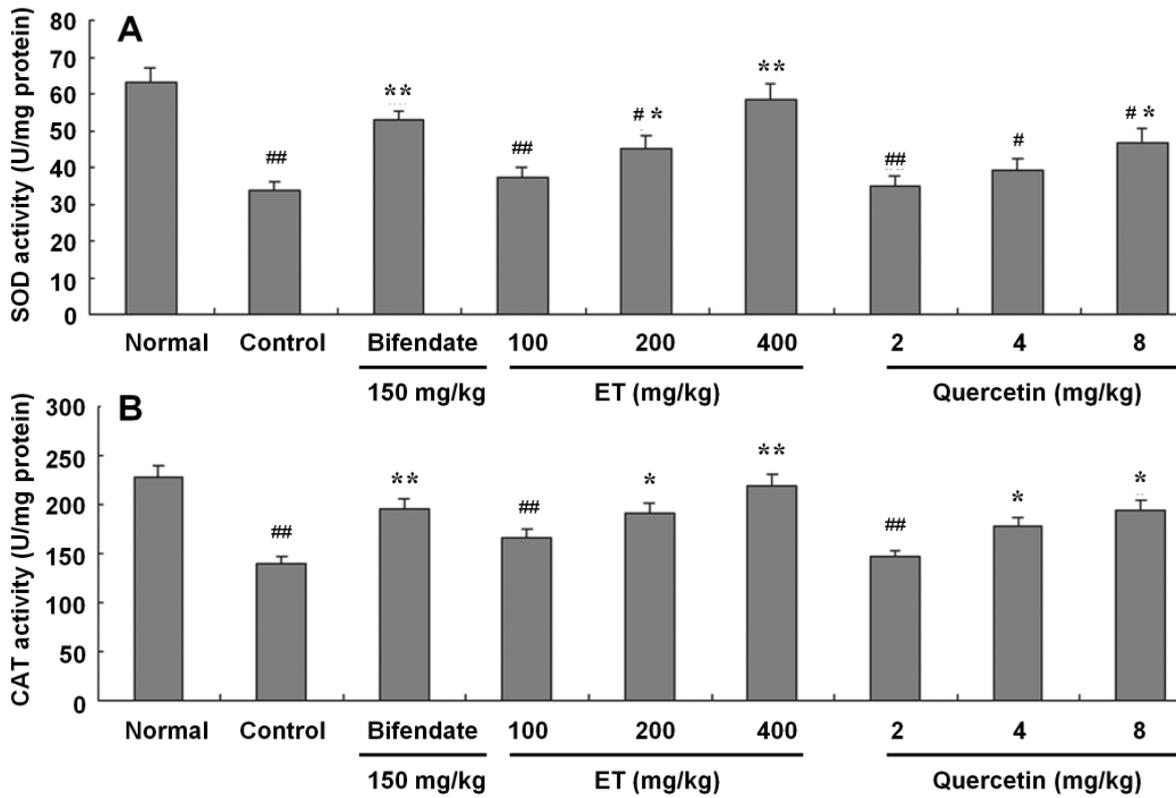


Figure 4. Effects of ET and quercetin isolated from *L. christinae* on liver main antioxidant enzyme activities. Data are presented as mean \pm S.E.M. ($n = 10$). Significant differences compared with normal (non-alcohol treated) group were designated as [#] $p < 0.05$ and ^{##} $p < 0.01$ and with control (alcohol alone) as ^{*} $p < 0.05$ and ^{**} $p < 0.01$.

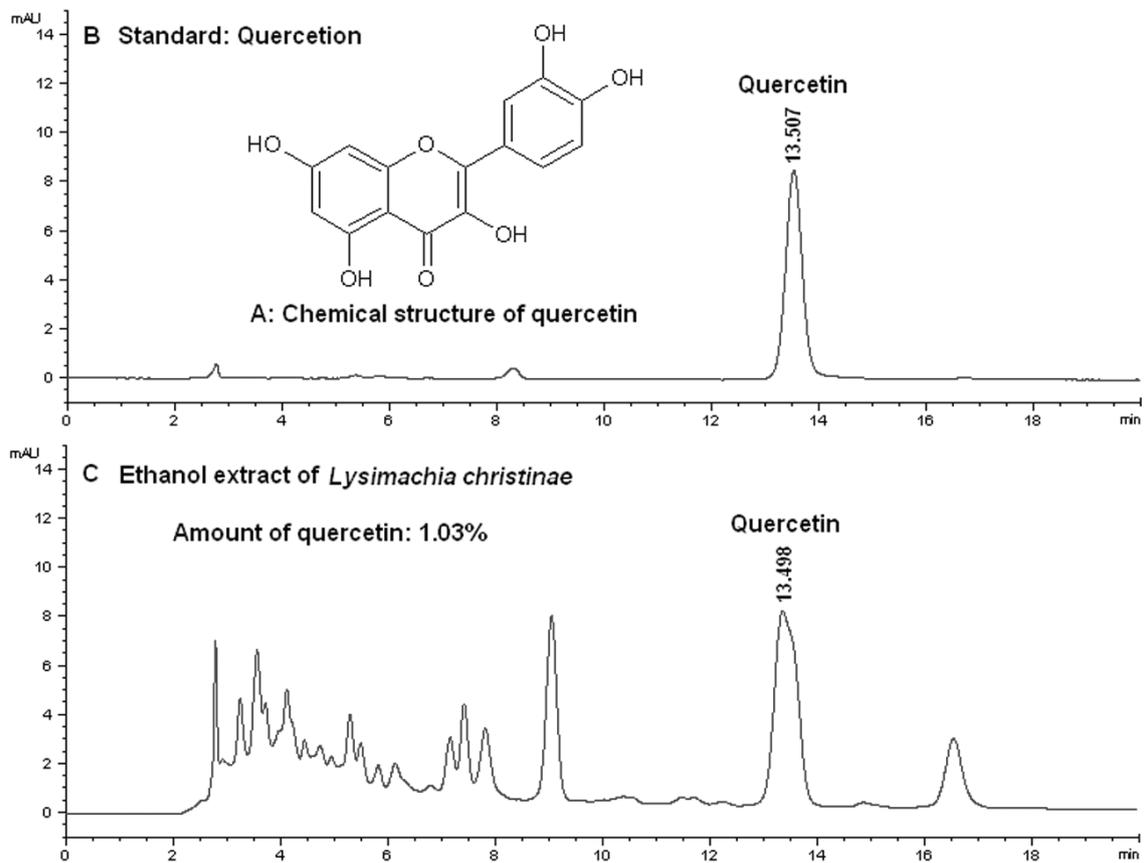


Figure 5. Chemical structure of quercetin, and HPLC chromatograms of ET and quercetin isolated from *L. christinae*. HPLC-DAD procedures are described in "Materials and Methods".

4. Discussion

ALT and AST are reliable biomarkers for liver function (15). It has been confirmed that AST can be found in liver, cardiac muscle, skeletal muscle, kidney, brain, pancreas, lung, leukocytes, and erythrocytes, whereas ALT is mainly present in liver (22). Significantly elevated levels of serum enzymes such as ALT and AST indicate increased permeability and damage and/or necrosis of hepatocytes (23). The results of the present study demonstrate that acute alcohol administration caused liver injury as evidenced by the elevation of serum ALT and AST levels, reflected early biochemical changes in alcoholic liver disease. ET (200, 400 mg/kg), or quercetin (4, 8 mg/kg) pretreatments offered significant protection against acute alcohol-intoxicated mice by attenuating ALT and AST elevation.

The role of oxidative stress in the development of alcoholic liver disease has been investigated since the early 1960s by Diluzio and Hartman (24,25), who observed that alcohol administration promoted oxidative breakdown of cell membranes. Studies using the intragastric feeding model have demonstrated that alcohol-induced liver injury was associated with increased LPO, formation of lipid radicals, and decrease of hepatic antioxidant defense, providing the most convincing evidence about the pathogenic role of oxidative stress (26-29). Among them, LPO is a free radical-related process (30). One of the main end products of LPO is MDA, which is characterized by cross-linking cellular macromolecules such as protein or DNA and induces widespread cellular damage (31). The results in Figure 2A showed that ET and quercetin isolated from *L. christinae* significantly inhibited alcohol-induced excessive amounts of MDA, indicating that ET and quercetin can prevent alcohol-induced LPO injury in liver.

SOD and CAT are believed to play key roles in the enzymatic defense of cells against oxidative stress injury. Because peroxisomes have an abundance of proteins, where oxidative stress always happens, CAT is a classical oxidative biomarker. SOD is a metalloenzyme that can convert O_2 , produced during oxidative stress, to hydrogen peroxide (18). CAT mainly exists in peroxisomes of all aerobic cells and serves to protect the cells against damage from hydrogen peroxide by catalyzing it into molecular oxygen and water without producing toxic free radicals (19). Our results showed that ET and quercetin significantly reversed SOD and CAT activities reduced by alcohol, suggesting that they could prevent alcohol-induced oxidative stress injury, while SOD and CAT participated in the protective effect of ET and quercetin against alcohol-induced liver oxidative injury.

Glutathione plays an important role in protecting hepatocytes against exogenous toxins, and there are lots of reports that depletion of cellular glutathione is

related to oxidative damage (32,33). Our results showed that ET and quercetin isolated from *L. christinae* significantly inhibited the alcohol-induced excessive exhaustion of glutathione amounts, suggesting that glutathione participated in the protective effect of ET and quercetin against alcohol-induced liver oxidative injury.

GST and GPx are both glutathione-related enzymes. Of them, the cytosolic GSTs exist in almost all aerobic species. It can catalyze the conjugation of electrophilic compounds produced during oxidative stress with glutathione (21). GPx catalyzes hydrogen peroxide decomposition to the stable form of hydroxides, specifically using reduced glutathione as the electron provider (20). In the present study, ET and quercetin significantly elevated the alcohol-induced decrease of GST and GPx activities in livers of mice, which further confirmed that liver glutathione-related enzymes were involved in the protective effects of ET and quercetin isolated from *L. christinae* against alcohol-induced liver oxidative injury.

Flavonoids have been reported to have many bioactivities including antioxidant (34), anti-inflammatory (35), antitumor (36) and so on. The main chemical components in *L. christinae* are flavonoids (37). As for the flavonoids in *L. christinae*, quercetin is relatively abundant (37) and has been found to have antioxidant activity in the previous study (38). However, it was still not clear until now that quercetin is the main hepatoprotective chemical compound of *L. christinae*. In the present study, we found that quercetin could protect against acute alcohol-induced liver injury isolated from *L. christinae* (as was shown in Figure 1B). Further, to determine whether quercetin was the main bioactive ingredient in ET, we converted the hepatoprotective doses of ET into quercetin based on the results of HPLC-DAD analysis. The result indicated that the converted amounts of quercetin were equivalent to the actual ones present in ET, suggesting that quercetin could be the main potential hepatoprotective compound from *L. christinae*.

In conclusion, the present study shows that ET and quercetin isolated from *L. christinae* can protect against alcohol-induced liver injury in mice and the underlying mechanisms may be related to inhibiting liver oxidative stress injury. We have also confirmed that quercetin is the major hepatoprotective component of *L. christinae* for the first time. Further studies are in progress in our laboratory to explore the protection of *L. christinae* against carbon tetrachloride- or drug-induced liver injury.

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