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Research Article

Hepatoprotective Activity of *Lophatherum gracile* Leaves of Ethanol Extracts Against Carbon Tetrachloride-induced Liver Damage in Mice

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Abstract

The ethanol extract of *L. gracile* leaves was screened for its ability of protection against carbon tetrachloride (0.3 mL kg^{-1} i.p.) induced hepatotoxicity on mice at the doses of 200 and 800 mg kg^{-1} b.wt. Fifty mice were divided into five groups (n = 10), group (Con): Normal control, group (CCl_4): CCl_4 (0.3 mL kg^{-1}), group (EEL): CCl_4+200 mg kg^{-1} b.wt., EE, group (EEH): CCl_4+800 mg kg^{-1} b.wt., EE, group (EE): 800 mg kg^{-1} b.wt., EE. Treatment duration was 15 consecutive days by gavage and the dose of CCl_4 was administered intraperitoneally (i.p.) to group (CCl_4), (EEL) and (EEH) after the experimental period. The results obtained showed that the pretreatment of mice with different doses of EE significantly decreased the serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL) and total cholesterol (TC) in a dose-dependent manner. Two doses of EE pretreatment also prevented the increase in MDA level and the reduce in abilities of SOD, CAT and GPX in the liver tissues of CCl_4 poisoned mice at different extent. A 800 mg kg^{-1} dose of EE pretreatment has little effect on the all observed biomarkers in normal mice. The histopathological examination also confirmed the hepatoprotective effect of EE. The present study adds to biological evidence that *L. gracile* leaves possess significant hepatoprotective and antioxidant activity.

Key words: Carbon tetrachloride, hepatotoxicity, antioxidant, *Lophatherum gracile*, ethanol extract

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Liver damage has become a cosmopolitan health problem which may develop into liver diseases with high morbidity. Liver is easily injured because it is a pivotal organ participating in the biotransformation and metabolism of xenobiotics. Carbon tetrachloride (CCl_4) is a typical hepatotoxin widely used for many years to induce acute/chronic hepatotoxicity in experimental studies for imitating the processes of hepatic damage in human (Guicciardi and Gores, 2005; Maling *et al*., 1974). The CCl_4 administration triggers the biotransformation of CCl_4 to highly reactive free radicals via the mixed function cytochrome P450 system (Weber *et al*., 2003) followed by a serious harmful reactions, such as accumulation of Reactive Oxygen Species (ROS), restraining free radical scavengers, causing lipid peroxidation as well as DNA damage (Khan *et al*., 2015).

The species *Lophatherum gracile* (*L. gracile*) belongs to the family of Gramineae and is widely distributed in Southern China. The leaves of this plant (LGL) popularly known as "Dan Zhu Ye" are used as a traditional Chinese herbal medicine for the curative effect of inflammation and fever. The LGL was also usually consumed for some herbal teas or soup cooking with other edible plants in China. In recent years, a wide range of pharmacological effects of LGL has been investigated, such as antiviral activity, vasorelaxation effect, antitumor and hyperglycemic activities (Kim *et al*., 2010). Currently, it has been revealed that LGL contains some antioxidant substances like phenolic acids, flavonoids, terpenoid, polyose and many other constituents (Shao *et al*., 2011). Among the identified functional components, flavonoids have attracted the most attention. For example, phytochemical screening showed two coumarins and eight flavonoids in *Lophatherum gracile* (Tang *et al*., 2015). Several flavonoids were identified from herba Lophatheri, namely isoorientin, orientin, swertisin, vitexin and luteolin-7-O-b-D-gl (Shao *et al*., 2014b). Wang *et al*. (2012) identified four new flavone C-glycosides, luteolin and apigenin were isolated from the leaves of *Lophatherum gracile* (Wang *et al*., 2012). Additionally, it was reported that ethanol extracts from *L. gracile* leaves with a high amount of total flavonoids showed good antioxidant activities confirmed by DPPH and ABTS *in vitro* methodologies (Shao *et al*., 2014a).

Although, the details mechanisms for the hepatoprotective activity of plant are not fully understood, it is accepted that inhibiting the generation of free radicals is considerable for protecting against CCl_4-induced hepatic damage (Gyamfi *et al*., 1999; Hsiao *et al*., 2003). In view of this based on the fact that LGL and its extracts have been reported to contain rich antioxidants and exhibit obvious free radical scavenging activities *in vitro*, it is easy to speculate that LGL would prevent against CCl_4-induced hepatotoxicity. However, no comprehensive study has so far been reported on the hepatoprotective activity of LGL until now. Therefore, this study was carried out to examine whether or not the flavonoids-rich extract obtained from LGL has any hepatoprotective activity with the aim to develop a new effective plant source for functional foods.

MATERIALS AND METHODS

Ethanol extract of *L. gracile* leaves: The herbs of LGL were bought from local market, Tianjin. The samples were completely dried and pulverized with an electric grinder and stored. The powered LGL was extracted exhaustively with ethanol (95%) at room temperature for 72 h. The obtained extract was concentrated under vacuum using a rotary vacuum evaporator (RE-2000A, Shengye Inc, Shanghai, China) at 50°C. The residues were lyophilized using a freeze drier (YB-FD-1, Yibei Inc, Shanghai, China) to obtain the ethanol extracts of *L. gracile* leaves (EE). The final samples were stored at -20°C until further use.

Animals: Fifty Kunming male mice (20-22 g) were provided by Academy of Military Medical Sciences. (Beijin, China). The animals were grouped and housed under standard laboratory conditions (23±2°C, 50% relative humidity and 12 h light/dark cycle). A standard commercial pellet diet and water were available *ad libitum*. All experimental procedures were strictly conducted in accordance with the Principles of Laboratory Animal Care and Use in Research stipulated by the Ministry of Health of China.

Experimental design: After acclimatizing for 3 days on the basal diet, mice were randomly divided into five groups of 10 animals each. Group (Con) was the normal control group and the mice received olive oil by gavage once a day from 1st-5th day. Group (CCl_4) was the CCl_4 control group. The mice in this group received olive oil by gavage once a day from 1st-5th day, then a single intraperitoneal injection of 0.3% CCl_4 (v/v in olive oil) at 0.1 mL/10 g b.wt., to induce hepatic damage after 12 h of the olive oil treatment on 5th day of study. Group (EEL) and Group (EEH) were EE pretreated group. All mice in group EEL and group EEH received a daily dose EE dissolved in olive oil (200 and 800 mg kg^{-1} b.wt., respectively) by gavage from 1st-5th day, then a single intraperitoneal injection of 0.3% CCl_4 at 0.1 mL/10 g b.wt., after 12 h of EE treatment on 5th day. All of

the mice were sacrificed under ether anesthesia after CCl_4 treatment for 12 h. The blood samples were collected and centrifuged (3000 rpm at 4°C for 15 min) to obtain serum. The serum samples were collected and stored at -20°C until further analysis. The fresh liver tissues were excised, blotted, weighed, stored at -80°C for further experiment research and thawed before use.

Hepatocellular injury assay: Serum samples were analyzed for alanine aminotransferase (ALT), aspartate amino transferase (AST), total bilirubin (TBIL), total cholesterol (TC) and total triglyceride (TG). All of the commercial kits were provided by BioSino Bio-technology and Science Inc, Beijing, China. The serum samples were analyzed on a Glamour 3000 Automatic Analyzer according to the instructions of the test kits.

Antioxidative enzyme activity in hepatocytes: The malondialdehyde (MDA) level and the enzymatic activities of superoxide dismutase (SOD), catalase (CAT) as well as glutathione peroxidase (GPX) were measured according to the commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing City, China). Total protein concentration was determined as described in the Bradford method (Bradford, 1976) with Bovine Serum Albumin (BSA) as the standard. The MDA concentration was expressed as nmol mg^{-1} protein and the enzymatic activities of SOD, GPX and CAT were expressed as U mg^{-1} protein.

Histological examination: The liver samples were collected and fixed in 10% neutral buffered formaldehyde, washed with running tap water for 30 min, dehydrated in gradual ethanol (50-99%), cleared in xylene and then embedded in paraffin to prepare 5 μm coronal sections using a Leica Microsystem microtome (Model RM 2235, Germany). Sections were deparaffinized with xylene and rehydrated using a graduated alcohol series (100-50%), then stained with hematoxylin and eosin (H and E) using a Leica Microsystem auto strainer (XL, Germany). The slides were viewed under an Olympus microscope (4X-1, Japan) for the observation of structural abnormality.

Statistical analysis: All Data were expressed as Mean±Standard Error of Mean (SEM). Statistical analysis was carried out using the SPSS version 13.0. Differences among groups were evaluated by one-way analysis of variance (ANOVA) followed by Duncan *post-hoc* test. Values with $p<0.05$ are considered as statistically significant.

RESULTS

Effects of EE on serum biochemical parameter: The levels of ALT and AST were significantly increased in the CCl_4-treated group compared with the control group ($p<0.05$). Pretreatment with EE (200 and 800 mg kg^{-1}) obviously decreased the activities of serum ALT and AST (Fig. 1). The serum levels of TG, TC and TBIL in CCl_4 group were substantially increased compared to those of the control group. Whereas, EE pretreatment (particularly at the dose of 800 mg kg^{-1}) significantly suppressed the increase in levels of TC and TBIL compared to that for the CCl_4 Control group (Fig. 2). Both of EE pretreated groups showed no significant difference in TC value as compared with the CCl_4 control group. The EE treatment alone has no effects on the observed serum biochemical parameters as compared with mice in normal control group.

Effects of EE on the mda levels in liver homogenates: The MDA was employed to estimate lipid peroxidation in liver issues. Figure 3 shows that CCl_4 administration significantly increases the MDA level ($p<0.05$), indicating the lipid peroxidation in liver tissues. In contrast, the EE pretreated groups exhibited decreased MDA levels in a dose-dependent manner compared to the CCl_4 control group. The group pretreated with EE (800 mg kg^{-1}) alone showed similar MDA level as the normal control group ($p>0.05$).

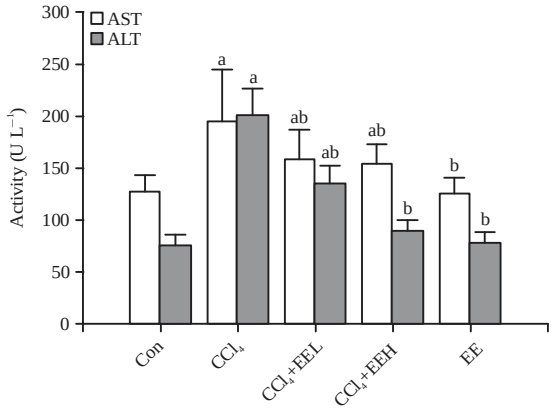

Fig. 1: Effect of ethanol extract of *L. gracile* leaves, (EE) on the activities of serum AST and ALT in CCl_4 induced liver damage mice. Results are presented as the Means±SE (n = 10 animals in each group). Group (Con): Normal control, Group (CCl_4): 0.3% CCl_4 (in olive oil) at 0.1 mL/10 g b.wt., CCl_4, Group (CCl_4+EEL): CCl_4+ 200 mg kg^{-1} EE, Group (CCl_4+EEH): CCl_4+800 mg kg^{-1} EE and Group (EE): 800 mg kg^{-1} EE. [a]Significant at $p<0.05$ compared with the control group, [b]Significant at $p<0.05$ compared with the CCl_4 group

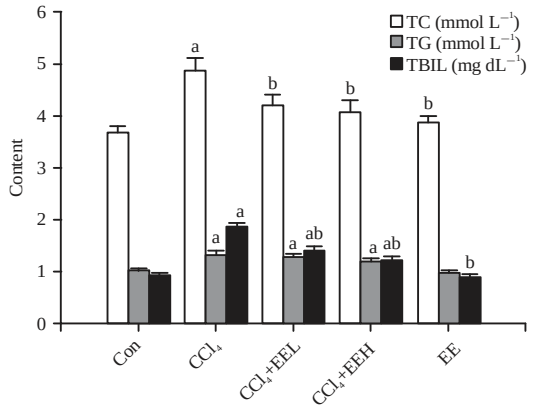

Fig. 2: Effect of ethanol extract of *L. gracile* leaves, (EE) on the activities of serum TG, TC and TBIL in CC_4-induced liver damage mice. Results are presented as the Means±SE (n = 10 animals in each group). Group (Con): Normal control, Group (CCl_4): 0.3% CCl_4 (in olive oil) at 0.1 mL/10 g b.wt., CCl_4, Group (CCl_4+EEL): CCl_4+200 mg kg^{-1} EE, Group (CCl_4+EEH): CCl_4+ 800 mg kg^{-1} EE, Group (EE): 800 mg kg^{-1} EE. [a]Significant at p<0.05 compared with the control group, [b]Significant at p<0.05 compared with the CCl_4 group

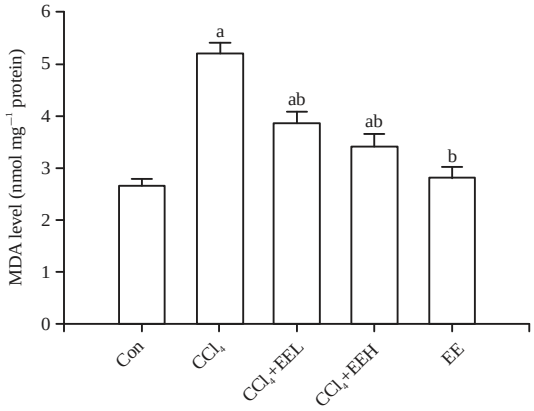

Fig. 3: Effect of ethanol extract of *L. gracile* leaves, (EE) on the MDA level in CCl_4-induced liver damage mice. Results are presented as the Means±SE (n = 10 animals in each group). Group (Con): Normal control, Group (CCl_4): 0.3% CCl_4 (in olive oil) at 0.1 mL/10 g b.wt., CCl_4, Group (CCl_4+EEL): CCl_4+200 mg kg^{-1} EE, Group (CCl_4+EEH): CCl_4+800 mg kg^{-1} EE, Group (EE): 800 mg kg^{-1} EE. [a]Significant at p<0.05 compared with the control group, [b]Significant at p<0.05 compared with the CCl_4 group

Effects of EE on the GPX, CAT as well as SOD activities in liver homogenates: Protective effects of EE on the activities of antioxidant enzymes are shown in Fig. 4. The activities of

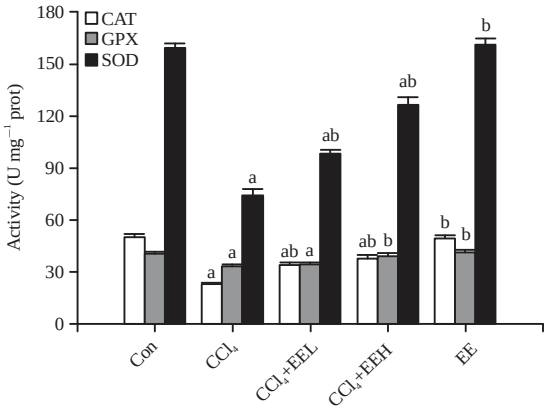

Fig. 4: Effect of ethanol extract of *L. gracile* leaves, (EE) on the activities of liver CAT, SOD and GPX in CC_4-induced liver damage mice. Results are presented as the Means±SE (n = 10 animals in each group). Group (Con): Normal control, Group (CCl_4): 0.3% CCl_4 (in olive oil) at 0.1 mL/10 g b.wt., CCl_4, Group (CCl_4+EEL): CCl_4 +200 mg kg^{-1} EE, Group (CCl_4+EEH): CCl_4+800 mg kg^{-1} EE, Group (EE): 800 mg kg^{-1} EE. [a]Significant at p<0.05 compared with the control group, [b]Significant at p<0.05 compared with the CCl_4 group

antioxidant enzymes, such as SOD, CAT and GPX all significantly decreased in the CCl_4-treated mice as compared to the control group (p<0.05). The reduced the activities of these enzymes indicated oxidative stress in liver tissue. Whereas, pretreatment with EE (200 and 800 mg kg^{-1}) had significantly prevented this trend in a dose-dependent manner as compared with the CCl_4 control group. The activities of antioxidant enzymes (SOD, CAT and GPX) in mice given the EE (800 mg kg^{-1}) alone were similar to those of the normal control group (p>0.05).

Histopathological examination of mice liver: Figure 5 shows that A, control group showed normal hepatic histological structure. However, histopathological examination of the liver of CCl_4 administered mice proved liver damages such as inflammatory cell infiltration, hemorrhage, hepatic lobular disorganisation and pycnotic nuclei (Fig. 5b). These pathological damages were significantly reduced with EE pretreatment at the doses of EE (Fig. 5c, d). The maximum protection was observed at a dose of 800 mg kg^{-1} of EE (Fig. 5d) and the liver sections of the mice from this group were almost comparable to the normal control group. This histopathological observation was in agreement with the results of serum aminotransferases activities and hepatic antioxidant enzyme activities. The liver histological alterations in mice treated with a dose of 800 mg kg^{-1} alone were basically normal (Fig. 5d).

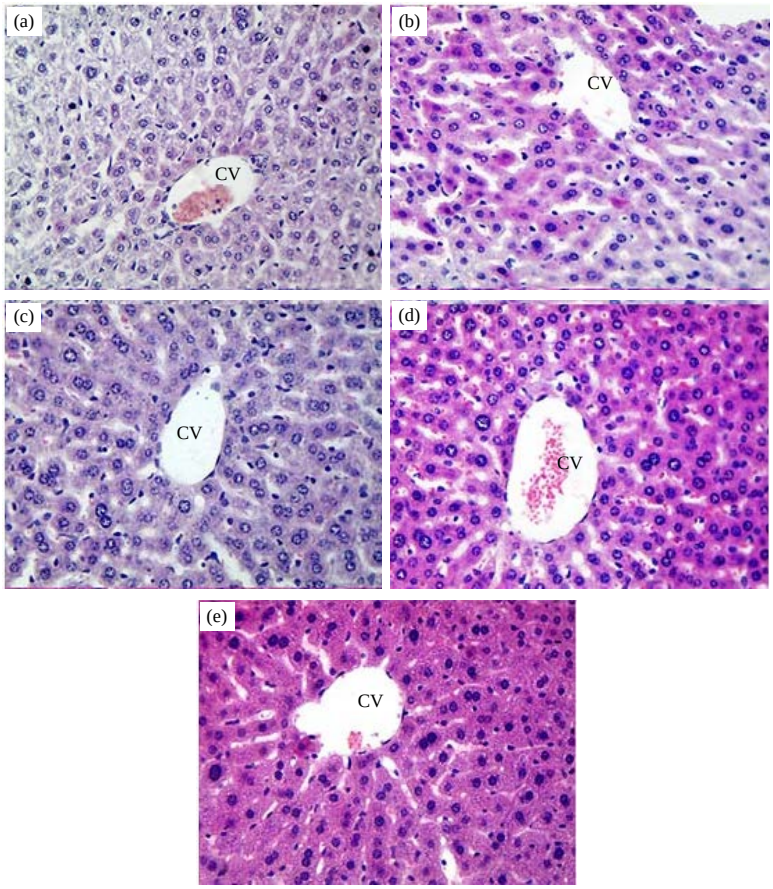

Fig. 5 (a-e): Protective effects of ethanol extract of *L. gracile* leaves, (EE) on $CC1_4$-induced hepatotoxicity in mice (original magnification of ×400), (a) Group con (normal control) showing normal histological structure of the Central Vein (CV) and normal cellular architecture with clear hepatic cell nucleus, (b) Group $CC1_4$ (CCl_4-treated group) showing a severe loss of hepatic architecture with multiple focal necroses in the hepatocytes, (c-d) Group EEL and EEH (CCl_4+200 mg kg^{-1} EE and CCl_4+800 mg kg^{-1} EE, respectively) showing ameliorative cellular architecture close to normal and (e) Group EE (800 mg kg^{-1} EE) showing a normal histological structure, CV: Central vein

DISCUSSION

During recent years, it has been widely proposed that the use of antioxidants can effectively counteract oxidative stress related liver damage (Basu, 2003). Because synthetic antioxidants have been reported to have adverse toxic and potential side effects, isolation and identifying new sources of natural antioxidants with few side effects have received considerable interests. The EE with a large amount of antioxidants seems to receive increased attention as a promising source of functional food which has the potential to attenuate the liver damage induced by CCl_4. However, little is known about EE for hepatoprotective effect. Therefore, the objective of this study was to evaluate the protective effect of EE against CCl_4-induced acute liver damage in mice.

The CCl_4 administration triggers the generation of trichloromethyl radical (CCl_3) that are further converted to trichloromethyl peroxy radical (CCl_3O_2). These highly reactive metabolites can bind covalently with macro-molecules, such as polyunsaturated fatty acids, DNA and protein, then initiate lipid peroxidation (Recknagel, 1967) generate peroxy and superoxide radicals (Lin *et al*., 2014) and ultimately cause the cell membrane damage that is responsible for hepatic acute damage or chronic necrosis (Recknagel *et al*., 1989). Due to the destroyed integrity of hepatocytic membrane under the attack of the generated free radicals, large amounts of enzymes (particularly ALT and AST) located in the hepatocytes leak into the bloodstream and the activities of ALT and AST in the plasma are sharply increased. Hence, the elevated activities of the serum aminotransferase have been generally regarded as

reference biochemical indicators in evaluating hepatic damage after CCl_4-induced intoxication. Moreover, CCl_4 administration can increase TBIL levels by inducing hepatocellular necrosis and cholestasis (Rajesh and Latha, 2004). What is more, this liver toxin can increase the levels of serum lipid by inducing the synthesis of fatty acids as well as decreasing the release of hepatic lipoproteins (Maling *et al*., 1962). Therefore, the evaluation of TBIL, TC and TG was frequently employed to form supplementary methods for investigating liver lesions. In this study, the loss of structure integrity of hepatocyte has been manifested by a significant elevation in the plasma levels of ALT, AST, TBIL and TC as other studies reported. The increasing trend of these serum parameters was reversed by EE pretreatment, indicating that EE improves the functional status of liver tissue and prevents against the hepatic damage caused by CCl_4. The hepatoprotective nature of EE was further confirmed by our histopathological examination.

The lipid peroxidation induced by the free-radical derivatives of CCl_4 has been regarded as one of the pathogenesis of cell membrane injuries (Danni *et al*., 1991). The MDA, a secondary product of lipid peroxidation is usually used as an indicator of free radical generation and lipid peroxidation (Ohkawa *et al*., 1979). In the present study, CCl_4 intoxication generated the formation of lipid peroxides as evidenced by the marked increase of hepatic MDA level. The EE significantly decreased the MDA level of CCl_4-poisoned mice close to its norm values, which implied a decrease in lipid peroxidation and demonstrated the efficacy of this extract in protecting hepatocyte membranes from peroxidation. Antioxidant enzymes, including SOD, CAT and GPX are one of the protection systems against oxidative damages in tissues (Halliwell and Gutteridge, 1990). The SOD catalyses the dismutation of superoxide anion to H_2O_2 and oxygen radical. The CAT and GPX further catalyse the decomposition of H_2O_2 to water. The GPX also oxidize GSH to GSSG that can then be reduced back to GSH by GR. In fact, it has been reported that free radicals, including Reactive Oxygen Species (ROS) by CCl_4 can change the antioxidant state of liver tissue by inhibiting the activities of defense antioxidant enzyme. This CCl_4induced defects in antioxidant enzyme system ultimately leads to oxidative stress that is believed to play a vital role in liver diseases (Slater, 1984). In this work, the activities of SOD, CAT and GPX were distinctly decreased in response to the CCl_4 intoxication, indicating the oxidative damage of hepatic tissue. The result is consistent with previous findings reported by Srivastava and Shivanandappa (2010). The loss of antioxidant enzyme activities was allegedly involved in protein inactivation by ROS (Kono and Fridovich, 1982). By comparison, EE pretreatment especially at doses of 800 mg kg^{-1}, markedly enhanced the impaired activities of the hepatic antioxidant enzymes, suggesting that EE acting as an antioxidant agent, exerts hepatoprotective effect by ameliorating the oxidative stress in the damaged liver tissue. However, it is interesting to note that pretreatment at a dose of 800 mg kg^{-1} EE alone not significantly increased the antioxidant enzyme activities as compared with the normal control mice, which implied that EE treatment was ineffective in creasing the hepatic antioxidant enzyme activities of health mice.

As a kind of polyphenolic compounds, flavonoids, universally existing in plants are categorized into flavones, flavonols, isoflavones, flavanones, catechins, anthocyanidins and chalcones. It is well known that one of the beneficial properties of flavonoids is protection against oxidative stress by direct scavenging free radicals and inducing antioxidant enzymes (Pietta, 2000; Robak and Gryglewski, 1988). Although, the flavonoids of LGL remain to be elucidated, various studies have indentified the presence of luteolin, isoorientin and apigenin in EE based on phytochemical screening. These three compounds have been reported to protect against CCl_4-induced microsomal lipid peroxidation (Cholbi *et al*., 1991). Luteolin has been con rmed to protect against CCl_4-induced liver damage (Domitrovic *et al*., 2009). Isoorientin from the ethanol extract of *Gentiana olivieri* Griseb has been reported to show obvious antihepatotoxic activity on subacute hepatotoxicity induced by CCl_4 (Orhan *et al*., 2003). Therefore, luteolin, Isoorientin and apigenin may be the important hepatoprotective ingredients in LGL and EE.

CONCLUSION

In conclusion, the present study was the first to examine the liver protective activity of EE. The results revealed that pretreatment of mice with EE attenuated hepatic damage produced by CCl_4 while, significantly reducing the alterations of ALT and AST activities, lipid profile and the observed histopathological parameters. Additionally, EE protected against increasing of MDA levels and reduction of SOD, CAT and GPX activities. These results suggest that EE might protect liver from the damage of CCl_4 by improving the activities of antioxidant enzymes. Although, this study did not analyze the composition of EE, this report did propose to investigate the whole ethanol extract of *L. gracile* leaves for hepatoprotective effect *in vivo*. This might enlarge the application fields of LGL and provide the clue for researching the new functional food for liver diseases. Further attempts are necessary to isolate major bioactive components from the extract and

examine their effects and clarify functional mechanisms responsible for the observed effect.

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