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Original Article

Evaluation of hepatoprotective activity of *Syringa oblata* leaves ethanol extract with the indicator of glutathione *S*-transferase A1

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ABSTRACT

The leaves of *Syringa oblata* Lindl., Oleaceae, had been extensively used as a folk medicine to treat various infections, heal inflammations, icteric hepatitis and acute mastitis. The study was designed to evaluate the hepatoprotective activity of *S. oblata* leaves ethanol extract against CCl_4-induced hepatotoxicity in primary hepatocytes and mice with the indicator of glutathione *S*-transferase alpha 1. The hepatoprotective effects of *S. oblata* leaves ethanol extract were evaluated on the basis of liver histopathology and biochemical parameters as well as hepatic oxidative stress markers. The results showed that CCl_4 negatively modulated biochemical parameters and liver antioxidant activities. However, the use of *S. oblata* leaves ethanol extract restored altered-serum biochemical parameters and liver antioxidant activities in a dose-dependent manner. Importantly, the trends in *S*-transferase alpha 1 were similar to alanine aminotransferase and aspartate aminotransferase level, and *S*-transferase alpha 1 was suggested to be a marker for the evaluation of hepatoprotective activity of *S. oblata* leaves ethanol extract. Histopathological examination showed that CCl_4 causes significant hepatic injury relative to control group. The above findings suggested that *S. oblata* leaves ethanol extract has hepatoprotective effects against CCl_4-induced hepatic injury and *S*-transferase alpha 1 may be an indicator to evaluate the protective effects of *S. oblata* leaves ethanol extract.

Introduction

Liver is an important digestive gland in the body, playing a major role in the metabolism, biotransformation and detoxification of toxins (Li et al., 2004). Meanwhile, liver is a susceptible organ to xenobiotic metabolisms, chemical substances, virus, alcohol and drugs (Zuo et al., 2014). Acute hepatic injury is very common induced by one of the several factors, including radical oxidative damage, viral exposure, alcohol consumption, drug and immune system issues (Andrade et al., 2015). Carbon tetrachloride (CCl_4), a highly toxic chemical agent, is widely used for hepatotoxicity model in animals (Weber et al., 2003). It is believed that CCl_4 in liver could form metabolic trichloromethyl radicals ($CCl_3\bullet$) and react with excess O_2 producing reactive oxygen species (ROS), which is the normal product of aerobic metabolic reaction (Zira et al., 2013). However, the high levels of ROS in the body could induce lipid peroxidation and protein oxidation in the cell membrane, causing liver enzyme leakage, mitochondrial injury and cellular disorders, as well as resulting in hepatic damage and apoptosis (Karakus et al., 2011).

Glutathione *S*-transferases (GST), a large family of multifunctional proteins that share similar dimer interactions and glutathione-(GSH) binding sites, play a critical role in protecting cells from xenobiotics and therapeutic compounds (Hayes et al., 2005). Glutathione *S*-transferase A1 (GSTA1), one isoenzyme of GST, is important in phase II metabolism by catalyzing the conjugation of reduced glutathione with a wide variety of electrophilic substances (Eaton and Bammler, 1999). Furthermore, the cytosolic concentration of GSTA1 is high and distribution of GSTA1 is uniform throughout the liver lobules. It is worth mentioning that GSTA1 could easily release to the blood due to its small molecular weight relative to alanine aminotransferase (ALT) and aspartate aminotransferase (AST) following liver damage. Consequently, GSTA1 is a more sensitive marker for monitoring the acute liver damage. (Bernuau et al., 2000). The sensitivity and specificity of GSTA1 as a marker have been tested in various conditions of acute viral, toxic and drug induced hepatitis (Liu et al., 2014).

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Syringa oblata Lindl., belongs to Oleaceae family and widely grows across China. The leaves of *S. oblata* have been extensively used as a folk medicine to treat various infections, inflammations, acute jaundiced hepatitis and acute mastitis. It is also used in China to treat vomiting, nausea, rheumatic pain and kidney deficiency (Xin et al., 2010; Su et al., 2015). To date more than 140 chemical constituents have been isolated from the bark, leaves and flowers of *Syringa* including phenylpropanoid, lignans, iridoid, crack iridoid, phenethyl alcohol and flavonoids *etc.* (Eriksson et al., 2008; Zhao et al., 2016). Previous reports demonstrated that *S. oblate* Lindl., *S. vulgaris* L., and *S. dilatata* Nakai leaves, flowers or fruit extract have potential hepatoprotective activities (Oh et al., 2003; Toth et al., 2016). There is evidence of hepatoprotective activity of *S. oblata* leaves due to the syringopicroside and flavonoids content, which have potent antioxidant properties (Erdogan et al., 2015). In addition, rutin has the effect of anti-lipid peroxidation, diastolic blood vessels as well as affects the activity of metabolic enzymes (Magalingam et al., 2013).

In the light of the traditional use of *S. oblata* leaves for the treatment of liver inflammation, this study explored the potential hepatoprotective activities of *S. oblata* leaves ethanol extract (SOLEE) against CCl_4-induced hepatotoxicity both *in vitro* and *in vivo*, and evaluated the possibility of GSTA1 being used as an indicator of hepatic injury.

Materials and methods

Reagents

CCl_4 was purchased from the Guoyao Chemical Reagent Co. LTD (Shanghai, China). Silymarin was purchased from Harbin Jiacheng Dispensary (Harbin, China). Type IV collagenase, dimethyl sulfoxide (DMSO), insulin, transferrin, heparin, dexamethasone, and trypan blue were bought from Sigma Chemical Co. (St. Louis, MO, USA). Mice feed (license no. SCXK (Liao) 2015-0003) was obtained from Changsheng Biotechnology (Liaoning, China). Rutin standard (lot no. 30225) was bought from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

Plant material and preparation of extracts

Syringa oblata Lindl., Oleaceae, leaves were purchased from Harbin Jiacheng Dispensary (Harbin, China) and authenticated in Heilongjiang University of Chinese Medicine. The specimen (accession no. 2015052006) has been deposited at Herbarium in the College of Veterinary Medicine, Northeast Agricultural University. SOLEE was prepared as previously described (Shi et al., 2017). In brief, 2000 g of fresh leaves were dried at 50 °C and ground to crude powder with a universal grinder (AK-1000A, Wenling, China), then the powder (500 g) was dissolved in 5000 ml ethanol (50%, v/v) in ultrasonic waves (KQ3200 Kunshan Ultrasonic Instrument Co., Ltd., China) for 60 min. After extraction, the solvent was filtered and evaporated at 60 °C till the extracts concentrated to a paste and dried by vacuum drying method at 35 °C.

Quantification of rutin bioactive compound from SOLEE

SOLEE was analyzed by using high performance liquid chromatography (HPLC) method. Rutin, one of the principal constituents of *S. oblata* leaves, was used as an external standard. The analysis were performed using Waters 2695 HPLC system (Waters, USA) and Venusil XBP C18 (L) column (250 mm × 4.6 mm, 5 μm, Bonna-Agela Technologies, China). The mobile phase consisted of methanol and acetonitrile in 80:20 (v/v) at a flow rate of 1 ml/min with an injection volume of 10 μl. The compounds were detected at a wavelength of 256 nm at a column temperature of 25 °C.

Mice

Adult male Kunming mice having the body weight 20 ± 2 g were obtained from Harbin Pharmaceutical Group CO., Ltd. General Pharm. Factory, laboratory animal center. The License number of mice was SCXK (black) 2012-007. Mice were housed under standard conditions at a temperature (20 ± 2 °C) and light (12 h light/12 h dark cycles), a relative humidity (40–60%), and allowed free access to food and water. Mice were acclimatized for at least 1 week before experiments. All procedures involving animals were carried out in accordance with the guidelines and approved protocols of Northeast Agricultural University and Harbin Veterinary Research Institute Animal Ethics Committee (approval number: SYXK (Hei) 2012-2067).

Primary hepatocytes

Hepatocytes were isolated from mice with the improved method of Seglen's two-step perfusion *in situ* as previously described (Chang et al., 2017). Briefly, the liver was perfused with preheated (37 °C) liver perfusion medium containing 1 ml heparin (2500 U/ml) and then digested by digesting medium including type IV collagenase (300 mg/ml). Primary hepatocytes were separated from liver tissue following centrifugation. The primary hepatocytes were seeded in 24 well plates for 6 h, and the cells were cultured in the growth medium without serum for 24 h. Then, the cells were again cultured at 37 °C under a humidified atmosphere of 5% (v/v) CO_2 for subsequent experiments.

Experimental protocol

Primary hepatocytes treatment. The primary hepatocytes were treated with or without SOLEE concentration (mass/volume) high (200 μg/ml), medium (100 μg/ml) and low concentration (50 μg/ml) of SOLEE for 12 h, and then incubated with CCl_4 (10 mmol/l) for 8 h. Silymarin was chosen as a positive control drug. Afterwards, the hepatocytes were centrifuged at 3000 × *g* for 10 min. Supernatant was collected for the detection of ALT, AST and GSTA1 enzyme activities, and the cells were tested for superoxide dismutase (SOD) and malonaldehyde (MDA) level.

Mice treatment. Forty eight mice were randomly assigned into six groups with eight animals per group. The animals of control group and model group (CCl_4 alone group) received only isopyknic physiological saline; positive control group were administered with silymarin (200 mg/kg), while the three experimental groups were administered with 100 mg/kg, 200 mg/kg and 300 mg/kg of SOLEE daily by gavage for 7 successive days. The administration volume of each mouse was 10 ml/kg. During experiments, mice were provided with a standard diet (composition shown in Table 1) and water *ad libitum*. After receiving treatments for 7 days, all groups fasted for 16 h. All groups were given 0.35% CCl_4 soya bean oil solution (10 ml/kg) to induce hepatic injury in mice except control group. The control group was only given soya bean oil (10 ml/kg). After 24 h of acute exposure, the mice were anesthetized (1.5% isoflurane), and the blood was collected from the retro-orbicular plexus. A piece of fresh liver was washed in ice-cold saline and dissected to prepare tissue homogenate. The remaining liver was stored at −80 °C for further analysis.

ALT, AST, MDA, SOD and GSTA1 assays for monitoring liver function

ALT (Kit No. C009-1), AST (Kit No. C010-1) activity in serum and MDA (Kit No. A003-1), SOD (Kit No. A001-1-1) concentration in tissue homogenates were detected according to the instructions of each kit. The kits were obtained from Jiancheng Institute

Table 1
The nutritive formula composition of the mice diet.

Leading indicator	Maintain food (/kg)	Leading indicator	Maintain food (/kg)	Leading indicator	Maintain food (/kg)
Crude protein	≥200 g	Fe	≥120 mg	VitamincB1	≥13 mg
Crude fat	≥40 g	Mn	≥75 mg	Vitamin B2	≥12 mg
Coarse fiber	≤50 g	Cu	≥10 mg	Vitamin B6	≥12 mg
Crude ash	≤80 g	Zn	≥30 mg	Vitamin B12	≥0.022 mg
Moisture	≤100 g	Vitamin A	≥14 000 IU	Lysine	≥13.2 g
Calcium	10–18 g	Vitamin D	≥1500 IU	Histidine	≥5.5 g
Phosphorus	6–12 g	Vitamin E	≥120 IU	Arginine	≥11 g
Calcium: phosphorus	1.2:1–1.7:1	Vitamin K	≥5 mg	Methionine andCystine	≥7.8 g

of Biotechnology (Nanjing, China). GSTA1 levels in serum and tissue homogenates were determined with a mouse glutathione *S*-transferase Alpha1 (GSTA1) ELISA Kit (American Rapidbio company, America. Kit No. DRE30790). The basic procedure was performed in accordance with the manufacturer's recommendations.

Histopathological examination of liver

The liver tissues from each group were collected and fixed in 10% (v/v) formalin for 24 h, dehydrated in graded ethanol solution varied in between 50% to 100%, cleared in xylene, and embedded in paraffin. Afterwards, 5-μm-thick sections were prepared, stained with hematoxylin and eosin dye, and observed for histopathological changes under a microscope.

Statistical analysis

Data is represented as mean ± standard deviation (SD). Statistically significant differences among all groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using the SPSS software 19.0 (SPSS, Inc., Chicago, IL, USA). A value of $p < 0.01/p < 0.05$ was considered as statistically significant.

Results

Validation of the extraction method

The chromatograms of [0] rutin standard and ethanol extract of *S. oblata* leaves are shown in Fig. 1. Rutin peaks appeared at a retention time of approximately 15 min.

Protective effects of SOLEE in vitro

Compared to control group, ALT, AST, GSTA1 ($p < 0.01$) and MDA ($p < 0.05$) level significantly increased in model group. Meanwhile, SOD level significantly ($p < 0.05$) decreased in model group (Table 2). Pretreated with the high concentration (200 μg/ml) of SOLEE, transaminase (ALT and AST) activity and GSTA1 level in the supernatant were effectively decreased ($p < 0.05$). The same high concentration markedly increased ($p < 0.05$) SOD level and obviously decreased ($p < 0.05$) MDA level in cells. However, there were no significant changes in the parameters above mentioned in the groups treated with the medium concentration (100 μg/ml) and low concentration (50 μg/ml) of SOLEE.

Effects of SOLEE on serum ALT and AST activities

Results of SOLEE on serum ALT and AST activities are shown in Fig. 2A. CCl_4 significantly ($p < 0.01$) increased serum ALT and AST activity, while pretreatment with silymarin and SOLEE (300 mg/kg), serum ALT and AST activity extremely ($p < 0.01$) reduced relative to model group. Treatment with SOLEE (200 mg/kg) caused significant

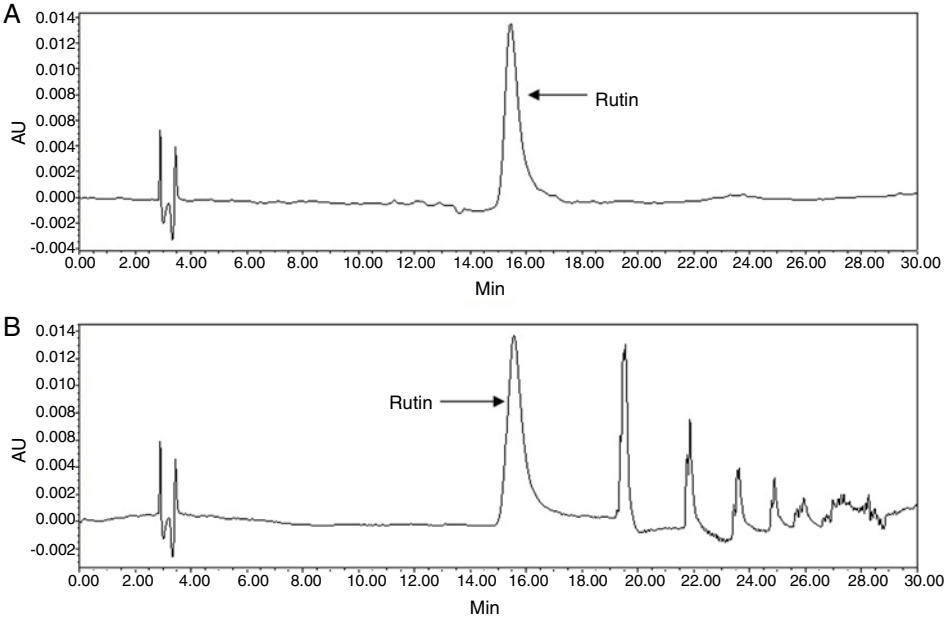

Fig. 1. (A) Chromatogram of rutin standard and (B) ethanol extract of *Syringa oblata* leaves.

Table 2
The changes of indicators in cell culture supernatant and in hepatocyte ($\bar{x} \pm s$, $n = 6$).

Group	ALT (IU l^{-1})	AST (IU l^{-1})	GSTA1 (ng ml^{-1})	MDA (nmol mg^{-1} prot)	SOD (U mg^{-1} prot)
Control	8.12 ± 0.99	9.18 ± 0.62	2.49 ± 0.03	2.81 ± 0.24	10.13 ± 0.87
Model	12.15 ± 1.13**	12.63 ± 1.58**	2.62 ± 0.04*	4.34 ± 0.56**	6.49 ± 0.55**
SOLEE(200 μg ml^{-1})	8.22 ± 0.95▲	10.24 ± 1.06▲	2.50 ± 0.08▲	3.84 ± 0.35▲	7.77 ± 0.61▲
SOLEE(100 μg ml^{-1})	11.56 ± 0.39	12.03 ± 1.29	2.55 ± 0.10	4.19 ± 0.45	6.42 ± 0.70
SOLEE (50 μg ml^{-1})	11.29 ± 1.56	12.37 ± 1.69	2.59 ± 0.07	4.22 ± 0.57	6.41 ± 0.93

Note: Values are expressed as mean ± standard deviation ($n = 6$). **$p < 0.01$ compared with the control group; *$p < 0.05$ compared with the control group; ▲$p < 0.05$ compared with the model group.

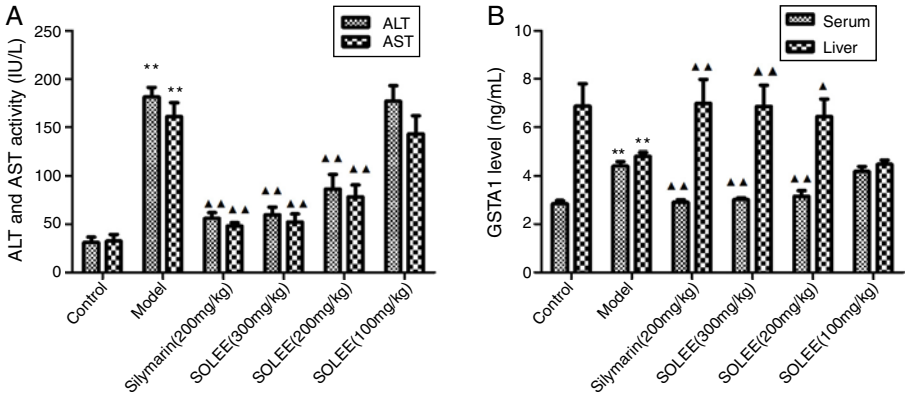

Fig. 2. (A) The changes of ALT and AST activity in the serum of mice. (B) The changes of GSTA1 level in mice serum and liver tissue ($n = 8$).

($p < 0.05$) decrease in serum ALT and AST activity. However, there was no statistically significant difference between low dose SOLEE group (100 mg/kg) and model group.

GSTA1 concentration in serum and liver tissue

Variations of GSTA1 in serum and liver tissue are presented in Fig. 2B. In the control group, GSTA1 concentrations in the liver tissues were almost 2.42 times higher than GSTA1 in the serum. CCl_4 treatment significantly ($p < 0.01$) increased GSTA1 concentration in serum and markedly decreased ($p < 0.01$) GSTA1 level in liver tissue. The ratio of GSTA1 concentrations in the liver and serum is 1:1. While, compared to model group, mice pre-treated with silymarin and SOLEE (300 mg/kg or 200 mg/kg) respectively, serum GSTA1 remarkably ($p < 0.01$) reduced and tissue GSTA1 significantly ($p < 0.05$ or $p < 0.01$) increased in a dose-dependent manner. Intriguingly, the GSTA1 levels of liver tissues were 2.40 and 2.27 times higher than the serum GSTA1 levels in silymarin group and SOLEE (300 mg/kg) group, respectively.

Effects of SOLEE on MDA and SOD levels

Compared to control group, the levels of MDA progressively increased to a high extent ($p < 0.01$), and the level of SOD obviously decreased ($p < 0.01$) in the model group (Fig. 3). The elevated level of MDA was significantly ($p < 0.01$–0.05) reduced and the depressed level of SOD was significantly ($p < 0.01$–0.05) increased in mice pretreated with silymarin and SOLEE (300 mg/kg, 200 mg/kg). The trend of MDA and SOD were similar in mice treated with silymarin and SOLEE (300 mg/kg). Furthermore, low dose SOLEE (100 mg/kg) showed no significant effect relative to model group.

Tissue sections analysis

As shown in Fig. 4, the photomicrographs from the control group (Fig. 4A) showed normal morphology, hepatic cords were neatly arranged and the structures of liver lobules were clearly seen. CCl_4-induced histopathological changes in liver were observed, including the obscure edge of the hepatic lobule, mussy hepatic cord, cloudy swelling of the hepatic cells, congestion and necrosis, significant acidophilic changes and obvious inflammatory infiltrates in focal areas. Compared to CCl_4 group (Fig. 4B), the sections from the groups treated with silymarin (Fig. 4C) and high dose (300 mg/kg) SOLEE (Fig. 4D) showed normal morphology. However, slight alteration and degeneration of the hepatocytes, mild inflammatory infiltration and congestion in the central retinal and hepatic sinusoids have been observed in the groups treated with silymarin (Fig. 4C).

Discussion

Oxidative stress is an important cause of liver dysfunction and also the pathophysiological basis of hepatic injury (Yang et al., 2015). Previous reports demonstrated that increase oxidative stress causes lipid peroxidation in cells, mitochondrial calcium overload, inflammatory reaction, abnormal function of organelles, oxidative DNA damage and even cancerization (Singh et al., 2017). The efficacy of any hepatoprotective drug is essentially dependent on its ability in reducing the harmful effects or maintaining the normal hepatic physiology that has been disturbed by a hepatotoxin. Numerous literatures demonstrated that *S. oblata* leaves had various therapeutic properties. Hyuncheol reported that activity-guided fractionation of the ethyl acetate and methyl alcohol extract of the leaves of *S. dilatata* could scavenge free radical (Oh et al., 2003). Gergő Tóth studied the polyphenolic composition of *S. vulgaris* flowers and fruits proving that the extracts and the constituents have radical scavenging activity (Toth et al., 2016). Rutin is a flavonoid glycoside found in fruits, tea, vegetables and medicinal plants (Liu et al., 2017). Studies reported that rutin have many pharmacological and therapeutic properties including anticarcinogenic, neuroprotective, nephroprotective, hepatoprotective and anti-inflammatory activities (Ghorbani, 2017). Besides, Khan et al., demonstrated that rutin possess radical scavenging activity and antioxidant potential that is useful in preventing CCl_4-induced liver damage (Khan et al., 2012). In the present study, the active ingredient of SOLEE is rutin that may be responsible for the prevention of CCl_4 induced liver injury in mice hepatocytes. Intriguingly, we obtained reasonably resolved and good chromatographic peaks

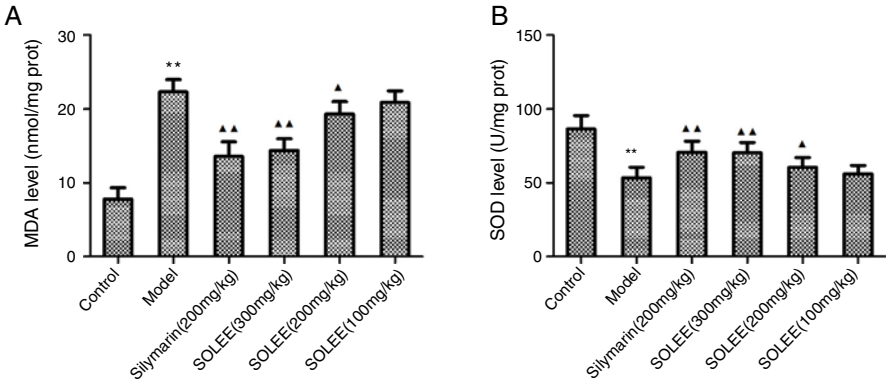

Fig. 3. The changes of MDA level (A) and SOD level (B) in liver tissue of mice ($n = 8$).

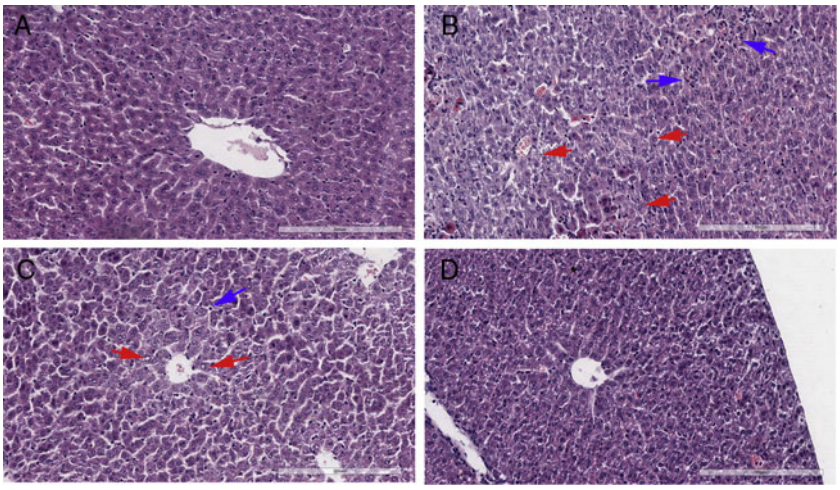

Fig. 4. (A) Control group, (B) Model group, (C) Group with silymarin (200 mg/kg), (D) Group with *Syringa oblata* leaves extract (300 mg/kg). Inflammatory infiltration was marked out by blue arrows. Necrosis was pointed out by the red arrows.

for standard rutin and SOLEE in HPLC analysis. In addition, silymarin is an important medicinal plant for alleviating hepatic damage, whose history of hepatoprotective activity could dates back to 2000 years ago and is used to treat jaundice and enlarged liver and spleen (Bahmani et al., 2015). Hence, we employed silymarin as a reference to study the hepatoprotective effects of SOLEE *in vivo*.

ALT and AST, as a traditional markers of hepatic damage, could reflect the effects of CCl_4-induced hepatotoxicity in mice (Knapen et al., 2000). Moreover, MDA is the byproduct of lipid peroxidation and the level of it in hepatic tissue is used as a biomarker of lipid peroxidation and a key feature in liver damage (Lin et al., 2006). Furthermore, cells have enzymatic antioxidant system to protect itself against oxidative damage. SOD is a major antioxidant enzyme and plays a crucial role in the conversion of superoxide radicals into hydrogen peroxide. Increase of oxidative stress in the cells could inactivate SOD enzyme (Escobar et al., 1996). Therefore, detecting oxidative stress parameters, such as MDA, SOD and GSH-Px can monitor CCl_4-induuced oxidative injury.

In our study, we found that CCl_4 treatment (model group) caused oxidative damage in liver tissues and negatively altered serum biochemical parameters. Interestingly, SOLEE significantly reversed CCl_4-induced changes in serum and liver biochemical parameters and antioxidant activities both *in vitro* and *in vivo*. The supernatant and serum levels of ALT, AST and GSTA1 as well as the cell and tissue level of MDA depressed significantly ($p < 0.01$ or $p < 0.05$) in the groups treated with high and medium dose of SOLEE. Meanwhile, the concentration of GSTA1 and SOD in liver tissues were elevated obviously ($p < 0.01$ or $p < 0.05$). We have noted similar trends in ALT, AST, GSTA1, MDA and SOD level in high dose SOLEE group and silymarin group, respectively. Hence, it is suggested that SOLEE had hepatoprotective activity, which was similar to silymarin, protecting the liver against CCl_4-induced injury.

Our histological findings were in general agreement with previous studies that CCl_4-induced liver injury in mice (Ma et al., 2017). We also noted that CCl_4 caused hepatocytes vacuolization, partial disruption in radial arrangement, fatty degeneration and inflammatory infiltrates. The pathological changes had the obvious uniformity with the variations of aminotransferases, SOD and GSTA1, demonstrating that SOLEE was a powerful antioxidant and prevents lipid peroxidation.

Previous studies demonstrated that GSTA1 was a more suitable marker of liver damage than ALT *in vivo* and *in vitro* (Liu et al., 2016; Li et al., 2017). Our data showed that the levels of GSTA1 in serum showed similar trend with that of ALT and AST, which is in agreement with previous studies (Liu et al., 2014). Interestingly, the trends of GSTA1 level were consistent with that of ALT and AST level in the experimental groups treated with SOLEE. Thus, GSTA1 could be used as an indicator to evaluate the hepatoprotective activity of *S. oblata* leaves extract. Nevertheless, higher molecular studies are needed to know the effect of *S. oblata* leaves extract on the role of GSTA1 and the mechanism involved in CCl_4-induced liver injury.

Conclusion

In summary, SOLEE had a free radical scavenging activity, prevented lipid peroxidation and enhanced antioxidant activities that prevented the hepatocytes from CCl_4-induced liver injury.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors have obtained the written informed consent of the patients or subjects mentioned in the article. The corresponding author is in possession of this document.

Authors contribution

FL supervised the whole experiments. YL and ZL performed the practical work and completed the experiments. CL, XM, YC, CS, JH and RL provided help during the experiments. IM helped in improving language expression.

Conflicts of interest

The authors declare no conflicts of interest.

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