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Original article

Hepatoprotective standardized EtOH–water extract from the seeds of *Fraxinus rhynchophylla* Hance

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ABSTRACT

Fraxinus rhynchophylla Hance (Oleaceae), its stem barks are known as *Cortex fraxini* (秦皮 qín pí) listed in Chinese Pharmacopoeia. Phytochemical study has indicated that methanol extracts from Qinpi has protective effect on acute liver injury. The present study investigates the hepatoprotective activity of EtOH–water extract from the seeds of *F. rhynchophylla* Hance against carbon tetrachloride-induced liver injury in mice. The EtOH–water extract significantly alleviated liver damage as indicated by the decreased levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), the malondialdehyde (MDA) content, and increased the levels of superoxide dismutase (SOD), glutathione (GSH) and glutathione peroxidase (GSH-Px), and reduced the pathological tissue injury induced by CCl_4. Quantitative analysis of seven major constituents (**1**–**7**) in EtOH–water extract (EWE) was developed by high performance liquid chromatography-diode-array detector (HPLC-DAD). The current research indicates that the EWE from the seeds of *F. rhynchophylla* Hance decreased liver index, inhibited the increase of serum aminotransferase induced by CCl_4, and decreased hepatic MDA content, SOD and GSH-Px activities. These results suggested that the pretreatment with EWE protected mice against CCl_4-induced liver injuries. Based on the results, the EtOH–water extract from the seeds of *F. rhynchophylla* Hance is efficacious for prevention and treatment of CCl_4-induced hepatic injury in mice. Secoiridoid and tyrosol glucosides might be the active ingredients responsible for the biological and pharmacological activities of hepatoprotection.

1. Introduction

The liver plays an important role in human metabolism and detoxification of endogenous and exogenous chemicals.[1] Liver injuries or dysfunctions have been recognized as serious health problem. Especially acute and chronic liver injuries resulted from the exposure to toxic chemicals, drugs, and virus infiltration from ingestion or infection, have gained more attention in recent years.[2–4] Corticosteroids and interferon has been used for the treatment of hepatic diseases, however, these synthetic chemical drugs are not well accepted by patients due to limited therapeutic efficacy and serious complications.[5] Therefore, more effective complementary and therapeutic drugs with low or no side-effects are needed for the treatment of liver diseases.[6–9] In recent years, some effective and safe dietary ingredients for liver-protection have been isolated from traditional medicinal plants, such as glycyrrhizin,[10] curcumin,[11] resveratrol,[12] as well as silybin and silymarin.[13,14] *Fraxinus rhynchophylla* Hance (Oleaceae) is a commonly used Chinese traditional medicinal plant, mainly distributed in China and Korea.[15] Its stem bark also known as *Cortex fraxini* (Qinpi) is Chinese herbal drug for treating diseases such as acute conjunctivitis and psoriasis; arresting discharges; curing chronic bronchitis; and bacillary dysentery, diuretic, antirheumatic, analgesic, antiperspiratory effects, and enhancing eyesight.[16] Phytochemical study has indicated that methanol extracts from Qinpi has protective effect on acute liver injury.[17] Many natural products such

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as secoiridoid glucosides, coumarins, lignans, sesquilignans, and coumarinolignans have been identified from this plant.[18–23] Recently, several pharmacological activities of phytochemical constituents isolated from the barks and leaves of *F. rhynchophylla* have been carried out, including anti-diabetes effects,[24–28] anti-*Toxoplasma gondii* effects,[29] including antioxidant enzymes,[30] inhibiting amyloid-β-induced neuronal cell damage,[31] and inhibiting nitric oxide synthesis activities.[32] Hydrangeside B, along with other secoiridoid glucosides showed hepatoprotective activities against DL-galactosamine-induced toxicity in human hepatocyte HL-7702 (HL-7702) cells.[33] Secoiridoid glycoside, oleuropein, showed anti-hepatitis B virus (HBV) activity and effectively blocked hepatitis B surface antigen (HBsAg) secretion with an IC_{50} of 23.2 μg/mL in HepG2.2.15 cells with no significant cytotoxicity.[34] The hepatoprotective activity of oleuropein against carbon tetrachloride (CCl_4)-induced liver damage in mice was achieved through the NF-E2-related factor 2-mediated induction of heme oxygenase-1.[35] Recently, Peng et al.[36] investigated the effect of *Fraxinus rhynchophylla* ethanol extract (FR_{EtOH}) on liver fibrosis induced by CCl_4 in rats. However, the hepatoprotective activity of the seeds of *F. rhynchophylla* Hance has not been evaluated so far.

The present study aimed to evaluate the hepatoprotective activity of EtOH–water extract from the seeds of *F. rhynchophylla* Hance employing a widely used CCl_4-induced liver damage model in mice and quantitative analysis of six secoiridoid glucosides (**1**–**6**) and one tyrosol glucoside (**7**) by high performance liquid chromatography-diode-array detector (HPLC-DAD) method.

2. Materials and methods

2.1. Chemicals and reagents

CH_3OH (HPLC grade), CH_3CH_2OH (HPLC grade), and CH_3CN (HPLC grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other organic solvents used in the current study, such as CH_3OH, ethyl acetate (EtOAc), acetone, and chloroform ($CHCl_3$) were of analytical grade. They are commercially available from Hengxing Chemical Reagent Co., Ltd. (Tianjin, China). Chemical standards of oleoside dimethyl ester (**1**), ligstroside (**2**), nuzhenide (**3**), 10-hydroxoleoside dimethyl ester (**4**), GI3 (**5**), GI5 (**6**), and salidroside (**7**) were prepared in our laboratory. The purity of each compound was >98%, determined by HPLC analysis. The chemical structures of these reference compounds are shown in Fig. 1.

2.2. Materials

The seeds of *F. rhynchophylla* Hance were provided in August 2013 from Baoji City, Shaanxi province, China. The herbariums of *F. rhynchophylla* Hance (FRH001) were deposited in Room 612, Department of Pharmaceutical Engineering, College of Chemical Engineering, Northwest University.

2.3. HPLC analysis conditions

HPLC analysis was performed on an Agilent 1260 separation module connected to a G1315D DAD detector using a Synergi 4u Hydro-RP 80R column (250 × 4.6 mm, 4 μm, 100 Å) with a flow rate of 1.0 mL/min. Solvent system: 0 min: 95% A (1% phosphoric acid) and 5% B (acetonitrile), 2 min: 95% A (1% phosphoric acid) and 5% B (acetonitrile), 5 min: 80% A (1% phosphoric acid) and 20% B (acetonitrile), 25 min: 75% A (1% phosphoric acid) and 25% B (acetonitrile), 27 min: 95% A (1% phosphoric acid) and 5% B (acetonitrile), 30 min: 95% A (1% phosphoric acid) and 5% B (acetonitrile). At the end of the run, 100% of acetonitrile was allowed to flush the column for 10 min, and an additional 10 min of post run time was set to allow for equilibration of the column with the starting eluant. The UV detector was operating at 230 nm, and the column temperature was maintained at 30 °C.

2.4. Calibration curves

Methanol stock solutions containing the seven standard compounds **1**–**7** were prepared and diluted to five different final concentrations. A calibration curve was constructed for each of the compounds by plotting peak areas versus compound concentrations.

2.5. Preparation of seeds of F. rhynchophylla Hance extract

The 5 kg air-dried seeds of *F. rhynchophylla* Hance were percolated twice with absolute ethyl alcohol at room temperature. The ethyl alcohol was evaporated under vacuum. The herb residue was then percolated twice with water at room temperature and made the SFR-water extracts. Finally, mixed the above two extracts, and the *in vivo* bioactivity study of seeds of *F. rhynchophylla* Hance was carried on by this sample. The HPLC spectrum of the extract is shown in Fig. 2.

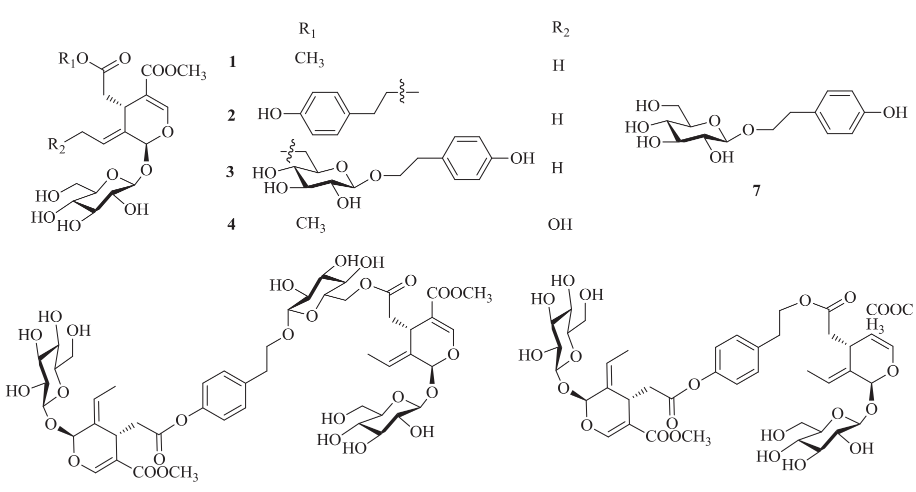

Fig. 1. Structures of compounds **1**–**7**.

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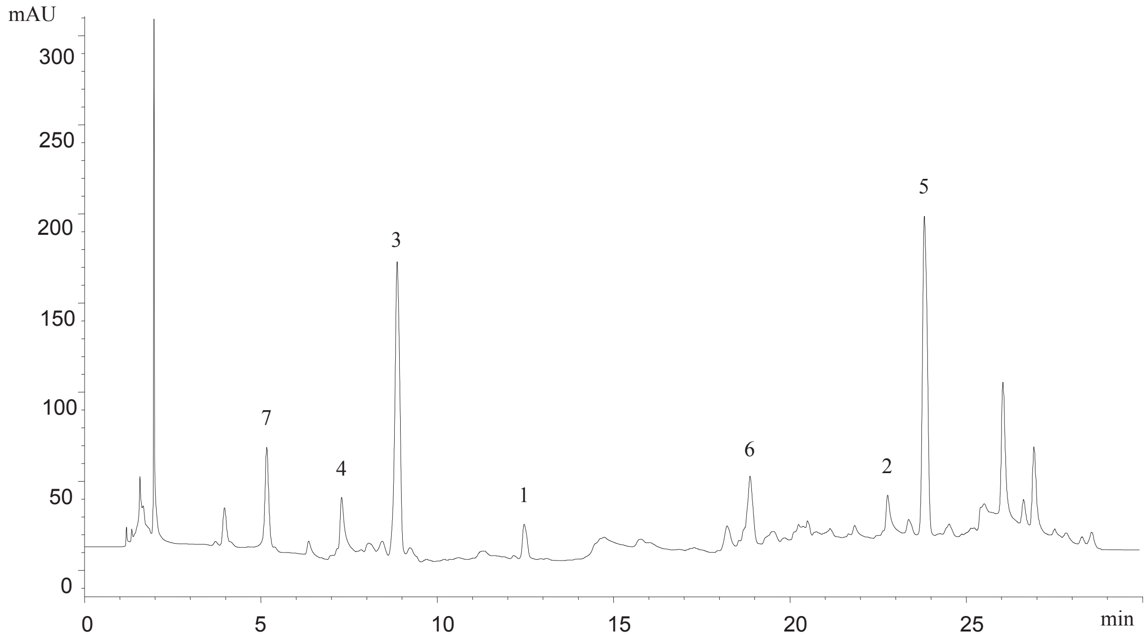

Fig. 2. The HPLC chromatogram of EtOH–Water extract.

2.6. The hepatoprotective activities assays

2.6.1. Animals

Male Kunming mice (weighing 18–22 g), obtained from the Experimental Animal Center of Xi'an Jiaotong University, were used. The animal ethical approval communication number is SCXK 2012-003 (Northwest University, Xi'an, Shaanxi, China). All animals were grouped and housed in polyacrylic cages (29 × 18 × 16 cm) with no more than six animals per cage and maintained under standard laboratory conditions (temperature 25 ± 2 °C, relative humidity 50 ± 10%) with dark and light cycle (14/10 h). The mice were acclimatized to laboratory condition for 5 days before commencement of experiment. All the experiments were performed in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of People's Republic of China.

2.6.2. CCl_4-induced hepatotoxicity model

Mice were randomly divided into five groups of 12 animals each. In the control group (group I) and CCl_4-intoxicated group (group II), mice were given a single daily dose of distilled water (0.2 mL/10 g, intragastrically (ig)). In the test groups, mice were given 2.0 g (group IV) and 4.0 g (group V) of EtOH–water extract (EWE) per kg body weight (BW) once daily. In group III, silymarin was served as a positive control, and mice were given 0.1 g/kg BW. All administrations were conducted for 7 weeks. On the 49th day, all mice except those in the control group were given simultaneously a CCl_4/peanut oil mixture (0.1:100, intraperitoneally, 0.1 mL/10 g, ig) 2 h after the last administration, while the control group received peanut oil alone. Then all the animals were fasted for 16 h and were subsequently tested for the following analysis.

2.6.3. Liver index

Liver index was determined as percent of wet liver weight to body weight.

2.6.4. Assessment of liver function

After blood collection, serum was separated by centrifugation at 604 g at room temperature for 20 min. The serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) values were measured with commercially available diagnostic kits produced by Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.6.5. Determination of MDA, SOD and GSH-Px activity

After the animals were sacrificed, livers were immediately excised. With the exception of a portion of the left lobe to be used for histopathological examination, the livers were homogenized in phosphate buffer (50 mM, pH 7.4) and centrifuged at 420 g for 20 min at 4 °C. The malondialdehyde (MDA) content, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities along with protein levels in the supernatant were measured according to commercially available diagnostic kits.

2.6.6. Histopathological examinations

A left lobe portion of the liver was incubated for 24 h in 10% neutral formalin solution. Based on standard procedures, we obtained 5 μm sections for histopathological studies using hematoxylin and eosin (H&E) staining.

2.6.7. Statistical analysis

The data were analyzed in triplicate, using SAS software, version 8.1 (SAS Institute, Cary, NC, USA). Tukey's test was used to determine statistical significance. The values were considered significantly different when the *P*-value was lower than 0.05.

3. Results

3.1. HPLC method validation

All calibration curves made by the HPLC method exhibited excellent linear regressions with the determination coefficients (r^2) ranging from 0.99 to 0.9999, and the calibration ranges adequately covered variations (Table 1).

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Table 1
Calibration curves and LOD and LOQ data of 7 compounds investigated by HPLC.

No.	Calibration curves[a]	r^2	Linear range (mg/mL)	LOD[b] (mg/mL)	LOQ[b] (mg/mL)
1	Y = −26.3302 + 15081.4372X	0.9998	0.02−0.48	0.0005	0.0016
2	Y = 40.3439 + 13692.4304X	0.9986	0.01−0.24	0.0048	0.0158
3	Y = 667.3964 + 4408.5855X	0.9999	0.07−3.50	0.0012	0.0035
4	Y = −1578.7230 + 15651.8470X	0.9993	0.10−1.20	0.0330	0.0992
5	Y = 481.0792 + 6299.1942X	0.9984	0.20−3.20	0.0010	0.0034
6	Y = −2.0423 + 1721.8740X	0.9999	0.05−1.20	0.0038	0.0119
7	Y = 1228.5054 + 2292.3870X	0.9999	0.20−2.40	0.0250	0.0840

[a] Y is the value of peak area, and X is the value of the reference compound's concentration (μg/mL).
[b] LOD and LOQ were determined at S/N of about 3 and 10, respectively.

3.2. Quantification of the seven compounds

According to these results, the content of compound **3** (377.280 mg/g) was higher than other compounds, and the content of compound **5** was 330.960 mg/g in EtOH−water extract from the seed of *F. rhynchophylla* Hance (Table 2). The content of total analytes is 778.086 mg/g or 77.81% in EtOH−water extract from the seed of *F. rhynchophylla* Hance.

3.3. The hepatoprotective activities assays

3.3.1. EtOH−water extract (EWE) decreased liver index

Liver swelling induced by CCl_4 was manifested on the increase of liver index. As shown in Fig. 3, in the model group (CCl_4-intoxicated group), liver index was 4.96, which was significantly higher than control group ($P < 0.05$). Pretreatment with **EWE** significantly decreased liver index compared with model group ($P < 0.05$).

3.3.2. EWE inhibited the increase of serum aminotransferase induced by CCl_4

The results of hepatoprotective effect of **EWE** on the serum ALT and AST activities are shown in Fig. 4. In CCl_4-intoxicated group, serum ALT and AST activities were 239.46 and 11.97 U/L, respectively, whereas the values of control group were only 46.79 and 2.34 U/L, respectively. Therefore, a significant increase in the activities of serum ALT and AST was observed when liver was exposured to CCl_4 ($P < 0.05$). Administration with different doses of **EWE** for 49 days significantly reduced the activities of serum ALT and AST as compared to the control group ($P < 0.05$) in a dose-dependent manner. Silymarin, which has been used as medicine for liver disease, had superior effects on decreasing serum ALT and AST activities.

3.3.3. EWE decreased hepatic MDA content, SOD and GSH-Px activities

The levels of MDA, SOD and GSH-Px are indicators of oxidative stress. A 30.77% increase of MDA content was observed in model group compared with control group (Fig. 5). Pretreatment with EWE and silymarin could completely inhibit CCl_4-induced increase of liver MDA content. As shown in Figs. 6 and 7, hepatic SOD and GSH-Px activities were decreased by 44.21% and 59.96%, respectively, after CCl_4 treatment. However, pretreatment with **EWE** significantly increased SOD and GSH-Px activities ($P < 0.05$).

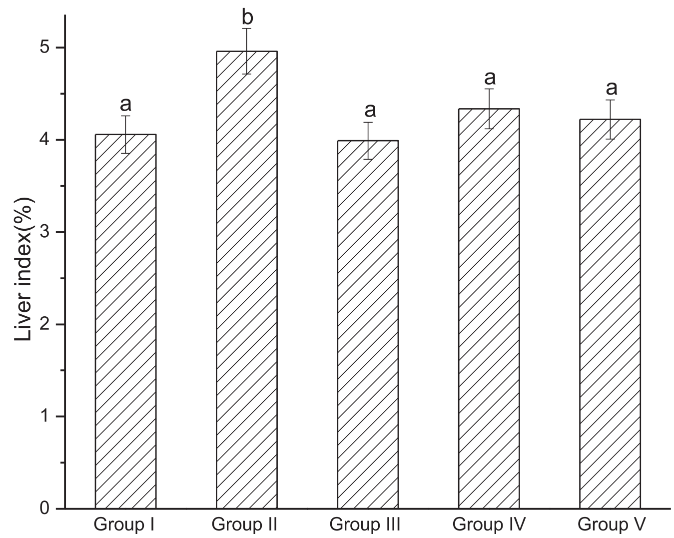

Fig. 3. Effects of **EWE** on liver index. Different lower case letters correspond to significant differences at $P < 0.05$. Group I was the control group. Group II was CCl_4-treated group. Group III was given CCl_4 + silymarin. Group IV and V were given CCl_4 + 2.0 and 4.0 g/kg BW of **EWE** respectively.

3.4. Histopatholgy

The histological observations of the hepatoprotective effects of **EWE** on CCl_4-induced liver damage are illustrated in Fig. 8. Liver sections from control mice displayed regular cellular morphology (Fig. 8A). Compared with the normal liver tissues of control mice, liver tissue in CCl_4-intoxicated mice revealed liver injury characterized by dilated sinusoidal spaces, and inflammatory cell infiltration (Fig. 8B). However, the hepatic lesions induced by treatment with CCl_4 were remarkably ameliorated in hypertrophy of hepatocytes, dilated sinusoidal spaces and inflammatory cell infiltration by treatment with silymarin and different dose of **EWE** (Fig. 8C−E).

Table 2
Contents of seven compounds in the EtOH−water extract of seeds of *F. rhynchophylla* Hance.

No	Contents of analytes[a] (mg/g, n = 3)							Extraction rate (*w* %)[b]
	1	2	3	4	5	6	7	
EtOH−water	2.247	2.930	377.280	11.244	330.960	27.368	26.057	9.08

[a] $\text{Content} = \frac{X \frac{V_1 \text{ (injection volume of standard solution)}}{V_2 \text{ (injection volume of sample solution)}} \times V \text{ (sample volume)}}{M \text{ (sample amount)}}$
[b] Extraction rate = solids content of the extract/sample volume.

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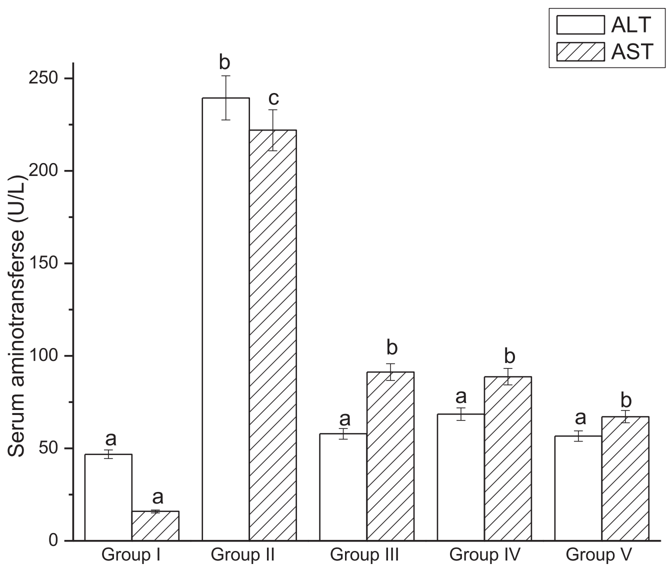

Fig. 4. Effects of **EWE** on serum ALT and AST. Different lower case letters correspond to significant differences at $P < 0.05$. Group I was the control group. Group II was CCl_4-treated group. Group III was given CCl_4 + silymarin. Group IV and V were given CCl_4 + 2.0 and 4.0 g/kg BW of **EWE** respectively.

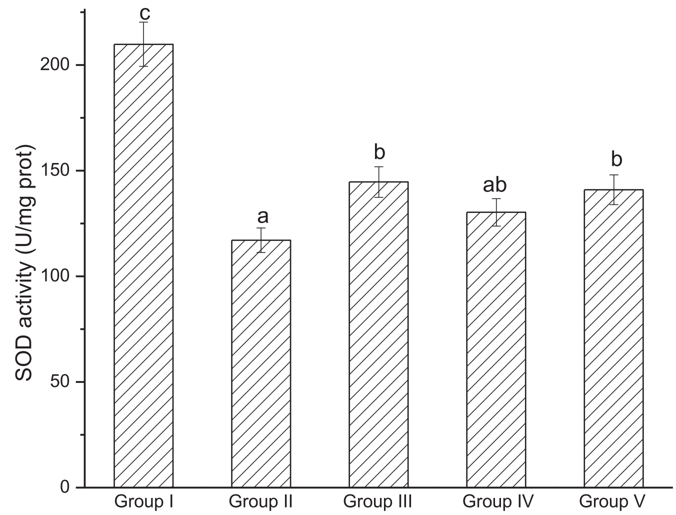

Fig. 6. Effects of **EWE** on hepatic SOD activity. Different lower case letters correspond to significant differences at $P < 0.05$. Group I was the control group. Group II was CCl_4-treated group. Group III was given CCl_4 + silymarin. Group IV and V were given CCl_4 + 2.0 and 4.0 g/kg BW of **EWE** respectively.

4. Discussion

The therapeutic benefits of Chinese herbal medicine have been recognized for centuries on the basis of clinical experience and practice. Qinpi, the stem barks of *F. rhynchophylla* Hance, is a traditional Chinese herbal drug for treating various types of chronic diseases.

ALT and AST are aminotransferases and associated with liver parenchymal cell. The enzymes normally present in the cytosol and are leaked out into the blood stream, when the hepatocellular plasma membrane is damaged. Thus, ALT and AST serum levels are very sensitive markers employed in the diagnosis of liver diseases. Rats administered once with CCl_4 exhibited liver injury, as indicated by significant elevation in serum markers for hepatic cell damage (ALT and AST).[37] At present study, in EWE-treated groups (2.0 and 4.0 g/kg) of rats, the studies showed that treatment with EWE significantly and dose-dependently decreased the elevations of serum levels of ALT and AST. Furthermore, the improvement of the hepatic impairment was achieved and could be observed by EWE intervention in CCl_4-induced liver injury. Thus, the results showed the protective effects of EWE on hepatic pathology and orally administered EWE exerted therapeutic effects on CCl_4-induced liver injury in rats.

Oxidative stress associated with lipid peroxidation is involved in liver development. The extracts and the identified compounds from *F. rhynchophylla* Hance had been reported anti-oxidative activity.[30–32] MDA is an aldehyde, which is a product of polyunsaturated fatty acid peroxidation, and is a highly toxic molecule considered as more than just a marker of lipid peroxidation. In this

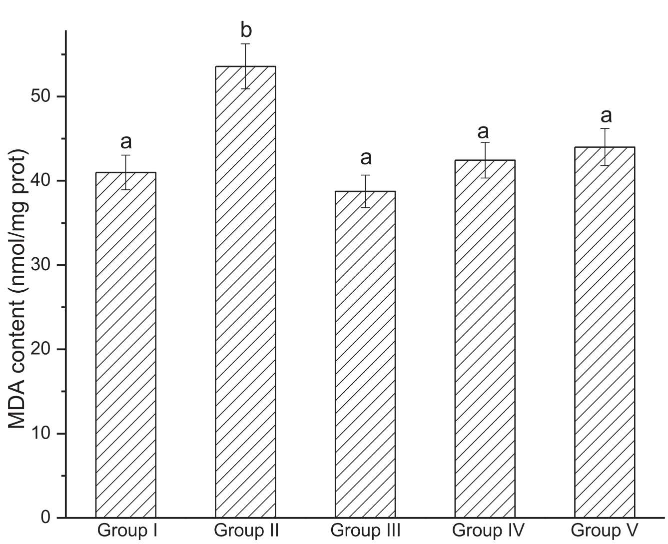

Fig. 5. Effects of **EWE** on hepatic MDA. Different lower case letters correspond to significant differences at $P < 0.05$. Group I was the control group. Group II was CCl_4-treated group. Group III was given CCl_4 + silymarin. Group IV and V were given CCl_4 + 2.0 and 4.0 g/kg BW of **EWE** respectively.

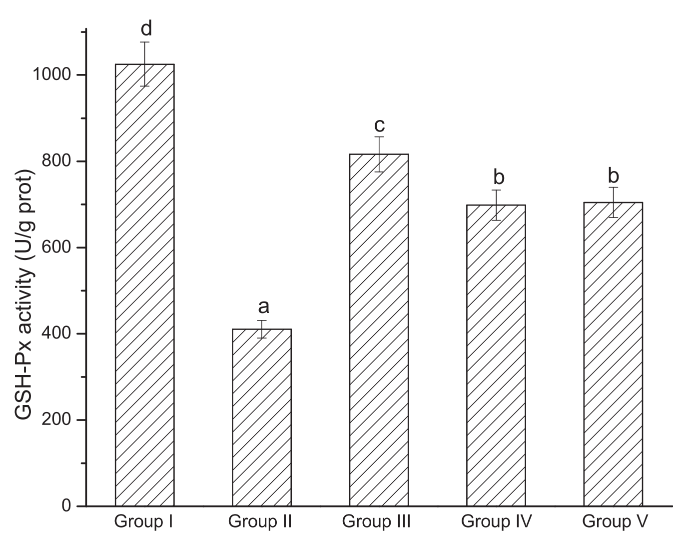

Fig. 7. Effects of **EWE** on hepatic GSH-Px activity. Different lower case letters correspond to significant differences at $P < 0.05$. Group I was the control group. Group II was CCl_4-treated group. Group III was given CCl_4 + silymarin. Group IV and V were given CCl_4 + 2.0 and 4.0 g/kg BW of **EWE** respectively.

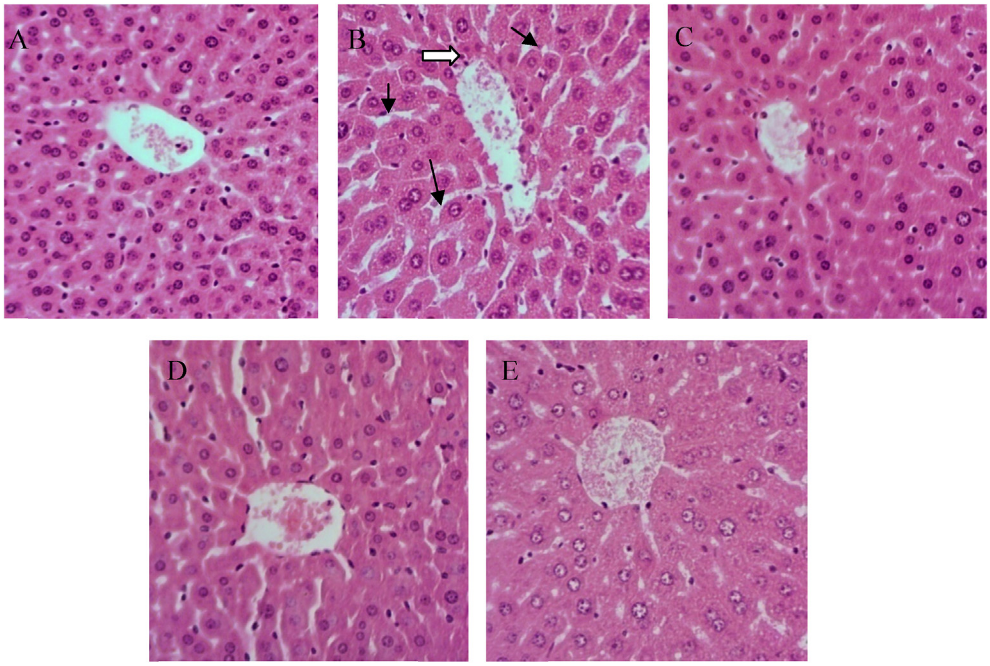

Fig. 8. Effects of **EWE** on hepatic morphological analysis (×200 H&E): control mice (A), CCl_4-treated mice (B), mice pretreated with silymarin prior to CCl_4 (C), mice pretreated with 2.0 (D) and 4.0 g/kg (E) BW of **EWE** respectively prior to CCl_4. (➡ dilated sinusoidal spaces, ⇨ inflammatory cell infiltration).

study, the elevated hepatic MDA level was decreased by pretreatment of EWE, so it indicated that EWE could protect the liver against CCl_4-induced lipid peroxidation. GSH and SOD are a host of antioxidant systems to protect the hepatocyte from oxidative stress. GSH, or GSH-Px, and SOD work in concert to control the cascades of uncontrolled lipid peroxidation and protect cell from oxidative damage by scavenging of reactive oxygen species (ROS) or the toxic free radicals.[38–40] In this study, the CCl_4-model rats present lower GSH-Px and SOD level than the control group. Thus, it was supposed that EWE possessed the properties to enhance GSH-Px and SOD, and could prevent hepatice impairment via inhibiting oxygen-free radicals and increasing anti-oxidation in CCl_4-induced liver injury in rats.

5. Conclusion

In conclusions, the current research indicates that the EtOH–-water extract (EWE) from the seeds of *F. rhynchophylla* Hance decreased liver index, inhibited the increase of serum aminotransferase induced by CCl_4, and decreased hepatic MDA content, SOD and GSH-Px activities. These results suggested that the pretreatment with EWE protected mice against CCl_4-induced liver injuries. Further hepatoprotective effect and the possible mechanism of active constituents of the seeds of *F. rhynchophylla* Hance will be studied.

Conflict of interests

The authors declare that they have no competing interest.

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