

Protective effect of seed oil of *Herpetospermum pedunculatum* against carbon tetrachloride-induced liver injury in rats

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ABSTRACT

الأهداف: دراسة الأثر الوقائي لزيت بذور نبتة كاسيه البذور ضد رابع كلوريد الكربون المسبب لضرر الكبد.

الطريقة: أجريت دراسة مخبرية في معهد شمال غرب معهد بلاتيو الحيوي، الأكاديمية الصينية للعلوم، جامعة يانتي، الصين خلال الفترة من نوفمبر 2012م حتى مايو 2013م. تم استخراج بذور نبتة كاسيه البذور باستخدام ثاني أكسيد فوق الحرج. تم فحص الأنشطة المضادة للأكسدة لزيت بذور نبتة كاسيه البذور في المختبر بواسطة مقايسة DPPH ومقايسة مانع انحلال الدم، وفحص بروكسيد الدهون، وفحص مانع انحلال الدهون. تم تقسيم الفئران البالغة إلى 6 مجموعات (10 فئران/مجموعة) والتي تشمل مجموعة الشاهد، ومجموعة CCl_4 ، ومجموعة CCl_4 مع البيفنديت، ومجموعة CCl_4 مع زيت بذور نبتة كاسيه البذور (3 جرعات مختلفة).

النتائج: تشمل آفات الكبد الناتجة عن CCl_4 على كلا من نخر الكبد وانحطاط التضخم، والتكلس والتليف، إضافة إلى ذلك، نتج ضرر مجموعة CCl_4 في ارتفاع ملحوظ في الدهون الثلاثية والدهن عالي الكثافة، والدهن قليل الكثافة، مالنديهولايد، و البيليروبين الكلي، ناقلة امين الالانين، و امين الاسبارتات، ونشاط فسفاتاز قلووية. إضافة إلى هذا، يقلل CCl_4 من نشاط من نشاط فوق الاكسيد الديسموتاز. وبالعكس، يحسن زيت بذور نبتة كاسيه البذور من آفات الكبد الناجمة من CCl_4 ويقلل من مستويات مصل علامات انزيم مصل الكبد ويزيد نشاط فوق الاكسيد الديسموتاز.

الخلاصة: تشير الدراسة أنه بالإمكان استخدام زيت بذور نبتة كاسيه البذور لحماية الكبد من الضرر التأكسدي الناجم من CCl_4 في الفئران وقد يكون التأثير على الكبد مرتبط مع مضادات الأكسدة والتأثير الجذري.

Objectives: To investigate the protective effect of *Herpetospermum pedunculatum* (*H. pedunculatum*) seed oil against carbon tetrachloride (CCl_4)-induced liver damage.

Methods: This experimental study was conducted at the Northwest Institute of Plateau Biology, Chinese

Academy of Sciences, and Yantai University, China from November 2012 to May 2013. The *H. pedunculatum* seed oil was extracted using supercritical carbon dioxide. The antioxidant activities of *H. pedunculatum* seed oil were assayed in vitro by 2,2-diphenyl-1-picrylhydrazyl assay, lipid peroxidation assay, and antihemolytic assay. Adult Sprague Dawley rats were randomly divided into 6 groups (10 rats/group) including control, CCl_4 , CCl_4 +bifendate, and CCl_4 +*H. pedunculatum* seed oil (3 different doses) groups.

Results: The CCl_4 -induced liver lesions include hepatocyte necrosis, ballooning degeneration, calcification, and fibrosis. Moreover, CCl_4 damage results in an obvious increase of serum triglycerides, high-density lipoprotein, low-density lipoprotein, malondialdehyde, total bilirubin, alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase activity. In addition, CCl_4 also significantly decreased the activities of superoxide dismutase (SOD). By contrast, *H. pedunculatum* seed oil administration significantly ameliorated the CCl_4 -induced liver lesions, lowered the serum levels of hepatic enzyme markers, and increased the activities of SOD.

Conclusion: The results of this study show that *H. pedunculatum* seed oil can be proposed to protect the liver against CCl_4 -induced oxidative damage in rats, and the hepatoprotective effect might be correlated with its potent antioxidant and free radical scavenging effect.

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Herpetospermum pedunculosum (*H. pedunculosum*) (Ser.) C. B. Clarke is an annual climber and is known as “Sejimeiduo” (meaning, auspicious beautiful flower) among Tibetan traditional healers. It is distributed and sometimes cultivated in the Himalayan region including China, Nepal, Bhutan, and India at elevations of 2000-3500 meters.¹ The seeds of *H. pedunculosum* are popularly accepted among traditional healer as “Bolenguazi”. In the past 30 years, many studies have been conducted using the contents of *H. pedunculosum* seeds, such as herpetal,² and others.³⁻¹⁰ Besides, *H. pedunculosum* seeds also contain rich fatty acids and unsaturated fatty acids (UFAs).^{11,12} In a previous study,¹² we have optimized the extracted conditions of *H. pedunculosum* seed oil by supercritical fluid of carbon dioxide (CO₂). The optimum parameters obtained from the orthogonal test and single factor experiments were as follows: extraction temperature 50°C; pressure 35 MPa; CO₂ flux 35 L/h; particle size of pulverized *H. pedunculosum* seed 80 mesh; and extraction time 3.5 hours.¹² The seeds of *H. pedunculosum* is a well-known heat-clearing and detoxicating herb, and may relieve “Chiba” diseases (one of the 3 main types of diseases in the Tibetan medicine, such as jaundice, hepatitis, intumescences, and inflammation, which are caused by functional disorder of the gallbladder and liver) by the Tibetan healers. Some researchers have studied the activity of lipophilic extracts from *H. pedunculosum* seeds including anti-inflammatory, antiviral, antioxidant, and hepatoprotective actions.^{13,14} However, there is no report on the activity of *H. pedunculosum* seed oil. The *H. pedunculosum* seed oil contains abundant UFA that play important roles in the regulation of a variety of physiological and biological functions. Essential fatty acids are important because of their role in diverse physiological processes affecting normal health and chronic diseases, such as, the regulation of plasma

lipid levels, cardiovascular and immune function, and liver function.¹⁵ The liver is the key organ in the metabolism, detoxification, and secretory function in the body.¹⁶ Liver diseases are mainly caused by toxic chemicals, excess consumption of alcohol, infections, and autoimmune disorders. Therefore, in the present study, we extracted the *H. pedunculosum* seed oil using supercritical CO₂. We assayed the antioxidant activities of *H. pedunculosum* seed oil in vitro, and evaluated the potential hepatoprotective effects of *H. pedunculosum* seed oil against carbon tetrachloride (CCl₄)-induced hepatic damage in rats.

Methods. This animal experimental study was conducted at the Key Laboratory of Tibetan Medicine Research, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, and Yantai University, China from November 2012 to May 2013.

Preparation of *H. pedunculosum* seed oil. The seeds of *H. pedunculosum* were collected from Linzhi, Tibet, China, and the plant was identified and authenticated by Prof. Changfan Zhou (Northwest Institute of Plateau Biology, Chinese Academy of Sciences). The seeds were cleaned, shade-dried, and powdered. The seed oil was extracted using the dried powder by supercritical CO₂ extraction as previously described.¹² The seed oil was mixed with water and Tween-80 at a ratio of 75:20:5(v/v/v) to feed the rats in the further experiments.

Animals. Adult Sprague Dawley (SD) rats of either gender weighing between 160 and 180 g were purchased from the Laboratory Center for Medical Science Lanzhou University. Animals had free access to standard diet and water, maintained under a controlled condition of temperature of 25±2°C, relative humidity 60-70%, and a 12 hour light:12 hour dark cycle. All treatments were conducted between 9:00 and 10:00 A.M. to minimize variations in animal response due to circadian rhythm. All studies were conducted according to the suggested ethical guidelines for the care of laboratory animals.

Chemicals. The CCl₄ and Tween-80 were obtained from Shanghai Chemical Reagent Co (Shanghai, China). Bifendate pills were purchased from Beijing Union Pharmaceutical Factory (Beijing, China). All the assay kits for serum triglycerides (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), superoxide dismutase (SOD), malondialdehyde (MDA), total bilirubin (TBIL), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline

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phosphatase (ALP) were purchased from Nanjing Jiancheng Biology Co., Nanjing, China. The other chemicals and reagents used were analytical grade.

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity. The free radical scavenging activity of the seed oil was examined using stable DPPH radical.¹⁷ Test samples (1-1000 µg/mL) were dissolved in methanol (MeOH [CH₃OH]), and mixed with ethanol solutions of DPPH (0.1 mM) in a 96-well microtiter plates following incubation at 37°C for 30 minutes. The DPPH reduction was estimated at 517 nm. Percentage inhibition by the sample treatment was determined by comparison with an MeOH-treated control group. All experiments were carried out in triplicate. The free radical scavenging activity was analyzed using linear regression analysis.

Lipid peroxidation assay. A modified thiobarbituric acid reactive species (TBARS) assay was used to measure lipid peroxide.¹⁸ Briefly, a 50 µl egg yolk homogenate (4% v/v in phosphate buffered saline (PBS [pH: 7.4]) and 50 µl of samples were added to a test tube. Then, 50 µl of ferrous sulfate (FeSO₄) (25 mM) were added, and made up to 0.5 ml with PBS, all were mixed and incubated for 15 min at 37°C to induce lipid peroxidation. Thereafter, 100 µl 20% trichloroacetic acid (TCA) was added in each test tube, and centrifuged for 10 min (3000×g). Then, 600 µl of supernatant and 300 µl of thiobarbituric acid (TBA) were mixed and heated for 15 min at 100°C. After cooling, the absorbance of supernatant was measured at 532 nm. Each assay was performed 3 times. Percentage inhibition of lipid peroxidation by various concentrations (1 to 1000 µg/mL) of seed oil was calculated.

Red blood cells hemolysis in vitro assay. Inhibition of peroxy radicals induced red blood cells (RBCs) hemolysis of seed oil was examined by in vitro method.¹⁹ Briefly, RBCs were isolated by centrifugation of heparinized blood obtained from healthy SD rats, and washed 3 times by normal saline solution. Washed RBCs were suspended to 10% hematocrit in PBS. Oxidative hemolysis of erythrocytes was induced by 2,2'-azobis dihydrochloride (AAPH), a peroxy radical initiator. One mL diluted RBC were incubated with 1 mL freshly prepared 25 mmol/L AAPH in the absence (negative control) and presence of different concentrations of seed oil (1 to 1000 µg/mL). The reaction mixture was shaken gently while being incubated at 37°C for 2 hours. After a 10-minute centrifugation (3000×g), the absorbance reading of supernatant was evaluated at 540 nm. Each assay was performed 3 times. The percentage of

inhibitory effect (X [%]) of each sample against RBCs hemolysis was calculated using the following formula:

$$X (\%) = (1 - A/B) \times 100$$

Where, A is the sample absorption and B is the negative control absorption.

Acute toxicity studies. The animals were randomly selected, and acclimatized to laboratory conditions for at least 5 days prior to the experiments. Seven animals were treated with 20 g/kg of seed oil as single treatment per oral, and the animals was observed continuously for the first 4 hours, and finally overnight mortality was observed.

Experimental treatments. The rats were randomly assigned to 6 groups (10 rats/group): Group 1 - normal control; Group 2 - CCl₄ control; Group 3 - bifendate positive control (200 mg/kg); Groups 4 (1 g/kg), 5 (2 g/kg), and 6 (4 g/kg) were seed oil treated rats. After 3 weeks administration, rats in Groups 2-5 were given a single intra-peritoneal (i.p) injection of CCl₄ (3.0 mL/kg). Then, the animals were deprived of food but allowed free access to tap water for 24 hours. All the rats were sacrificed by chloroform anesthesia injection.

Determination of biochemical parameters from serum. After the animals were sacrificed by chloroform anesthesia injection, blood was collected by cardiac puncture, and the blood samples of each animal were allowed to clot for 45 min at room temperature. Serum was separated by centrifugation at 2,500 rpm at 4°C for 15 min. Various biochemical parameters including TG, HDL, LDL, SOD, MDA, TBIL, AST, ALT, and ALP in serum were determined by an automatic blood biochemical analyzer (Model 7080, Hitachi High-Technologies Corp., Tokyo, Japan).

Histopathological studies. Liver specimens were preserved in 10% neutral buffered formalin and dehydrated in a graded alcohol series. Following xylene treatment, the specimen was then embedded in paraffin blocks, and cut into 5 µm thick sections. Sections were stained with hematoxylin and eosin (H&E), and examined for histopathological changes under the microscope (BX41TF, Olympus Microsystems Corp., Tokyo, Japan). Images were taken at original magnification of 200× (Eclipse E600 Microscope, Nikon, Japan, and Leica DMIL, Leica Microsystems, Germany).

Statistical analysis. Results were presented as mean ± standard deviation. The Statistical Package for Social Sciences for Windows version 19 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Mann-Whitney U test and Kruskal-Wallis test were

used for non-parametric data comparisons and $p < 0.05$ was considered statistically significant.

Results. The *H. pedunculosum* seed oil in this study richly contains UFA, such as, 12-octadecenoic acid (46.9%), 9,12-octadecadienoic acid (27.7%), and 8,11,14-octadecatrienoic acid (8.9%). Besides the above components, it also contains hexadecanoic acid (9.4%), and eicosoic acid (5.5%) (Figure 1). The antioxidant activities of the *H. pedunculosum* seed oil were measured in different systems of assay, for example, DPPH assay, lipid peroxidation assay, and antihemolytic assay (Figure 2). The results demonstrated

that *H. pedunculosum* seed oil possessed high antihemolytic ability and lipid peroxidation inhibitory activity with a concentration range from 1 µg/mL to 1000 µg/mL, the inhibitory concentration 50 (IC₅₀) values were 20.3 ± 2.5 µg/mL and 178.5 ± 16.3 µg/mL. While the IC₅₀ of *H. pedunculosum* seed oil on DPPH radicals was 601.7 ± 76.8 µg/mL, which indicated that seed oil has no obvious inhibitory effects on DPPH. In acute toxicity studies, the oral administration of seed oil caused neither any behavioral changes, nor mortality up to 20 g/kg. The LD₅₀ of seed oil was thus found to be more than 20 g/kg. The SOD is an effective defense enzyme that catalyzes the dismutation

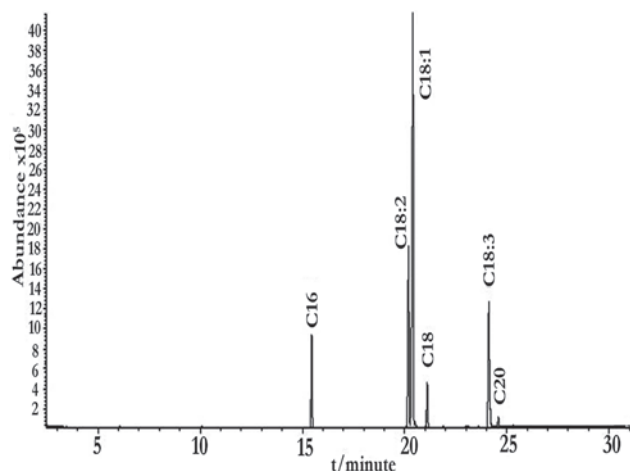


Figure 1 - The total ion chromatogram of methyl esterifying derivatives of fatty acids in *Herpetospermum pedunculosum* seed oil. C16 - hexadecanoic acid, C18:2 - 9,12-octadecadienoic acid, C18:1 - 12-octadecenoic acid, C18 - octadecanoic acid, C18:3 - 8,11,14-octadecatrienoic acid, C20 - eicosoic acid

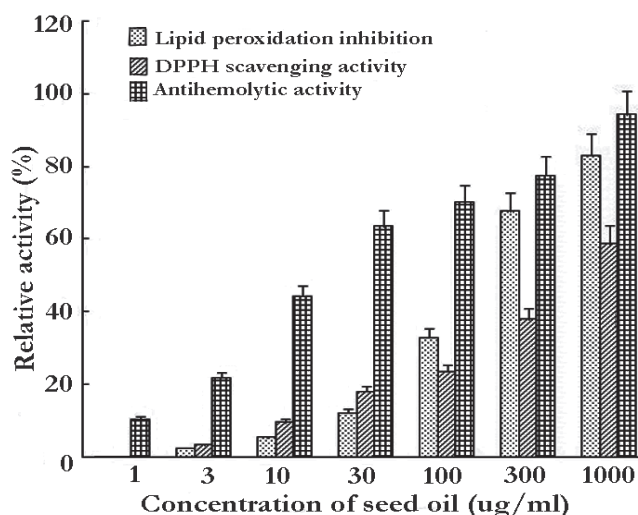


Figure 2 - Antioxidant assay of *Herpetospermum pedunculosum* seed oil in vitro by lipid peroxidation inhibition, 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging and red blood cells hemolysis (\pm s, n=3)

Table 1 - Effects of *Herpetospermum pedunculosum* seed oil on serum biochemical parameters of rats intoxicated with CCl₄ (mean \pm standard deviation n=10).

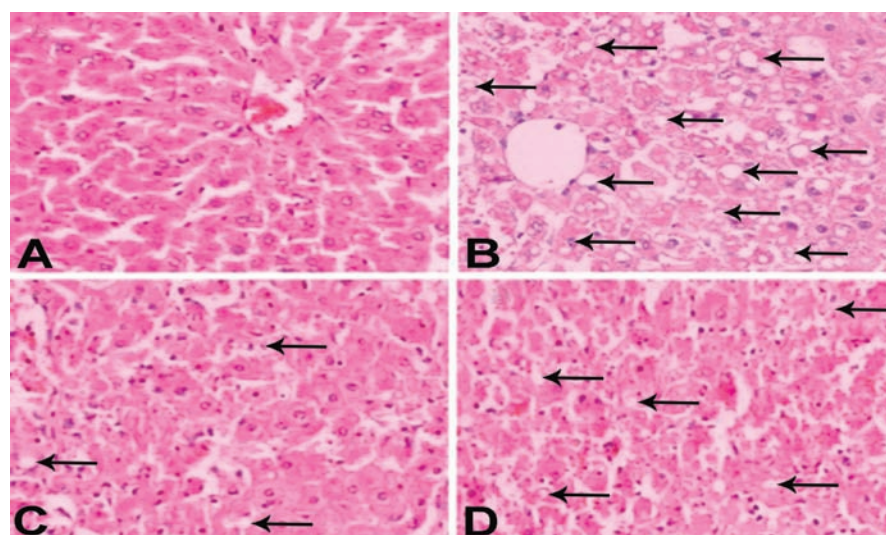
Treatments	Superoxide dismutase (U/L)	Malondialdehyde	Triglycerides (nmol/L)	High-density lipoprotein	Low-density lipoprotein
Group 1 (control)	57.69 \pm 15.08	20.84 \pm 6.66	0.67 \pm 0.21	0.99 \pm 0.19	1.03 \pm 0.24
Group 2 (CCl ₄)	42.86 \pm 10.76	28.68 \pm 8.32	2.18 \pm 0.53	1.30 \pm 0.11	1.65 \pm 0.10
<i>P</i> -value	0.043*	0.047*	<0.001*	0.02*	0.019*
Group 3 (CCl ₄ + bifendate)	100.57 \pm 21.66	12.96 \pm 3.59	1.44 \pm 0.34	0.57 \pm 0.11	1.06 \pm 0.19
<i>P</i> -value	0.005 [†]	0.004 [†]	0.027 [†]	<0.001 [†]	<0.001 [†]
Group 4 (CCl ₄ + 1 g/kg seed oil)	74.28 \pm 17.91	14.66 \pm 3.89	1.10 \pm 0.38	0.43 \pm 0.32	1.11 \pm 0.37
<i>P</i> -value	0.031 [†]	0.009 [†]	<0.001 [†]	<0.001 [†]	0.03 [†]
Group 5 (CCl ₄ + 2 g/kg seed oil)	97.30 \pm 12.51	15.44 \pm 5.43	1.03 \pm 0.40	0.50 \pm 0.13	1.08 \pm 0.33
<i>P</i> -value	<0.001 [†]	0.031 [†]	<0.001 [†]	0.02 [†]	0.04 [†]
Group 6 (CCl ₄ + 4 g/kg seed oil)	102.69 \pm 29.39	8.55 \pm 1.94	0.82 \pm 0.10	0.59 \pm 0.12	1.14 \pm 0.20
<i>P</i> -value	<0.001 [†]	<0.001 [†]	<0.001 [†]	0.017 [†]	0.09 [†]

CCl₄ - carbon tetrachloride. *compared to normal group, [†]compared to CCl₄ group

Table 2 - Effects of *Herpetospermum pedunculosum* seed oil on liver damage markers activities in serum of rats intoxicated with CCl₄ (mean ± standard deviation, n=10).

Treatments	Total bilirubin (Umol/L)	Alanine aminotransferase	Aspartate aminotransferase (U/L)	Alkaline phosphatase
Group 1 (control)	1.15 ± 0.98	39.60 ± 5.41	229.00 ± 35.03	136.7 ± 23.3
Group 2 (CCl ₄)	11.89 ± 3.87	1263.43 ± 361.30	1084.86 ± 289.13	281.6 ± 36.3
<i>P</i> -value	<0.001*	0.001*	0.001*	<0.001*
Group 3 (CCl ₄ + bifendate)	9.7 ± 2.6	1394.20 ± 278.11	886.00 ± 204.82	191.4 ± 29.4
<i>P</i> -value	0.198 [†]	0.372 [†]	0.088 [†]	0.001 [†]
Group 4 (CCl ₄ + 1 g/kg seed oil)	6.54 ± 1.58	1285.38 ± 322.05	1181.38 ± 178.33	220.8 ± 34.3
<i>P</i> -value	0.047 [†]	0.766 [†]	0.321 [†]	0.007 [†]
Group 5 (CCl ₄ + 2 g/kg seed oil)	5.67 ± 2.07	1109.14 ± 365.50	1039.43 ± 244.18	185.0 ± 21.3
<i>P</i> -value	0.003 [†]	0.776 [†]	0.909 [†]	<0.001 [†]
Group 6 (CCl ₄ + 4 g/kg seed oil)	4.75 ± 1.09	397.40 ± 76.87	310.10 ± 33.99	141.3 ± 27.8
<i>P</i> -value	<0.001 [†]	0.001 [†]	0.002 [†]	<0.001 [†]

CCl₄ - carbon tetrachloride. *compared to normal group, [†]compared to CCl₄ group.

**Figure 3** - Histopathology of rat liver after administration of carbon tetrachloride (CCl₄) in different groups: A) normal group; B) CCl₄ group; C) CCl₄+ bifendate group; and D) CCl₄+ 4 g/kg seed oil group, (stained with Hematoxylin & Eosin) original magnification ×200.

of superoxide anions into hydrogen peroxide.²⁰ In the present study, the hepatic antioxidant enzymatic activity of SOD was significantly decreased in CCl₄-intoxicated rats compared with control rats, implying increased oxidative damage to the liver (Table 1). On the contrary, SOD levels were significantly elevated by the administration of *H. pedunculosum* seed oil to CCl₄-intoxicated rats, suggesting that it has the ability to restore the activity of SOD in CCl₄-damaged liver. The MDA levels of serum were also determined as an indicator of lipid peroxidation. The administration of *H. pedunculosum* seed oil could significantly reduce the production of MDA generated in CCl₄-damaged liver.

These results demonstrated that the antioxidant ability of *H. pedunculosum* seed oil was very critical for its hepatoprotective effects.

In the present study, we also found that treatment with *H. pedunculosum* seed oil markedly inhibits CCl₄-induced liver damage as evidenced by reduced serum concentration of TG, HDL, and LDL, and decreased serum activities of AST, ALT, and ALP (Table 2). In addition, histopathological examination of the liver was performed after H & E staining (Figure 3). Severe vacuolization (arrows) and necrotic hepatocytes clearly appeared in CCl₄ intoxicated group (Figure 3B). Figure 3C showed the liver sections of rats

treated with CCl₄ and bifendate, which manifested slight fatty changes. Liver cells of rats treated with CCl₄ and 4g/kg *H. pedunculosum* seed oil also showed slight fatty changes (Figure 3D).

Discussion. In this study, we investigated the effects of *H. pedunculosum* seed oil on liver injury induced by CCl₄ in rats. The CCl₄ is a xenobiotic that produces hepatotoxicity in various experimental animals.²¹ The CCl₄ is metabolized by cytochrome P450 to form a reactive trichloromethyl radical (CCl₃) and a trichloromethyl peroxy radical (CCl₃O₂).²² Both radicals are capable of binding to DNA, lipids, proteins or carbohydrates, which will cause the loss of integrity of all membranes, necrosis of hepatocytes, and deposition of collagen resulting in liver fibrosis.²³⁻²⁵ Many studies have demonstrated that an important mechanism of the hepatoprotective effects may be related to an antioxidant capacity to scavenge reactive oxygen species.^{26,27}

First, the TG, HDL, and LDL content of the serum were increased after CCl₄ administration, and the increase was suppressed by the administration of *H. pedunculosum* seed oil. Farber et al²⁸ has proven that CCl₄ can influence the secession of ribosome and liver proteosynthesis, and blocks lipoprotein secretion. It suggested that *H. pedunculosum* seed oil may regulate lipid accumulation in the liver. Since the lipid accumulations are associated with deactivation of metabolizing enzymes AST, ALT, TBIL, and ALP activities in rats serums were assayed (Table 2). Both AST and ALT activities in serum were increased by administration of CCl₄, but they were not effectively reduced by the administration of bifendate. However, AST and ALT activity were significantly decreased after administration of 4 g/kg seed oil. It is known that ALT activity increases as a result of destruction and necrosis of hepatocytes. As ALT activity was inhibited by administration of *H. pedunculosum* seed oil, hepatocyte necrosis might have been suppressed. Meanwhile, the TBIL and ALP activity were significantly increased in the CCl₄ group, and *H. pedunculosum* seed oil had a significant suppressive effect, especially in 4 g/kg seed oil group. It has been reported that UFA could ameliorate fatty liver damage induced by CCl₄.²⁹ The biochemical observations are also supported by the histopathological examination of the rats' liver (Figure 3) treated with 4 g/kg *H. pedunculosum* seed oil could significantly reduce the vacuolization and slight fatty changes. Although our research had proven the protective effects of *H. pedunculosum* seed oil on liver injury, the role of single component of UFA was still unclear, and this limits our study.

In conclusion, the results of this study demonstrate that *H. pedunculosum* seed oil is effective in preventing CCl₄-induced hepatic injury in rats. Our results show that *H. pedunculosum* seed oil mainly consisted of several UFA. The hepatoprotective effects of *H. pedunculosum* seed oil is attributed to these UFA by increasing the antioxidant enzymes activity, and decreasing the liver enzymes activity. Further studies should be carried out in order to assess the role and elucidate the action mechanisms.

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