Research Journal of Pharmacognosy 5(1), 2018: 47-54
Received: 4 Sep 2017
Accepted: 12 Dec 2017
Published online 27 Dec 2017

Original article

The Ameliorative Potential of *Sophora alopecuroides* Essential Oil on CCl_4-Induced Hepatotoxicity in Mice; a Stereological Study

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Abstract

Background and objectives: *Sophora alopecuroides*, one of the most important herbal medicines is widely grown in west of Iran, and has a long history for treatment of gastrointestinal diseases, leucorrhea, eczema, and psoriasis. The aim of this study was to investigate the ameliorative effect of *Sophora alopecuroides* essential oil on CCl_4-induced hepatotoxicity in mice. **Methods:** Thirty five male mice were divided into five groups; group I as the negative control, received olive oil intraperitoneally and distilled water orally. Group II as the positive control, received CCl_4 mixed with olive oil in the ratio of 5:5, intraperitoneally and distilled water orally. Group III, IV and V received CCl_4 and 200, 800 and 1600 µg/kg of *S. alopecuroides* essential oil through gavages for 45 consecutive days. **Results:** The results showed that low and intermediate doses of *S. alopecuroides* essential oil significantly decreased the raised levels of Alanine aminotrasferase and Aspartate aminotransaminase toward control levels ($p<0.05$). Alkaline phosphatase was improved with tree examined doses of *S. alopecuroides* essential oil. The volume and weight of the liver, as well as the volume of hepatocytes and sinusoids which had increased significantly in the positive control group ($p<0.05$), decreased significantly following treatment by low dose of *S. alopecuroides* essential oil ($p<0.05$). **Conclusion:** It was concluded that although *Sophora alopecuroides* essential could protect liver against CCl_4-induced toxicity at lower doses, further studies would be needed to define the selective dose of this plant against CCl_4-induced hepatotoxicity.

Keywords: hepatocyte; liver; medicinal plants; *Sophora alopecuroides*; volume density

Introduction

By virtue of its unique structure and high metabolic rate, the liver is the first organ to be subjected to many toxic agents. Therefore, protection of the liver functions is of major importance [1]. On the other hand, it has been established that free radicals and reactive oxygen species (ROS) play a pivotal role in the progression of liver diseases regardless of their exact etiologies [2,3].

Carbon tetrachloride (CCl_4) is a known toxic substance for most cells especially hepatocytes. Hence, it is used routinely for inducing acute hepatotoxicity in experimental designs and evaluating hepatoprotective features of different

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medicinal plants [4]. Due to the adverse side effects of synthetic drugs and their expensive cost, the interest in herbal medicine is increasing and currently it is accepted that most of the herbal drugs are safe, more accessible and more affordable [5].

New methods in the preparation of herbal remedies such as essential oil extraction are attractive for plant researchers [6]. Essential oil is a condensate hydrophobic liquid including aromatic compounds from plants and could be extracted from several parts such as roots, stems, leaves, and flowers. In recent years, interest in essential oils has been enhanced for pharmacological studies which claim that the essential oils have useful effects for inhibiting and treating liver disease [7].

Iran is one of the richest countries in the world for medicinal plants. *Sophora alopecuroides* is one of the most important herbal medicines which is widely grown in west of Iran. It belongs to the Fabaceae family and *Faboideae* subfamily [8]. This plant has a long history for treatment of gastrointestinal diseases, leucorrhea disorder, eczema, *psoriasis, etc.* [9]. Despite the long history of use in traditional medicine, there is no evidence related to its hepatoprotective properties; therefore, the present study was aimed to determine thechemical composition of the plant grown in west of Iran and to investigate its hepatoprotective effects in CCl_4-induced toxicity through stereological procedures and biochemical analysis.

Material and Methods

Plant material

Sophora alopecuroides was collected from Kermanshah, west of Iran. The plant was identified and a voucher specimen (no. KF1275) was deposited at the Herbarium of research Center of Faculty of Agriculture, Razi University, Kermanshah, Iran. The essential oil from aerial part was extracted by Clevenger apparatus. Briefly, 150 g of plant was added to the distillation flask, the steam containing the essential oil was compressed through a cooling system for 3 h and the obtainedessential oil was kept in a refrigerator.

Gas chromatography/mass spectrometry (GC/MS)

GC/MS (Shimadzu capillary GC-quadrupole MS system QP 5000) with two fused silica capillary column DB-5 (30 μm, 0.25 mm i.d, film thickness 0.25 μm) and a flame ionization detector (FID) run in EI mode at 70 eV were used. The injector temperature was 220 °C and the detector temperature was set at 250 °C. Helium was employed as the carrier gas (1 mL/min). one μL of the essential oil was injected and analyzed with the column held initially at 60 °C for 2 min. NIST standard reference database (AMDIA version 2.70) was used to interpret the mass spectral data.

Animals

Male Balb/c mice weighing between 30 and 40 g were procured from laboratory animal center of Kermanshah University of Medical Sciences, Kermanshah, Iran. The animals were housed in an air-conditioned room (22±2 °C) with 12 h light/dark cycle and had free access to standard pellet diet and water. All animal procedures were approved by standards of Payame Noor University of Kermanshah (No. 01/Z/G 1395/12/01) on Humane Care and Use of Laboratory Animals, in accordance with the Research Ethics Committee of the Ministry of Health and Medical Education in Iran (adopted on April 17, 2006), based on the Helsinki Protocol (Helsinki, Finland, 1975).

Experimental design

In the present study, a total of 35 mice were used. The mice were divided into five groups of seven mice each;

Group I (negative control): received 1 mL/kg olive oil intraperitoneally and 0.5 mL distilled water through gavages.

Group II (positive control): received 1 mg/kg CCl_4 mixed with olive oil in the ratio of 5:5 intraperitoneally + 0.5 mL distilled water through

gavages.
Group III, IV, V: received CCl_4 mixed with olive oil in the ratio of 5:5 intraperitoneally + ethanol extract of *Sophora alopecuroides* 200, 800 and 1600 µg/kg body weight through gavages, respectively.
The animals were treated twice a week for 45 consecutive days. At the end of the experimental period, the blood samples were collected directly from animals' heart for enzyme assessment. Then the subjects were sacrificed with deep chloroform inhalation.

Assessment of serum biochemical markers
Blood samples were centrifuged at 10,000 rpm for 15 min for serum separation. The values of liver enzymes namely, alkaline phosphatase (ALP), aspartate aminotransaminase (AST) and alanine aminotrasferase (ALT) were measured using commercially available kits (Pars Azmun CO, Iran).

Stereological study
The whole liver of each animal was dissected out and washed with ice cold saline to remove blood. Primary volume of the livers was measured using Archimedes principles [10]. Then the livers were fixes in 10% neutral buffered formaldehyde. After 5 days, due to the tissue shrinkage following fixation, the value of tissue shrinkage should be estimated. Estimation of shrinkage requires isotropic uniform random sections [11]. These sections were achieved by the orientator method by and 7-10 slabs were collected from each liver. A circle was punched from a liver slab by a trocar. The diameters of the circular piece of the liver were measured by a micrometer and the area of the circle was estimated using a usual formula for calculating the area of a circle. The slabs and circular piece were embedded in paraffin and the sections (5 µm thickness) were prepared and stained with hematoxyline and eosin (H&E). After staining, the area of the circular piece was measured again and volume shrinkage was calculated as [12]:

$$\text{Volume shrinkag} = 1 - \left(\frac{AA}{AB}\right)^{1.5}$$

Where AA and AB are the area of the circular piece after and before processing, sectioning and staining, respectively. After estimating the shrinkage, the final volume of the liver (the reference space) was corrected using:

$$V_{final} = V_{primary} \times (1 - \text{volume shrinkage})$$

Volume density of the liver structures including hepatocytes, sinusoids, central veins, portal veins, hepatic arteries, and bile ducts estimated with point counting rule were briefly as follows (figure 1).

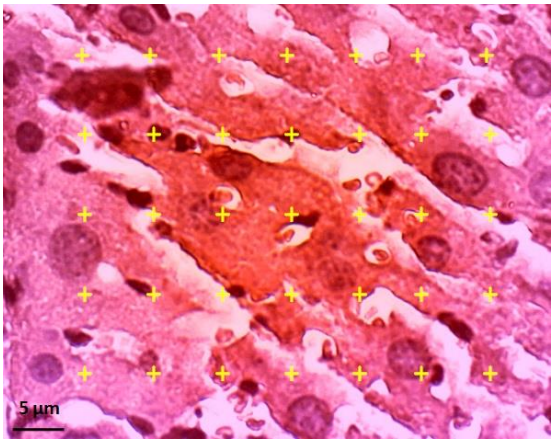

Figure 1. Microscopic section of mice liver. To estimate volume density of hepatocytes, sinosoids, central veins, portal veins, hepatic arteries, and bile ducts a point probe was used. The total number of points hitting each component was divided by the total number of the points hitting the reference space. (H&E, 1000×).

The images of microscopic fields from each section were projected on point probe (frame 15 cm×15 cm) by video projector via microscope equipped with a camera (Dinocapture ver.5, dino-lit.com 30.5 mm) attached to a computer.
At total magnification of 1000×, points that hit desired structures were counted and volume density was estimated using following formula:

$$V_v = \frac{P_{structure}}{P_{reference}}$$

Where $P_{stucture}$ and $P_{reference}$ were the number of

points hitting the structure's profile and on the reference space, respectively. 10-14 microscopic fields were examined in each liver. The absolute volume of the structures was estimated by multiplying the fractional volume by the final volume of the liver to prevent the reference trap [11,12].

Statistical analysis

The obtained results were expressed as means±standard deviation (SD). Statistical comparison between the means groups was done using one- way ANOVA followed by Tukey's post-hoc test and a *p* value less than 0.05 was considered significant.

Results and Discussion

Chemical composition of *Sophora alopecuroides* essential oil has been presented in table 1. The main constituents of the essential oil were sophoridine (32.12%), matrine (25.51%), sophocarpine (8.25%) and 12β-hydroxysophocarpine (5.98%) and sophoranol (5.42%).

Table 1. The components of *Sophora alopecuroides* essential oil that analyzed by GC/MS

No.	Compound	Percentage
1	**α-Isosparteine**	**0.49**
2	11,12-Dehydrosparteine	3.21
3	N-Methylcytisine	1.21
4	Cytisine	2.1
5	5,6-Dehydrolupanine	0.38
6	7,11-Dehydromatrine	1.81
7	Lehmannine	1.3
8	Sophocarpine	8.25
9	Matrine	25.51
10	Sophoridine	32.12
11	Sophoridine-N-oxide	1.1
12	Sophoramine	2.31
13	5,17-Dehydromatrine	2.71
14	Oxymatrine	0.62
15	14β-Hydroxymatrine	0.59
16	Anagyrine	0.91
17	Sophocarpine-N-oxide	0.82
18	12β-Hydroxysophocarpine	5.98
19	Sophoranol	5.42
20	Sophoranol-N-oxide	0.51
21	Baptifoline	0.5
22	**Total**	**97.85**

The estimated values of the liver enzymes have been presented in figure 2. CCl_4- induced hepatotoxicity increased liver enzymes significantly ($p<0.05$) as compared to the negative control group. The level of ALP decreased significantly ($p<0.05$) with different doses of *S. alopecuroides* essential oil as compared to the CCl_4-treated group. However, AST and ALT levels improved with low and intermediate doses of *S. alopecuroides* essential oil. High dose of the essential oil could not decreased these enzymes and there was no significant differences between the CCl_4-treated group and fifth group ($p>0.05$).

The estimated values for the weigh and volume of the liver and volume of the liver structures have been presented in figure 3. The weight and volume of the liver and volume of the hepatocytes and sinusoids significantly increased in CCl_4- treated group as compared to the negative control group ($p<0.05$). These parameters significantly decreased at low dose (200 µg/kg) of *S. alopecuroides* essential oil in comparison with the positive control group ($p<0.05$). The volume of central veins, portal veins, hepatic arteries, and bile ducts did not significantly change (figure 4) ($p>0.05$).

In the negative control group, the liver sections showed a normal architecture with intact and organized hepatocyts, central vein, portal vein and bile ducts in portal area. The liver of CCl_4-treated mice showed extended hepatic necrosis with hemorrhage, infiltration of inflammatory cells and cell swelling. Accordingly, the mice treated with *S. alopecuroides* essential oil revealed markedly milder hepatic lesions.

In this experimental study the hepatoprotective effects of *Sophora alopecuroides* essential oil was investigated in CCl_4 - induced hepatotoxicitiy mice model. The obtained data showed that treatment of CCl_4-intoxicated mice with *S. alopecuroides* essential oil resulted in marked decrease in liver enzymes and low dose of the essential oil could normalize weight and volume of the liver in treated mice versus untreated mice.

It is now generally accepted that hepatotoxicity of CCl_4 is the result of reductive dehalogenation,

which is catalyzed by its specific isoenzyme of cytochrome P 450 2E1, and which forms the highly reactive trichloromethyl free radical. Therefore, the suppression of P 450 2E1 could result in reduced levels of reactive metabolites, and thus decreased tissue injury [14,15]. The detoxification pathway involves protective physiological moieties (glutathione, a-tocopherol, etc.). A previous study on the mechanism of CCl_4-induced hepatotoxicity has shown that endogenous antioxidants such as GSH (glutathione) play a crucial role in detoxifying the reactive toxic derivatives of CCl_4 and that liver necrosis begins when antioxidant stores are markedly depleted. It has been suggested that one of the principal causes of CCl_4-induced liver injury was lipid peroxidation caused by its free radical derivatives [16]. Since free radicals and reactive oxygen species play a central role in liver disease pathology and progression, dietary antioxidants have been proposed as therapeutic agents to counteract liver damage [2]. The extent of hepatic damages is assessed by the elevated serum levels of cytoplasmic enzymes as well as by histological examination. The increased serum enzyme levels have been attributed to the damaged structural integrity of the liver, because these are cytoplasmic in location and are released into the circulation after cellular damage [14,16]. Administration of CCl_4 induced liver damage in mice which led to an increase in serum levels of AST, ALT and ALP enzymes. In the present study, the raised level of ALP and ALT significantly decreased following administration of different doses of *S. alopecuroides* essential oil. However, stereological findings indicated that better result was achieved by low dose of the essential oil.

The protective effect of *S. alopecuroides* essential oil against CCl_4-induced hepatotoxicity in the present study may be attributed to its anti-inflammatory and antioxidant constituents. Based on the GC/MS results, sophoridine and matrine were identified as the main components of *S. alopecuroides* essential oil.

Hanung et al. investigated the anti-inflammatory effects of sophoridine and revealed that sophoridine had inhibitory effects on inflammatory cytokines including TNF*α*, IL-6, and IL-8 and inflammation media prostaglandin both in cell culture supernatant (*in vitro*) and in the local inflammatory exudates (*in vivo*) [17]. Based on the the results, the anti-inflammatory effect of sophoridine might be attributed to its ability to inhibit the production of cytokines such as TNF*α*, IL-8, and LTB_4 and inflammation media (PGE_2 and histamine) in the initial phase of inflammation.

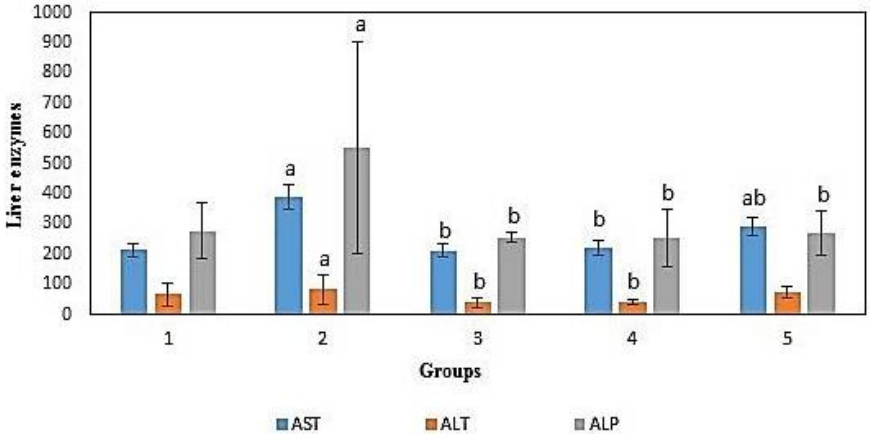

Figure 2. Liver enzymes values in the control and experimental groups treated with different doses of *Sophora alopecuroides* essential oil (n=7). AST: aspartate transaminase; ALT: alanine aminotrasferase; ALP: Alkaline phosphatase. 1- control group, 2- CCl_4 group, 3,4 and 5- received 200, 800 and 600 µg/kg of *S. alopecuroides* essential oil, respectively. [a]$p<0.05$ compared to control group and [b]$p<0.05$ compared to CCl_4 group

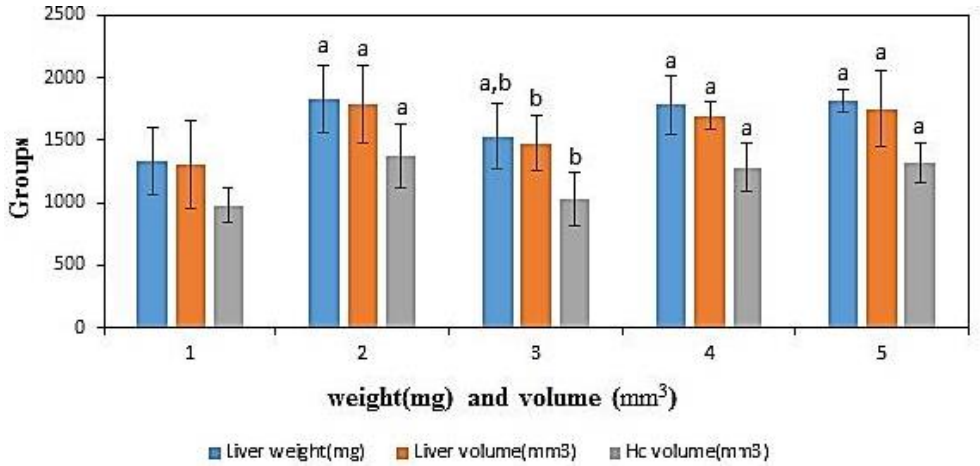

Figure 3. Weight (mg) and total volume (mm^3) of the liver and hepatocytes in the control and experimental groups treated with different doses of *Sophora alopecuroides* essential oil (n=7). Hc; hepatocyte, 1- control group, 2- CCl_4 group, 3,4 and 5- received 200, 800 and 600 µg/kg of *S. alopecuroides* essential oil, respectively. [a]p<0.05 compared to control group and [b]p<0.05 compared to CCl_4 group

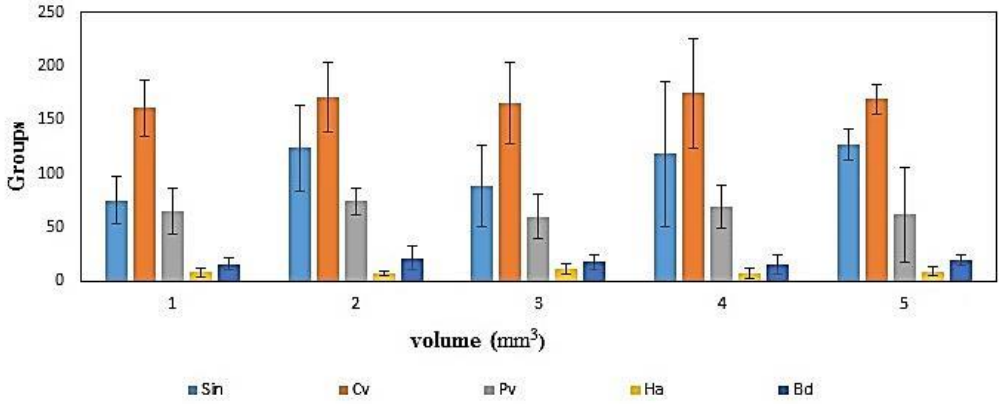

Figure 4. Total volume (mm^3) of the liver structures in the control and experimental groups treated with different doses of *Sophora alopecuroides* essential oil (n=7). Sin; sinusiod, Cv; central vein, Pv; portal vein, Ha; hepatic artery, Bd; bile duct. 1- control group, 2- CCl_4 group, 3,4 and 5- received 200, 800 and 600 µg/kg of *S. alopecuroides* essential oil, respectively. [a]p<0.05 compared to control group and [b]p<0.05 compared to CCl_4 group

Matrine and oxymatrine are two alkaloids which are found in the *S. alopecuroides* essential oil. Their main applications were in treatment of cancer, cardiac disease and viral hepatitis. For the first time, these alkaloids were released in tablet form by Institute for Traditional Medicine of China in 1998 [18]. In recent years, Matrine and oxymatrine were utilized for treating hepatitis C and hepatitis B, successfully. Moreover, in some experimental studies, matrine was shown to inhibit hepatic fibrosis due to chemical damages. However, the mechanism of this function has not yet been determined [19]. The present findings indicated that CCl_4 intoxiation results in remarkable hypertrophy in the liver which leads to increase in weight and volume of the liver as well as volume of the hepatocytes and sinusoids. These changes were ameliorated perfectly in third group which received low dose of *S. alopecuroides* essential oil. However, intermediate and high dose of *Sophora alopecuroides* essential oil could not reduce increased volume of the liver. This study suggested that *S. alopecuroides* essential oil

could be used warily for amelioration hepatic structural changes due to CCl_4-induced toxicity. As a conclusion, although *Sophora alopecuroides* essential oil could protect the liver structure against CCl_4-induced hepatocellular injuries at selected low dose and improved raised levels of hepatic enzymes at low and intermediate examined doses, further studies is needed to define the selective dose against CCl_4-induced hepatotocicity.

Acknowledgements
The authors would like to thank Mr. Moradi and Dr. Massomi for their technical assistances.

Declaration of interest
The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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