

## Original Article:

# Effects of Hydroalcoholic Extract of *Lepidium Sativum* L. on Carbon Tetrachloride-Induced Hepatotoxicity in Mice



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## ABSTRACT

**Background:** Carbon tetrachloride (CCl<sub>4</sub>) as an organic solvent causes symptoms of acute and chronic liver injury, including necrosis, fat changes, liver cancer, and cirrhosis. *Lepidium sativum* contains flavonoids, alkaloids, and antioxidant components.

**Objectives:** This study aims to investigate the hepatic protection of *L. Sativum* Extract (LSE) on CCl<sub>4</sub>-induced hepatotoxicity in mice.

**Methods:** A total of 25 male mice were randomly divided into five groups (n=5): control (olive oil), CCl<sub>4</sub>, and 3 LSE groups. Except for the control group, all the mice received CCl<sub>4</sub> (50%, 0.5 mL/kg) intraperitoneally twice a week for 4 weeks. The mice in the LSE groups were treated daily with LSE (200, 400, and 600 mg/kg) via IP injection. The animals were sacrificed 24 h after the last dose, and liver function parameters, such as Alkaline Phosphatase (ALP), Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), and Glutathione (GSH) were determined. Furthermore, 0.1 g of liver tissue was removed for histochemical analysis.

**Results:** Significant differences were observed in GSH, ALP, AST, and ALT levels between the CCl<sub>4</sub> and the control groups. Compared to the CCl<sub>4</sub> group, LSE treatment significantly decreased plasma ALT (P<0.05), AST in all doses (P<0.001), and ALP in 600 mg/kg (P<0.001). In addition, LSE treatment significantly increased GSH in 400 mg/kg (P<0.01) and 600 mg/kg (P<0.001).

**Conclusion:** LSE has hepatic protective activity against CCl<sub>4</sub>-induced injuries. The possible anti-hepatotoxic mechanisms may be related to the presence of flavonoids, triterpenes, alkaloids, tannin, and coumarins in the LSE by inhibiting the free radicals mediated damage.

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## Introduction

The liver is responsible for the absorption, metabolism, and excretion of drugs and environmental contaminants in the body. During detoxification and metabolic activation by the cytochrome P450 in the liver microsomes, active secondary metabolites are produced that can cause tissue damage in various organs. Alanine transferase, aspartate transferase, alkaline phosphatase, and reduced glutathione levels are used to diagnose liver diseases and damage; their increased activity indicates hepatocyte injury and the presence of active liver disease [1, 2]. Carbon tetrachloride ( $\text{CCl}_4$ ) is one of the most widely used organic solvents in the industry and is highly toxic to the liver and mainly metabolized by cytochrome P450. Trichloromethyl and peroxy tetrachloromethyl are the 2 most toxic compounds produced by the  $\text{CCl}_4$  metabolism that can cause acute liver injury, including cirrhosis and hepatic necrosis [3]. Antioxidants are beneficial compounds that, in small quantities, can protect cell membranes and other living structures against oxidants [1]. Glutathione (GSH) is one of the body's natural antioxidants that is produced in the liver and protects important cellular components against oxygenated functional groups, such as peroxides [4]. Polyphenols are one of the natural compounds with good antioxidant effects that can protect against damage caused by liver toxins and free radicals [5]. Phenolic compounds are widely found in plants. The most abundant phenolic compounds present in virtually all parts of the plant are flavonoids that can effectively detoxify and neutralize hydroxy and peroxy radicals, complexing with heavy metals and preventing lipid oxidation [6]. Meanwhile, the use of herbs for treatment has been prevalent since ancient times. Herbal compounds have always been considered a substitute for chemical drugs given their ease of access, fewer side effects, low toxicity, and low cost.

*Lepidium sativum* (LS) is a member of the Brassicaceae family whose geographical distribution is in the north, northwest, south, southeast, and central parts of Iran and is commonly used for disorders, such as rheumatism, osteoarthritis, wounds, sexual disorders, and menstruation [7]. It has also been reported to have antihypertensive [8], diuretic [9], hypoglycemia [10], and antioxidant properties [11-13]. The major constituents of LS include alkaloids, flavonoids, glycosides, sterols, and tannins [11]. Studies have shown that flavonoids have numerous pharmacological effects, such as anti-inflammatory, anti-cancer, anti-diabetic, and hepatoprotective effects.

Since liver disease is one of the current world's health problems and viruses, bacteria, parasites, toxins, and drugs have a significant role in its development, prevention and treatment of this disease are important. It is vital to find effective compounds to eliminate the toxicity of drugs and common toxins that are more prevalent in liver toxicity; particularly, these materials can be obtained from a natural and accessible source [14]. Although the hepatoprotective effects of LS on acetaminophen-induced hepatotoxicity were reported, to the best of our knowledge, no studies have evaluated the effects of LS on  $\text{CCl}_4$ -induced hepatotoxicity. Therefore, given the rich phenol and flavonoid content of LS, in this study, we aim to investigate the effects of LS on  $\text{CCl}_4$ -induced hepatotoxicity in mice.

## Materials and Methods

### Study chemicals

The following are the study chemicals used in this research: diammonium ethylenediaminetetraacetic acid ( $\text{C}_{10}\text{H}_{22}\text{N}_4\text{O}_8$ , Merck & Co., Inc., USA), ethanol (Dr. Mojallali Industrial Chemical Complex Co., Inc., Iran), GSH ( $\text{C}_{10}\text{H}_{17}\text{N}_3\text{O}_6\text{S}$ , Merck & Co., Inc., USA), sodium chloride 0.9% (Samen Pharmaceutical Co., Inc., Iran), tromethamine ( $\text{C}_4\text{H}_{11}\text{NO}_3$ , Merck & Co., Inc., USA), and 5,5'-dithiobis (2-nitrobenzoic acid) ( $\text{C}_{14}\text{H}_8\text{N}_2\text{O}_8\text{S}_2$ , Merck & Co., Inc., USA).

### Preparation of the plant extract

Aerial parts of LS were collected from the farms of Mazandaran Province, Iran, and dried in  $37^\circ\text{C}$ . A botanist approved the plant taxonomy and confirmed its species (herbarium number: E1-26-231; stored in the faculty of pharmacy of the Mazandaran University of Medical Sciences). The dried plant was powdered and extracted at  $45^\circ\text{C}$  with ethanol (70% v/v) by the maceration method. The extract was freed from the solvent under vacuum by a rotary evaporator.

### Determination of total phenolic content

Total phenol content was determined by the Folin-Ciocalteu reagent using standard spectrophotometry and standard curve drawing with some modifications [15]. First, 1 mL of the extract solution at a concentration of 1 mg/mL was mixed with 5 mL of diluted (1 in 10) Folin-Ciocalteu reagent and incubated at room temperature. After 10 min, 4 mL of sodium bicarbonate solution was added at a concentration of 75 mg/mL and incubated at room temperature for 60 min (3 replicates). The absor-

bance of all samples was measured at 765 nm using a Shimadzu UV-mini1240 UV-vis spectrophotometer. The data are represented as mg of gallic acid equivalent per g of dried extract weight.

### Determination of total flavonoid content

We used the aluminum chloride colorimetric method with slight modifications [15]. First, we added 0.5 mL of 1mg/mL extract, 1.5 mL of 95% ethanol, then 0.1 mL of 10% aluminum chloride solution and 0.1 mL of 1M potassium acetate was added, and finally, we added 2.8 mL of distilled water and we mixed them. We incubated the mixture at room temperature for 30 min and finally read the absorbance of the sample at 415 nm against the blank solution. The data are represented as mg of quercetin equivalent per g of dried extract weight.

### Animal experimentation

A total of 25 BALB/c male mice weighing 20 to 25 g were obtained from the Mazandaran University of Medical Sciences Laboratory. They were acclimatized in an air-conditioned room at  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ , with a relative humidity of  $81\% \pm 2\%$ , and 12/12 h light/dark cycles. All the animals were free to use the standard laboratory feed and tap water before the experiment. The studies were confirmed by the Animal Ethics Committee of the [Mazandaran University of Medical Science](#) (ethics code: IR.MAZUMS.REC.1394.671)

After one week of dietary adaptation, animals were divided into groups (n=5) as follows: (A)  $\text{CCl}_4$  2 times a week (0.5 mL/kg); (B)  $\text{CCl}_4$  (2 times a week)+LS extract (LSE) (200 mg/kg daily); (C)  $\text{CCl}_4$  (2 times a week)+LSE (400 mg/kg daily); (D)  $\text{CCl}_4$  (2 times a week)+LSE (600 mg/kg daily); (E) olive oil ( $\text{CCl}_4$  solvent) 2 times a week as a control. All injections were given intraperitoneally for 28 days [16].

To follow the ethical considerations, the animals were anesthetized 24 h after the last injection. After cleavage of the animal's abdomen and chest, 1.5 - 2 mL of blood was taken from the animal's heart with an insulin syringe (heparinized) and then centrifuged at 3000 rpm for 15 min (at  $4^{\circ}\text{C}$ ). The serum was separated and transferred to sterile falcon tubes and stored in a freezer. Finally, the activity of the serum aminotransferases, including Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), and Alkaline Phosphatase (ALP), were measured. The liver tissue GSH levels were also measured.

The Elman method was also used to measure the reduced GSH. For this purpose, 0.1 g of the mice's liver was transferred to a homogenizer tube, 1 mL of ethylenediaminetetraacetic acid was added, and homogenization was repeated several times to obtain a uniform mixture. The contents were then transferred to a centrifuge tube, and 0.5 mL of ethylenediaminetetraacetic acid was added. Next, 1.5 mL of trichloroacetic acid (with 10% purity) was added to the centrifuge tube. The tubes were then centrifuged at 3000 rpm for 15 min, and then 1 mL of the supernatant was transferred to another centrifuge tube with 2.5 mL buffer 0.4 M (pH=8.9) and 0.5 mL of Ellman's reagent. The tube was shaken gently to give a uniform yellow color. Finally, the absorbance of the resulting solution was read at 412 nm. By comparing the absorbance with the standard curve, GSH concentration was calculated and reported in micromoles per gram of liver weight [17].

### Histological analysis

Appropriate tissue samples were cut from the liver and placed in a 10% formalin solution. After fixation, the tissues were dehydrated by alcohol. Then, the samples were clarified in xylene, and sections were prepared in 5-micron diameters after the preparation of paraffin blocks. Finally, the samples were stained with hematoxylin-eosin and studied by light microscopy, and the rates of tissue necrosis, inflammation, and edema in different parts of the tissue were investigated [18].

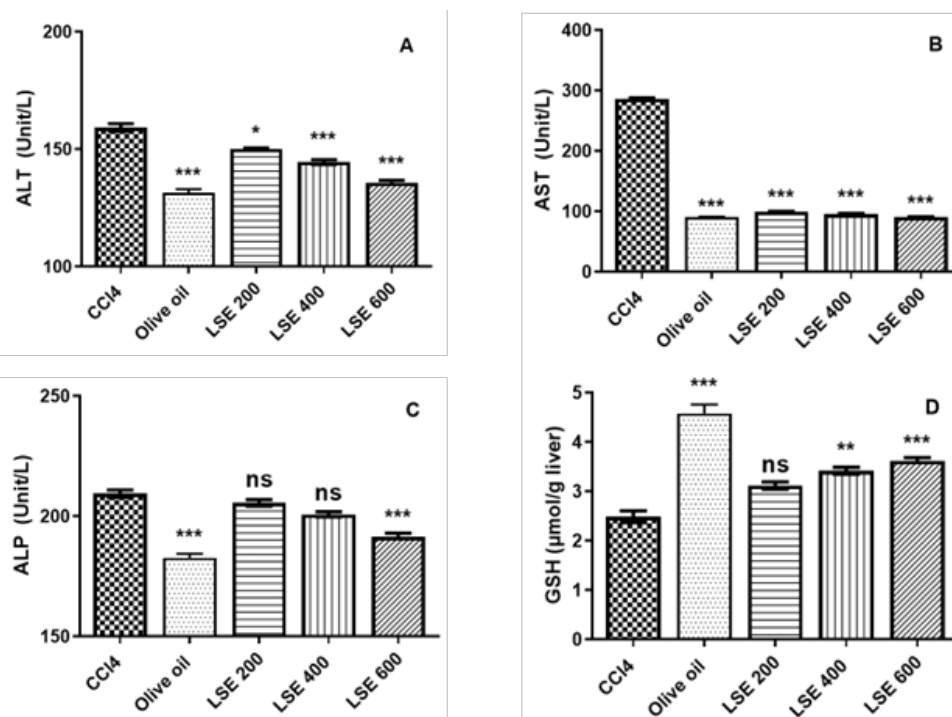
### Statistical analysis

The data were analyzed using the SPSS software, v. 16, a 1-way analysis of variance, and the Tukey test. The results were reported as Mean $\pm$ Standard Error of the Mean. The results were considered significant at  $P < 0.05$ .

### Results

The yield of the extract was 21% w/w. The total phenolic compound of the LSE was  $34.3 \pm 0.32$  mg gallic acid/g extract and the total flavonoid compound was  $8.6 \pm 0.07$  mg quercetin/g extract.

The amount of ALT, AST, ALP, and GSH in different groups is reported in [Figure 1](#). Determining the amount of ALT and AST in serum showed that the LSE could significantly reduce the amount of these enzymes in the mice serum at all doses. The plant extract at a dose of 200 mg/kg could reduce AST to  $99.78 \pm 0.67$  Unit/L ( $P < 0.001$ ) and ALT to  $150.20 \pm 0.55$  Unit/L ( $P < 0.05$ ). At a dose of 400 mg/kg, AST was reduced to  $95.52 \pm 1.56$



**Figure 1.** The Activity of Serum Aminotransferases and Glutathione in Mice

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Notes: A) ALT, B) AST, C) ALP, D) GSH. The data are expressed as mean±standard deviation (n=5), (ns, not significant; \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, compared to the carbon tetrachloride [CCl<sub>4</sub>] as the control group).

Histopathologic findings of the transverse sections prepared from the liver tissue in the control group showed central venous hyperemia with a sinusoid, the structure of liver lobules and hydropic hepatocytes, and single necrosis (Figure 2-1). In the CCl<sub>4</sub> group, in addition to central venous hyperemia and hydropic changes in hepatocytes, severe inflammation in the port space (Figure 2-2) and hepatocyte single and group necrosis and lymphocyte cell infiltration have occurred (Figure 2-3). At a dose of 600 mg/kg of the extract, the lobular structure was preserved, and mild inflammation of the port space, single hepatocyte necrosis with focal lymphocyte accumulation (Figure 2-4), nucleus enlargement with dual nucleation (Figure 2-5), inflammation of the port space and focal, and brief hyperplasia were observed (Figure 2-6).

Unit/L (P<0.001) and ALT was reduced to 144.56±1.75 Unit/L (P<0.001). In addition, the amount of ALT and AST in serum at a dose of 600 mg/kg (135.74±1.85 Unit ALT/L, P<0.001 and 90.56±1.10 Unit AST/L, P<0.001) was not significantly different from the olive oil group (131.44±1.48 Unit ALT/L, P<0.001 and 90.04±1.02 Unit AST/L, P<0.001).

LSE at a dose of 600 mg/kg (191.42±2.71 Unit/L, P<0.001) could significantly reduce serum ALP; however, at doses of 200 mg/kg (205.60±2.42 Unit/L, P>0.05) and 400 mg/kg (200.82±1.79 Unit/L, P>0.05) it showed no significant effect. The lowest ALP was observed in the olive oil group (182.84±1.59 Unit/L, P<0.001).

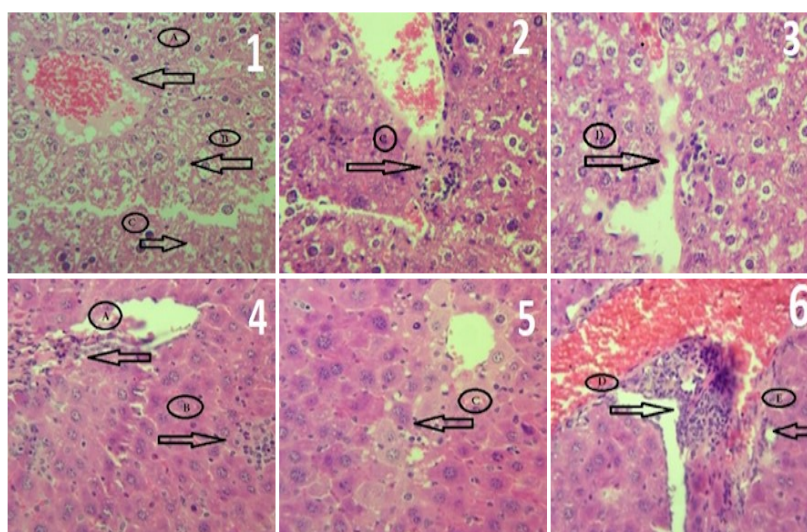
The results of determining the amount of GSE in the mice liver did not show any significant difference between the group receiving the extract at a dose of 200 mg/kg (3.12±0.13 μmol/g liver, P=0.052) and the CCl<sub>4</sub> group (2.49±0.20 μmol/g liver). However, the extract in doses of 400 mg/kg (3.42±0.12 μmol/g liver, P<0.01)

and 600 mg/kg (3.62±0.11 μmol/g liver, P<0.001) could significantly increase hepatic GSH. The highest amount of GSH was observed in the olive oil group (4.58±0.18 μmol/g liver, P<0.001).

## Discussion

Nowadays, the side effects of drugs used in treating liver diseases have necessitated more novel research to find suitable herbal medicines given their ease of access, fewer side effects, less toxicity, and cheaper price [19]. In addition to its metabolic function, the liver detoxification of environmental contaminants and chemical drugs in some cases causes the production of secondary metabolites that lead to cell damage and liver disease [20]. The results of various studies show that intraperitoneal injection of CCl<sub>4</sub> causes hepatotoxicity in mice. In the presence of CCl<sub>4</sub>, the number of liver enzymes, such as ALT, ALP, and AST is significantly increased compared to the control group. These enzymes are naturally present inside the cells and enter the serum when cell





**Figure 2.** Histological analysis of liver tissues in studied groups

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Notes: Liver tissue of the control group is shown in Part 1 (1-A: central venous hyperemia with sinusoid, 1-B: hydropic hepatocytes, 1-C: single necrosis). Liver tissue of the carbon tetrachloride group is shown in parts 2 and 3 (2-C: severe inflammation in the port space, 3-D: lymphocyte cell infiltration). Liver tissue of extract 600 mg /kg + CCl<sub>4</sub> group is shown in parts 4, 5, and 6 (4-A: mild inflammation in the port space, 4-B: single hepatocyte necrosis with focal lymphocyte accumulation, 5-C: nucleus enlargement).

damage occurs; therefore, it can be concluded that CCl<sub>4</sub> causes liver cell damage [16, 21-22]. The cause of this toxicity is the breaking of the bond between carbon and chlorine in the CCl<sub>4</sub>, which results in the formation of a free and unstable radical, trichloromethyl. Trichloromethyl reacts rapidly with cell membrane compounds, especially unsaturated fatty acids, to produce a radical lipid and chloroform. As a result of the reaction of radical lipids and chloroforms with oxygen, phospholipids in the endoplasmic reticulum are broken and liver enzymes are released [23]. As the major non-protein thiol in aerobic organisms, GSH is the most abundant non-enzymatic antioxidant in the cells, formed in the human liver, and protects important cellular components against oxygenated functional groups, such as free radicals and peroxides [2]. Reducing the presence of liver enzymes in serum and the concentration of bilirubin, while increasing the concentration of GSH, total protein, and albumin are the main indicators of liver treatment and restoring the health of this important organ in the body [23].

Abuelgasim et al. evaluated the effects of methanolic extract of LS seeds on hepatotoxicity induced by CCl<sub>4</sub>. The results showed that CCl<sub>4</sub> increased the level of liver parameters (ALT, AST, and ALP) compared to the control group ( $P < 0.05$ ). In the groups that received the extract with doses of 200 and 400 mg/kg, the level of liver enzymes decreased. The function of this extract has been attributed to the presence of flavonoids, alkaloids, tan-

nins, and coumarins that are involved in inhibiting free radicals. In the evaluation of liver histopathology, severe liver fat changes caused by CCl<sub>4</sub> were reduced in the extract groups, which is similar to the function of leaf extract in our study on some liver factors [24]. Heidarian et al. evaluated the protective effects of LS aerial parts extract on acetaminophen poisoning in mice. Serum ALT, AST, and ALP levels were significantly and dose-dependently decreased in the extract groups compared to the acetaminophen group ( $P < 0.001$ ). Histopathological studies showed that necrosis and lymphocytic infiltration decreased in the liver of the extract groups compared to the acetaminophen group [7], which is in line with our study.

According to the results of this study, LS aerial parts extract has liver protection effects against CCl<sub>4</sub> poisoning, which may be related to flavonoid and phenolic compounds. It mainly contains polyphenolic and flavonoid compounds, which are the most important antioxidants. Previous studies have confirmed that the presence of phenolic compounds can improve hepatic markers in hepatotoxicity [25, 26]. 5'-dimethoxy-2',3'-methylenedioxy-7-C-β-D-glucopyranosyl isoflavone (an isoflavone C-glycoside) was isolated from LS seeds, which reduces serum lipids profile, free radicals, and improves hepatic function in hepatotoxicity with paracetamol [27]. Also, the desulfo-glucosinolate fraction of *L. perfoliatum* seeds (glucoerucin was the major ingredient) improved hepa-

tototoxicity by increasing GSH and decreasing Reactive Oxygen Species (ROS), and malondialdehyde [28]. Inflammation is one of the most important factors in liver toxicity. LS reduces inflammation by reducing TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and NF- $\kappa$ B [29, 30]. These effects seem to be due to the presence of  $\alpha$ -linoleic acid (which has anti-inflammatory effects) in LS [25].

## Conclusion

LSE balances biochemical factors such as GSH, ALP, AST, and ALT. It also minimizes changes due to CCl<sub>4</sub> administration. The results of this study show that hydroalcoholic extract of LS aerial parts has a protective role in CCl<sub>4</sub> hepatotoxicity and this effect is dose-dependent.

## Ethical Considerations

### Compliance with ethical guidelines

This study was approved by the Ethics Committee of Mazandaran University of Medical Sciences (Code: IR.MAZUMS.REC.1394.671).

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### Authors' contributions

All authors contributed equally to preparing this article.

### Conflict of interest

The authors declared no conflict of interest.

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