



## Original Article

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# *Levisticum officinale* extract protects against CCl<sub>4</sub>-induced hepatotoxicity through anti-inflammatory, anti-fibrotic, and antioxidant properties in rats

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## ABSTRACT

**Objective:** To investigate the hepatoprotective effects of *Levisticum officinale* extract on CCl<sub>4</sub>-induced hepatotoxicity.

**Methods:** Different doses of *Levisticum officinale* extract were given orally to rats for 10 days, then rats received a single dose of CCl<sub>4</sub> (2.5 mL/kg, 50% v/v in liquid paraffin). Biochemical and histopathological assays were performed to assess the effects of the extract on liver function and architecture. Moreover, antioxidant and oxidative markers as well as inflammatory and fibrotic indicators were measured.

**Results:** Pretreatment with *Levisticum officinale* extract significantly mitigated CCl<sub>4</sub>-induced damage to liver structure, improved serum levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, urea, total bilirubin, and total protein, enhanced glutathione content and superoxide dismutase and catalase activities in the liver, as well as decreased plasma and hepatic malondialdehyde levels. Immunohistochemical results demonstrated that the extract reduced Ki-67 and  $\alpha$ -SMA expression and Masson's trichrome staining revealed decreased liver collagen in rats treated with *Levisticum officinale* extract. Moreover, *Levisticum officinale* extract markedly decreased the gene expressions of *TNF- $\alpha$* , *IL-6*, *TGF- $\beta$ 1*, *MCP-1*, and *COX-2*.

**Conclusions:** *Levisticum officinale* extract exerts hepatoprotective effects on CCl<sub>4</sub>-induced hepatotoxicity through antioxidant, anti-inflammatory, and anti-fibrotic activities.

**KEYWORDS:** *Levisticum officinale*; CCl<sub>4</sub>; Inflammation; Liver toxicity; Antioxidant

it is susceptible to damage from drug metabolites and toxic agents. Carbon tetrachloride (CCl<sub>4</sub>) as a well-known hepatotoxin is frequently used to induce acute liver injury in experimental studies[1,2]. CCl<sub>4</sub> metabolism in the liver is believed to lead to the accumulation of lipid-lethal trichloromethyl ( $\bullet$ CCl<sub>3</sub>) and peroxy trichloromethyl ( $\bullet$ OOCCl<sub>3</sub>) reactive radicals, thereby activating liver cell necrosis and apoptosis[3]. Also, CCl<sub>4</sub> activates Kupffer and hepatic stellate cells (HSCs) to release proinflammatory mediators such as interleukins (IL-6) and cytokines[4]. It seems that there is a strong relationship between oxidative stress, cytotoxic cytokines, and liver injury. According to the pathogenesis of hepatotoxicity, antioxidant therapy is useful for prevention, treatment, or

## Significance

*Levisticum officinale* (Apiaceae) is a wild, edible medicinal plant that possesses antioxidant, antibacterial, nephroprotective, neuroprotective, and anti-inflammatory properties. There is no report about the hepatoprotective potential of *Levisticum officinale* extract on CCl<sub>4</sub>-induced acute liver toxicity and the possible underlying mechanisms. The present study showed that *Levisticum officinale* exerted hepatoprotective effects on CCl<sub>4</sub>-induced liver toxicity by exerting antioxidant, anti-inflammatory, and anti-fibrotic properties.

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## 1. Introduction

Since the liver is a major organ for detoxification of xenobiotics,

improvement of liver dysfunctions. Plants are a main source of natural antioxidants and recently herbal therapeutic resources have attracted a lot of attention for treatment or protection against different diseases.

*Levisticum officinale* (*L. officinale*) (Apiaceae) is one of the endemic, wild, aromatic, and edible plants that grow in the Hezar mountain, in Kerman province of Iran. It is locally called “Karafs -e-Koohi” and its fragrant leaves and stems are used as a spice and culinary herbs for flavoring foods. Moreover, roots, fruits, and leaves of this valuable plant have many medicinal properties. It has been reported that *L. officinale* has antioxidant, anticancer[5], antibacterial[6], and nephroprotective[7] properties. Also, *L. officinale* showed a protective effect on paraquat-exposed hepatocytes[8]. Recently we reported that *L. officinale* has considerable neuroprotective, anti-inflammatory, and anti-dyslipidemia activities[9–11]. However, there is no report about the hepatoprotective potential of *L. officinale* on CCl<sub>4</sub>-induced acute liver toxicity, so this study aimed to evaluate this and clarify the possible underlying mechanisms.

## 2. Materials and methods

### 2.1. Extract preparation

Aerial parts of *L. officinale* were collected from May to June, from the Hezar Mountain in Kerman province, southeast of Iran, and were taxonomically identified by a botanist. A voucher specimen of samples was kept in the herbarium (voucher no. 2046). The stems and leaves of the plant were air-dried at ambient temperature, powdered, and extracted with 80% methanol by maceration. The extract was then evaporated using a rotary evaporator under reduced pressure at a temperature not exceeding 42 °C. The concentrated extract was dried in an oven at 40 °C. The final extraction yield was about 22%. The obtained extract was stored at 4 °C to be used in the experimental studies.

### 2.2. Extract standardization

The extract was standardized using a high-performance liquid chromatography (HPLC) system (Knauer; Germany) for apigenin and silibinin as flavonoids. The chromatographic system conditions were optimized to quantify the amount of these flavonoids in the extract.

Stock standard solutions of apigenin  $\geq 95\%$  and silibinin  $\geq 98\%$  were prepared and diluted with HPLC grade methanol, and the detector (Knauer-UV K2501: 288 nm) was adjusted for them at 340 and 288 nm, respectively. Furthermore, mobile phases, including A: solution of phosphoric acid, water (0.5:99.5, *v/v*) and B: phosphoric acid, acetonitrile (0.5:99.5, *v/v*), were used for apigenin, and a system composed of phosphoric acid, methanol and water as solution A (0.5:35:65, *v/v/v*) and solution B: phosphoric acid, methanol and

water (0.5:50:50 *v/v/v*) was applied for silibinin. The flow rate was 0.8 mL/min, and a reversed-phase analytical Eclipse-XBD-C18 column (12.5 cm, 4.6 cm in length, 15  $\mu$ m) as the stationary phase at (20  $\pm$  1) °C was used. Dissolved *L. officinale* extract (in methanol 80%) was centrifuged, filtered, diluted, and injected (volume: 20  $\mu$ L) into the system. For determination of the phytochemical content of *L. officinale* extract, the relative peak area and retention time of each sample chromatogram were related to the standard reference peak for quantification and data acquisition through ChromGate software.

### 2.3. Animals

Adult male Wistar rats (200-230 g) were procured from the animal house of Biology Department at Shahid Bahonar University of Kerman and acclimated to the laboratory environment conditions [temperature: (23  $\pm$  2) °C, humidity: 50%-55%, and a 12 h light/dark cycle]. They were allowed free access to a standard pellet diet and tap water.

### 2.4. Ethical statement

Animal procedures were approved by Research Ethics Committees of Veterinary Faculty of Shahid Bahonar University of Kerman (approval ID: IR.UK.VETMED.REC.1401.022, Date: 2023-02-11) according to “The 3Rs protocol” guidelines (defined in 1960 and promoted in 2017 by the NC3Rs).

### 2.5. Experimental design

To study the hepatoprotective effects of *L. officinale* extract, adult male rats were randomly divided into five groups (six rats in each group) including:

Group I (control): received distilled water (DW) as extract solvent (1 mL) orally for 10 d and liquid paraffin (as CCl<sub>4</sub> solvent) 30 min after the administration of DW on day 11.

Group II: received DW for 10 d and 50% CCl<sub>4</sub> in liquid paraffin (2.5 mL/kg b.w., orally) 30 min after the administration of DW on day 11.

Groups III and IV: received *L. officinale* extract (300 or 500 mg/kg b.w., orally, once daily) respectively, for 10 consecutive days and 30 min before the administration of the CCl<sub>4</sub> on day 11. According to our pilot studies, two doses 300 and 500 mg/kg were selected in this study.

Group V: (positive control), received Legalon containing 70% silymarin (420 mg/kg b.w., orally, once daily) for 10 consecutive days and 30 min before the administration of the CCl<sub>4</sub> on day 11.

Twenty-four hours after the CCl<sub>4</sub> or its solvent administration, fasting rats were anesthetized by inhalation of CO<sub>2</sub>, sacrificed by decapitation and blood samples were collected for the biochemical assays. Liver tissue samples of each group were washed with cold saline, divided into segments, and stored at –70 °C for the next

enzymatic and molecular studies. Furthermore, some of the liver tissue samples were fixed in 10% formaldehyde for histological studies.

## 2.6. Histopathological examination

Liver tissue samples were fixed in 10% formaldehyde for 24 h, dehydrated with graded ethanol, cleared with xylene, and embedded in paraffin. Paraffin blocks were cut into 5- $\mu$ m thick sections using a rotary microtome (Microtec, Cu4050, Germany). The tissue sections were stained with hematoxylin and eosin dyes. To study the histopathological changes, all specimens were examined under blinded conditions by a pathologist (Dr. M.M) through a light microscope (Olympus BX-41, Olympus Corporation, Tokyo, Japan). Sections ( $n=6$ ) in different groups were scored according to hepatic injury indices, including, degeneration in hepatocytes (fatty hydropic changes), degeneration in hepatic cords, deformation in hepatocytes, focal necrosis, congestion in a central vein, congestion in sinusoids, infiltration of lymphocytes, Kupffer cells proliferation and bleeding area in hepatic lobes. According to our scoring system, the histopathological changes were assessed as follows: score 0: no visible cell damage; score 1: focal hepatocyte damage less than 25%; score 2: 25%-50%; score 3: 50%-75% and score 4: 75%-100% tissue damage. Six slides were examined with respect to the above injury indices and the mean score of liver injury was determined in different groups.

In addition, for detecting tissue fibrosis changes, Masson's trichrome staining protocol was used for some of the 5  $\mu$ m-thick sections. This stain imparts a blue color to collagen fibers against tissue background.

## 2.7. Serum biomarkers of liver injury

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin, total protein, and urea were determined by spectrophotometry (CS-T240, ADVANCE, China) using commercially available detection kits (Pars Azmoon, Tehran, Iran).

## 2.8. Assessment of antioxidant and lipid peroxidation markers

For determination of oxidative stress biomarkers, liver tissues were homogenized and centrifuged; then supernatants were collected and used for the determination of protein content and antioxidant enzyme activities as described below.

Protein content in liver tissue samples was determined by the method of Bradford[12] using bovine serum albumin as standard. Catalase activity was determined using the method described by Aebi[13] with some modifications. Superoxide dismutase (SOD) activity was determined with the protocol of Giannopolitis and Ries[14]. Glutathione (GSH) content in liver tissue was evaluated as

described by Sedlak and Lindsay[15] and malondialdehyde (MDA) level was measured by the method of Ohkawa *et al.*[16] modified by Jamall and Smith[17]. Also, plasma MDA level was determined according to the method of Kurtel *et al.*[18].

## 2.9. Gene expression level determination by real-time PCR

Total RNA of homogenized liver specimens was isolated by using a total RNA purification kit (GeneAll; Korea). Concentration and purity of RNA samples were determined at OD 260/280 ratio using a spectrophotometer (Nanodrop 2000c; Thermo Fisher Scientific; USA) and RNA integrity was confirmed by running 5  $\mu$ L RNA on 1.5% agarose gel containing Gel Red (10 ng/mL). First-strand cDNA was synthesized from a certain amount of total RNA by reverse transcription using the Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific; UK) according to the manufacturer's guidelines.

The PCR system program was set to run for one cycle at 95 °C for 2 min (initial denaturation) followed by 35 cycles at 95 °C for 15 s (denaturation) and at annealing temperature for 1 min.  $\beta$ -Actin was used as the internal control for qRT-PCR. Specific primers were designed using Gene Runner Program Version 3.0, Oligo 7 software, and NCBI Primer-Blast. The sequences of primers used in this study were *TNF- $\alpha$*  forward primer: 5'-AACACACGAGACGCTGAAGT-3', reverse primer: 5'-TCCAGTGAGTTCCGAAAGCC-3' (93 bp); *TGF- $\beta$ 1* forward primer: 5'-CCCCTACATTTGGAGCCTGG-3', reverse primer: 5'-TTGCGACCCACGTAGAAGAC-3' (141 bp); *IL-6* forward primer: 5'-CTGGTCTTCTGGAGTTCCGT-3', reverse primer: 5'-TGGTCCTTAGCCACTCCTTCT-3' (219 bp); *COX-2* forward primer: 5'-CTCAGCCATGCAGCAAATCC-3', reverse primer: 5'-GGGTGGGCTTCAGCAGTAAT-3' (172 bp); *MCP-1* forward primer: 5'-TGTCTCAGCCAGATGCAGTT-3', reverse primer: 5'-AGCCGACTCATTTGGGATCAT-3' (79 bp); and  $\beta$ -actin forward primer: 5'-GCTGAGAGGGAAATCGTGC-3', reverse primer: 5'-TCAACGTCACACTTCATGATGG-3' (253 bp). All reactions were performed in duplicate and included the following materials: cDNA samples, a specific pair of primers, 2 $\times$  SYBR Green PCR Master Mix, and nuclease-free water until the final volume of 20  $\mu$ L. The gene expression levels were calculated using 2<sup>- $\Delta\Delta$ Ct</sup> method through melting curves analysis[19].

## 2.10. Immunohistochemical studies (IHC)

Hepatic staining for Ki-67 (a marker of regeneration after tissue injury) was done using a standard immunohistochemical protocol as described before[20]. Immunohistochemical procedures were done on 5  $\mu$ m tissue sections to characterize  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) in liver tissue according to Domitrović *et al.*[21]. Briefly, deparaffinized and rehydrated sections were immersed in citrate buffer (0.01 M, pH = 6.0) in a microwave for 20 min and passed through a routine protocol for antigen retrieval and followed

by incubation with 0.3% H<sub>2</sub>O<sub>2</sub> for 30 min to block endogenous peroxidase activity. After washing in phosphate buffered saline (PBS), the sections were incubated overnight at 4 °C with diluted (1:100) primary antibody, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG. The sections were then washed three times with PBS and color developed using substrate-chromogen diaminobenzidine (DAB) mixed solution. After washing, slides were counterstained with hematoxylin and were processed until mounted with entellan. The abovementioned immunohistochemical protocols resulted in a brown-colored precipitate at antigen sites. Permanent slides were analyzed by light microscopy and results were presented based on the percent of brownstained Ki-67 and  $\alpha$ -SMA immunopositive cells.

### 2.11. Statistical analysis

Statistical analyses of all the data were performed using one-way ANOVA followed by Tukey *post hoc* test with the SPSS software program (SPSS v.16). The difference between groups was considered significant at  $P < 0.05$ .

## 3. Results

### 3.1. Extract standardization

HPLC analysis determined the amount of some of the bioactive flavonoids in the extract. The chromatograms of these standard

flavonoids and *L. officinale* samples are shown in Figure 1. Phytochemical analysis of *L. officinale* extract showed the presence of 11.4 mg/g of apigenin and 2.2 mg/g of silibinin as flavonoids of the extract.

### 3.2. Histopathological findings

Histopathological examination of H & E-stained liver sections showed normal lobular architecture with radiating hepatic cords (Figure 2A), but in the CCl<sub>4</sub> intoxicated group, severe morphological changes, including hepatic architecture disruption, significant cytoplasm reduction, cytoplasmic vacuolization, infiltration of inflammatory cells, fatty degeneration and necrosis were observed (Figure 2B). Interestingly, hepatic damages caused by CCl<sub>4</sub> were significantly mitigated in rats receiving *L. officinale* extract pretreatment (500 mg/kg) and Legalon (Figure 2D and E). Also, the mean score of injury in CCl<sub>4</sub> intoxicated tissues was significantly higher than that in the control group. The groups pretreated with *L. officinale* extract, especially at 500 mg/kg, or with Legalon showed significantly decreased scores of injury (Table 1). According to the above histological findings and serum biomarkers of liver function (Table 1), 500 mg/kg was the most effective dose of *L. officinale* extract. Therefore, this dose was selected for doing the next subsidiary assessments.

### 3.3. Masson's trichrome staining findings

Normal liver sections stained with Masson's trichrome showed a

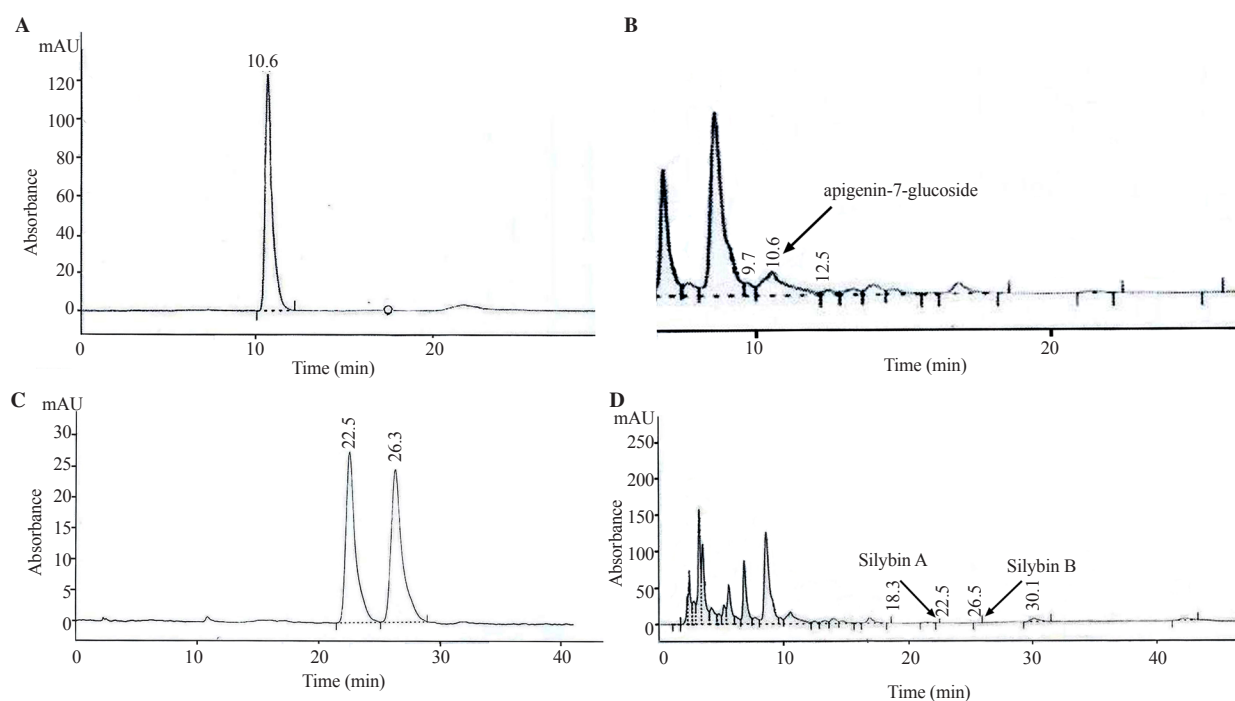


**Figure 1.** HPLC analysis of *Levisticum officinale*. HPLC chromatograms of standard apigenin-7- $\alpha$ -glucosidase (A) and silibinin (C) which includes silybin A and silybin B, as well as *Levisticum officinale* extract samples (B and D).

thin rim of collagen around central veins and portal tracts (Figure 3A). However, CCl<sub>4</sub> intoxication caused a marked increase in collagen deposition in liver sections especially around the central veins and portal tracts (Figure 3B). *L. officinale* extract (500 mg/kg) and Legalon pretreatment decreased CCl<sub>4</sub>-induced fibrosis (Figure 3C and D).

### 3.4. Effect of *L. officinale* extract on serum biochemical markers in rats with CCl<sub>4</sub>-induced liver injury

The results of the biochemical tests showed that the serum levels of ALT, AST, ALP, urea, and total bilirubin were significantly increased and serum total protein level was decreased in the CCl<sub>4</sub>-exposed group in comparison to the control group ( $P<0.01$ ). In contrast, the

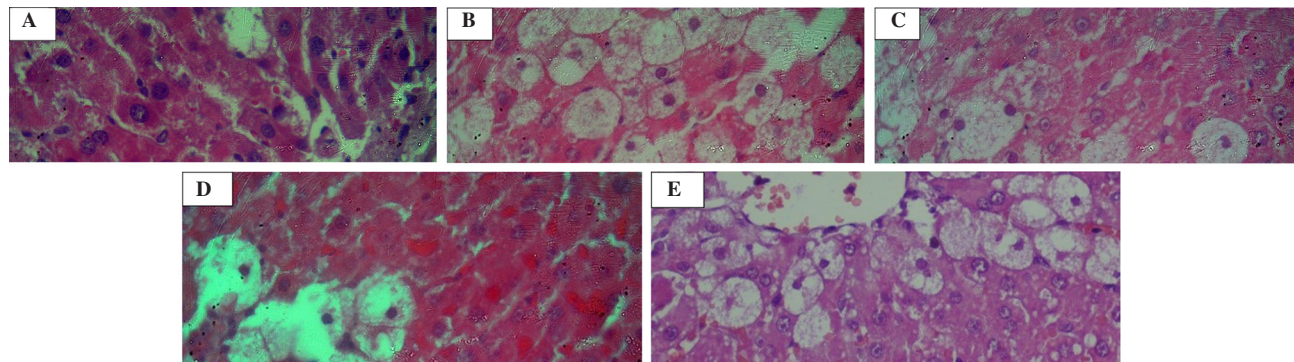


**Figure 2.** Photomicrographs of liver sections stained with hematoxylin and eosin (magnification:  $\times 400$ ). (A): The normal control group shows normal liver architecture with cords of hepatocytes radiating from the central vein. (B): Sections obtained from the liver of CCl<sub>4</sub>-intoxicated rats show loss of hepatic architecture with inflammatory cell infiltration, cytoplasmic vacuolization, fatty degeneration, and vacuolar fatty change. Sections obtained from the liver of CCl<sub>4</sub>-intoxicated rats pretreated with *Levisticum officinale* extract at 300 mg/kg (C) and 500 mg/kg (D) or Legalon at 420 mg/kg (E), show an improvement in CCl<sub>4</sub>-induced liver injuries.

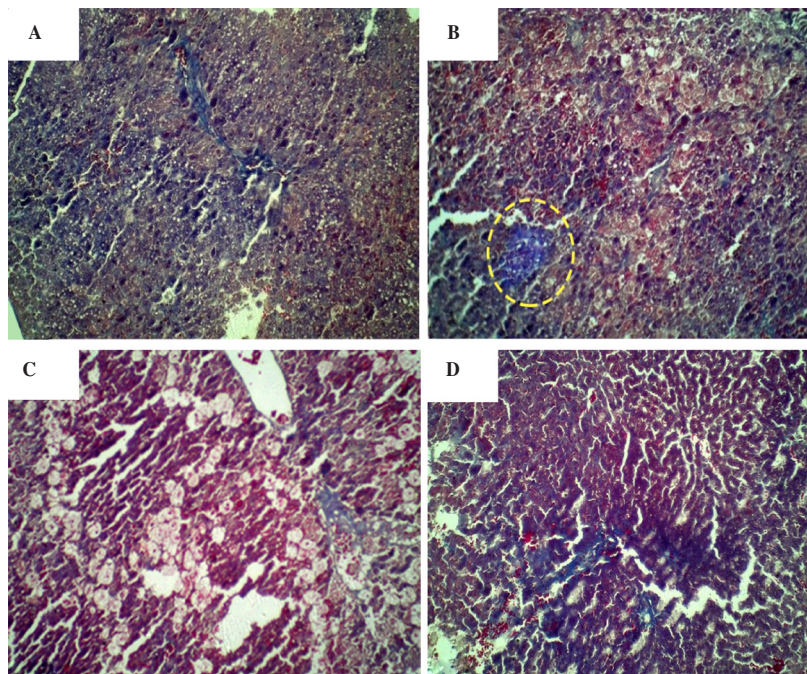


**Figure 3.** Representative micrographs of rat liver sections stained by Masson's trichrome (magnification:  $\times 100$ ). (A): Sections obtained from the liver of control rats show a thin rim of collagen fibers stained blue around the central vein and portal space. (B): Sections from the liver of CCl<sub>4</sub>-intoxicated rats show excessive collagen fibers deposition (yellow circle). (C-D): Sections from the liver of rats intoxicated with CCl<sub>4</sub> and pretreated with *Levisticum officinale* extract at 500 mg/kg or Legalon at 420 mg/kg show minimal deposition of collagen fibers.

**Table 1.** Effects of *Levisticum officinale* extract on serum parameters of liver function and mean score of liver injury in rats.

| Groups                                 | ALT<br>(IU/dL)                  | AST<br>(IU/L)                   | ALP<br>(U/L)                   | Urea<br>(mg/dL)              | Total protein<br>(mg/dL)    | Total bilirubin<br>(mg/dL)    | Mean score of<br>liver injury |
|----------------------------------------|---------------------------------|---------------------------------|--------------------------------|------------------------------|-----------------------------|-------------------------------|-------------------------------|
| Control                                | 190.2 $\pm$ 1.2                 | 362.6 $\pm$ 5.8                 | 410.2 $\pm$ 3.8                | 45.7 $\pm$ 1.4               | 7.4 $\pm$ 0.2               | 0.70 $\pm$ 0.01               | 3.5 $\pm$ 0.5                 |
| CCl <sub>4</sub> (2.5 mL/kg)           | 5107.5 $\pm$ 388.5**            | 5235.0 $\pm$ 66.1**             | 1231.5 $\pm$ 13.3**            | 65.5 $\pm$ 1.0**             | 5.5 $\pm$ 0.2**             | 2.60 $\pm$ 0.01**             | 17.9 $\pm$ 1.0**              |
| Extract (300 mg/kg) + CCl <sub>4</sub> | 1834.5 $\pm$ 18.6 <sup>##</sup> | 1385.0 $\pm$ 88.5 <sup>##</sup> | 1100.7 $\pm$ 6.5 <sup>##</sup> | 50.2 $\pm$ 0.6 <sup>##</sup> | 6.2 $\pm$ 0.1               | 1.90 $\pm$ 0.06 <sup>##</sup> | 14.1 $\pm$ 1.5 <sup>#</sup>   |
| Extract (500 mg/kg) + CCl <sub>4</sub> | 1029.0 $\pm$ 8.2 <sup>##</sup>  | 832.5 $\pm$ 29.0 <sup>##</sup>  | 741.5 $\pm$ 6.5 <sup>##</sup>  | 47.5 $\pm$ 0.2 <sup>##</sup> | 6.7 $\pm$ 0.1 <sup>##</sup> | 1.00 $\pm$ 0.05 <sup>##</sup> | 11.4 $\pm$ 1.0 <sup>##</sup>  |
| Legalon (420 mg/kg) + CCl <sub>4</sub> | 487.5 $\pm$ 17.0 <sup>##</sup>  | 357.7 $\pm$ 38.9 <sup>##</sup>  | 543.7 $\pm$ 7.5 <sup>##</sup>  | 46.0 $\pm$ 0.4 <sup>##</sup> | 6.8 $\pm$ 0.2 <sup>##</sup> | 0.90 $\pm$ 0.00 <sup>##</sup> | 8.5 $\pm$ 1.0 <sup>##</sup>   |

Values are presented as mean  $\pm$  SEM,  $n=6$ . \*\* $P<0.01$  compared to the control group. <sup>#</sup> $P<0.05$ , <sup>##</sup> $P<0.01$  compared to the CCl<sub>4</sub>-intoxicated group. ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase.

group pretreated with *L. officinale* extract at 300 and 500 mg/kg significantly ( $P<0.01$ ) prevented the rise in serum level of the above parameters (ALT, AST, ALP, urea, and total bilirubin) and improved serum level of total protein at 500 mg/kg ( $P<0.01$ ) when compared with the CCl<sub>4</sub>-intoxicated group (Table 1).

### 3.5. Effect of *L. officinale* on antioxidant indices

CCl<sub>4</sub> intoxication markedly decreased SOD and catalase activities and GSH content ( $P<0.01$ ) in the liver tissue samples compared to the control group. *L. officinale* (500 mg/kg) pretreatment significantly increased activities of the above antioxidant enzymes ( $P<0.05$ ) and GSH content ( $P<0.01$ ) in liver tissue in comparison to the CCl<sub>4</sub> group (Table 2).

### 3.6. Effect of *L. officinale* on MDA level

Results showed a significant increase of MDA level in the plasma and liver samples in the CCl<sub>4</sub> group in comparison to control ( $P<0.01$ ), *L. officinale* pretreatment significantly ( $P<0.01$ ) alleviated

this lipid peroxidation indicator in both plasma and liver tissue samples in comparison to the CCl<sub>4</sub> group (Table 2).

### 3.7. Effect of *L. officinale* on mRNA expression of inflammatory and fibrosis factors

As shown in Figure 4, in the CCl<sub>4</sub>-intoxicated rats, gene expression levels of *TNF- $\alpha$* , *TGF- $\beta$ 1*, *IL-6*, *MCP-1*, and *COX-2* were notably increased ( $P<0.05$ ) compared to the control group. By contrast, *L. officinale* pretreatment (500 mg/kg) significantly decreased the mRNA level of *TNF- $\alpha$* , *TGF- $\beta$ 1*, *IL-6*, *COX-2*, and *MCP-1* (Figure 4,  $P<0.01$ ).

### 3.8. Immunohistochemical results

The percent of immunopositively stained cells in tissue slides was a criterion for estimation of expression level of Ki-67 and  $\alpha$ -SMA markers. Results show that there was a significant increase in Ki-67 positivity in the CCl<sub>4</sub> group in comparison with the control group ( $P<0.01$ ), while Ki-67 immunosignals were markedly alleviated

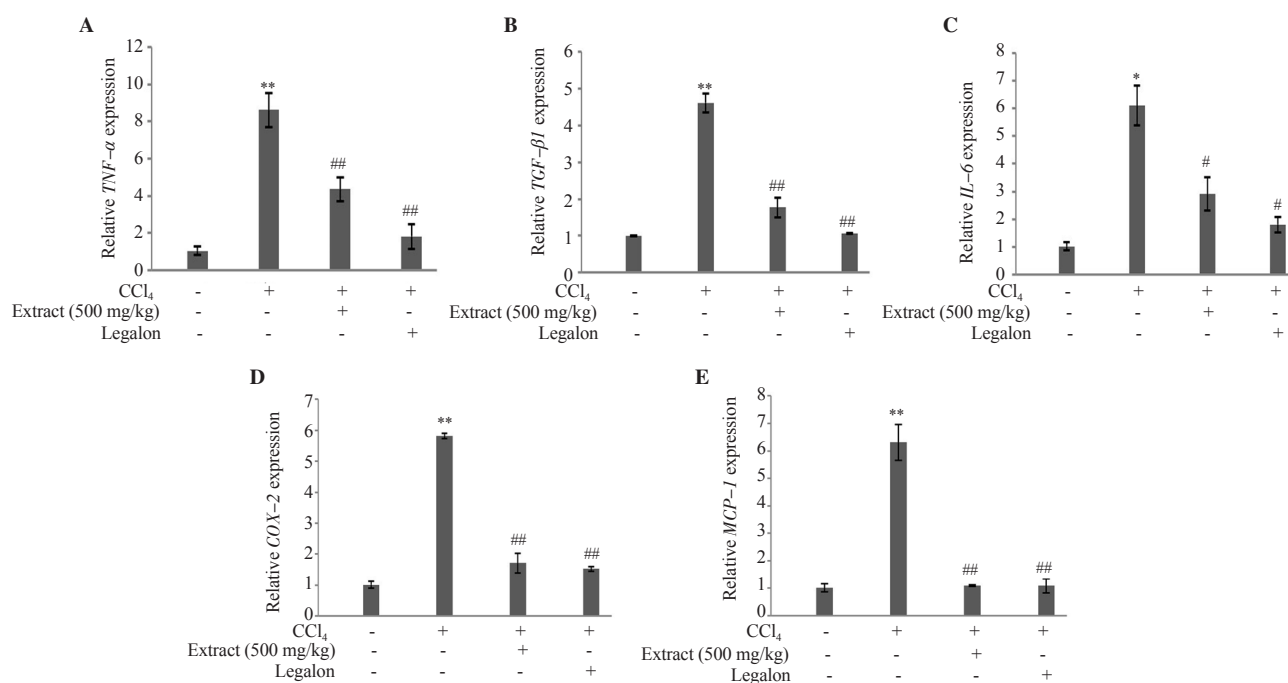


**Figure 4.** Effect of *Levisticum officinale* extract on mRNA expression levels of *TNF- $\alpha$*  (A), *TGF- $\beta$ 1* (B), *IL-6* (C), *COX-2* (D), and *MCP-1* (E) in CCl<sub>4</sub>-intoxicated rats. Data are presented as mean  $\pm$  SEM ( $n = 6$ ).  $\beta$ -Actin was used as an internal control for calculating mRNA fold change. \* $P<0.05$ , \*\* $P<0.01$  compared to the control group. # $P<0.05$ , ## $P<0.01$  compared to the CCl<sub>4</sub>-intoxicated group.

**Table 2.** Effects of *Levisticum officinale* extract on liver and plasma antioxidant biomarkers.

| Groups                                 | SOD (U/mg protein) | Catalase (U/g protein) | GSH ( $\mu$ mol/g tissue) | MDA (nmol/g tissue) | Plasma MDA (nmol/mL) |
|----------------------------------------|--------------------|------------------------|---------------------------|---------------------|----------------------|
| Control                                | 17.2 $\pm$ 0.5     | 209.9 $\pm$ 1.7        | 10.8 $\pm$ 0.0            | 3.6 $\pm$ 0.2       | 0.9 $\pm$ 0.0        |
| CCl <sub>4</sub> (2.5 mL/kg)           | 5.4 $\pm$ 0.6**    | 147.2 $\pm$ 3.5**      | 4.6 $\pm$ 0.1**           | 15.9 $\pm$ 0.1**    | 6.2 $\pm$ 0.0**      |
| Extract (500 mg/kg) + CCl <sub>4</sub> | 11.2 $\pm$ 0.2###  | 167.6 $\pm$ 2.8#       | 9.9 $\pm$ 0.1##           | 6.6 $\pm$ 0.5##     | 3.0 $\pm$ 0.1##      |
| Legalon (420 mg/kg) + CCl <sub>4</sub> | 16.8 $\pm$ 0.7###  | 184.5 $\pm$ 3.5###     | 10.3 $\pm$ 0.0##          | 4.4 $\pm$ 0.1##     | 2.1 $\pm$ 0.0##      |

Values are presented as mean  $\pm$  SEM,  $n=6$ . \*\* $P<0.01$  compared to the control group. # $P<0.05$ , ## $P<0.01$  compared to the CCl<sub>4</sub>-intoxicated group. SOD: superoxide dismutase; GSH: glutathione; MDA: malondialdehyde.

in the *L. officinale* and Legalon pretreated groups ( $P<0.01$ ) in comparison with the CCl<sub>4</sub> group indicating reduced hepatocellular injury (Figure 5).

Moreover, as shown in Figure 6, significant  $\alpha$ -SMA immunopositivity was found in the livers of CCl<sub>4</sub>-intoxicated rats compared to the control group ( $P<0.01$ ). However, pretreatment with *L. officinale* (500 mg/kg) and Legalon significantly decreased  $\alpha$ -SMA immunostaining ( $P<0.05$ ). Additionally, according to the obtained photomicrographs, the livers of control rats exhibited a thin rim of collagen fibers stained blue around the central vein. In contrast, liver

sections from CCl<sub>4</sub>-intoxicated rats showed excessive collagen fiber deposition. However, sections from rats pretreated with the extract or Legalon demonstrated a lesser amount of collagen fiber deposition compared to the CCl<sub>4</sub> group (Figure 6).

#### 4. Discussion

In the present study, we demonstrate for the first time that *L. officinale* pretreatment significantly alleviated biochemical and

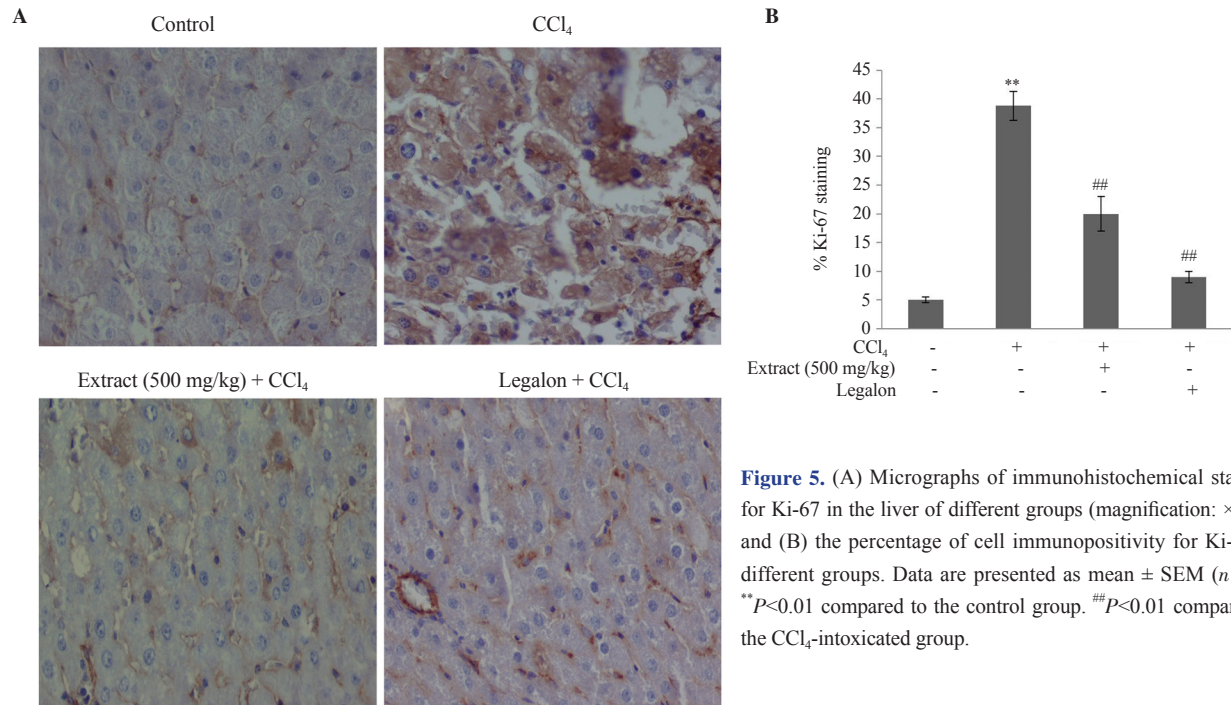


**Figure 5.** (A) Micrographs of immunohistochemical staining for Ki-67 in the liver of different groups (magnification:  $\times 400$ ) and (B) the percentage of cell immunopositivity for Ki-67 in different groups. Data are presented as mean  $\pm$  SEM ( $n = 6$ ). \*\* $P<0.01$  compared to the control group. ## $P<0.01$  compared to the CCl<sub>4</sub>-intoxicated group.

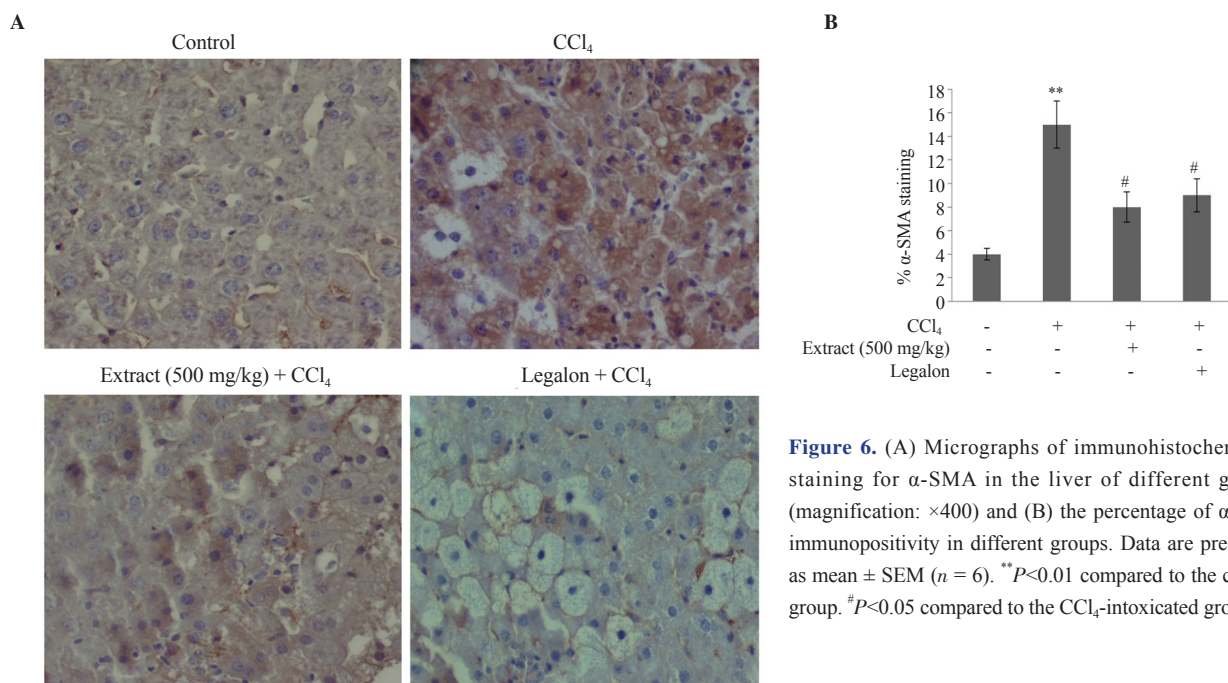


**Figure 6.** (A) Micrographs of immunohistochemistry staining for  $\alpha$ -SMA in the liver of different groups (magnification:  $\times 400$ ) and (B) the percentage of  $\alpha$ -SMA immunopositivity in different groups. Data are presented as mean  $\pm$  SEM ( $n = 6$ ). \*\* $P<0.01$  compared to the control group. # $P<0.05$  compared to the CCl<sub>4</sub>-intoxicated group.

histological changes in rats with CCl<sub>4</sub>-induced hepatotoxicity. Moreover, *L. officinale* could modulate mRNA expression of pro-fibrotic and pro-inflammatory agents such as *TNF-α*, *TGF-β1*, *IL-6*, *MCP-1*, and *COX-2*. It also mitigated  $\alpha$ -SMA expression as an indicator of fibrosis and Ki-67 as a regenerative biomarker, indicating reduced hepatocellular injury.

CCl<sub>4</sub>-induced injury is an experimental model that promotes hepatic pathology similar to that observed in humans and is widely used for screening the effectiveness of different agents against hepatic diseases. The mechanism underlying CCl<sub>4</sub>-induced liver injury seems to be multifactorial. Several factors, including oxidative stress, pro-inflammatory cytokines, and fibrogenesis are implicated[4,21].

CCl<sub>4</sub> as a potent hepatotoxin activates phase I cytochrome P450 system in the smooth endoplasmic reticulum of hepatocytes to produce the highly reactive  $\bullet\text{CCl}_3$  and  $\bullet\text{OCCl}_3$  free radicals. High levels of these radicals in the liver cells lead to peroxidative degradation of hepatocytes and an increase in their membranes' permeability, which consequently cause a rise in serum levels of ALT, AST, ALP, and total bilirubin. Besides, free radicals that result from CCl<sub>4</sub> metabolism lead to lipid peroxidation and a decrease in antioxidant enzyme activities. It is known that oxidative stress is linked to decreased GSH levels in the liver of CCl<sub>4</sub>-treated rats. GSH as a non-enzymatic antioxidant, acts as a substrate for glutathione S-transferase (GST) and glutathione peroxidase (GPx) and prevents the deleterious effect of oxygen radicals. A significant decrease in activities of liver SOD and catalase in hepatocytes and an increase in MDA level have been reported in CCl<sub>4</sub>-intoxicated rats as a result of enhanced ROS generation in liver cells[22–25]. Also, CCl<sub>4</sub> and excessive ROS activate liver Kupffer cells, which release *TNF-α*, *TGF-β1*, *IL-6*, and other pro-inflammatory cytokines. These act on hepatocytes, HSCs, and endothelial cells and subsequently recruit immune cells to initiate inflammation process. *TNF-α*, as a proinflammatory cytokine, stimulates the release of other cytokines such as *TGF-α*, *IL-1β*, and *IL-6* from macrophages. *IL-6* plays a potential role in the pathogenesis of hepatic diseases[26]. *COX-2* is an inducible, rate-limiting enzyme that is responsible for production of a large number of prostaglandins and pro-inflammatory cytokines. It plays an important role in the pathogenesis of inflammation and hepatic fibrosis[27]. Also, activated HSCs contribute to excessive hepatic expression of *MCP-1* and this may be upregulated by other members of cytokines such as *TNF-α* and *IL-1*. *MCP-1* upregulation is an important factor in the primary stages of inflammation[28]. *TGF-β1* is one of the potent pro-fibrogenic cytokines in hepatic diseases. It is generated by activated HSCs and plays a major role in the fibrogenic response of HSCs to CCl<sub>4</sub> toxicity through stimulation of fibrogenic gene expression[4].  $\alpha$ -SMA is an indicator of HSC activation and liver fibrosis. HSCs increase  $\alpha$ -SMA expression and produce extracellular matrix components such as collagen which has a main role in hepatic fibrosis. In support of this, overproduction of type I collagen has been reported following CCl<sub>4</sub> administration[4]. Furthermore, a lot of studies reported that CCl<sub>4</sub> caused fibrosis and

increased  $\alpha$ -SMA levels in liver cells[29]. High levels of mRNA transcripts of inflammatory and fibrosis mediators, including *TNF-α*, *TGF-β1*, *MCP-1*[4], *IL-6*, and *COX-2*[25] were reported in CCl<sub>4</sub>-induced hepatotoxicity.

In this study, a remarkable decrease of above pro-fibrogenic and pro-inflammatory mRNA expression such as *TNF-α*, *TGF-β1*, *IL-6*, *COX-2*, and *MCP-1* was observed after *L. officinale* pretreatment in CCl<sub>4</sub>-intoxicated rats. Besides, the present immunohistochemical observations showed CCl<sub>4</sub>-induced  $\alpha$ -SMA expression in liver tissue which is in line with the previous studies. Interestingly, *L. officinale* mitigated CCl<sub>4</sub>-stimulated  $\alpha$ -SMA expression. In line with recent findings, our previous studies revealed antioxidant and anti-inflammatory properties of *L. officinale*[9–11]. Thus, the hepatoprotective effects of this plant can be related to its ability to reduce oxidative stress and the inflammatory and fibrogenic cytokines in liver tissue.

In a recent study, a phytochemical analysis detected apigenin and silybinin as bioactive flavonoids in *L. officinale*. Many pharmacological studies have reported that apigenin has anti-oxidative and anti-inflammatory properties[30]. Also, apigenin can exert protection against liver injuries caused by CCl<sub>4</sub>[31]. Apigenin improves deficits related to mouse serum levels of ALT, AST, MDA, liver myeloperoxidase activity, GSH, and ROS content levels and subsequently exerts protective effects on acetaminophen-induced liver damage[32]. In the methotrexate-induced hepatotoxicity model, apigenin pretreatment showed a significant reduction in hepatic content of MDA, NO, *TNF-α*, and *IL-1β*, and the serum levels of AST, ALT, and ALP through prevention of oxidative stress and inflammation. Furthermore, histological assessment of the rat livers confirmed the hepatoprotective effects of apigenin[33].

Silibinin as the other known ingredients of *L. officinale*, is a mixture of silybin A and B (ratio 1:1) and is widely known as a strong antioxidant. It has free radical scavenging activity, which can improve cellular antioxidant components such as GSH content and SOD activity[34]. In addition to ROS scavenging activity, it mitigated *TNF-α*, *PGE<sub>2</sub>*, and *IL-1β* release through inhibition of nuclear factor-kappa B (NF- $\kappa$ B) expression in the inflamed liver[35]. Additionally, another study reported that silibinin exerted anti-inflammatory effects by suppressing the NF- $\kappa$ B signaling pathway and inhibiting *TNF-α*[36]. This compound also can improve liver circulation by preventing sinusoidal congestion and endothelial damage and restoring serum liver function markers such as AST, ALT, and ALP through ROS production inhibition[37]. Moreover, previous studies show that phenolic acids such as rosmarinic acid, caffeic acid, and chlorogenic acid, and flavonoids such as luteolin, quercetin[11], rutin, and catechin[9] are present in *L. officinale*. Additionally, GC-MS analysis detected thymol and phytol in its extract[11].

It has been reported that rosmarinic acid[38], caffeic acid[39], chlorogenic acid[40], quercetin[41], rutin[42], catechin[43], thymol[44], and phytol[45] possess antioxidant, anti-inflammatory, and hepatoprotective properties. With regards to the above findings, *L.*

*officinale* includes different valuable active ingredients that play an important role in its hepatoprotective properties against CCl<sub>4</sub>-intoxication.

However, this study has some limitations. It did not provide information about efficacy, toxicity, and side effects of long-term *L. officinale* administration for hepatoprotection against CCl<sub>4</sub>. Although our recent studies tried to characterize some effective chemical constituents of *L. officinale*, further investigations are needed to identify bioactive compounds of the extract responsible for its hepatoprotective activity. Moreover, further mechanisms underlying its hepatoprotective properties remain to be fully elucidated.

In summary, the present study demonstrated that *L. officinale* significantly protected against CCl<sub>4</sub>-induced hepatotoxicity by exerting antioxidant, antifibrotic, and anti-inflammatory activities. Therefore, it may be used as a therapeutic agent for hepatotoxicity.

## Conflict of interest statement

The authors declare that there is no conflict of interest.

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## Data availability statement

The data supporting the findings of this study are available from the corresponding author upon request.

## Authors' contributions

IP conceptualized the study and performed data analysis and interpretation. NG, MM and MMM collected the data and drafted the article. All the authors critically revised the article, and approved the final version of the manuscript.

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