Arch Biol Sci. 2026;78(2):151-165 https://doi.org/10.2298/ABS260206009M

# Hepatoprotective effects of the *Centaurea pungens n*-butanol extract against carbon tetrachloride-induced liver injury in rats

Hichem Mezdour[1,3],*, Ahmed Menad[2,3], and Souad Amedah[2,3]

[1]*Department of Biology, Faculty of Life Sciences and of Earth and Universe Sciences, University of Mohamed El Bachir El Ibrahimi*, Bordj Bou Arreridj, 34000, Algeria

[2]*Department of Animal Biology, Faculty of Natural and Life Sciences, University of Constantine 1 Frères Mentouri*, Aïn El Bey Road, 25000, Constantine, Algeria

[3]*Laboratory of Biology and Environment, Department of Animal Biology, Faculty of Natural and Life Sciences, University of Constantine 1 Frères Mentouri*, Aïn El Bey Road, 25000, Constantine, Algeria

***Corresponding author**: hichem.mezdour@univ-bba.dz

**Received**: February 6, 2026; **Revised**: May 13, 2026; **Accepted**: May 15, 2026; **Published online**: May 18, 2026

**Abstract:** The study evaluated the antioxidant and hepatoprotective potential of the n-butanol extract of *Centaurea pungens* (BECP) against carbon tetrachloride ($CCl_4$)-induced hepatotoxicity in Wistar rats. The safety of BECP was demonstrated through acute cytotoxicity tests and MTT assays. BECP showed *in vitro* radical scavenging activity. Pretreatment with BECP (100, 200, and 400 mg/kg) significantly alleviated liver markers in a dose-dependent manner, restoring antioxidant status. The results suggested that *Centaurea pungens* exerts strong hepatoprotective effects mediated by bioactive compounds identified by total phenolic content and LC-MS analysis, supporting its potential in liver disorder treatment.

**Keywords:** *Centaurea pungens,* carbon tetrachloride, hepatotoxicity, antioxidant activity, polyphenols

## INTRODUCTION

The genus *Centaurea* (Asteraceae) is a large and taxonomically complex group comprising approximately 500 species distributed throughout the Old World [1]. These species are mainly found in the Mediterranean basin and the Middle East, where they represent an important component of the regional flora [2]. In Algeria, the genus is represented by approximately 47 species, including several taxa adapted to arid and semi-arid environments, with seven species reported in Saharan regions [3].

Several *Centaurea* species have long been used in traditional medicine to treat various ailments, including fever, inflammatory disorders, diabetes, and gastrointestinal diseases such as peptic ulcers [4-6]. Recent pharmacological studies have demonstrated that many species of this genus possess significant antioxidant and hepatoprotective activities, mainly attributed to their high content of phenolic compounds and flavonoids capable of scavenging reactive oxygen species and reducing oxidative stress [7-9]. For example, the n-butanol extract of *Centaurea sphaerocephala* significantly attenuated valproic acid-induced hepatotoxicity by decreasing lipid peroxidation and restoring antioxidant enzyme activities [10]. Similarly, the ethanolic extract of *Centaurea behen* showed protective effects against carbon tetrachloride-induced liver injury through normalization of biochemical parameters and improvement of liver histology [11]. In addition, extracts of *Centaurea incana* exhibited strong antioxidant activity and enhanced endogenous antioxidant defenses in experimental models of hepatic injury [12].

*Centaurea pungens*, commonly known as the common purple star thistle, is widely distributed in the Algerian steppe and is traditionally used in folk medicine for the treatment of respiratory disorders and inflammatory conditions [13]. Despite its ethnomedicinal importance and its belonging to a genus rich in bioactive phytochemicals, the pharmacological properties of this species remain poorly investigated.

The liver plays a central role in maintaining physiological homeostasis through its involvement in metabolism, detoxification, and immune functions. Various factors, including viral infections, alcohol consumption, toxins, and metabolic disorders, can impair hepatic function and lead to acute or chronic liver injury [14]. Carbon tetrachloride ($CCl_4$) is widely used as an experimental hepatotoxin to induce liver damage in animal models. Its toxicity results from metabolic activation by the cytochrome P450 system, generating highly reactive trichloromethyl ($CCl_3•$) radicals that initiate lipid peroxidation and cause oxidative damage to cellular membranes and macromolecules [15]. This injury is reflected by increased serum levels of hepatic enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST), along with disruption of the antioxidant defense system, including enzymes such as superoxide dismutase (SOD) and catalase (CAT), and increased lipid peroxidation measured by malondialdehyde (MDA).

Antioxidants play an important role in protecting biological systems against oxidative damage caused by free radicals, which are implicated in numerous pathological conditions, including inflammation, tissue injury, and neurodegenerative diseases [16]. However, concerns regarding the potential toxicity of synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have encouraged the search for safer natural alternatives, particularly plant-derived phytochemicals rich in phenolic compounds [17].

Based on the phytochemical richness reported for species of the genus *Centaurea*, it can be hypothesized that the bioactive constituents present in *C. pungens* may exert protective effects against oxidative stress-mediated liver injury. These compounds could potentially neutralize free radicals generated during $CCl_4$ metabolism and enhance endogenous antioxidant defenses, thereby limiting hepatic damage.

To date, no scientific study has investigated the hepatoprotective activity of *C. pungens* growing in Algeria. Therefore, the present study aimed to evaluate the antioxidant activity and hepatoprotective potential of the n-butanol extract of the aerial parts of *C. pungens* (BECP) against $CCl_4$-induced liver injury in rats by assessing serum biochemical markers (AST, ALT, ALP), total bilirubin (TBIL), and hepatic antioxidant parameters including glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and malondialdehyde (MDA).

## MATERIALS AND METHODS

### Ethics statement

All experimental studies were approved by the Algerian MESRS and DGRSDT Ethics Committee (D01N01UN250120230010) and were conducted in compliance with the National Academy of Sciences guidelines for the care and use of laboratory animals (1996) and the European Union Directive 2010/63/EU on the protection of experimental animals.

### Preparation of extract

The dried aerial parts of *C. pungens* (1,285 g) were macerated using a hydroalcoholic solution containing methanol/water (80/20, v/v) at room temperature for 72 h four times. After filtration, the filtrate was concentrated and dissolved in 514 mL of water. The resulting solution was extracted successively with petroleum ether (1×200 mL), chloroform ($CHCl_3$) (3×200 mL), ethyl acetate (EtOAc) (3×200 mL), and *n*-butanol (*n*-BuOH) (1×200 mL). Combined solutions were concentrated under reduced pressure and dried (PE: 3 g, $CHCl_3$: 8 g, EtOAc: 7 g, *n*-BuOH: 61 g). The resulting fractions were stored at -20°C pending further analysis. In our investigation, we focused on the *n*-BuOH fraction. The n-BuOH fraction (BECP) was selected due to its enrichment in polar compounds, including polyhydroxylated flavonoids and glycosides [13]. The n-BuOH fraction yielded the highest extract amount (61 g), sufficient for the entire treatment period.

### Total phenolic content assay

Total phenolics in the aerial parts of BECP were determined using the Folin-Ciocalteu method with gallic acid as the standard [18]. An aliquot (0.1 mL) containing 1 mg of extract was mixed with 46 mL of distilled water and 1 mL of Folin-Ciocalteu reagent in a volumetric flask and thoroughly shaken. After 3 min, 3 mL of a solution of 7% Na2CO3 was added, and the mixture was allowed to stand for 2 h with intermittent shaking. Absorbance was measured at

765 nm. Phenolic concentration was determined from a gallic acid calibration curve over the linear range of 25-200 μg/mL. Results were expressed as equivalent gallic acid (μg GAE/mg dried extract).

**Total flavonoid content assay**

The total flavonoid content of each extract was determined by a colorimetric method [19]. Each sample (1 mL) was mixed with 1 mL of aluminum chloride (AlCl3) solution (2%) and allowed to stand for 15 min. Absorbance of the mixture was then determined at 430 nm versus a prepared methanol blank. Flavonoid concentration was determined from a quercetin calibration curve over the linear range of 5-400 μg/mL. Results were expressed as equivalent quercetin (μg QE/mg dried extract).

**LC-MS analysis**

We performed liquid chromatography (LC) analyses of BECP using an Accela system (Thermo Fisher Scientific) equipped with a quaternary pump and a refrigerated plate autosampler. A Luna HST column (100 × 2.0 mm, 2.5 μm; Phenomenex, Torrance, CA, USA) was used. Gradient elution was performed using water/0.1% formic acid (solvent A) and acetonitrile/0.1% formic acid (solvent B) at a flow rate of 0.4 mL/min with a 5 μL injection volume. An increasing linear gradient of solvent B was used. The separation was carried out in 15 min under the following conditions: Gradient (t (min), %B) = (0, 0), (0.5, 0), (10, 50), (12, 50), (25, 95), (28, 95), (28.5.5, 0), (35, 0). The column was equilibrated for 5 min before each analysis. An LTQ Orbitrap Velos triple quadrupole mass spectrometer was used to obtain mass spectrometry (MS) and MS/MS data. The Turbo Ion spray source settings were as follows: capillary voltage, -3500 V; nebulizer gas (N2), 10 (arbitrary units); curtain gas (N2), 12 (arbitrary units); collision gas (N2), 4 (arbitrary units); focusing potential, -200 V; input potential, 10 V; drying gas (N2), heated to 400°C and introduced at a flow rate of 8000 mL/min. Depriming potential and collision energy were optimized in the perfusion experiments: individual standard solutions (10 mg/mL) dissolved in 50:50 (v/v) mobile phase were infused at a constant flow rate of 5 mL/min using a syringe pump (Harvard Apparatus, Holliston, MA, USA). Full-scan acquisition was performed in profile mode over an m/z range of 100-1000 using a 2 s cycle time, 0.1 u step size, and 2 ms interscan pause. The wavelengths used during the analysis were 280 and 320 nm. Data analyses were performed using XCalibur™ software.

***In vitro* MTT assay**

Cytotoxicity studies were conducted using the MTT (3-[4,5-dimethylthiazole-2-yl]-2,5 diphenyltetrazolium bromide) assay [20]. Human hepatocellular carcinoma (HepG2) cells were used as an *in vitro* model of hepatotoxicity. They were seeded at a density of $3\times10^5$ cells/mL in 96-well plates. Cells were kept in an incubator for 20 h at 37°C and 5% $CO_2$ for proper adhesion before being exposed to the extract. Cells were cultured in MEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin. The extract was applied at the concentrations of 3, 30, 300, and 3,000 μg/mL for 24 h. Three wells were used per concentration, six for the negative control, and three for the medium control. After exposure, cells were washed with PBS and exposed to 0.5 mg/mL of MTT solution for 3 h. The MTT solution was then removed by washing twice with PBS. The formazan salt produced in the MTT reaction was dissolved in 0.1 mL of DMSO. Finally, the absorbance of each well was read at 570 nm with an automatic 96-well plate spectrophotometer. The percentage of mortality was calculated by subtracting the percentage of cell viability from 100%. Means and standard deviations (SD) of mortality were calculated.

***In vitro* antioxidant activity**

***DPPH assay***

The DPPH free radical scavenging activity was estimated according to [21]. One mL of 1,1-diphenyl-2-picrylhydrazyl (DPPH) prepared in methanol (0.1 mM) was added to 1 mL of extracts in different concentrations (50-1,200 μg/mL) and left to stand in the dark for 10 min. The absorbance of the final solution was taken spectrophotometrically at 520 nm against a blank of alcohol. Vitamin C was used as a reference antioxidant. DPPH scavenging activity was expressed as $IC_{50}$ values (μg extract/mL). The $IC_{50}$ value of each sample was defined as the concentration of the sample

required for a 50% decrease in absorbance of the blank and calculated using the following formula:

% scavenging = [Absorbance of control - Absorbance of test sample/Absorbance of control] × 100

**Hydroxyl radical (OH•) scavenging activity**

Hydroxyl radical scavenging activity was determined according to the method of [22]. OH• was generated by incubating 1.4 mM $H_2O_2$, 100 µM $FeCl_3$, 2.8 mM deoxyribose, 100 µM EDTA, and 100 µM ascorbic acid in 1.2 mL of 10 mM $KH_2PO_4$-KOH buffer (pH 7.4) at 37°C for 60 min, with or without (control) the extract. Ascorbic acid was added at the end to start the reaction. Degradations of deoxyribose sugar induced by OH• were determined by the addition of 1 mL TBA (1% w/v), 1 mL TCA (5.0% w/v), and heating at 100°C for 20 min. The resulting pink chromogen was quantified by measuring absorbance at 535 nm. Trolox was used as a reference. Hydroxyl radical scavenging activity was expressed as $IC_{50}$ values (µg extract/mL), defined as the concentration required to reduce blank absorbance by 50%, calculated using the following formula:

% scavenging = [Absorbance of control - Absorbance of test sample/Absorbance of control] × 100

**Reducing power assay**

The reducing power of BECP was determined according to the method of [23]. Various concentrations of BECP were mixed with phosphate buffer (2.5 mL) and potassium ferricyanide (2.5 mL) and incubated in a water bath at 50°C for 20 min. After cooling, 2.5 mL of 10% trichloro acetic acid was added and centrifuged at 1,000 ×g for 10 min. The upper layer (2.5 mL) was mixed with distilled water (2.5 mL) and freshly prepared ferric chloride solution (0.5 mL). The absorbance was measured at 700 nm. The control was prepared similarly without adding samples. Various concentrations of ascorbic acid were used as a standard. Increased absorbance of the reaction mixture indicated an increase in reducing power. Reducing power was evaluated at different extract concentrations and incubation times.

**Animal material**

Male Wistar rats (100-150 g), 5-7 weeks old, were procured from the animal house of the Faculty of Life Sciences, Mentouri Brothers, Constantine, Algeria. The animals were kept under controlled conditions of temperature (22±3°C) and humidity (60±5%). They were given pellet food and drinking water *ad libitum*. A 12 h day and night cycle was maintained in the animal house.

**Oral toxicity studies**

An acute oral toxicity study was conducted in accordance with the Organization for Economic Cooperation and Development (OECD) guidelines [24]. Male Wistar rats were randomly divided into groups (n=6 per group). To determine the safety profile and establish the $LD_{50}$, an escalating dose design was utilized up to the standard OECD limit dose. BECP was administered orally as a single dose of 250, 500, 1,000, 1,500, or 2,000 mg/kg b.w. The animals were continuously monitored for the first 24 h (with special attention given during the first 4 h) for any behavioral changes, physical anomalies, or signs of acute distress, and thereafter monitored daily for 14 days to record any delayed toxicity or mortality. BECP was safe up to 2,000 mg/kg; therefore, 100, 200, and 400 mg/kg b.w. (1/20, 1/10, and 1/5 of this dose, respectively) were selected for hepatoprotective evaluation.

**$CCl_4$-induced hepatotoxicity model**

To induce hepatic damage, $CCl_4$ was dissolved in olive oil (1:1) and administered intraperitoneally at 1.5 mL/kg. Based on previously published hepatoprotection studies using similar models [25], the Wistar rats were randomly divided into six groups (n=6 per group). Group I (untreated control) received distilled water (1 mL/kg, p.o.) for 14 days and olive oil on day 14. Group II ($CCl_4$ control) received distilled water for 14 days, followed by intraperitoneal administration of $CCl_4$/olive oil (1:1, 1.5 mL/kg) on day 14. Group III received vitamin E (200 mg/kg) for 14 days, followed by $CCl_4$ treatment. Groups IV-VI received BECP (100, 200, or 400 mg/kg/day, dispersed in distilled water) for 14 days, followed by $CCl_4$ treatment on day 14.

After the last treatments, the rats were fasted overnight. The following day, all animals from the

experimental groups were deeply anesthetized using isoflurane inhalation, and blood samples were taken by puncture in the retro-orbital venous plexus; the animals were subsequently sacrificed by decapitation. Blood samples were collected into sterile dry centrifuge tubes, and the separated serum was used for liver enzyme analysis. For histopathological studies, portions of the liver were removed immediately and kept in 10% formalin solution. All experimental groups underwent the same tissue and blood collection procedure.

**Blood collection**

The collected blood samples were immediately centrifuged at 400 ×g for 15 min to separate serum from blood. Serum samples were stored at -80°C for analysis of hepatic injury markers, including ALT, AST, ALP, and TBIL.

**Preparation of tissue lysate**

Liver tissue was sectioned into small pieces and homogenized (10% w/v) by Ultra-Turrax basic homogenizer in ice-cold PBS (pH 7.4 and 50 mM), then centrifuged at 400 ×g for 15 min. The resulting supernatant was kept at -80°C for analysis of antioxidant enzyme activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx). Protein concentration was determined according to the Bradford method [26] using bovine serum albumin as a standard, and enzyme activities were expressed per mg of protein.

**Biochemical markers**

Biochemical parameters were assayed by standard methods. The levels of aspartate aminotransferase(AST), alanine-aminotransferase (ALT), alkaline phosphatase (ALP), and total bilirubin (TBIL) were evaluated using commercial kits from Spinreact Laboratories, Spain (AST, ref. 1001160 and 1001161; ALT, ref. 1001170 and 1002171; ALP, ref. 1001130 and 1001131; total bilirubin, ref. 1001044).

**Determination of hepatic malondialdehyde (MDA) level**

MDA levels in liver tissues were measured using the method as described by [27]. Briefly, the liver tissues were promptly excised, rinsed in cold saline, and homogenized using an Ultra-Turrax homogenizer in 10% 0.1 M potassium phosphate buffer (pH 7.4). The homogenate was then centrifuged (400 ×g for 10 min) to obtain supernatant. Four hundred µL of 1.2% TBA was added, and the mixture was placed in a water bath (95-100°C) for 30 min. The solution was then cooled. Absorbance was measured spectrophotometrically at 532 nm. The MDA level was expressed as nmol/g tissue.

**Determination of catalase (CAT) activity**

CAT activity was measured as described by [28]. The CAT assay mixture consisted of 0.05 mL extract, 1.5 mL phosphate buffer (100 mM buffer, pH 7.0), 0.5 mL $H_2O_2$, and 0.95 mL distilled water. Change in absorbance was noted six times at 30 s intervals at 240 nm. The CAT activity was expressed as µmol of $H_2O_2$ oxidized per minute per milligram protein.

**Determination of intracellular glutathione (GSH) levels**

Reduced glutathione (GSH) levels were determined by the Ellman method [29] using DTNB reagent (400 mg 5,5´-dithiobis(2-nitrobenzoic acid) in 100 mL 1% sodium citrate). Absorbance was measured at 412 nm using a spectrophotometer. GSH level was expressed as nmol/g tissue.

**Determination of superoxide dismutase (SOD) activity**

Measurement of liver cytosolic SOD was performed spectrophotometrically by inhibition of pyrogallol (2 mM) autooxidation in Tris buffer [30]. Results were expressed as unit/min/mg protein. One unit (U) of SOD activity was defined as the amount of enzyme required to inhibit the oxidation of 50% pyrogallol.

**Determination of glutathione peroxidase (GPx) activity**

GPx activity was assessed by the method of [31]. Based on the degradation of H2O2 in the presence of GSH, the GPx activity was expressed as nmol/min/mg protein.

### Histological evaluation

Liver tissues were fixed in 10% neutral buffered formalin and processed for paraffin embedding following standard histological procedures [32]. Paraffin-embedded samples were sectioned at a thickness of 4 μm and stained with hematoxylin and eosin (H&E). Histopathological alterations were examined by light microscopy (Leica, Germany) and evaluated using the semi-quantitative scoring system of Meyerholz and Beck [33]. Liver sections were assessed for hepatocellular degeneration, sinusoidal dilation, inflammatory cell infiltration, necrotic areas, central vein congestion, and hemorrhage. Each parameter was graded on a scale ranging from 0 (absence of lesion) to 8 (severe lesion).

### Statistical analysis

All results are presented as mean±standard deviation (SD). *In vitro* experiments were conducted in triplicate (n=3). For *in vivo* experiments, each group comprised 6 animals (biological replicates, n=6); biochemical and oxidative stress parameters were measured in technical triplicate for each rat, and the resulting mean value was used for subsequent statistical analysis. Group comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey's test. All statistical analyses were carried out using GraphPad Prism version 8.2.0 for Windows, GraphPad Software (San Diego, California, USA). Differences were considered statistically significant at $P<0.05$.

**Table 1.** Tentatively identified compounds in the n-BuOH extract of *Centaurea pungens*

| Peak N | Rt (min) | Molecular weight (g/mol) | Observed (*m/z*) | Proposed compound | Chemical composition |
|---|---|---|---|---|---|
| 1 | 2.91 | 170.120 | 169.0135 | Gallic acid | $C_7H_6O_5$ |
| 2 | 3.69 | 154.120 | 153.0186 | Gentisic acid | $C_7H_6O_4$ |
| 3 | 3.78 | 478.400 | 477.1231 | Isorhamnetin O-glucoside | $C_{22}H_{22}O_{12}$ |
| 4 | 4.11 | 180.160 | 181.1275 | Caffeic acid | $C_9H_8O_4$ |
| 5 | 4.25 | 312.230 | 311.0395 | Caftaric acid | $C_{13}H_{12}O_9$ |
| 6 | 4.83 | 354.310 | 353.2714 | Chlorogenic acid | $C_{16}H_{18}O_9$ |
| 7 | 5.07 | 272.257 | 272.0914 | Naringenin | $C_{15}H_{12}O_5$ |
| 8 | 5.49 | 286.230 | 285.1672 | Kaempferol | $C_{15}H_{10}O_6$ |
| 9 | 5.91 | 164.047 | 165.0957 | *p*-Coumaric acid | $C_9H_8O_3$ |
| 10 | 6.53 | 610.189 | 609.1035 | Hesperidin | $C_{28}H_{34}O_{15}$ |
| 11 | 7.25 | 302.236 | 301.0822 | Quercetin | $C_{15}H_{10}O_7$ |

RT – retention time, *m/z* – mass-to-charge ratio of ions

## RESULTS

### Total phenolic content

BECP exhibited a high total phenolic content of 211.47 ± 2.91 μg GAE/mg.

### Total flavonoid content

The total flavonoid content of BECP was quantified at 21.67±1.61 μg QE/mg.

### LC-MS analysis

LC-MS analysis of BECP extract tentatively identified 11 major phenolic constituents, characterized by their retention times, molecular weights, and observed m/z values (Table 1). The chromatographic profile revealed the presence of several low-molecular-weight phenolic acids, including gallic acid (Rt = 2.91 min, m/z 169.0135), gentisic acid (Rt = 3.69 min, m/z 153.0186), caffeic acid (Rt = 4.11 min, m/z 181.1275), and *p*-coumaric acid (Rt = 5.91 min, m/z 165.0957). In addition, hydroxycinnamic acid derivatives were detected, notably caftaric acid (Rt = 4.25 min, m/z 311.0395), and chlorogenic acid (Rt = 4.83 min, m/z 353.2714). The extract also contained several flavonoid compounds, including the flavanone naringenin (Rt = 5.07 min, m/z 272.0914), the flavonol kaempferol (Rt = 5.49 min, m/z 285.1672), and quercetin (Rt = 7.25 min, m/z 301.0822). Furthermore, glycosylated flavonoids were identified, such as isorhamnetin O-glucoside (Rt = 3.78 min, m/z 477.1231) and hesperidin (Rt = 6.53 min, m/z 609.1035). The LC-MS profile indicated that BECP is characterized by a chemically diverse polyphenolic composition, including phenolic acids, hydroxycinnamic acid esters, flavonoid aglycones, and flavonoid glycosides.

### MTT assay

Results obtained in the MTT assay indicated that BECP had a low cytotoxicity to HepG2 cells (low percentage of cell mortality) in all tested

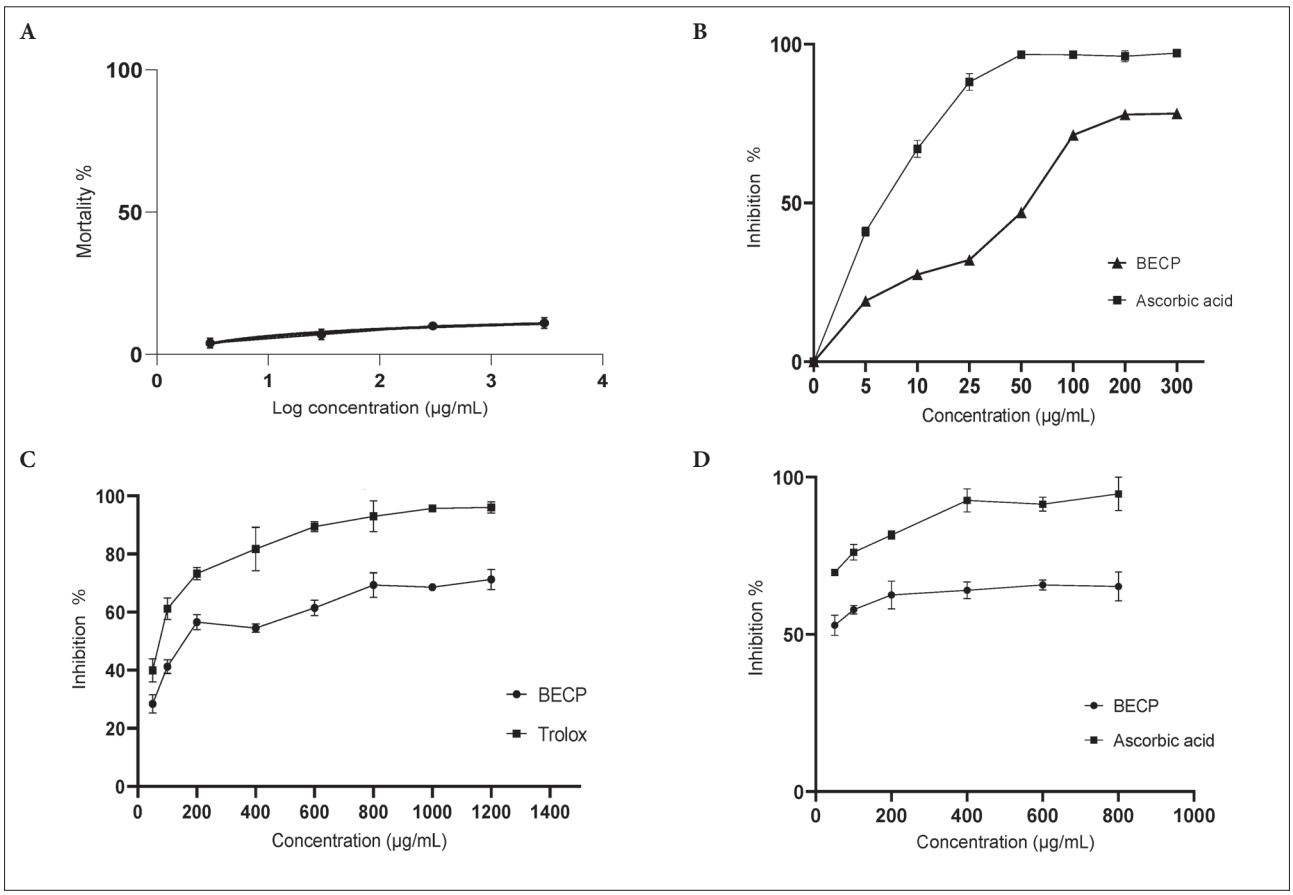



**Fig. 1.** *In vitro* antioxidant activity and cytotoxicity of the n-butanol extract of *Centaurea pungens* (BECP). Concentration-dependent antioxidant responses were evaluated using four complementary assays. **A** – Dose-response cytotoxicity curve of BECP on HepG2 cells (human hepatocellular carcinoma cell line) assessed by the MTT assay after 24 h exposure at concentrations of 3, 30, 300, and 3,000 µg/mL; cell mortality (%) remained below 11% at all tested concentrations, indicating low cytotoxicity and an $IC_{50}$ exceeding the maximum tested concentration. **B** – DPPH radical scavenging activity (%) of BECP and ascorbic acid (standard) at concentrations ranging from 50 to 1200 µg/mL; the $IC_{50}$ of BECP was 44.48±2.7 µg/mL vs. 6.44±0.61 µg/mL for ascorbic acid. **C** – Hydroxyl radical (OH•) scavenging activity (%) of BECP and Trolox (standard) at concentrations of 50-1200 µg/mL; $IC_{50}$ of BECP was 218.23±1.02 µg/mL vs. 69.67±1.44 µg/mL for Trolox. **D** – Reducing power of BECP and ascorbic acid (standard) expressed as absorbance at 700 nm as a function of concentration (50-600 µg/mL). All values are presented as mean±SD (n=3).

concentrations (3, 30, 300, and 3,000 µg/mL), as illustrated in Fig. 1A. It was not possible to establish an $IC_{50}$, and it is estimated to exceed the maximum concentration tested.

## DPPH free radical scavenging activity

The DPPH radical scavenging activity of BECP extract increased in a concentration-dependent manner (Fig. 1B). The extract exhibited a maximum inhibition of 78.74±1.15% at a concentration of 200 µg/mL. The calculated $IC_{50}$ value for BECP was 44.48±2.7 µg/mL, indicating a notable free radical scavenging capacity. In comparison, the reference antioxidant ascorbic acid demonstrated stronger activity, with an $IC_{50}$ value of 6.44±0.61 µg/mL and a maximum inhibition of 97.18±0.02% at 100 µg/mL. The scavenging activity of BECP was significantly lower than that of ascorbic acid ($P<0.05$), but still demonstrated substantial antioxidant potential.

## Hydroxyl radical scavenging activity

Hydroxyl radicals (OH•) are among the most reactive oxygen species and can induce severe oxidative damage to biomolecules such as proteins, DNA, and membrane lipids. As shown in Fig. 1C, BECP exhibited a dose-dependent hydroxyl radical scavenging activity over

the tested concentration range (50-1,200 μg/mL). The $IC_{50}$ value of BECP was 218.23±1.02 μg/mL, whereas the standard antioxidant Trolox showed significantly stronger activity with an $IC_{50}$ value of 69.67±1.44 μg/mL (P<0.05). Although BECP displayed lower activity than the reference compound, the results clearly demonstrated its ability to neutralize hydroxyl radicals.

**Reducing power activity**

The reducing power was assessed to evaluate the electron-donating capacity of antioxidant compounds. As illustrated in Fig. 1D, both BECP extract and ascorbic acid showed a concentration-dependent increase in reducing power activity. For BECP, the reducing activity increased from 52.89±3.22% at 50 μg/mL to 65.72±1.61% at 600 μg/mL. In contrast, ascorbic acid exhibited higher reducing capacity, increasing from 61.27±2.38% at 50 μg/mL to 92.61±3.67% at 400 μg/mL. Although the reducing power of BECP remained lower than that of the reference antioxidant (P<0.05), the extract demonstrated a clear dose-dependent electron-donating ability, which reflects its potential antioxidant activity.

***In vivo* experiments**

***Acute toxicity studies***

The acute toxicity study showed no signs of toxicity or mortality in rats orally treated with BECP at doses of 250-2,000 mg/kg. Therefore, the oral $LD_{50}$ of the BECP extract is considered to be greater than 2,000 mg/kg b.w., indicating a high margin of safety.

**Liver enzymes and total bilirubin**

Serum hepatic biomarkers are presented in Fig. 2A (liver enzymes) and 2B (total bilirubin). Administration of $CCl_4$ resulted in marked hepatic injury, as evidenced by a significant elevation (P<0.001) in serum activities of ALT, AST, and ALP, as well as TBIL levels, compared with the normal control group. Specifically, ALT, AST, ALP, and TBIL increased to 528.85±7.84 U/L, 428.35±17.03 U/L, 475.36±12.69 U/L, and 0.34±0.03 mg/dL, respectively, whereas the corresponding values in the normal control group were 109.46±5.12 U/L, 58.33±2.40 U/L, 156.72±10.27 U/L, and 0.18±0.01 mg/dL. However, oral administration of BECP extract (100, 200, and 400 mg/kg) significantly attenuated the $CCl_4$-induced elevation of these hepatic biomarkers in a dose-dependent manner (P<0.001 vs. $CCl_4$ group). At the highest dose (400 mg/kg), BECP exhibited pronounced hepatoprotective activity, reducing serum ALT, AST, ALP, and TBIL levels to 225.24±9.71 U/L, 139.23±8.35 U/L, 258.61±9.05 U/L, and 0.20±0.02 mg/dL, respectively. Furthermore, the positive control group treated with vitamin E demonstrated the greatest hepatoprotective effect, showing a highly significant reduction (P<0.001) in all evaluated biochemical parameters compared with the $CCl_4$-treated group.

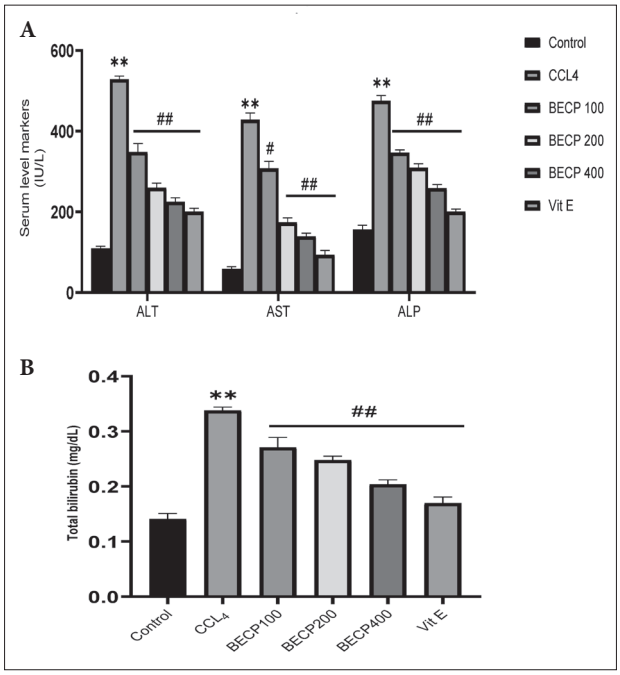



**Fig. 2.** Effect of BECP pretreatment on serum hepatic injury markers in $CCl_4$-intoxicated rats. Wistar rats were assigned to six groups (n=6 per group): Group I, healthy control (distilled water by gavage for 14 days + olive oil i.p. on day 14); Group II, $CCl_4$-intoxicated control (distilled water by gavage for 14 days + $CCl_4$/olive oil 1:1 v/v, 1.5 mL/kg i.p. on day 14); Group III, standard treatment (vitamin E, 200 mg/kg/day p.o. for 14 days + $CCl_4$ on day 14); Groups IV-VI, BECP at 100, 200, and 400 mg/kg/day p.o. for 14 days + $CCl_4$ on day 14. **A** – Serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP), expressed in U/L. **B** – Serum total bilirubin (TBIL) levels, expressed in mg/dL. All values are presented as mean±SD (n=6). Statistical differences were assessed by one-way ANOVA followed by Tukey's multiple comparison test. **P<0.01: $CCl_4$-intoxicated control (Group II) vs. healthy control (Group I); #P<0.05, ##P<0.01: treatment groups (Groups III-VI) vs. $CCl_4$-intoxicated control (Group II).

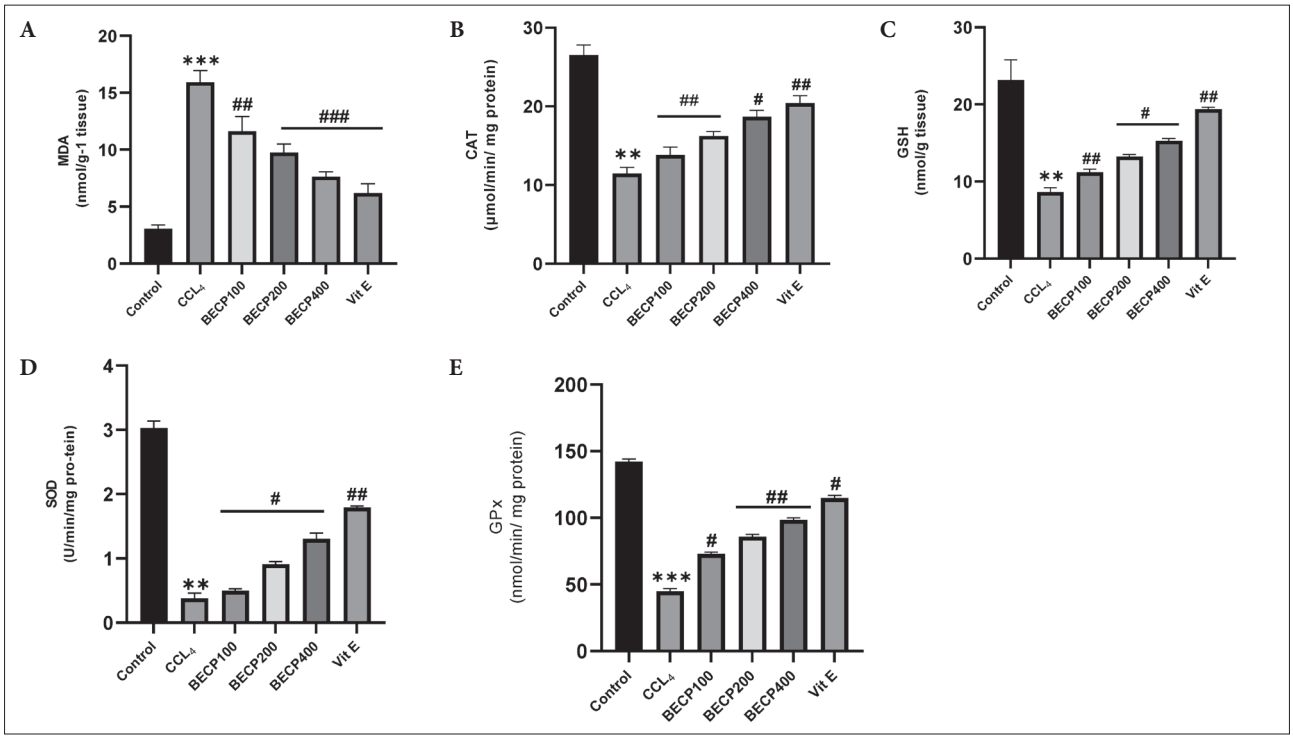



**Fig. 3.** Effect of BECP pretreatment on hepatic oxidative stress markers in $CCl_4$-intoxicated rats. Experimental groups are as described in Fig. 2 (n=6 per group; each biochemical assay was performed in technical triplicate). Hepatic malondialdehyde (MDA) level, expressed as nmol/g tissue, used as an index of lipid peroxidation. Hepatic catalase (CAT) activity, expressed as µmol $H_2O_2$ oxidized/min/mg protein. Reduced glutathione (GSH) level, expressed as nmol/g tissue. Superoxide dismutase (SOD) activity, expressed as U/min/mg protein. Glutathione peroxidase (GPx) activity, expressed as nmol/min/mg protein. All values are presented as mean±SD (n=6; technical triplicate per rat). One-way ANOVA, Tukey's post hoc test. *P<0.05, **P<0.01, ***P<0.001 vs. healthy control (Group I); #P<0.05, ##P<0.01, ###P<0.001 vs. $CCl_4$-intoxicated control (Group II).

## Antioxidant parameters

$CCl_4$ administration significantly increased hepatic MDA levels (15.914±0.589 nmol/g tissue) compared with the normal control group (3.058±0.186 nmol/g tissue) (P<0.001). Pretreatment with BECP reduced MDA levels in a dose-dependent manner. MDA values were 11.604±0.748, 9.756±0.424, and 7.643±0.228 nmol/g tissue at 100, 200, and 400 mg/kg, respectively. The highest dose (400 mg/kg) showed a highly significant reduction compared with the $CCl_4$ group (P<0.001). Vitamin E treatment also significantly reduced MDA levels (6.169±0.490 nmol/g tissue) (Fig. 3A). $CCl_4$ significantly decreased CAT activity (11.47±0.76 µmol/min/mg protein) compared with the control group (26.52±1.27 µmol/min/mg protein) (P<0.001). BECP pretreatment increased CAT activity to 13.81±0.98, 16.24±0.55, and 18.69±1.79 µmol/min/mg protein at 100, 200, and 400 mg/kg, respectively. Vitamin E restored CAT activity to 20.40±0.95 µmol/min/mg protein (Fig. 3B). In addition, GSH levels were significantly decreased in the $CCl_4$ group (8.63±0.55 nmol/g tissue) compared with controls (23.15±2.64 nmol/g tissue) (P< 0.01). BECP treatment significantly elevated GSH levels to 11.20±0.38, 13.23±0.27, and 15.28±0.30 nmol/g tissue at 100, 200, and 400 mg/kg, respectively. Vitamin E treatment increased GSH levels to 19.38±0.04 nmol/g tissue (Fig. 3C).

SOD activity was markedly reduced in the $CCl_4$ group (0.38±0.04 U/min/mg protein) compared with controls (3.02±0.84 U/min/mg protein) (P<0.001). BECP administration significantly increased SOD activity to 0.49±0.03, 0.91±0.02, and 1.30±0.08 U/min/mg protein at 100, 200, and 400 mg/kg, respectively. Vitamin E treatment resulted in SOD activity of 1.79±0.13 U/min/mg protein (Fig. 3D). Similarly, GPx activity was significantly reduced following $CCl_4$ administration (62.92±1.90 nmol/min/mg protein) compared with the control group (112.11±2.01 nmol/min/mg protein) (P<0.001). BECP pretreatment

increased GPx activity in a dose-dependent manner to 69.93±1.52, 76.68±0.85, and 83.09±1.65 nmol/min/mg protein at 100, 200, and 400 mg/kg, respectively. Vitamin E restored GPx activity to 96.03±2.13 nmol/min/mg protein (Fig. 3E).

### Histopathological examination

Histopathological examination provided substantial evidence of $CCl_4$-induced liver injury and its attenuation by BECP treatment. Liver sections from the normal control group exhibited typical hepatic architecture (score: 0.3±0.01; Supplementary Figs. S1A, S2), with well-preserved hepatocytes, intact cytoplasm, and clearly defined nuclei. In contrast, $CCl_4$-intoxicated rats displayed severe hepatic damage (score: 7.5±0.8; $P<0.001$ vs. normal control; Supplementary Figs. S1B, S2), characterized by diffuse hepatocellular necrosis, moderate cytoplasmic vacuolization, and mild inflammatory cell infiltration. Pretreatment with vitamin E markedly attenuated these pathological alterations (score: 2.52±0.2; $P<0.001$ vs. $CCl_4$ group; Supplementary Figs. S1F, S2), with only mild hepatocellular necrosis, minimal lymphocytic infiltration, and reduced ballooning degeneration. Administration of BECP at 100, 200, and 400 mg/kg progressively reduced hepatic injury scores to 6.20±0.60, 4.33±0.4, and 2.8±0.1, respectively ($P<0.001$ vs. $CCl_4$ group; Supplementary Figs. S1C-E, S2), with dose-dependent reductions in hepatocellular necrosis, inflammatory cell infiltration, and ballooning degeneration.

## DISCUSSION

The present study evaluated the hepatoprotective potential of the butanolic extract of *Centaurea pungens* (BECP) against induced $CCl_4$ liver injury. Prior to *in vivo* testing, cytotoxicity was assessed using the MTT assay on HepG2 cells. Even at the highest tested concentration (3,000 µg/mL), BECP showed minimal cytotoxicity, with cell mortality (4-11%) comparable to untreated controls (3-9%), confirming its safety for further investigation. In addition, an acute oral toxicity study was conducted to evaluate the safety profile of BECP *in vivo*. Administration of BECP at doses up to 2,000 mg/kg produced no mortality, no observable behavioral abnormalities, and no signs of systemic toxicity during the observation period. These findings indicate that BECP possesses a wide safety margin and can be considered practically non-toxic at the tested doses, supporting its suitability for subsequent hepatoprotective evaluation.

$CCl_4$ was then employed as a standard hepatotoxic agent to model oxidative liver damage *in vivo*. Its toxicity is initiated by metabolic activation through cytochrome P450 enzymes, particularly CYP2E1, leading to the formation of the highly reactive trichloromethyl radical ($CCl_3•$), which can further react with oxygen to generate the trichloromethylperoxy radical ($CCl_3OO•$). These reactive species initiate lipid peroxidation, disrupt membrane integrity, and impair cellular function, ultimately leading to hepatocellular damage [34]. Because oxidative stress plays a central role in $CCl_4$-induced hepatotoxicity, this experimental model is widely used to evaluate the hepatoprotective potential of antioxidant compounds.

As expected, $CCl_4$ administration significantly increased serum ALT, AST, ALP, and total bilirubin (TBIL), indicating oxidative stress-induced hepatocellular damage and membrane permeability. These markers are well-established indicators of hepatic pathophysiology [33]. Pre-treatment with BECP at all tested doses (100, 200, and 400 mg/kg) substantially normalized these serum markers, suggesting that the extract preserved membrane integrity and prevented intracellular enzyme leakage. These findings are consistent with previously reported hepatoprotective effects of other *Centaurea* species [35,36]. Biochemical results were further corroborated by histopathological evaluation: liver sections from $CCl_4$-treated animals showed pronounced pathological alterations, while BECP pre-treatment markedly reduced these lesions and restored normal hepatic architecture, providing direct morphological confirmation of its protective capacity.

Hepatic lipid peroxidation, assessed through MDA levels, was significantly elevated following $CCl_4$ treatment, confirming oxidative membrane injury [36]. BECP dose-dependently reduced MDA levels, indicating effective suppression of lipid peroxidation, consistent with findings reported for *Centaurea cyanus* L. petal extract [8]. In parallel, $CCl_4$ significantly depleted key antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), due to excessive reactive oxygen species production overwhelming endogenous antioxidant

defenses [38]. These enzymes constitute the primary enzymatic defense system against ROS: SOD converts superoxide radicals into hydrogen peroxide ($H_2O_2$), which is subsequently detoxified into water by CAT and GPx [39]. Pre-treatment with BECP restored the activities of these antioxidant enzymes in a dose-dependent manner, suggesting that the extract reinforces endogenous antioxidant defense mechanisms. The dose-dependent improvement in biochemical and oxidative stress parameters further supports the pharmacological efficacy of BECP. Similar observations were reported by Mostafa et al. [40] for *Centaurea pumilio* L. extract, which significantly enhanced SOD activity and reduced oxidative stress markers *in vivo*.

Similarly, hepatic glutathione (GSH), a critical non-enzymatic antioxidant that plays a central role in cellular redox homeostasis, was significantly reduced in $CCl_4$-treated animals. Depletion of hepatic GSH is considered an early indicator of oxidative liver injury and may contribute to hepatocellular necrosis [41]. In the present study, BECP treatment significantly restored GSH levels, which may reflect improved redox balance and possible stimulation of glutathione synthesis or regeneration.

The protective effects observed *in vivo* are consistent with the rich phytochemical LC–MS analysis of BECP tentatively identified several phenolic constituents, including gentisic, caffeic, and *p*-coumaric acids, as well as hydroxycinnamic acid derivatives such as caftaric and chlorogenic acids. The extract also contained several flavonoid compounds, including the flavanone naringenin, the flavonol kaempferol, and quercetin. Furthermore, glycosylated flavonoids were identified, such as isorhamnetin *O*-glucoside and hesperidin. Polyphenolic compounds are known to exert hepatoprotective effects through multiple mechanisms, including direct scavenging of reactive oxygen species, inhibition of lipid peroxidation, metal ion chelation, and modulation of endogenous antioxidant defense systems [42]. Therefore, the hepatoprotective activity of BECP may largely be attributed to its high content of phenolic and flavonoid compounds, which are known to counteract oxidative stress and stabilize cellular membranes [43].

*In vitro* antioxidant assays further supported this hypothesis. BECP exhibited concentration-dependent DPPH radical scavenging activity through proton donation, significant ferric-reducing power, and hydroxyl radical (OH•) scavenging activity [44,45]. The latter is particularly important since hydroxyl radicals, generated through the Fenton reaction in iron-rich tissues such as the liver, are among the most reactive ROS and play a crucial role in initiating lipid peroxidation and cellular damage [46,47]. The strong antioxidant activity of BECP observed *in vitro* may contribute to the hepatoprotective effects observed *in vivo*, since attenuation of oxidative stress plays a key role in $CCl_4$-induced liver injury.

Previous studies on *Centaurea* species have demonstrated significant correlations between phenolic and flavonoid contents and antioxidant capacity, supporting their synergistic contribution to hepatoprotective activity [48-50] and the mechanistic basis of the present findings.

## CONCLUSION

The n-BuOH extract of *Centaurea pungens* protected against $CCl_4$-induced liver injury, as demonstrated by improved biochemical, oxidative stress, and histopathological parameters. These effects are likely mediated by its strong antioxidant activity, supported by *in vitro* and *in vivo* findings, and its high phenolic content. Collectively, the results identify *C. pungens* as a safe and promising source of bioactive compounds with potential therapeutic value in oxidative stress-related liver disorders.

**Funding:** The authors received no specific funding for this work.

**Acknowledgments:** The authors gratefully acknowledge the Algerian Ministry of Higher Education and Scientific Research (MESRS) and the General Directorate for Scientific Research and Technological Development (DGRSDT) for their support of this study.

**Author contributions:** Conceptualization, SA; methodology, HM; software, AM; validation, SA, AM; formal analysis, AM; investigation, HM; resources, SA; data curation, HM; writing – original draft preparation, HM; writing – review and editing, SA; visualization, HM; supervision, SA. All authors have read and agreed to the published version of the manuscript.

**Conflict of interest disclosure:** The authors declare no conflict of interest.

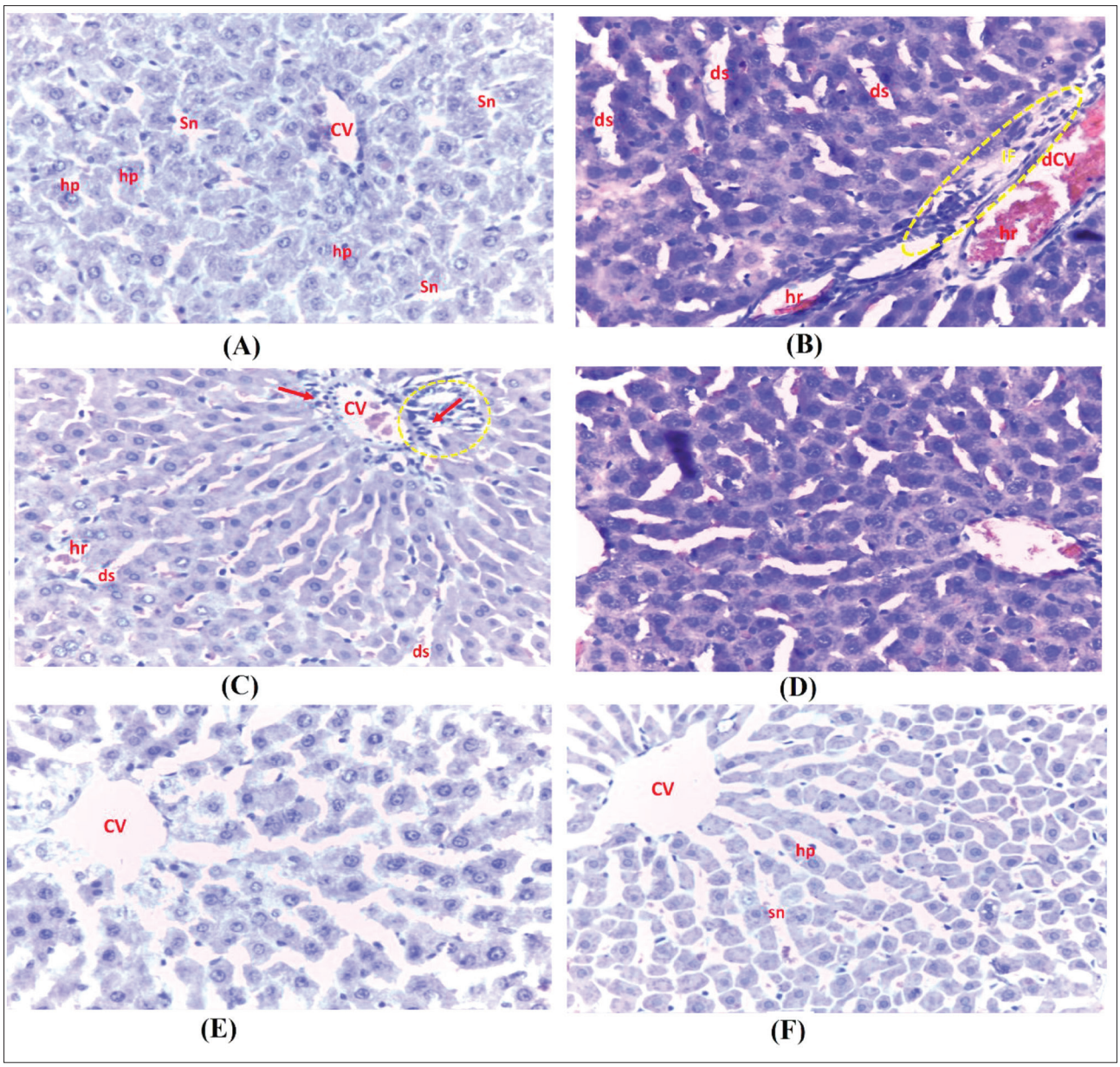
**Data availability:** The data supporting this article are available in the online dataset: https://www.serbiosoc.org.rs/NewUploads/Uploads/Mezdour%20et%20al_12140-Dataset.xlsx

## REFERENCES

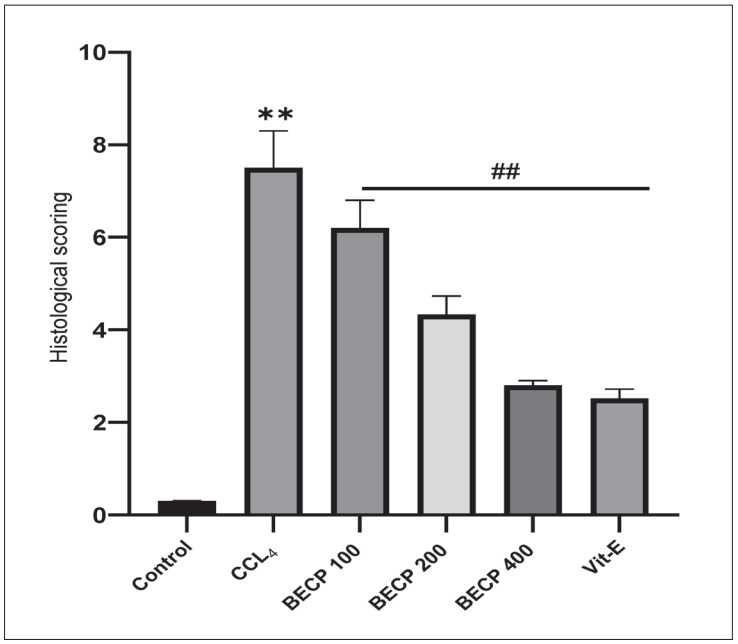

1. Brummitt RK. Report of the Committee for Spermatophyta: 54. Taxon. 2004;53(3):813-25. https://doi.org/10.2307/4135458
2. Hilpold A, Garcia-Jacas N, Vilatersana R, Susanna A. Taxonomical and nomenclatural notes on Centaurea: A proposal of classification, a description of new sections and subsections, and a species list of the redefined section Centaurea. Collect Bot. 2014;33: e001. https://doi.org/10.3989/collectbot.2013.v33.001
3. Quézel P, Santa S. Nouvelle flore de l'Algérie et des régions désertiques méridionales. Paris :Editions du Centre National de la recherche scientifique ; 1962. 626 p.
4. Inngjerdingen K, Nergård CS, Diallo D, Mounkoro PP, Paulsen BS. An ethnopharmacological survey of plants used for wound healing in Dogonland, Mali, West Africa. J Ethnopharmacol. 2004;92(2-3):233-44. https://doi.org/10.1016/j.jep.2004.02.021
5. Khanavi M, Ahmadi R, Rajabi A, Jabbari Arfaee S, Hassanzadeh G, Khademi R, Hadjiakhoondi A, Beyer C, Sharifzadeh M. Pharmacological and histological effects of *Centaurea bruguierana* ssp. belangerana on indomethacin-induced peptic ulcer in rats. J Nat Med. 2012;66(2):343-9. https://doi.org/10.1007/s11418-011-0598-7
6. Fattaheian-Dehkordi S, Hojjatifard R, Saeedi M, Khanavi M. A Review on Antidiabetic Activity of *Centaurea* spp.: A New Approach for Developing Herbal Remedies. Evid Based Complement Alternat Med. 2021; 2021:5587938. https://doi.org/10.1155/2021/5587938
7. Aktumsek A, Zengin G, Guler GO, Cakmak YS, Duran A. Screening for *in vitro* antioxidant properties and fatty acid profiles of five *Centaurea* L. species from Turkey flora. Food Chem Toxicol. 2011;49(11):2914-20. https://doi.org/10.1016/j.fct.2011.08.016
8. Escher GB, Santos JS, Rosso ND, Marques MB, Azevedo L, do Carmo MAV. Chemical study, antioxidant, anti-hypertensive, and cytotoxic/cytoprotective activities of *Centaurea cyanus* L. petals aqueous extract. Food Chem Toxicol. 2018;118:439-53. https://doi.org/10.1016/j.fct.2018.05.046
9. Bensaad MS, Dassamiour S, Hambaba L, Saidi A, Melakhsou MA, Nouicer F, Baghiani A, Khennouf S, Kahoul MA, Kadrine N. *In vivo* investigation of antidiabetic, hepatoprotective, anti-inflammatory and antipyretic activities of *Centaurea tougourensis Boiss.* & Reut. J Physiol Pharmacol. 2021;72(3):439-49. https://doi.org/10.26402/jpp.2021.3.12
10. Lahneche AM, Boucheham R, Boubekri N, Bicha S, Bentamene A, Benayache F, Benayache S, Zama D. Protective role of n-butanol extract of *Centaurea sphaerocephala* against sodium valproate-induced hepatotoxicity in rats. *Int J* Pharmacogn Phytochem Res. 2017;9(10):1335-43. https://doi.org/10.25258/phyto.v9i10.10458
11. Chougule P, Joshi Y, Jain A. Protective effect of ethanol extract of *Centaurea behen* Linn in carbon tetrachloride-induced hepatitis in rats. Int J Pharm Pharm Sci. 2014;6(8):197-200. https://doi.org/10.22159/ijpps.2014v6i8.1501
12. Boubellouta H, Khelifi-Touhami F, Mahdi D. *In vivo* and *in vitro* hepatoprotective effect of three endemic plants against carbon tetrachloride-induced liver damage in rats. Acta Sci Nat. 2021;8(1):15-36. https://doi.org/10.2478/asn-2021-0003
13. Labed F, Masullo M, Mirra V, Nazzaro F, Benayache F, Benayache S, Piacente S. Amino acid-sesquiterpene lactone conjugates from the aerial parts of *Centaurea pungens* and evaluation of their antimicrobial activity. Fitoterapia. 2019;133:51-5. https://doi.org/10.1016/j.fitote.2018.12.001
14. Gan C, Yuan Y, Shen H, Gao J, Kong X, Che Z, Guo Y, Wang H, Dong E, Xiao J. Liver diseases: epidemiology, causes, trends and predictions. Signal Transduct Target Ther. 2025;10(1):33. https://doi.org/10.1038/s41392-024-02072-z
15. Cheng N, Ren N, Gao H, Lei X, Zheng J, Cao W. Antioxidant and hepatoprotective effects of Schisandra chinensis pollen extract on $CCl_4$ -induced acute liver damage in mice. Food Chem Toxicol. 2013;55:234-40. https://doi.org/10.1016/j.fct.2012.11.022
16. Halliwell B. Free radicals, reactive oxygen species and human disease: a critical evaluation with special reference to atherosclerosis. Br J Exp Pathol. 1989;70(6):737-57. https://pmc.ncbi.nlm.nih.gov/articles/PMC2040729/
17. Botterweck AAM, Verhagen H, Goldbohm RA, Kleinjans J, van den Brandt PA. Intake of butylated hydroxyanisole and butylated hydroxytoluene and stomach cancer risk: results from analyses in the Netherlands Cohort Study. Food Chem Toxicol. 2000;38(7):599-605. https://doi.org/10.1016/S0278-6915(00)00042-9
18. Singleton VL, Rossi JA. Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. Am J Enol Vitic. 1 janv 1965;16(3):144-58. https://doi.org/10.5344/ajev.1965.16.3.144
19. Zhishen J, Mengcheng T, Jianming W. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem. 1999;64(4):555-9. https://doi.org/10.1016/S0308-8146(98)00102-2
20. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65(1-2):55-63. https://doi.org/10.1016/0022-1759(83)90303-4
21. Cheung LM, Cheung PCK, Ooi VEC. Antioxidant activity and total phenolics of edible mushroom extracts. Food Chem. 2003;81(2):249-55. https://doi.org/10.1016/S0308-8146(02)00419-3
22. Halliwell B, Gutteridge JM, Aruoma OI. The deoxyribose method: a simple « test-tube » assay for determination of rate constants for reactions of hydroxyl radicals. Anal Biochem. 1987;165(1):215-9. https://doi.org/10.1016/0003-2697(87)90222-3
23. Oyaizu M. Studies on products of browning reaction. Antioxidative activities of products of browning reaction prepared from glucosamine. Jpn J Nutr Diet. 1986;44(6):307-15. [Japanese]. https://doi.org/10.5264/eiyogakuzashi.44.307
24. OECD. Test No. 423: Acute Oral Toxicity - Acute Toxic Class Method [Internet]. Paris: OECD Publishing; 2002 [cited 2025 Jan 25]. (OECD Guidelines for the Testing of Chemicals, Section 4). Available from: https://www.oecd.org/content/dam/oecd/fr/publications/reports/2002/02/test-no-423-acute-oral-toxicity-acute-toxic-class-method_g1gh294f/9789264071018-fr.pdf.

25. Khan RA, Khan MR, Sahreen S. $CCl_4$-induced hepatotoxicity: protective effect of rutin on p53, CYP2E1 and the antioxidative status in rat. BMC Complement Altern Med. 2012;12:178. https://doi.org/10.1186/1472-6882-12-178
26. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-54. https://doi.org/10.1016/0003-2697(76)90527-3
27. Draper HH, Hadley M. Malondialdehyde determination as index of lipid peroxidation. Methods Enzymol. 1990;186:421-431. http://doi.org/10.1016/0076-6879(90)86135-i
28. Aebi H. Catalase in vitro. In: Oxygen Radicals in Biological Systems [Internet]. Academic Press; 1984. p. 121-6.(Methods Enzymology; Vol. 105). http://doi.org/10.1016/S0076-6879(84)05016-3
29. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys. 1959;82(1):70-7. http://doi.org/10.1016/0003-9861(59)90090-6
30. Marklund S, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem. 1974;47(3):469-74. http://doi.org/10.1111/j.1432-1033.1974.tb03714.x
31. Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG. Selenium: biochemical role as a component of glutathione peroxidase. Science. 1973;179(4073):588-90. http://doi.org/10.1126/science.179.4073.588
32. Meharie BG, Amare GG, Belayneh YM. Evaluation of Hepatoprotective Activity of the Crude Extract and Solvent Fractions of Clutia abyssinica Euphorbiaceae Leaf Against $CCl_4$ -Induced Hepatotoxicity in Mice. J Exp Pharmacol. 2020; 12:137-50. https://doi.org/10.2147/JEP.S248677
33. Meyerholz KD, Beck PA. Fundamental concepts for semiquantitative tissue scoring in translational research. ILAR J. 2019;59(1):13-7. https://doi.org/10.1093/ilar/ily025
34. Ponmari G, Annamalai A, Gopalakrishnan VK, Lakshmi PTV, Guruvayoorappan C. NF-κB activation and proinflammatory cytokines mediated protective effect of Indigofera caerulea Roxb. on $CCl_4$ induced liver damage in rats. Int Immunopharmacol. 2014;23(2):672-80. https://doi.org/10.1016/j.intimp.2014.10.021
35. Torres-González L, Elsa Muñoz-Espinosa L, María Rivas-Estilla A, Trujillo-Murillo K, Garza-Rodríguez ML, Ortiz-López R, Páez-Martínez N. Protective effect of four Mexican plants against $CCl_4$ -induced damage on the Huh7 human hepatoma cell line. Ann Hepatol. 2011;10(1):73-9. https://doi.org/10.1016/S1665-2681(19)31590-X
36. Abdallah HM, Mohamed MA, Abdou AM, Hamed MM, Abdel-Naim AB, Ashour OM. Protective effect of *Centaurea pallescens* Del. against $CCl_4$ -induced injury on a human hepatoma cell line (Huh7). Med Chem Res. 2013;22(12):5700-6. https://doi.org/10.1007/s00044-013-0563-y
37. Ayala A, Muñoz MF, Argüelles S. Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal. Oxid Med Cell Longev. 2014;2014:1-31. https://doi.org/10.1155/2014/360438
38. Juan CA, Pérez de la Lastra JM, Plou FJ, Pérez-Lebeña E. The Chemistry of Reactive Oxygen Species (ROS) Revisited: Outlining Their Role in Biological Macromolecules (DNA, Lipids and Proteins) and Induced Pathologies. Int J Mol Sci. 2021;22(9):4642. https://doi.org/10.3390/ijms22094642
39. Gaetani GF, Ferraris AM, Rolfo M, Mangerini R, Arena S, Kirkman HN. Predominant Role of Catalase in the Disposal of Hydrogen Peroxide Within Human Erythrocytes. Blood. 1996;87(4):1595-9. https://doi.org/10.1182/blood.V87.4.1595.bloodjournal8741595
40. Mostafa E, Fayed MAA, Radwan RA, Bakr RO. *Centaurea pumilio* L. extract and nanoparticles: A candidate for healthy skin. Colloids Surf B Biointerfaces. 2019;182:110350. https://doi.org/10.1016/j.colsurfb.2019.110350
41. Abdel-Moneim AM, Al-Kahtani MA, El-Kersh MA, Al-Omair MA. Free Radical-Scavenging, Anti-Inflammatory/ Anti-Fibrotic and Hepatoprotective Actions of Taurine and Silymarin against $CCl_4$ Induced Rat Liver Damage. PLoS One. 2015;10(12): e0144509. https://doi.org/10.1371/journal.pone.0144509
42. Csepregi K, Neugart S, Schreiner M, Hideg É. Comparative evaluation of total antioxidant capacities of plant polyphenols. Molecules. 2016;21(2):208. https://doi.org/10.3390/molecules21020208
43. Phang CW, Malek SNA, Ibrahim H. Antioxidant potential, cytotoxic activity and total phenolic content of Alpinia pahangensis rhizomes. BMC Complement Altern Med. 2013;13(1):243. https://doi.org/10.1186/1472-6882-13-243
44. Qingming Y, Xianhui P, Weibao K, Hong Y, Yidan S, Li Z, Yuzhu Y. Antioxidant activities of malt extract from barley (Hordeum vulgare L.) toward various oxidative stress *in vitro and in vivo*. Food Chem. 2010;118(1):84-9. https://doi.org/10.1016/j.foodchem.2009.04.094
45. Akar Z, Küçük M, Doğan H. A new colorimetric DPPH $^{\bullet}$ scavenging activity method with no need for a spectrophotometer applied on synthetic and natural antioxidants and medicinal herbs. J Enzyme Inhib Med Chem. 2017;32(1):640-7. https://doi.org/10.1080/14756366.2017.1284068
46. Fang YZ, Yang S, Wu G. Free radicals, antioxidants, and nutrition. Nutrition. 2002;18(10):872-9. https://doi.org/10.1016/S0899-9007(02)00916-4
47. Dorman HJD, Koşar M, Kahlos K, Holm Y, Hiltunen R. Antioxidant Properties and Composition of Aqueous Extracts from Mentha Species, Hybrids, Varieties, and Cultivars. J Agric Food Chem. 2003;51(16):4563-9. https://doi.org/10.1021/jf034108k
48. Shoeb M, Jaspars M, MacManus SM, Celik S, Nahar L, Kong-Thoo-Lin P, Sarker SD. Anti-colon cancer potential of phenolic compounds from the aerial parts of *Centaurea gigantea* (Asteraceae). J Nat Med. 2007;61(2):164-9. https://doi.org/10.1007/s11418-006-0117-4
49. Zengin G, Cakmak YS, Guler GO, Aktumsek A. In vitro antioxidant capacities and fatty acid compositions of three *Centaurea* species collected from Central Anatolia region of Turkey. Food Chem Toxicol. 2010;48(10):2638-41. https://doi.org/10.1016/j.fct.2010.06.033
50. Erel SB, Karaalp C, Bedir E, Kaehlig H, Glasl S, Khan S, Krenn L. Secondary metabolites of *Centaurea calolepis* and evaluation of cnicin for anti-inflammatory, antioxidant, and cytotoxic activities. Pharm Biol. 2011;49(8):840-9. https://doi.org/10.3109/13880209.2010.551538

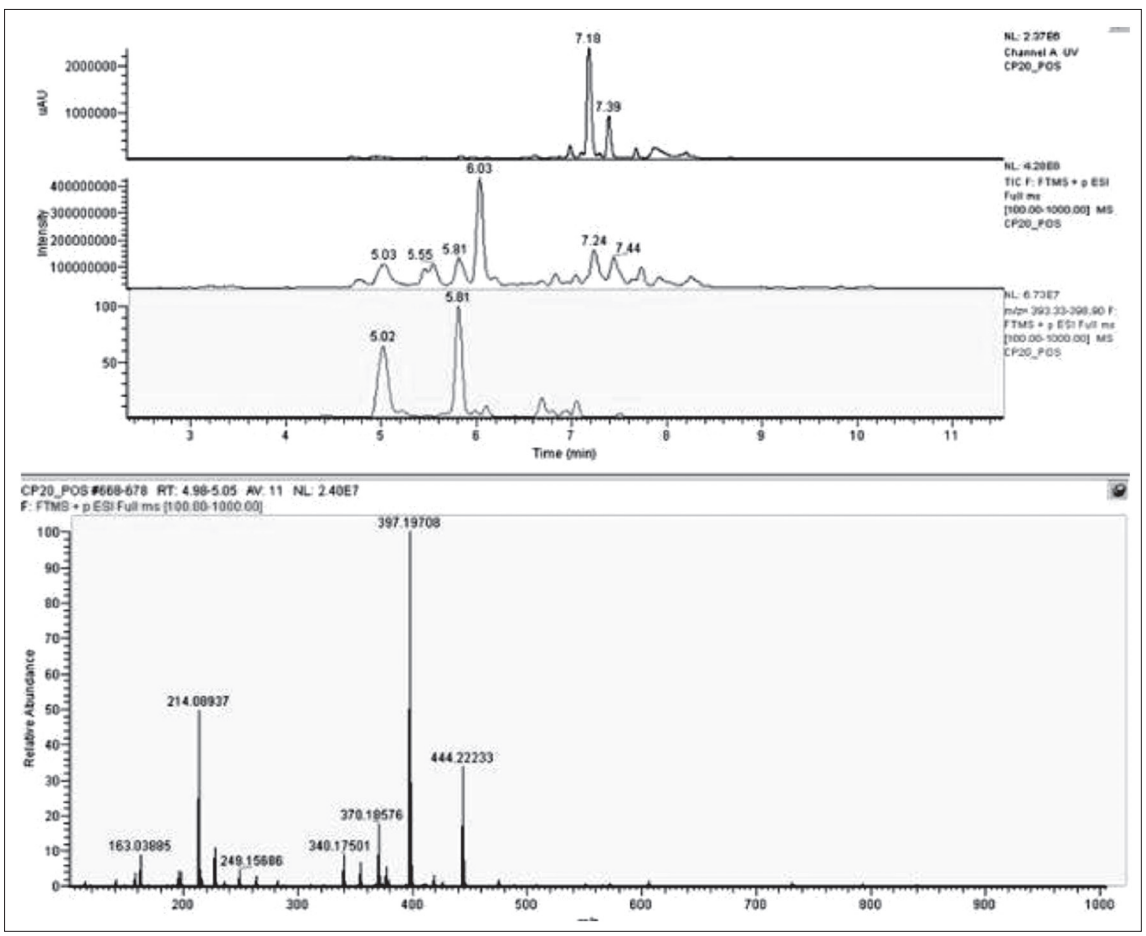
## SUPPLEMENTARY MATERIALS




**Supplementary Fig. S1.** Histopathological examination of rat liver tissues (H&E staining, 400 ×). **A** – Control group: normal hepatic architecture (hp) with well-preserved hepatocyte cords radiating around the central vein (cv) and patent sinusoidal spaces (sn). **B** – $CCl_4$-intoxicated group: severe histoarchitectural disruption, including centrilobular necrosis, extensive hepatocellular vacuolation, dilated and congested sinusoids (ds), severe degree of haemorrhage (hr), irregularly dilated central vein (dcv), and marked inflammatory cell infiltration (inf). **C** – $CCl_4$ + BECP 100 mg/kg: partial recovery with mild-to-moderate hepatocellular degeneration, moderate necrotic foci, and reduced sinusoidal dilation. **D** – $CCl_4$ + BECP 200 mg/kg: clear attenuation of hepatic damage, with only mild hepatocellular degeneration and markedly reduced necrotic areas. **E** – $CCl_4$ + BECP 400 mg/kg: near-normal hepatic architecture with minimal degeneration and no overt necrosis. **F** – $CCl_4$ + vitamin E (standard treatment): well-preserved hepatic architecture with regular hepatocyte cords and no significant pathological alterations.




**Supplementary Fig. S2.** Effect of BECP on histological scoring. All values are presented as mean±SD (n=6). One-way ANOVA, Tukey's post hoc test. *P<0.05, **P<0.01, ***P<0.001 vs. healthy control (Group I); #P<0.05, ##P<0.01, ###P<0.001 vs. $CCl_4$-intoxicated control (Group II).




**Supplementary Fig. S3.** LC–MS chromatogram of the n-butanol extract of *Centaurea pungens* showing the separation of several putative polyphenolic constituents, with well-defined peaks detected at distinct retention times corresponding to individual compounds.