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ORIGINAL ARTICLE

Hepatoprotective Effects of Nonpolar Extracts from Inflorescences of Thistles *Cirsium vulgare* and *Cirsium ehrenbergii* on Acute Liver Damage in Rat

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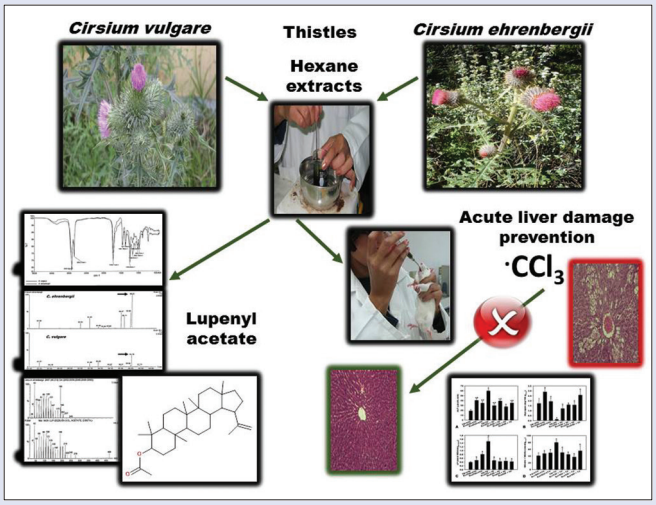
ABSTRACT

Background: Drugs for the treatment of liver diseases are scarce and not effective enough. Some species of the genus *Cirsium* possess hepatoprotective activity. There are no studies on the hepatoprotective effects of nonpolar extracts from inflorescences of thistles *Cirsium vulgare* and *Cirsium ehrenbergii*, and there are few reports on their chemical composition. **Objective:** The aim is to obtain the hexane extract from inflorescences of both thistles and to identify preliminarily their main chemical component, and to evaluate the hepatoprotective properties of the extracts. **Materials and Methods:** Hexane extracts were obtained using a Soxhlet apparatus. The chemical composition was analyzed using infrared spectra and gas chromatography-mass spectrometry. Two doses (250 and 500 mg/kg, p.o.) of both extracts were administered to assess their hepatoprotective effect on acute carbon tetrachloride (TC)-induced liver damage in rats using biochemical markers of necrosis, cholestasis, functionality, oxidative stress, and histological analysis. **Results:** Extracts were shown to have a very similar chemical profile. Their major constituent seems to be lupeol acetate. The two doses of both extracts demonstrated comparable hepatoprotective properties because they significantly diminished all the liver injury indicators ($P < 0.05$) and were corroborated using histopathology. **Conclusion:** This is the first study on the hepatoprotective effects of nonpolar extracts from inflorescences of thistles *C. vulgare* and *C. ehrenbergii*. Hexane extracts administration totally prevented the acute TC-induced liver damage. The preliminary chemical analysis strongly suggests the lupeol acetate as their major constituent. Lupeol and its derivatives have been previously reported as antiinflammatory and hepatoprotective agents.

Key words: *Cirsium ehrenbergii, Cirsium vulgare*, hepatoprotective, liver damage, lupeol acetate

SUMMARY

- Hexane extracts of both thistles kept normal liver functionality and glycogen store in carbon tetrachloride-induced acute liver damage
- Hexane extracts of both thistles showed anti-necrotic and anti-cholestatic effects, also diminished the lipid peroxidation and nitric oxide levels on the carbon tetrachloride-induced acute liver damage
- The two doses of hexane extracts administered (250 and 500 mg/kg) prevented the liver injury in a very similar extent
- Both nonpolar extracts are chemically very similar and their main compound seems to be lupeol acetate.



Abbreviations used: TC: Carbon tetrachloride; FT-IR: Fourier transform Infrared spectroscopy; GC-MS: Gas chromatography – Mass spectrometry; V: Vehicle; E: Extract; Ecv: Extract of *Cirsium vulgare*; Ece: Extract of *Cirsium ehrenbergii*; AP: Alkaline phosphatase; GGTP: γ-Glutamyl transpeptidase; ALT: Alanine aminotransferase; DB: Direct bilirubin; TB: Total bilirubin; LP: Lipid peroxidation; MDA: Malondialdehyde; NO: Nitric oxide; TNF-α: Tumor necrosis factor-α.

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INTRODUCTION

Liver diseases are one of the leading causes of death; they are caused by viral infections, chronic alcoholism, nonalcoholic fatty liver disease, and autoimmune illnesses, which may chronically be precursors of cirrhosis. Cirrhosis is the late-stage liver disease, which can proceed to hepatocellular carcinoma.[1,2] Pharmacological treatment options for liver diseases and cirrhosis are limited, expensive, and not quite effective.[1,3,4] The use of medicinal herbs and plant-derived compounds is growing worldwide.[5,6]

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Herbal medicines for liver diseases therapy require pharmacological valuation of their efficacy as novel hepatoprotective phytodrugs.[7,8]

Genus *Cirsium* Mill. (*Asteraceae* or Compositae) comprises of flowering plants commonly known as thistles, which are despised just as weedy invasive herbs. Genus *Cirsium* is widely distributed and original from Europe, Asia, and Africa, also it was introduced in America and Australia.[9-14] Diverse pharmacological effects have been described for species of *Cirsium*, anti-microbial, anti-cancer, anti-oxidant,[15-20] anti-diabetic,[21,22] hypolipidemic,[23,24] pro-cognitive, neuroprotective,[25,26] analgesic, and anti-inflammatory activities.[27,28] There are reports about the hepatoprotective effects of some species of *Cirsium* from Asia evaluated *in vitro* and *in vivo*,[29-32] as well as of their isolated major constituents.[33,34] Nevertheless, only polar extracts (water, methanol, and ethanol as solvents) from the thistles were used in all these studies to obtain polar compounds, such as flavonoids, which are very well-known as anti-oxidant and anti-inflammatory agents, like those of silymarin from *Silybum marianum* that has proven effects on chronic liver diseases.[35] Thus, to assess the hepatoprotective activity of nonpolar extracts from thistles should be of interest because some authors have pointed out that the bioactive molecules remain in nonpolar extracts rather than in the polar ones from *Cirsium*.[36-38] In addition, there are no studies on the hepatoprotective effects of *Cirsium vulgare* (Savi) Ten. and *C. ehrenbergii* Sch. Bip., besides there are few reports on their chemical composition.[37,39-41]

Inflorescences and leaves of *C. vulgare* are used in Polish folk medicine as diuretic, astringent, anti-inflammatory, and anxiolytic agents.[39,40,42] Inflorescences and roots of *C. ehrenbergii* are used in Mexico for the treatment of gastritis, diabetes, hemorrhoids, cough, and vaginal bleeding.[37,43,44] Therefore, the aim of this study was to obtain the hexane extracts from inflorescences of both thistles, to identify preliminarily their main chemical component, and to evaluate the hepatoprotective properties of these extracts.

MATERIALS AND METHODS

Chemicals

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Solvents and the carbon tetrachloride (CCl_4, TC) were analytical grade and purchased from J.T. Baker (Phillipsburg, NJ, USA).

Plant materials and preparation of nonpolar extracts

C. vulgare inflorescences were collected from municipality of "Mineral de la Reforma" (20° 09' 00 N latitude; 98° 26' 00 W longitude) and *C. ehrenbergii* inflorescences were collected from municipality of "Mineral del Chico" (20° 12' 11" N latitude; 98° 44' 52" W longitude), in Hidalgo's State, Mexico, during the summer season. The identity of the plants was taxonomically confirmed and voucher specimens (15598IMSSM and 15597IMSSM, respectively) were deposited at the "Herbario del Centro Médico Siglo XXI" of the "Instituto Mexicano del Seguro Social", Mexico. Inflorescences were dried and milled. Hexane extracts were carried out by exhaustive extraction in a Soxhlet apparatus. The solvent was removed by total evaporation in vacuum at 40°C, and the extracts were obtained as dark green honeys with characteristic odors. The extraction yield was 2.05% and 7.19% for *C. vulgare* and *C. ehrenbergii*, correspondingly.

Preliminary chemical analysis of the nonpolar extracts by infrared spectra and gas chromatography-mass spectrometry

To compare the nonpolar compounds, present in both hexane extracts, an initial characterization of their functional groups was performed using Fourier transform infrared spectra (FT-IR, Perkin-Elmer Spectrum version 10.4.00, Norwalk, CT, USA). The characterization was carried out by recording the transmittance (%T) of the extracts in the frequency range of 4000 cm^{-1}–400 cm^{-1}. The samples were pelletized with KBr for the infrared (IR) spectroscopy.

Gas chromatography-mass spectrometry (GC-MS) analyses of the hexane extract samples were performed using an AutoSystem XL-GC equipped with a EN5MS column (30 m × 0.25 mm ID × 0.25 μm film thickness) and coupled to a selective quadrupole TurboMass-MS detector with an electron impact (EI) ionization system at 70 eV (Perkin-Elmer, Norwalk, CT, USA). The diluted extract samples were manually injected (1.0 μL) and transferred in splitless mode. The flow rate of helium as the carrier was 1.0 mL/min at 8 psi. The oven temperature started at 60°C and was gradually increased up to 330°C at a rate of 8°C/min, where it was maintained for 30 min. The mass spectra were set and recorded in scan mode from 15 to 600 m/z.

The preliminary identification of only the major detected compound (maximum peak height for that compound, %) from each hexane extract gas chromatogram was accomplished by comparing its retention time and mass spectra with those available from the computerized spectral database of National Institute of Standard and Technology (NIST MS Search 1.7, Gaithersburg, MD, USA) and from published literature.

Animals

Male Wistar rats weighing approximately 200–250 g were housed in standard plastic cages at a temperature of 22°C–24°C, under a 12 h light–dark cycle. They had free access to food (standard Purina chow diet, USA) and purified water. All the animals received humane care according to the Institution's guidelines, the Mexican Official Norm (NOM-062-ZOO-999) for the production, care and use of laboratory animals, and the criteria outlined in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health 1985).

Acute tetrachloride-induced liver damage *in vivo*: Experimental groups, treatment, and doses

Rats were divided into eight groups (n = 7). The first group was the normal control group (vehicle for extracts + vehicle for TC [VE + VTC]) that received three p.o. doses (12 h in between each dose) of 1 mL of olive oil as the VE; 2 h after the second administration of VE, the rats were orally given 1 mL of mineral oil as the vehicle for TC (VTC). Remaining groups underwent the same administration schedule. The second (Ecv500 + VTC) and third (Ece500 + VTC) groups received a dose of 500 mg/kg of the *C. vulgare* and *C. ehrenbergii* extracts, respectively, thrice orally dissolved in their vehicle and received the VTC p.o. too; both were control groups intended to show any adverse effect of the thistles *per SE* on normal rats. The fourth group was the damaged control (VE + TC), wherein rats received the VE thrice orally, and a single dose of 4 g/kg TC dissolved in its vehicle (1:1, v/v). The fifth (Ecv250 + TC) and sixth (Ecv500 + TC) groups were administered with their respective doses of 250 and 500 mg/kg of *C. vulgare* extract and were injured by TC. Finally, seventh (Ece250 + TC) and eighth (Ece500 + TC) groups were administered their respective doses of 250 and 500 mg/kg of *C. ehrenbergii* extract and were damaged by TC.[45]

The two assayed extract doses are considered to be middle doses and are based on previous reports wherein the hepatoprotective effects of *Cirsium* species were assessed in rats and mice.[29,30,34] Animals were sacrificed by exsanguination under light ether anesthesia 24 h after TC administration; blood sample was collected by cardiac puncture using a syringe containing sodium heparin as an anticoagulant. The liver was rapidly removed and rinsed in saline. Samples were either kept on ice for immediate use or frozen at −70°C until analyzed.

Plasma enzyme activities and bilirubin determinations

Plasma was obtained for the determination of the canalicular membrane enzyme activities of cholestasis markers, such as alkaline phosphatase (AP) and γ-glutamyl transpeptidase (GGTP), and for the cytosolic activity of the necrosis indicator alanine aminotransferase (ALT) as well as for the quantification of the cholestasis and liver functionality marker direct (DB) and total (TB) bilirubin concentration (TECO Diagnostics kit, CA, USA).[46,47]

Glycogen determination

Small liver pieces (0.5 g) were separated for glycogen measurement using anthrone-sulfuric acid reagent.[48]

Assessment of lipid peroxidation

The extent of lipid peroxidation (LP) was estimated in liver homogenates by measuring malondialdehyde (MDA) formation using the thiobarbituric acid method.[49] Protein was determined according to Bradford using bovine serum albumin as a standard.[50]

Hepatic nitric oxide determination

Hepatic nitric oxide (NO) amounts were determined from liver extracts obtained as previously reported[46] using a commercial nitrate/nitrite enzyme colorimetric assay kit (Cayman Chemical Co., MI, USA). Hepatic NO amounts are expressed as nitrite and nitrate (NO^{2-} + NO^{3-}) nmol/g of wet tissue.

Histology

Liver samples were taken and fixed with 10% formaldehyde in phosphate-buffered-saline for 24 h. They were washed with tap water, dehydrated in alcohols and embedded in paraffin. Sections of 6–7 μm were mounted on glass slides covered with silane, previous elimination of paraffin, and were used for hematoxylin/eosin staining for histological examinations using light microscopy.[47]

Statistical analysis

An ANOVA with the Student–Newman–Keuls test was used to compare groups. The resulting data are expressed as the means ± standard error of the mean and were analyzed using Sigma Stat software version 3.1 (Systat Software Inc., San Jose, CA, USA). The difference was considered statistically significant when $P < 0.05$.

RESULTS

Hexane extracts of both thistles are very similar and share the same major constituent

The initial characterization of the nonpolar major secondary metabolite present in the hexane extracts of the thistles was achieved by FT-IR spectra [Figure 1a]. The spectra of *C. vulgare* and *C. ehrenbergii* were overlaid and show very similar patterns of characteristic absorption bands of functional groups assigned as follows, IR ν_{max} (KBr): 2923 and 2853 (C-H, alkane), 1729 (C = O, ester carbonyl), 1462 and 1378 (-$CH(CH_3)_2$, dimethyl groups), 1245 (C-O, ester), 1168, 1097, 1025, 980 and 881 (-CH = CH_2), 707 cm^{-1}. GC chromatograms of the hexane extracts evidenced that they share at least eight compounds proportionally in a similar abundance; however, the same major constituent (highest peak) was present in both extracts at the average retention time of 40.19 min [Figure 1b]. Just the fragmentation pattern of the main compound from *C. ehrenbergii* is presented [Figure 1c], EI-MS (m/z): 249, 207, 203, 190, 189 (90%), 175, 147, 121 (57%), 109 (60%), 95 (65%), 81 (57%), 69, 55 (50%), 43 (100%), and 41, 28. That fragmentation pattern was compared using the NIST database which found a high match with the lupeol acetate fragmentation pattern.

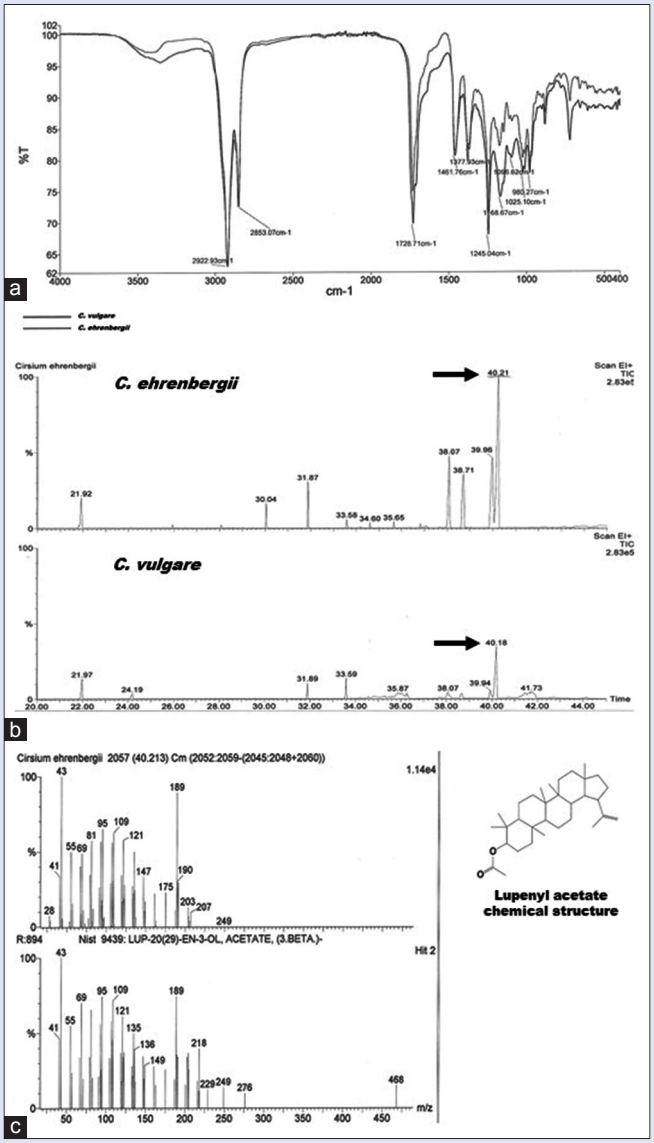

Figure 1: Preliminary chemical analysis of hexane extracts of *Cirsium vulgare* and *Cirsium ehrenbergii*. (a) Overlaid Fourier transform infrared spectra; (b) gas chromatography chromatograms, major constituent average retention time 40.19 min; (c) fragmentation pattern of the main compound compared to lupenol (lupenyl) acetate

Effect of nonpolar extracts from *Cirsium vulgare* and *Cirsium ehrenbergii* on acute tetrachloride-induced liver damage *in vivo*

Figure 2 depicts the plasma enzyme activities of AP and GGTP as well as DB and TB concentrations. Due to TC-provoked injury, the AP activity [Figure 2a] was increased almost two-fold in the damaged group (VE + TC) when compared with the normal control group (VE + VTC). Administration of the hexane extracts did not elevate that marker *per se* even using 500 mg/kg. However, the treatment with the two doses of both thistles decreased the enzyme activity during the liver injury compared with VE + CT, although only the doses of *C. ehrenbergii* diminished it significantly ($P < 0.05$) in a dose-dependent manner, which shows an anticholestatic effect.

GGTP enzyme activity [Figure 2b] was increased 5-fold by TC administration ($P < 0.05$) with respect to that in VE + VTC control

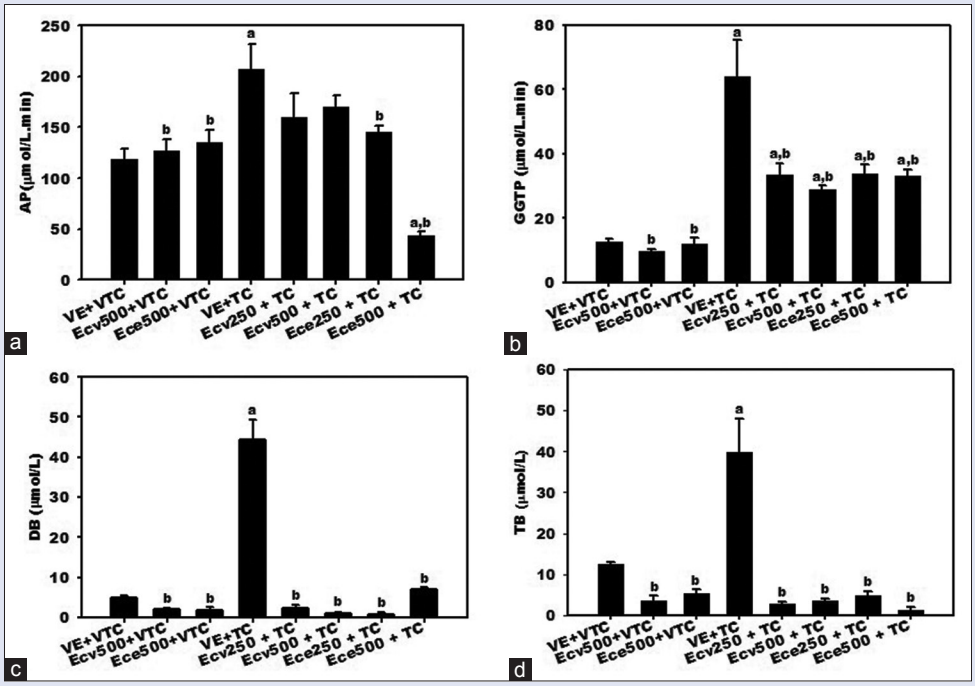

Figure 2: Plasma cholestasis markers: (a) AP and (b) GGTP activities; (c) DB and (d) TB concentrations from rats treated with vehicles, TC, extracts (250 and 500 mg/kg) or combined. (a) Different versus VE + VTC, (b) different versus VE + TC, $P < 0.05$. AP: Alkaline phosphatase; GGTP: γ-glutamyl transpeptidase; DB: Direct bilirubin; TB: Total bilirubin; TC: Tetrachloride; VE: Vehicle for extract; VTC: vehicle for tetrachloride

group; hexane extracts administration did not modify the normal levels. Regarding the TC-injured groups treated with the respective doses of both extracts, their GGTP activity increases were lessened in a 50%; besides, no differences were found among the treated groups due to either the doses or the thistle as anticholestatic agents.

DB and TB concentrations [Figure 2c and d] showed a very similar pattern because TC-induced liver damage increased these two bilirubins in a statistically significant way; both bilirubins were diminished in a small degree in extract control groups. In contrast, the two doses of both extracts completely lowered the DB and TB levels in damaged groups ($P < 0.05$).

Figure 3 shows the markers of liver injury and oxidative stress: ALT, glycogen, LP, and NO. ALT is a cytoplasmic enzyme marker of necrosis; its activity [Figure 3a] was significantly augmented by the control high doses of *C. vulgare* and *C. ehrenbergii* (Ecv500 + VTC and Ece500 + VTC groups). As expected, TC-induced a notable increment in ALT activity ($P < 0.05$) while treatments with both doses of the two thistles lowered such elevation of activity, indicating their anti-necrotic properties; particularly, the low dose (250 mg/kg) of both extracts had a better effect than the high dose.

The control groups of hexane extracts showed normal levels of glycogen with respect to the normal control group [Figure 3b] while the TC-damaged group exhibited depleted glycogen content ($P < 0.05$). Treatment with both doses of these extracts completely prevented the depletion of glycogen in a dose-dependent manner.

LP represents the oxidative stress of hepatocellular membranes. This indicator [Figure 3c] was augmented several-fold through TC administration in contrast with the VE + VTC and the control groups of extracts, which increased the normal level of LP *per se* without statistical significance. In contrast, the two doses of both thistles abolished the TC-induced oxidative injury by lowering the LP to normal levels.

NO (nitrate + nitrite) represents oxidative stress and is an inflammation mediator. Normal liver NO amount [Figure 3d] was augmented by TC administration ($P < 0.05$) while hexane extracts administration did not modify it. In addition, treatment with the two doses of either *C. vulgare* or *C. ehrenbergii* extracts completely prevented the TC-induced hepatic NO elevation in a similar way.

Liver damage was also evaluated by histopathology through hematoxylin/eosin staining, staining nuclei a black/dark blue and parenchymal hepatocyte cytoplasm a pink/magenta [Figure 4]. Normal hepatic cell population and tissue homogeneity are shown in a representative liver sample from the VE + VTC normal control group [Figure 4a]. In contrast, type TC-injured liver sample showed important damage zones, diffuse ballooning necrosis, pyknotic nuclei, and high hepatic steatosis with hepatocyte vacuolization and inflammation [Figure 4b]. In control groups treated with the high dose of thistle extracts [Figure 4c and d] the liver tissues are apparently healthy, but only some few areas show a very slight inflammatory reaction. When 250 mg/kg doses of both thistles were administered concomitantly with TC, the livers presented minor diffuse inflammation and vascular congestion [Figure 4e and f]; although, the hepatic tissues are considered healthy. Liver samples from animals damaged by TC and treated with 500 mg/kg doses of *C. vulgare* or *C. ehrenbergii* extracts resulted in a similar case; however, hepatic tissues also demonstrated some focal swollen hepatocytes and scarce micro-vacuolization accompanied by neutrophil infiltration and slight perivascular inflammation [Figure 4g and h].

DISCUSSION

Phytochemical studies have reported that *C. vulgare* contains polar compounds such as flavonoids and phenolic compounds,[12,40] found also in other species of genus *Cirsium*.[51,52] Concerning compounds of low-polarity of nonpolar extracts from *C. vulgare*, its essential oil contains volatile terpenes and fatty acids,[39] found also in seeds of this plant,[53] and in other *Cirsium* species.[36,54,55] This thistle also contains sterols and triterpenes such as β-sitosterol, stigmasterol, and

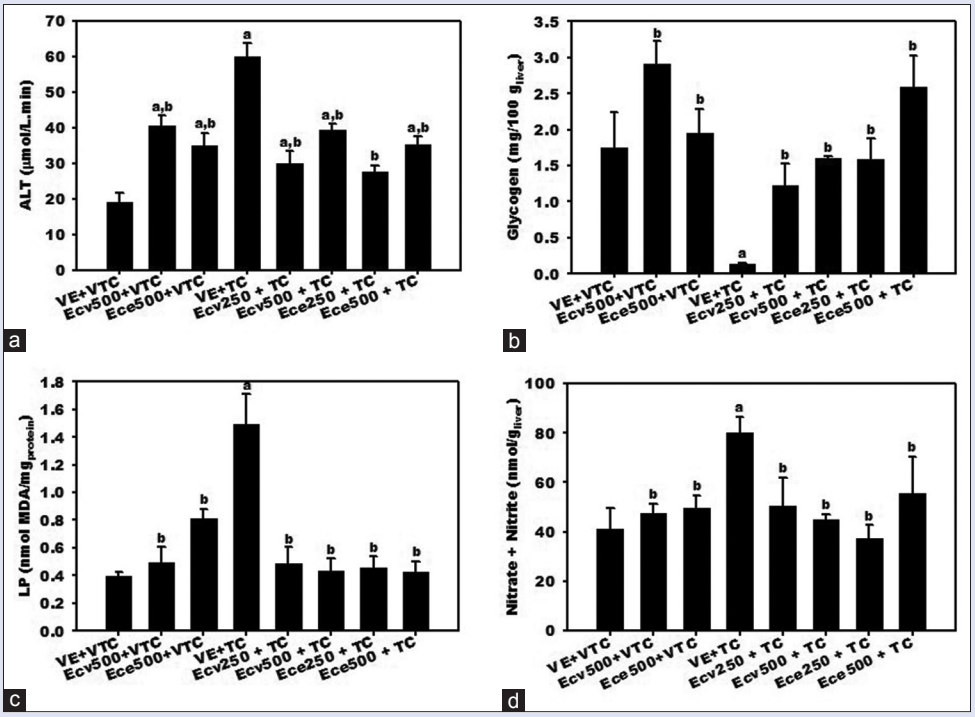

Figure 3: Necrosis, functionality, and oxidative stress markers: (a) plasma ALT activity; (b) hepatic glycogen, (c) LP, and (d) NO from rats treated with vehicles, TC, extracts (250 and 500 mg/kg) or combined. (a) Different versus VE + VTC, (b) different versus VE + TC, $P < 0.05$. TC: Tetrachloride; VE: Vehicle for extract; VTC: vehicle for tetrachloride; LP: Lipid peroxidation; NO: Nitric oxide; ALT: Alanine aminotransferase

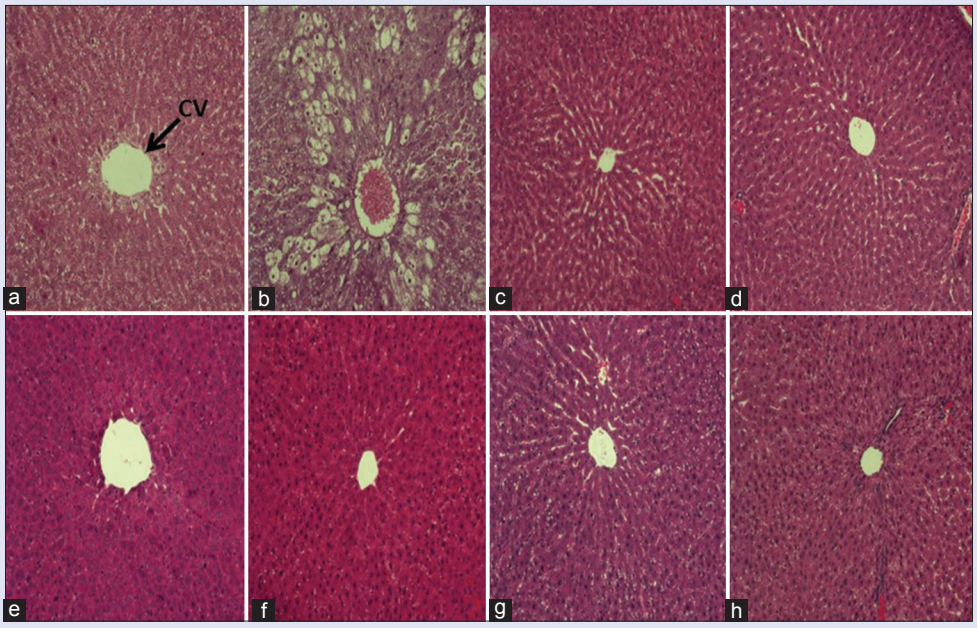

Figure 4: Hematoxylin/eosin staining of representative liver sections from: (a) normal VE + VTC; (b) damaged VE + TC; (c) Ecv500 + VTC; (d) Ece500 + VTC; (e) Ecv250 + TC; (f) Ece250 + TC; (g) Ecv500 + TC; (h) Ece500 + TC. Extract doses (250 and 500 mg/kg). Central vein, (×10). TC: Tetrachloride; VE: Vehicle for extract; VTC: vehicle for tetrachloride

aliphatic aldehydes;[12,39] in fact, most of them have been described in hexane extracts from other *Cirsium* species.[55-57] However, the precise constituents of *C. ehrenbergii* inflorescences have not yet been reported.[37] In this study, the preliminary chemical analysis on both hexane extracts strongly suggested lupeol acetate as the major constituent. Indeed, lupeol acetate, lupeol, and lupenone have been found in *C. setosum* and *C. japonicum*.[31,58] Besides, the IR spectra and the pattern of fragmentation of GC-MS of this study are in agreement with several reported spectral analyses of lupeol acetate.[59-62] Furthermore, this family of lupeol derivatives have recognized hepatoprotective activity in diverse models

of liver damage.[31,63,64] Lupeol acetate has been reported to diminish the TC-induced liver injury markers, ALT, LP as MDA, NO, and reduced the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α).[65]

Cholestasis is the mechanical or functional stoppage of bile flow in the intrahepatic or extrahepatic bile ducts, with bile components passing into the blood.[66] The cholestasis indicators AP and GGTP were increased by TC as well as the necrosis marker ALT. TC elevated plasma DB and TB as sign of impaired excretory hepatic functions by cholestasis.[66] However, the administration of the extracts showed anti-cholestatic and anti-necrotic effects on all the biochemical markers. Although ALT activity was significantly increased by high dose of extracts, such dose-dependent elevation was neither synergized with TC administration nor replicated in other liver damage indicator; perhaps, some metabolites augment its enzyme activity *per se* These results are in agreement with various reports wherein the administration of diverse *Cirsium* extracts prevented the elevation of ALT and AP in liver damage models, although polar extracts rich in flavonoids were used in those studies.[29,30,33] Constituents of the hexane extracts may have similar cholagogue effects, perhaps through activating canalicular membrane transporters or by inducing the bilirubin conjugation to improve the excretion of bile products. Anti-cholestatic and anti-necrotic properties may also be related to the immunomodulatory effects of lupeol derivatives.[63-65]

Hepatic glycogen is the main source of energy in the body and is indicative of metabolism and functionality. NO is either a mediator of liver injury or a protective mechanism and is induced by pro-inflammatory cytokines such as TNF-α.[46,67] Glycogen synthesis and glycogenolysis are affected by TNF-α and NO; therefore, this marker is very sensitive to liver stress. The treatment with thistle extracts completely prevented the TC-induced depletion of glycogen, possibly because hepatic NO was inhibited too. These activities may be due to the lupeol acetate content;[65] besides, some lupeol derivatives have activities as NO and pro-inflammatory cytokine inhibitors as well as glucose uptake stimulatory promoters,[68,69] because glycogen repletion is prompted by an increased uptake of glucose in the liver.[70]

Several hepatotoxic chemicals cause liver damage through free radicals that provoke oxidative stress.[47,71] Hexane extracts prevented the TC-induced LP; this anti-oxidant effect may be also associated with the lupeol derivatives content.[63,65] These results suggest that despite the nonpolar constituents of hexane extracts are not free radical scavengers, as flavonoids of *Cirsium* polar extracts,[33,34] a different anti-oxidant pathway may be implicated at nuclear receptors level due to the biological activities of sterols and lipids from nonpolar extracts.[72] The hepatoprotective properties demonstrated by the biochemical markers for the thistle extracts were corroborated through histopathological analysis, wherein hepatic parenchymal damage was prevented.

CONCLUSION

This is the first study on the hepatoprotective effects of nonpolar extracts from inflorescences of thistles *C. vulgare* and *C. ehrenbergii*. The preliminary chemical analysis strongly suggests the lupeol acetate as their major constituent. The hexane extracts of these thistles deserve further chemical and pharmacological studies as hepatoprotective agents.

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Conflicts of interest

There are no conflicts of interest.

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