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The hepatoprotective effects of the polyphenol-enriched n-butanol fraction of *Cnicus benedictus* against carbon tetrachloride-induced liver fibrosis in rats: *In vivo* study

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ABSTRACT

Liver fibrosis is a continuous wound-healing response to chronic injury caused by various chemical, virus, and pathological disorders; the lack of approved drugs or methods to reverse or prevent liver fibrosis makes it an interesting area of research. This study investigates the potential hepatoprotective effects of the phenolic extract of *Cnicus benedictus* in rat's module of liver fibrosis. Liver fibrosis was induced by intraperitoneal injection of carbon tetrachloride (CCl_4) for six consecutive weeks; the butanol fraction of *Cnicus* and silymarin was administered orally concurrently with CCl_4. After six weeks, all animals were euthanized. Rat liver tissue levels of malondialdehyde (MDA) and glutathione (GSH) were measured, and serum liver enzymes and protein were measured using the ELISA technique. Histopathological study and immunohistochemistry of liver tissue for transforming growth factor (TGF-β1), alpha-smooth muscle actin (α-SMA), and hydroxyproline were assessed. In HPLC analysis, Cnicus extract showed several components, including quercetin, gallic acid, rutin, kaempferol, silibinin, and apigenin. Treatment with *Cnicus* butanol extract reduces serum ALT, AST, bilirubin, and albumin levels compared to induction. Additionally, *Cnicus* extract increases liver GSH levels and decreases liver MDA levels compared to induction. Liver tissue of TGF-β1, α-SMA, and hydroxyproline expression was downregulated in rats receiving *Cnicus* extract. Liver tissue histopathology showed improvement in its features compared to the induction group. In conclusion, oral administration of the polyphenol-enriched n-butanol fraction of Cnicus benedictus showed a protective effect on liver fibrosis caused by CCl4, possibly through antioxidant and anti-inflammatory mechanisms.

1. Introduction

Liver fibrosis is a pathophysiological outcome of continuous wound-healing response to chronic injury from repeated extracellular matrix (ECM) protein accumulation. Fibrosis is a dynamic process that involves intercommunication between hepatic stellate cells (HSCs), parenchymal cells (hepatocytes), sinusoidal endothelial cells, and both infiltrating and resident immune cells [1]. This progressive and continuous displacement of hepatocytes with ECM and fibrous scars could ultimately end in cirrhosis [2]. The occurrence rate and mortality of hepatic fibrosis are high throughout the world. Liver fibrosis and the following cirrhosis represent serious medical concerns. Yet, there is still a lack of approved drugs or methods to reverse or prevent fibrosis, except for liver transplantation or removal of the underlying cause of injury. Therefore, effective hepatic antifibrotic drugs are needed urgently [2,3]. Hydroxyproline is a non-proteinogenic amino acid produced during collagen synthesis through post-translational proline hydroxylation [4]. It provides unique amino acids of collagens, being fibrillar collagen's primary constituent of all given types of collagen [5].

Phenolic compounds (PC) are plants' most generous secondary metabolites , showing multiple biological roles; recently, they have received more attention [6]. PCs contain many subunits, such as phenolic acids, flavonoids, tannins, anthocyanins, procyanins, and lignins. The Shikimate pathway in plants synthesizes these compounds and has many important biological activities, specifically

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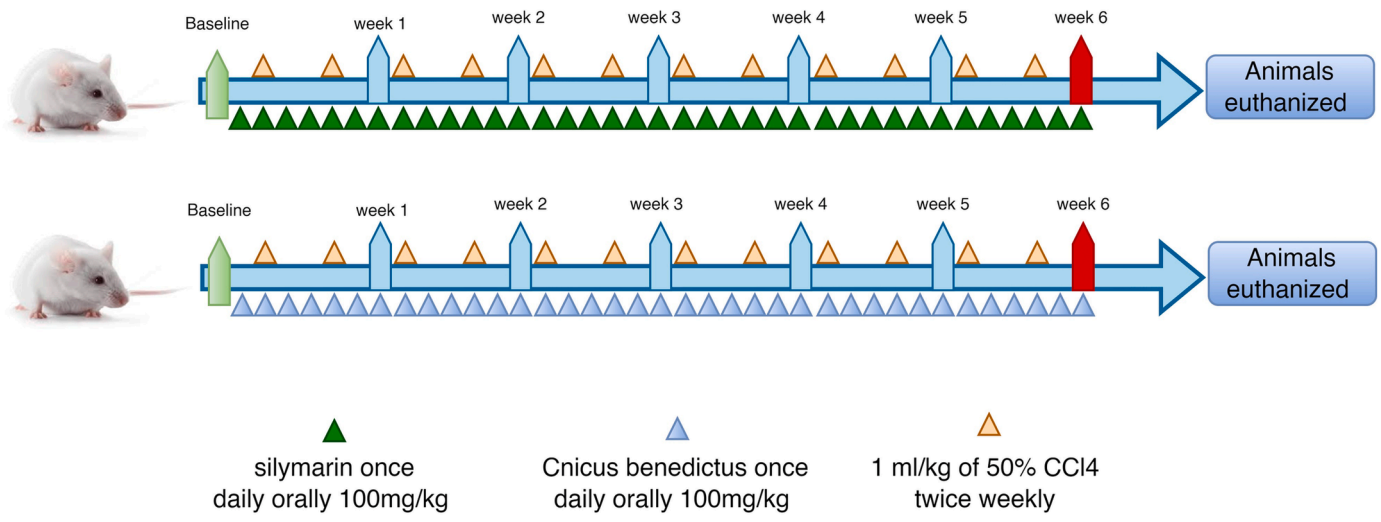
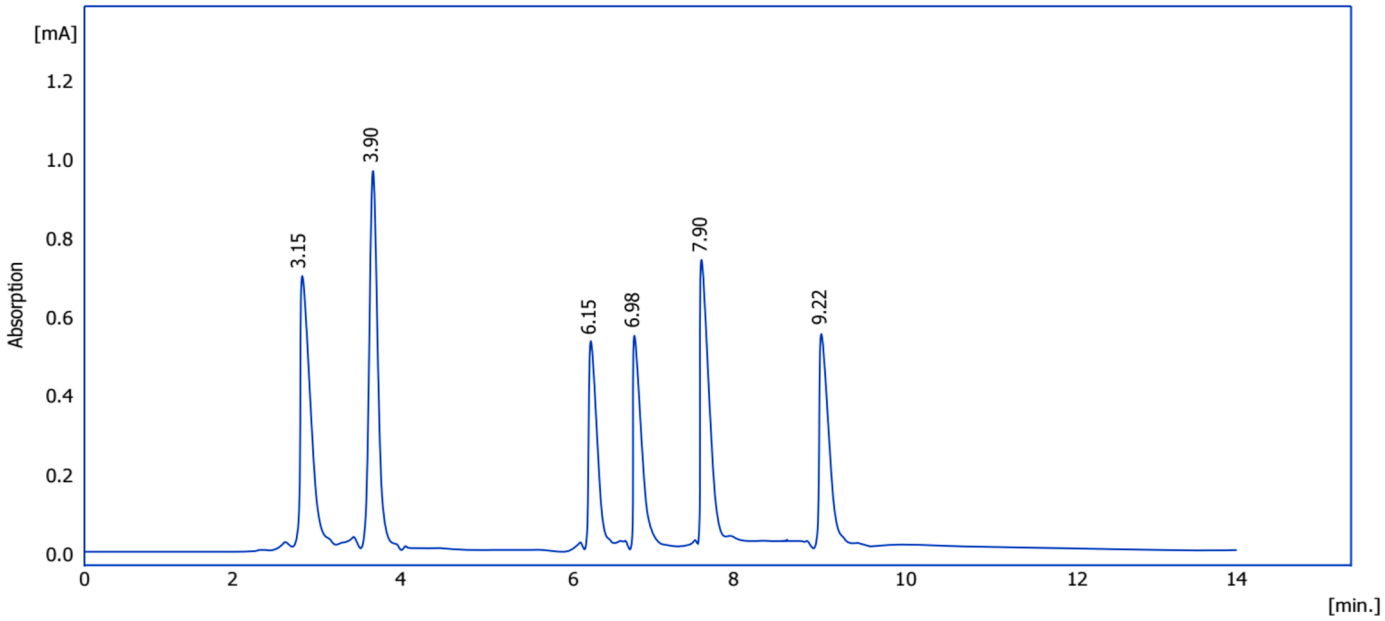


Fig. 1. Flow chart of the study.



Result chromatography Table (Uncal - F:\ sample 1)

No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	3.15	5412.56	701.25	17.00	17.00	0.10	
2	3.90	8520.00	960.25	21.00	21.00	0.15	
3	6.15	3214.25	501.23	15.00	15.00	0.08	
4	6.98	3652.41	502.33	15.00	15.00	0.08	
5	7.90	5011.98	704.65	17.00	17.00	0.10	
6	9.22	3452.16	503.11	15.00	15.00	0.08	
	Total	29263.59	3872.19	100.00	100.00		

Fig. 2. HPLC analysis of *Cnicus benedictus* leaves (no 1: quercetin, no 2: gallic acid, no 3: rutin, no 4: Kaempferol, no 5: silibinin, no 6: apigenin).

anti-inflammatory, ↱antioxidant, anti-microbial, anti-viral, and anti-tumoral effects. ↱PC are reducing, scavenging, stabilizer, chelating, surfactant, and stimulant agents; therefore, many uses can be considered [7]. PCs can treat common conditions, including ↱metabolic problems, diabetes mellitus, hypertension, infections, ↱and neurodegenerative diseases. Because of their anti-inflammatory ↱properties, they have also treated rheumatoid arthritis and inflammatory ↱bowel disease [8], skin diseases like atopic dermatitis ↱↱[9]. Furthermore, they have an antiangiogenic activity ↱that may benefit cancer treatment [10].

Recent animal studies reveal that natural sources, particularly ↱PC, may represent a new therapeutic strategy to prevent liver ↱fibrosis [11]. The effects of PC in preventing liver fibrosis may ↱manifest as a reorganization of the tissue or inflammation pathways and the ↱defense against oxidative stress [11]↱. Gradually, phenolic compounds are attracting more attention to become ↱the focus for providing a strategy for liver fibrosis treatment [12].

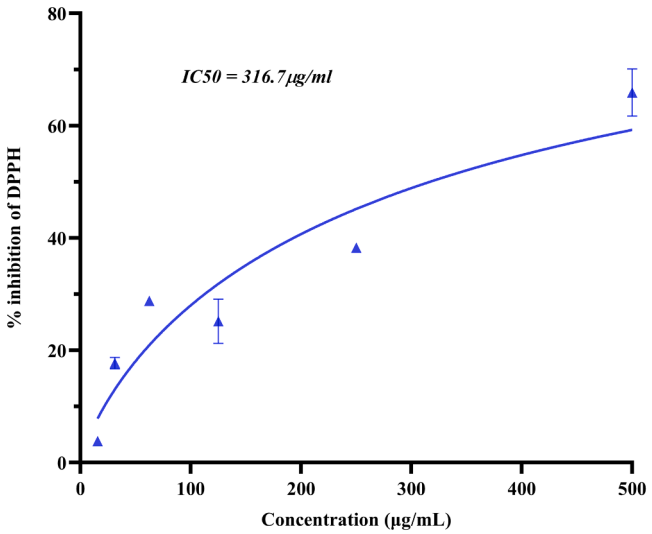


Fig. 3. antioxidant activity of Cnicus extract using DPPH assay.

Cnicus benedictus, popularly known as "blessed thistle," the sole ↱species in the genus *Cnicus*, is a thistle-like medicinal plant in the family ↱*Asteraceae*. The leathery leaves ↱of the plant are stomachic, diuretic, bitter, astringent, and diaphoretic [13]. The blessed thistle is native to the ↱Mediterranean. It was also considered native worldwide [14]. ↱Ethanolic extract of the *Cnicus benedictus* leaves contains many active ↱compounds, alkaloids, flavonoids, phenols, tannins, and terpenes ↱and has a significant inhibitory effect against some species of pathological ↱bacteria [15].

Silymarin is a phytoconstituent of a plant named *Silybum ↱marianum* (family *Asteraceae*). It is extracted and isolated from different ↱plant parts such as fruits, seeds, and flowers. Silymarin inhibits lipid ↱peroxidation because of its strong antioxidant potential for the liver ↱cells and increases hepatocyte protein synthesis, which helps them ↱regenerate in case of a diseased condition [16].↱ A range of *in vitro* and *in vivo* animal studies proved that silymarin is an ↱important standard hepatoprotectant used singly or combined with other drugs or nutrients to manage multiple liver ↱disorders [17]. Silymarin acts as a free radical ↱scavenger and modulates enzymes associated with the development of ↱cellular damage, fibrosis, and cirrhosis. These hepatoprotective effects ↱were observed in clinical studies in patients with NAFLD or NASH, including patients with cirrhosis [18].

Evaluation of the effect of *Cnicus benedictus* on liver fibrosis was not reported previously; as discussed above, it traditionally was used to improve ↱liver function and some gastrointestinal problems, and the natural extract ↱like phenolic compounds of *Cnicus benedictus*, which is from the same ↱family of *Silybum marianum* (*Asteraceae*), was proven to possess anti-inflammatory and antioxidant characteristics that may benefit in ↱protecting against fibrosis.↱ This study investigated the potential ↱hepatoprotective effects of polyphenol-enriched n-butanol fraction of leaves ↱*Cnicus benedictus* against ↱CCl_4-induced liver fibrosis in rats.↱

2. Materials and methods

2.1. Materials

All materials and chemicals were pharmaceutical grade, CCl_4 (THOMAS BAKER® PVT, India↱), silymarin (MADUAS®, Germany↱), ketamine (Rotex-Medica, Germany), xylazine (Alpha, India), ethanol (Haymankimia®, UK↱), petroleum ether (Chem lab NV®, Belgium↱), n-hexane (Chem lab NV®, Belgium↱), and butanol (THOMAS BAKER® PVT. India↱).

2.2. Plant material collection

Leaves of *Cnicus benedictus* were obtained from Erbil (11' 28.0068" N and 44° 0' 33.0012") in the north of Iraq. A certified botanist authenticated them in the College of Science, University of Baghdad, Iraq. The voucher specimen of *C. benedictus* leaves was deposited in the College of Science, University of Baghdad, under the accession numbers 44,856 and 44,858. The leaves were cleaned, dried in the shade at room temperature, and ↱then pulverized and stored for further use.↱

2.3. Method of extraction

A 650 gm of air-dried powder of the leaves is weighed and defatted with ↱petroleum ether overnight to remove chlorophyll and waxy material, then ↱extracted in Soxhlet with 90 % ethanol; the extracts are combined and dried by ↱rotary evaporator. The resulting dry extract is dissolved in 100 ml of distilled water. ↱Then, partitioned with 100 ml of n-hexane using a separatory funnel ↱three times, all the upper layers are discarded. The lower part (aqueous ↱part) is partitioned with butanol; the butanol fraction was separated from the aqueous part to obtain the butanol part. A rotary evaporator then dried the butanol fraction to obtain the final extraction yield↱, representing the dried plant's total phenolic compounds [19].

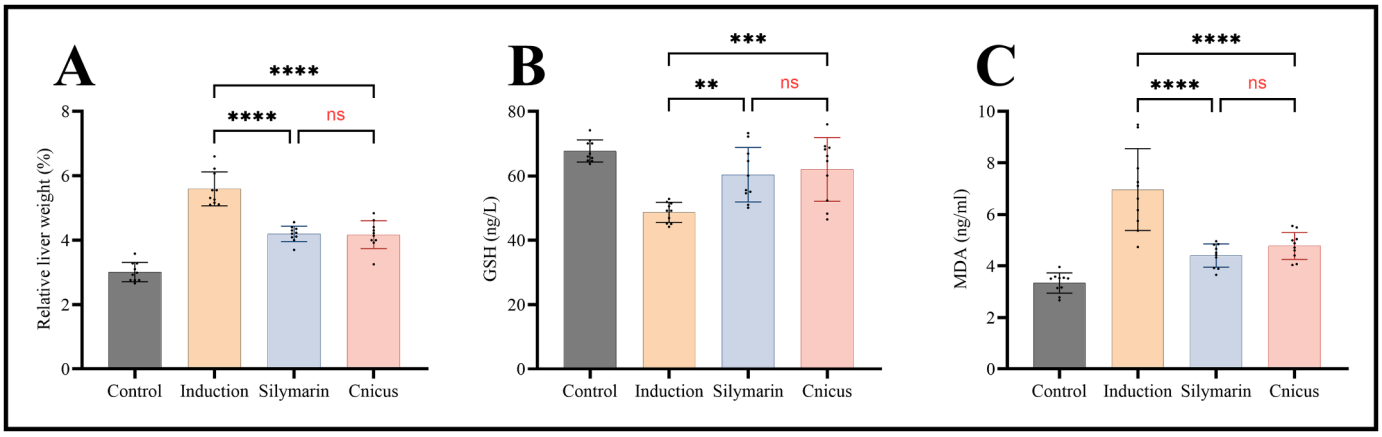


Fig. 4. Assessment of the protective effect of phenolic extract of *Cnicus benedictus* against carbon tetrachloride-induced liver fibrosis in rats on liver tissue homogenate, (B) liver glutathione levels, and (C) liver MDA levels. Data presented as mean ± standard deviation. One-way ANOVA with *post hoc* Tukey test was used.

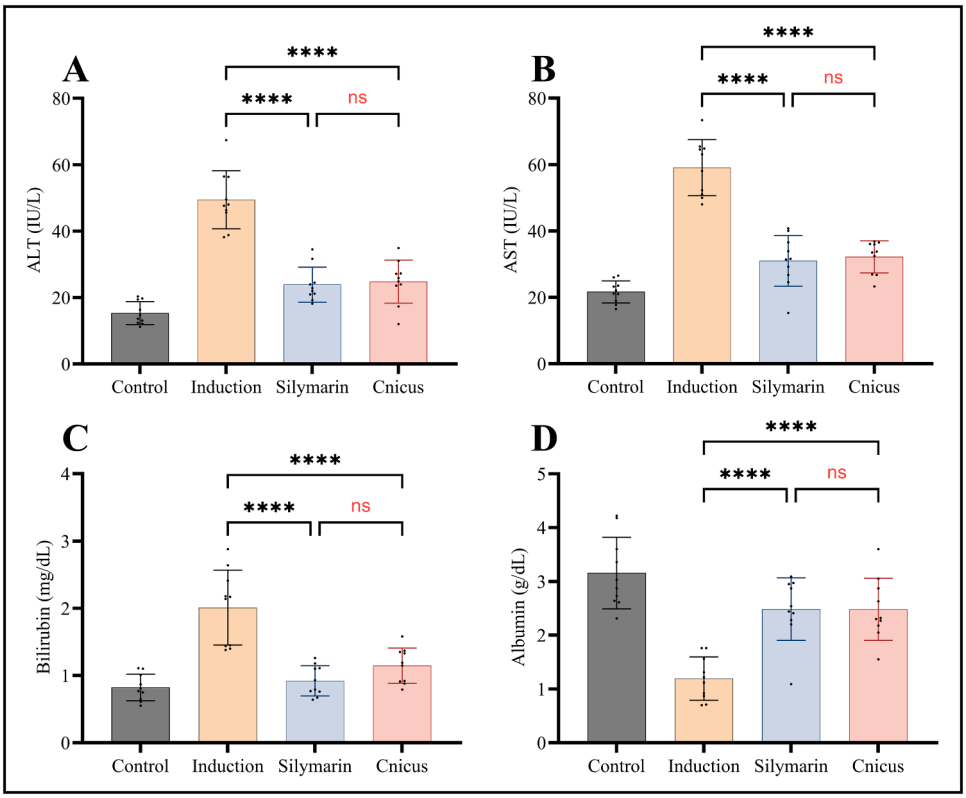


Fig. 5. Assessment of the protective effect of phenolic extract of *Cnicus benedictus* against carbon tetrachloride-induced liver fibrosis in rats on serum liver enzymes and protein. (A) serum ALT, (B) serum AST, (C) serum total bilirubin, and (D) serum albumin. Data presented as mean ± standard deviation. One-way ANOVA with *post hoc* Tukey test was used.

2.4. Identification of leaves of Cnicus benedictus Components

Samples were analyzed using the high-performance liquid chromatography HPLC model (SYKAM, Germany). The mobile phase was Methanol: deionized distilled water: formic acid (70: 25: 5), the column is C_{18}-ODS (25 cm × 4.6 mm), and the detector UV – 280 nm at a flow rate of 1.0 ml/min [20].

2.5. Determination of antioxidant capacity by DPPH (2,2 diphenyl-1-picrylhydrazyl) method

Cnicus extract has the antioxidant ability to neutralize free radicals; DPPH assessment is an in vitro assay utilized to assess such capacity. 0.1 ml of the extract at various concentrations (from 500 to 15.625 µg) were mixed with 200 µl of 0.1 mM DPPH dissolved in methanol; each concentration tested in triplicate, and the absorbance was measured at 519 nm after 30 min of reaction [21,22]. The negative control consisted of 100 µl of methanol and 200 µl of DPPH [23].

2.6. Animals housing and ethical considerations

Forty apparently healthy male albino rats, weighing between ↱↱200–250 gm, ranging in age between 8 and 9 ↱weeks,↱ were utilized in the study; the study started in March 2023 and was completed in September 2023. The study was approved by the Research Ethical Committee of the College of Medicine Al-Nahrain University (approval number: UNCOMIRB20240626, approval date: 8th January 2023).

The animal house of the Veterinary Medicine College, Tikrit University supplied these animals. The animals are kept in plastic cages at the animal ↱house of the College of Pharmacy, Al-Nahrain University. These animals were kept under standard ↱conditions at 23 ± 2 °C and relative humidity of 50-↱↱60 %, with a 12/12-hour light-dark cycle applied. Animals were ↱subjected to acclimatization for two weeks before starting the experiment.

2.7. Induction of Liver Fibrosis (Model of Hepatic Fibrosis)

Rat liver fibrosis was induced by intraperitoneal (IP) injection of 1 ml/kg of 50 % CCl_4 ↱in olive oil twice weekly (every Saturday and Wednesday) for six consecutive weeks [24,25]. The 50 % CCl_4 solution was prepared by mixing an equal ↱volume of CCl_4 and olive oil in a glass container. ↱

2.8. Pilot study to determine the dose of Cnicus

The dose of *Cnicus benedictus* was determined based on a pilot study. We used three doses: 25, 50, and 100 mg/kg once daily orally for six weeks concurrently with CCl_4. At the end of the pilot study, a serum sample from the rats' tail was taken, and we analyzed the rat serum ALT, AST, total bilirubin, and albumin. Based on the results, the 100 mg/kg dose showed the best protective effect and was thus chosen for further analysis (results are illustrated in supplementary Fig. S1).

2.9. Animals grouping and study design

The animal was divided into four groups according to the treatment protocol. In the normal control group, 10 apparently healthy rats received an IP injection of 0.5 ↱ml/kg of olive oil (vehicle only) twice weekly ↱for six weeks. ↱Induction Group: 10 rats received an IP injection of ↱↱1 ml/kg of 50 % CCl_4 solution in olive oil twice weekly for six weeks ↱[3,26–28].

Silymarin Group: 10 rats received an IP injection of 1 ml/kg of ↱↱50 % CCl_4 solution in olive oil twice weekly and silymarin ↱↱(hepatoprotective agent) given once daily orally 100 mg/kg for six

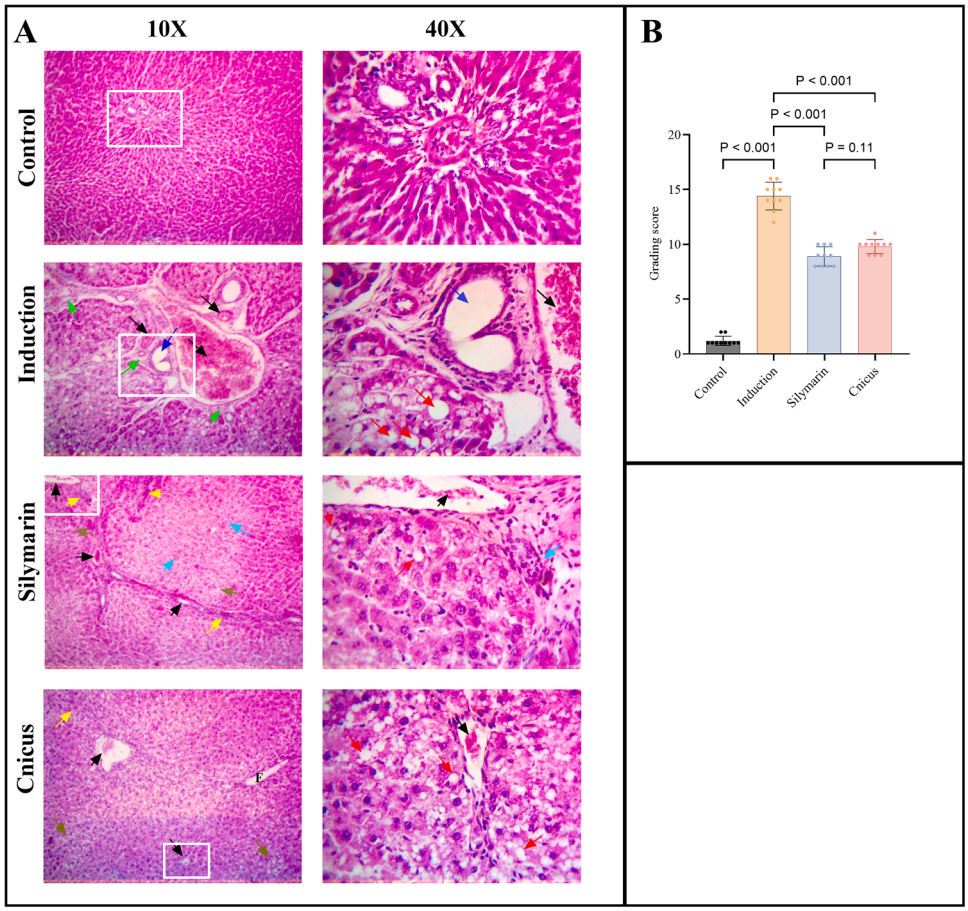


Fig. 6. (A) Histopathological examination of necroinflammatory grading of liver tissue showing the protective effect of phenolic extract of *Cnicus benedictus* against CCl_4-induced liver fibrosis in rats; induction group at low-power shows vascular congestion (←), bile duct hyperplasia ((←)), and along with bile duct proliferation ((←)). The induction group at high power shows fatty change ((←)), bile duct hyperplasia ((←)), and congested blood vessels (←). The Silymarin group at low power shows mild periportal infiltration of inflammatory cells ((←)), severely congested blood vessels (←), coagulative necrosis in different areas ((←)), and vascular degeneration of hepatocytes ((←)). The Silymarin group at high power shows mildly congested blood vessels (←), with mild periportal infiltration of inflammatory cells ((←)) and coagulative necrosis of hepatocytes ((←)). The Cnicus group at low power shows mild infiltration of inflammatory cells ((←)), mildly congested blood vessels (central vein) (←), vascular degeneration ((←)), and intact endothelial cell lining blood vessels (E). The Cnicus group at high power shows mild fatty changes ((←)) along with congested central veins (←). Slides stained with hematoxylin and eosin stain under 10x and 40x power. (B) The total necroinflammatory grading score. Data presented as mean ± standard deviation. One-way ANOVA with *post hoc* Tukey test was used.

weeks ↱concurrently with CCl_4 [29,30]. Cnicus Group: 10 rats received an IP injection of 1 ml/kg of ↱↱50 % CCl_4 solution in olive oil twice weekly and phenolic extract of *Cnicus benedictus* at a dose of 100 mg/kg once daily orally for six weeks concurrently with CCl_4 [31].

Twenty-four hours after the end of the experimental phase (the six weeks of induction and treatment), all animals were kept ↱withholding from food overnight; they were weighed individually, and then they were anesthetized with 50 mg/kg of Ketamine and 5 mg/kg of Xylazine, injected intramuscularly in the limb muscle [32,33], following total anesthesia, all rats were terminated by exsanguination (cardiac puncture) following AVMA guidelines [34], the relative liver weight percentage and liver tissue samples were collected for further analysis. The flow chart of the experiment is illustrated in Fig. 1.

2.10. Measurement of serum markers

Blood samples were collected slowly by cardiac puncture using 5 ml syringes. Blood was then collected in a clot activator tube to separate serum and centrifuged at 5000 rpm for 10 min. Serum samples were kept at −20 °C in Eppendorf tubes, which were then used to determine liver function parameters.

ELISA Procedures used to determine the serum liver functions and tissue homogenate parameters: All ELISA kits used in this study were purchased from Sunlong Biotech®, China. The purchased sandwich ELISA kits have similar procedures in the instructions that were strictly followed while measuring the study parameters. The manufacturer's kit was used to test biomarker levels, and a microplate reader recorded absorbance at 450 nm. Each manufacturing kit provided a standard curve to determine sample concentration.

2.11. Measurement of hepatic oxidative stress

A piece of liver tissue was cut into small pieces, washed with ↱phosphate-buffered saline, and then pulverized in a ratio of 1:10 (the tissue sample weight to the homogenization ↱buffer solution containing 1 % protease inhibitor portions, respectively). The mix was ↱sonicated in an ice bath to prevent overheating for 15 seconds, followed ↱by 5 min centrifugation at 12,000 rpm and 4 °C. The supernatant was ↱aliquoted, and levels of MDA (Cat# SL1135Hu, SunLong, China) and GSH (Cat# SL0998Ra, SunLong, China) in hepatic tissue homogenates ↱were

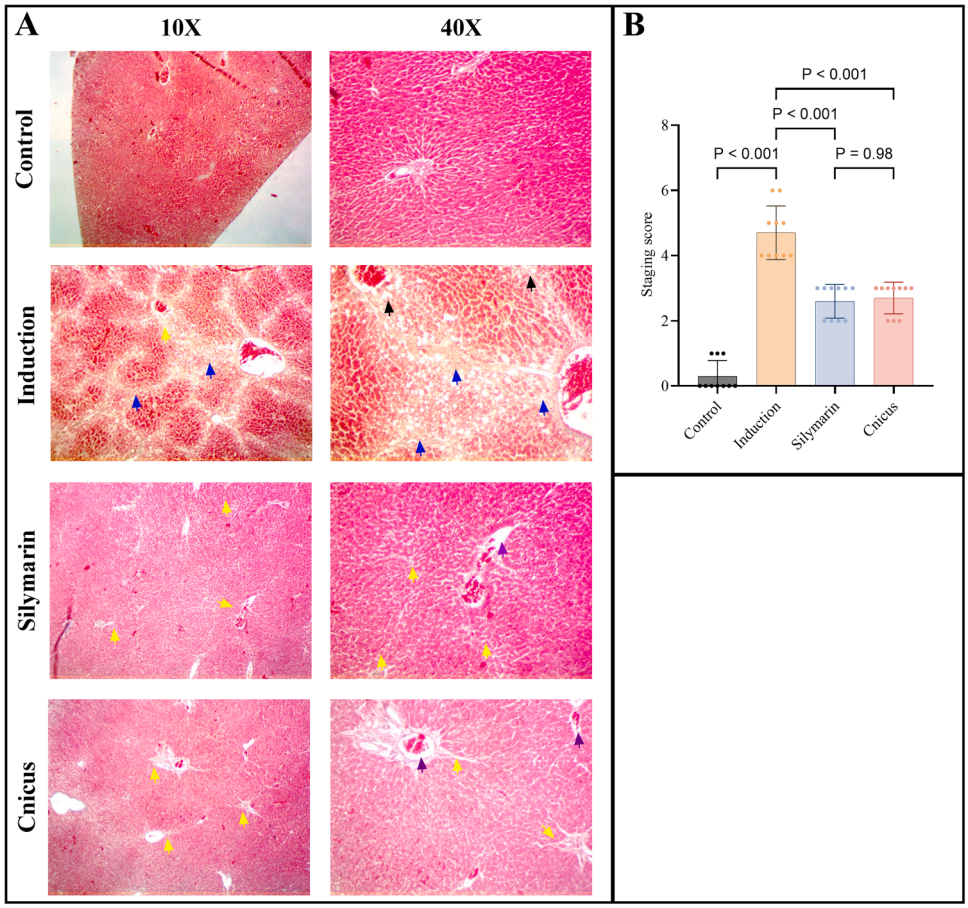


Fig. 7. (A) Histopathological examination of fibrosis staging of liver tissue showing the protective effect of phenolic extract of *Cnicus benedictus* against CCl_4-induced liver fibrosis in rats. The induction group at low power shows the fibrous expansion of portal-to-portal fibrosis and portocentral fibrosis ((←)), as well as bridging fibrosis ((←)). The induction group at high power shows portal and periportal fibrosis (←) and bridging fibrosis ((←)). The silymarin group at low power shows a fibrous expansion of hepatic tissue at most portal areas ((←)). The silymarin group at high power shows congested blood vessels ((←)) with fibrous expansion ((←)). The Cnicus group at low power shows a fibrous expansion of hepatic tissue at most portal areas ((←)). The Cnicus group at high power shows congested blood vessels ((←)) and fibrous expansion of hepatic tissue at most portal areas ((←)). Slides stained with Masson Trichrome under 10x and 40x power. (B) bars for fibrosis staging. Data presented as mean ± standard deviation. One-way ANOVA with *post hoc* Tukey test was used.

determined using enzyme-linked immunosorbent assay (ELISA) (Biotech, USA). The principle of the analysis is based on Sandwich-ELISA methods, in which The Microelisa strip plate provided in this kit has been pre-coated with an antibody specific to MDA, standards or samples are added to the appropriate Microelisa strip plate wells and combined to the specific antibody. Then, a Horseradish Peroxidase (HRP)- conjugated antibody specific to MDA or GSH is added to each Microelisa stripplate well and incubated. The TMB (Tetramethylbenzidine) substrate solution is added to each well. Only those wells that contain MDA or GSH and HRP conjugated MDA- or GSH- antibodies will appear blue and then turn yellow after adding the stop solution. The optical density is measured spectrophotometrically at a wavelength of 450 nm [28,35,36].

2.12. Histological assessment

Liver tissue specimens were fixed in 10 % neutral buffered formalin and then embedded in paraffin; after being deparaffinized, 5-μm-thick slices were processed for hematoxylin and eosin (H&E) and Masson trichrome (MT) stains, the slides were examined under the light microscope (Novel Biological Microscope, China) in which H&E stain used to assess the general liver architecture and inflammation grade, while the MT stain used to assess collagen deposition and fibrosis stage.

Scoring of necroinflammation grade and fibrosis stage is done according to the semiquantitative scoring of Ishak – HAI system, which is based on grading (describe the intensity of necroinflammatory activity in chronic hepatitis) and staging (measure of fibrosis and architectural alteration) of hepatic tissues [37].

The modified HAI system (Ishak) uses a seven-point scale for the fibrosis stage (0 – 6) and an 18-point scale for grading (0 – 18), which is divided into four features: A) periportal or perceptual interface hepatitis (piecemeal necrosis) from 0 to 4 score, B) confluent necrosis from 0 to 6 score, C) focal (spotty) lytic necrosis, apoptosis, and focal inflammation from 0 to 4 score, and D) portal inflammation from 0 to 4 score [37].

Three blinded pathologists from the Department of Pathology and Poultry Diseases at the College of Veterinary Medicine, University of Mosul, examined all histopathological slides and individually provided their reports for each slide, without the use of any software.

2.13. Immunohistochemical assessment

The samples were fixed in 10 % neutral buffered formalin for 48 hours and then stained with H & E stain. The tissue slices were subjected to immunohistochemistry (IHC) by dewaxing in xylene, rehydration in ethanol, and washing in phosphate-buffered saline. A solution of hydrogen peroxide in methanol (3 %) suppressed endogenous peroxidase activity for 30 minutes. Following that, pieces of tissue were frozen at 25 °C for an hour, and then the slices were incubated

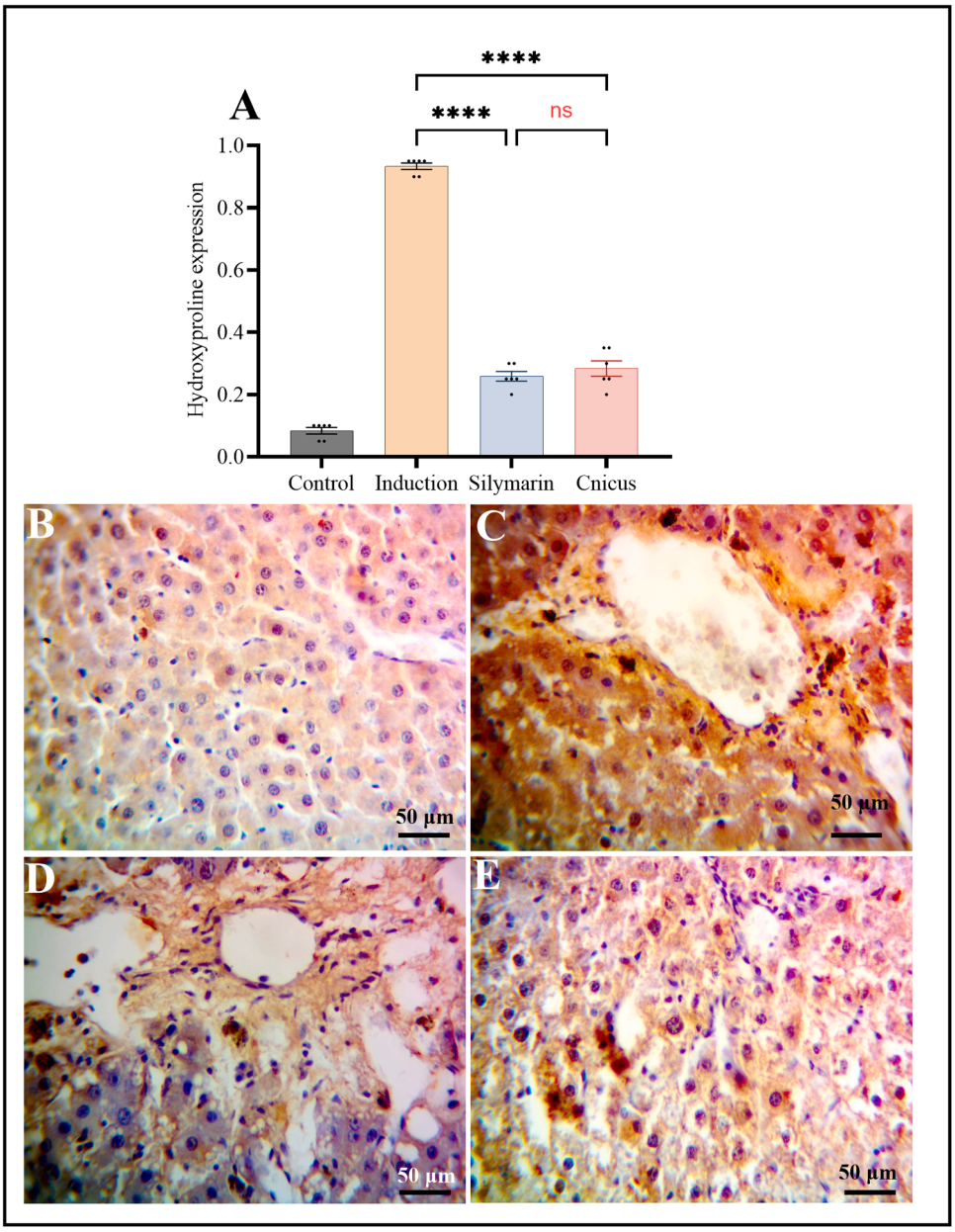


Fig. 8. Immunohistochemical staining for hydroxyproline in the liver shows the protective effect of phenolic extract of *Cnicus benedictus* against carbon tetrachloride-induced liver fibrosis in rats. (A) Relative Hyp expression in various groups, (B) Hyp expression in the control group, (C) Hyp expression in the induction group, (D) Hyp expression in the silymarin-treated group, (E) Hyp expression in the Cnicus-treated group (40X power)⁋. Data presented as mean ± standard deviation. One-way ANOVA with *post hoc* Tukey test was used.

with primary antibodies at 4 °C for one ⁋night [38,39].

The primary antibodies included anti-rat ⁋IHC of paraffin-embedded using α-SMA polyclonal ⁋antibody (cat# E-AB-34268, Elabscience, USA⁋) at dilution of 1:200; the α-SMA ⁋proteins are extremely conserved and have a role in many forms of cell ⁋mobility in eukaryotic cells [40]. The hydroxyproline antibody enables ⁋the visualization of hydroxy-proline in paraffin-embedded tissues. The hydroxyproline antibody (cat# 73812, Elabscience, USA) at dilution 1:200 was used in this experiment. The IHC ⁋test enables the detection of collagen throughout the tissue while ⁋preserving contextual information typically absent in conventional. It can also be used with other antibodies to ⁋identify additional relevant indicators [41,42]. Regarding TGF-β1, which is a group of produced polypeptide ⁋factors that have been preserved throughout evolution [43]; TGF-β receptor I polyclonal antibody (Cat#E-AB-16094 Elabscience, USA), IHC of paraffin-embedded ⁋tissue using a polyclonal antibody at dilution 1:70. The sections received ⁋a triple washing process, followed by treatment with poly-HRP goat anti-mouse IgG (diluted at a ratio of 1:200, Wuhan Biotech, China) for 60 ⁋minutes at 37°C. A mixture of avidin and biotin was ⁋utilized for detection. After staining with hematoxylin for 60 seconds, the ⁋sections were dried and then coated.⁋

Quantification of IHC was performed according to the following ⁋semiquantitative scores based on the percentage of positively stained ⁋cells: score 1 (≤25 % positive cells), score 2 (26–50 % positive cells), score 3 (51–75 % positive cells), and score 4 (76–100 % positive cells) [44,45].

2.14. Ethical approval

The study was approved by the Research Ethical Committee of the

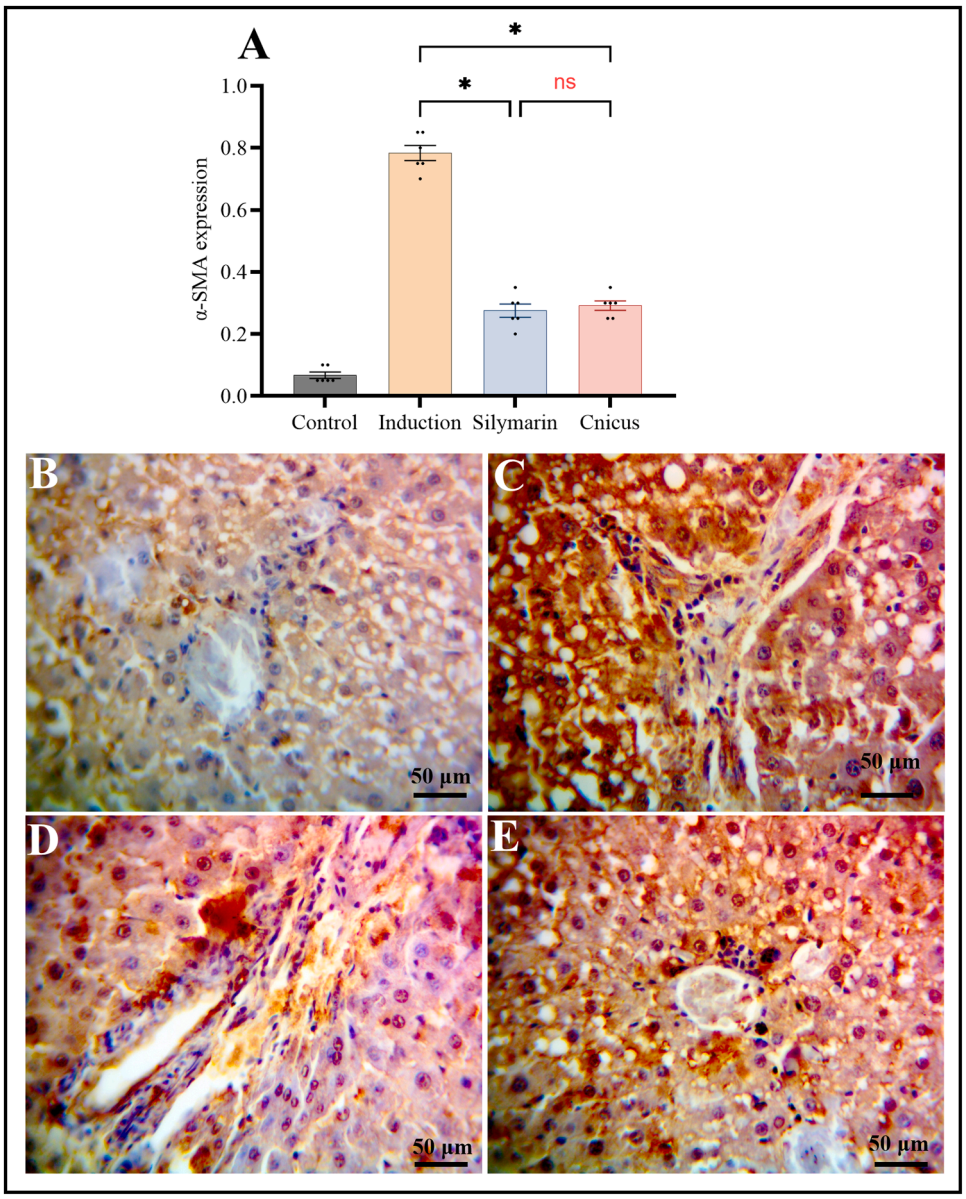


Fig. 9. Immunohistochemical staining for α-SMA in the liver shows the protective effect of phenolic extract of *Cnicus benedictus* against carbon tetrachloride-induced liver fibrosis in rats. (A) Relative α-SMA expression in various groups, (B) α-SMA expression in control group, (C) α-SMA expression in induction group, (D) α-SMA expression in silymarin treated group, (E) α-SMA expression in Cnicus treated group (40X power)⁋. Data presented as mean ± standard deviation. One-way ANOVA with *post hoc* Tukey test was used.

College of Medicine, Al-Nahrain University, approval number (UNCO-MIRB20240522), data (8 January 2023), following the American Veterinary Association Guidelines (AVMA) [34].

2.15. Sample size calculation

A post hoc sample size was done with an effect size of 0.5 and an alpha level of 0.05, F-family tests with a total ⁋sample size of 40 for each group of 10 animals.

2.16. Statistical analysis

Anderson Darling test of normality was done, and for variables that adhere to the normal distribution, one-way ANOVA and post hoc Tukey tests were employed to compare groups. In contrast, if data were non-normally distributed, the Kruskal-Wallis and post hoc Dunn tests were employed. The data underwent analysis using GraphPad Prism 10.3, which generated graphs and figures.

3. Results

3.1. HPLC analysis of Cnicus benedictus leaves

Cnicus benedictus leaves showed several components in HPLC analysis, including quercetin (reference compound shown in Fig. S2A), gallic acid (reference compound shown in Fig. S2B), rutin (reference compound shown in Fig. S2C), kaempferol (reference compound shown in Fig. S2D), silibinin (reference compound shown in Fig. S2E), and apigenin (reference compound is shown in Fig. S2F), as illustrated in Fig. 2.

3.2. In-vitro antioxidant activity of Cnicus extract

Cnicus extract reduces the DPPH free radical in a dose-dependent pattern; as seen in Fig. 3, the IC_{50} of the extract was 316.7 ± 39.98

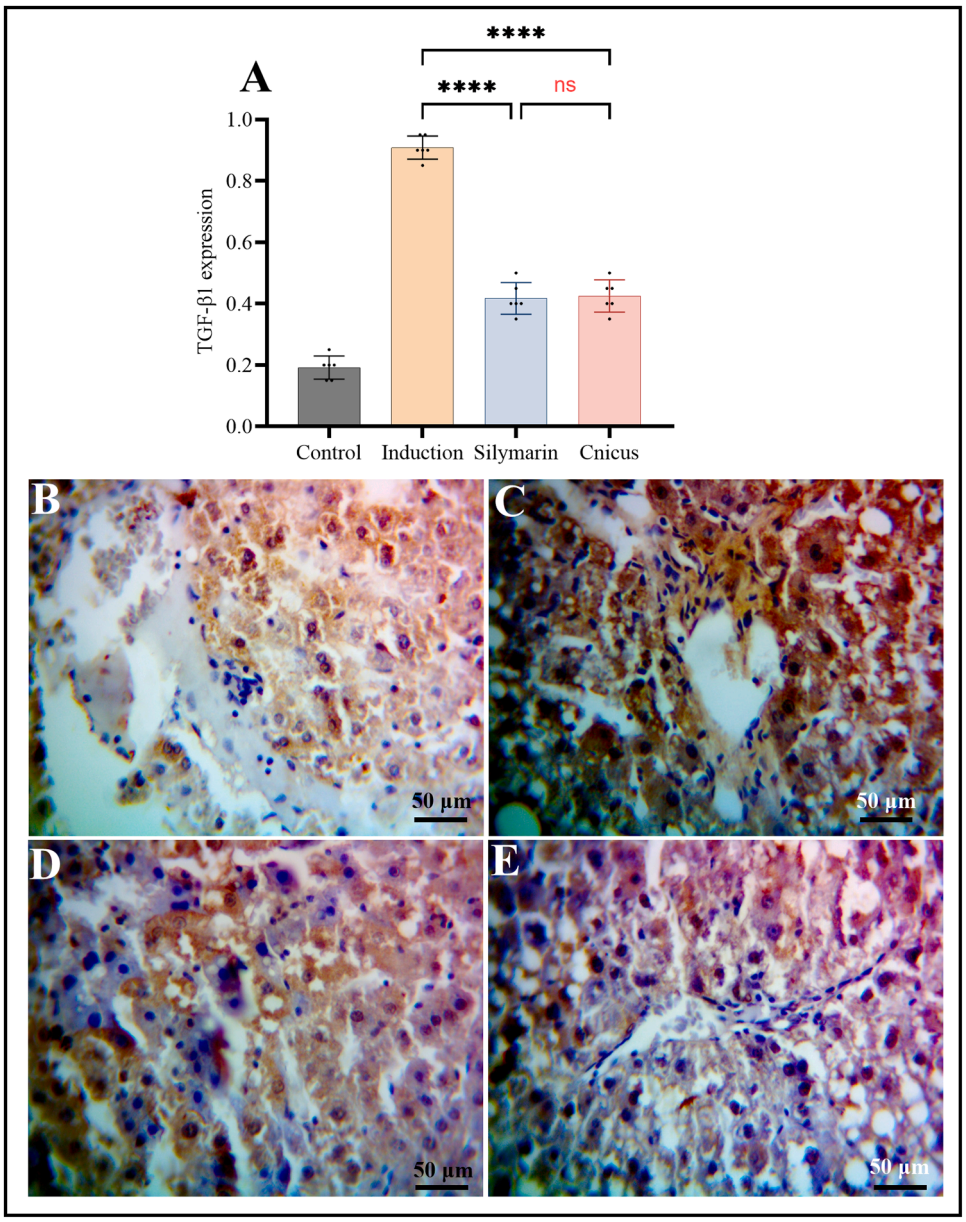


Fig. 10. Immunohistochemical staining for TGF-β1 in the liver shows the protective effect of phenolic extract of *Cnicus benedictus* against carbon tetrachloride-induced liver fibrosis in rats. (A) Relative TGF-β1 expression in various groups, (B) TGF-β1 expression in the control group, (C) TGF-β1 expression in the induction group, (D) TGF-β1 expression in the silymarin-treated group, (E) TGF-β1expression in Cnicus treated group (40X power)↱. Data presented as mean ± standard deviation. One-way ANOVA with *post hoc* Tukey test was used.

(µg/ml).

3.3. Effect of the study group on liver weight and oxidative stress parameters

The induction group showed significant elevation in relative liver weight and MDA levels and a significant reduction in liver GSH levels compared to the control group. Both silymarin and Cnicus extract showed significant protective effects against CCl_4 induction compared to the induction group (there was no significant difference between silymarin and Cnicus benedictus extract), as illustrated in Fig. 4.

3.4. Effect of phenolic extract of Cnicus benedictus ↱on serum levels of liver enzyme and proteins

The induction group showed significant elevation in serum levels of ALT, AST, and total bilirubin and a significant reduction in serum albumin compared to the control group, which indicates successful induction by CCl_4.

As illustrated in Fig. 5, both silymarin and Cnicus extract showed significant protective effects against CCl_4 induction compared to the induction group (there was no significant difference between silymarin and Cnicus benedictus extract).

3.5. Effect of study groups in histopathological examinations

H & E staining was utilized↱to view necroinflammation grades, and stained slides of the control group showed normal hepatic architecture. In ↱contrast, induction group sections displayed hepatocellular degeneration with the loss of hepatic ↱coordination, a periportal infiltration of inflammatory cells, fatty change, congested blood ↱vessels, bile duct hyperplasia, congested blood vessels, necrosis of endothelial cell layers,

accompanied by necrosis of hepatocytes. Silymarin and Cnicus groups showed mild periportal infiltration of inflammatory cells with mildly congested blood vessels, mild necrosis of hepatocytes in different areas but with intact endothelial cell lining blood vessels, and little fatty changes, as seen in Fig. 6.

MT staining is used to view the fibrosis stage (collagen deposition). In MT-stained control group sections, no fibrous expansion was detected. Induction group sections showed fibrous expansion of portal areas with marked bridging (portal to portal, portal to central). Silymarin and Cnicus groups showed fibrous expansion of most portal areas with short fibrous septa without fibrous bridging, as seen in Fig. 7.

3.6. Effect of study groups in IHC

The induction group showed a significant elevation in the relative expression of Hyp, α-SMA, TGF-**β**1 compared to the control group. Both silymarin and Cnicus showed a significant reduction in the relative expression of Hyp, α-SMA, TGF-**β**1 compared to induction (no significant difference between silymarin and Cnicus), as illustrated in Figs. 8-10.

4. Discussion

The lack of FDA-approved anti-fibrotic therapy that attenuates hepatic fibrosis, the nature of liver fibrosis in terms of its morbidity and mortality, and the complications of untreated liver fibrosis (being irreversible if untreated or progressed) need urgent new pharmacological moieties to halt back hepatic fibrosis [2,46]. The effectiveness of natural products against hepatic fibrosis is currently increasing, and they are very valuable in the discovery of novel therapeutic agents for liver fibrosis [47].

The ability of phenolic compounds to modulate the intracellular pathways of mitochondrial biogenesis makes them have wide-ranged therapeutic functions like antioxidant, anti-inflammatory, anticancer, and anti-aging properties, which suggests that polyphenol components of plants could be a potential choice for the treatment or prevention of many diseases [48]. Clinical trials and nutritional evidence support the idea that certain PC can improve liver disease and associated metabolic disorders. However, further fundamental clinical trials and research are warranted to validate the efficacy of dietary PC [49]. All parts of *Cnicus benedictus* traditionally have been used in gastrointestinal problems, strengthen the liver, diminish jaundice, stimulate appetite, enhance bile secretion, aid digestion, and decrease flatulence [14]. The HPLC results of our study's phenolic extract detected six phenolic compounds: silibinin, rutin, apigenin, gallic acid, quercetin, and kaempferol.

Regarding oxidative stress markers (MDA and GSH), the present study demonstrated that *Cnicus benedictus* extract significantly reduces MDA and elevates GSH in the liver homogenate of tested animals in comparison to the CCl_4-induction group, representing a property of hepatoprotection against induced liver fibrosis via antioxidant effects. A previous study identified the phenolic and flavonoid content of *Cnicus benedictus* and its antioxidant activities. Different parts of the plant showed ferric-reducing effects, which are related to its antioxidant capacity, and the leaf was found to have higher activity than the root. They also observed that free radical scavenging activity in the leaves was more pronounced than in the roots; these results agree with and support our study findings regarding oxidative stress markers [50]. These findings, coupled with the current study findings, support our selection of the extraction of phenolic compounds from *Cnicus benedictus* leaves rather than the other parts of the plant.

The antioxidant ability of phenolic compounds is due to their ability to donate a hydrogen atom and/or an electron to free radicals, causing the break of the chain reaction of oxidation. The antioxidant effect depends on the number and position of the hydroxyl groups [51].

The findings of this study revealed that the phenolic extract of *Cnicus benedictus* significantly reduces the histopathological necroinflammation grade scores and immunohistochemical expression of TGF-β1 (inflammation markers) of liver tissue slides in the treated group in comparison to the induction group, representing an anti-inflammatory characteristic that benefits suppression of liver fibrosis progression. Paun et al. (2019) found that the phenolic extract of *Cnicus benedictus* could be utilized as a potential anti-inflammatory agent and support traditional anti-inflammatory actions [21]. In liver fibrosis models in rats, rutin mitigates hepatic fibrogenesis and inflammation process through inhibition of signaling pathways underlying TGF-β stimulation in activated HSCs [52,53]. Quercetin, apigenin, and gallic acid inhibited liver fibrosis by attenuating HSC activation and reducing autophagy by regulating the TGF-β1/Smads pathways [54–56]. In a study of CCl_4-mediated liver fibrosis in mice, kaempferol significantly decreased the necro-inflammatory scores of the treated group compared to the induction group [57]. The findings of the mentioned phenolic compounds related to liver inflammation support our results. In the liver fibrosis model in rats, rutin exhibits a reduction of fibrosis score, enhancement of apoptosis, and inhibition of the proliferation of impaired hepatocytes. Moreover, it has a beneficial role in decreasing α-SMA expression levels and the amount of hydroxyproline [52,53].

Silibinin is useful in preventing hepatic steatosis, inflammation, and fibrosis. This beneficial effect is due to the alteration of lipid metabolism-related gene expression. Lower levels of collagen fiber proliferation and staining were observed in the silibinin treatment animals. The α-SMA expression was significantly lower after silibinin treatment than in the NASH group [58]. Silibinin exhibits hepatoprotective, antioxidant, free radical scavenging, membrane stabilizing, and anti-fibrotic activity against dimethyl nitrosamine-induced hepatic fibrosis [59]. Hydroxyproline and collagen deposition were prevented by silibinin, reflecting hepatoprotective properties in the liver tissue in N-nitrosodimethylamine-induced fibrotic rats. Recovery of rat liver tissue against N-nitrosodimethylamine-induced hepatocellular necrosis, inflammatory changes, and hepatic fibrosis by silibinin treatment agrees with current study findings by both H&E and MT-stained histopathological evaluation of liver tissue.

Kaempferol exhibits various pharmacological properties, including anti-inflammatory, anti-lipid metabolizing, and antioxidant effects. It was found that kaempferol significantly reduced the expression of vascular endothelial growth factor, α-SMA, and collagen-I proteins in a model of CCl_4-induced liver fibrosis in rats in addition to inhibition of HSC activation; so, kaempferol has a positive role in liver fibrosis management [60]. Kaempferol may be a novel agent for treating liver fibrosis or other fibroproliferative diseases. It significantly suppressed collagen synthesis and HSC activation both in vivo and in vitro [57]. All these studies' findings about the mentioned phenolic compounds agree with our results concerning fibrosis markers.

4.1. Study limitations

The study's limitations were that the extract contained six compounds rather than a purified one, and the genetic parameters, which were not used, may have another beneficial role in studying the potential protective use of this extract against liver fibrosis.

5. Conclusion

Oral administration of phenolic compounds of *Cnicus benedictus* improved defense against oxidative stress and inflammation caused by CCl_4, reducing the degree of liver fibrosis.

CRediT authorship contribution statement

Mohammed Jasim Mohammed: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Haitham Mahmood Kadhim:** Writing – review & editing,

Writing – original draft, Visualization, Supervision, Methodology, Conceptualization.

Ethical approval

The study was approved by the Research Ethical Committee of the College of Medicine, Al-Nahrain University, approval number (UNCO-MIRB20240522), data (8 January 2023), following the American Veterinary Association Guidelines (AVMA) [34].

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2024.101850.

Data availability

Data will be made available on request.

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