

## Original Research Article

# Chicory abrogates oxidative stress, inflammation and caspase-dependent apoptosis in acute hepatic injury model induced by acetaminophen in rats.

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## Abstract

In this study the protective effect of chicory leaves hydroalcoholic extract (CIE) against acute liver injury induced by a single dose of acetaminophen (700 mg/kg, i.p.) was investigated in rats. The CIE and silymarin treatment (standard reference) were given in a dose of (100 mg/kg, p.o.) for 3 days before and at 1 and 12 h following acetaminophen administration. Treatment with CIE significantly reduced the levels of serum ALT, AST, alkaline phosphatase, bilirubin, total cholesterol, triglycerides, urea, creatinine, TNF- $\alpha$  and hepatic contents of malondialdehyde (MDA), nitric oxide, caspase-3 and hydroxyproline, with significant increases in serum total protein, albumin, HDL-cholesterol and hepatic activities of reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) as compared with the acetaminophen group. The histopathological alterations mediated by acetaminophen were ameliorated by CIE. It was concluded that CIE protects rat liver against acetaminophen hepatotoxicity, most probably through abrogation of oxidative stress, inflammation and caspase-3 dependent apoptosis.

**KEYWORDS:** Acetaminophen; Antioxidant; Apoptosis; Cichorium intybus; Hepatotoxicity; Silymarin.

## Introduction

Among vegetables, chicory (*Cichorium intybus* L.), a typical vegetable indigenous to Europe and North and Western America, has gained attention for its content of phytochemicals such as phenolic acids (e.g. cichoric acid), flavonoids, sesquiterpene lactones (e.g. lactucin, lactucopicrin), oligofructose, inulin, tannins and anthocyanins [1,2]. Historically, chicory was grown by the ancient Egyptians as a medicinal plant, coffee substitute, and vegetable crop. To date, chicory is grown for the production of inulin on an industrial scale [3]. Various studies demonstrate the ability of different chicory parts to counteract various free radicals, as well as a linear correlation between phytochemical content and antioxidant capacity of this vegetable [4-7]. The CIE is reported to possess anti-diabetic [8, 9], antiviral [10], anti-hyperlipidemic [11], wound healing [12], hepato and neuroprotective effects [11, 13].

Acetaminophen (N-acetyl-p-aminophenol; APAP) a widely used analgesic and antipyretic drug that is safe when taken at therapeutic dose, has become one of the most common cause of acute liver failure in western countries when taken at acute or cumulative overdose [14]. It is well documented that the toxic effects of

acetaminophen are initiated by the formation of a reactive metabolite NAPQI, which under normal conditions is readily detoxified by conjugation with glutathione (GSH). However, acetaminophen overdose saturate detoxification pathways, resulting in GSH depletion, subsequently excess NAPQI, binds to cellular proteins especially in the mitochondria. The resulting mitochondrial oxidative stress leads to mitochondrial dysfunction, ATP depletion, increased MPT and nuclear DNA fragmentation, which contribute to hepatocellular necrosis. In addition, NAPQI can increase the formation of ROS such as superoxide anion, hydroxyl radical and hydrogen peroxide, and RNS such as peroxynitrite. Excess levels of ROS and RNS can attack hepatocellular macromolecules such as DNA, protein, and phospholipids, which lead to lipid peroxidation and depletion of the antioxidant enzymes causing an imbalance between pro-oxidants and antioxidants that further results in oxidative stress [15, 16]. Several studies highlight the importance of hepatic macrophages, so-called Kupffer cells, for initiating and driving the inflammatory processes following APAP-induced hepatotoxicity through the release of chemokines and the pro-inflammatory mediators, such as nitric oxide (NO) and tumor necrosis factor-alpha (TNF- $\alpha$ ), interleukins as well as activating other non-parenchymal liver cells, e.g. endothelial or hepatic stellate



cells. Many of these pro-inflammatory cytokines can trigger hepatocyte cell death pathways and promote tissue damage [17]. The acetaminophen-induced hepatotoxicity serves as an example of the interrelationship between apoptotic and necrotic cell death and their common origin in mitochondrial dysfunction. There is increasing evidence that apoptosis plays a critical role in the pathogenesis of hepatic injury caused by acetaminophen [18]. It has been reported that APAP hepatotoxicity is caused by the mitochondrial apoptosis pathway and facilitated by chemokine CXCR2 signaling [19]. It was reported that APAP triggered the release of cytochrome C from mitochondria into the cytosol, activation of caspase-3 and degradation of DNA [20].

Previous studies have extensively demonstrated that antioxidants and anti-inflammatory agents effectively can protect against the acute hepatic injury induced by APAP administration.

*Cichoriumintybus* presents a little investigated plant in terms of phytochemistry and pharmacology, most of the pharmacological studies on this plant document the testing of the roots and few studies tested the pharmacological effects of the leaves.

Therefore, the objective of the present study is to examine possible protective effects of CIE against APAP-induced liver damage in an acute injury model.

## Material and Method

### Drugs and Chemicals

Acetaminophen, silymarin, cremophor EL, bovine serum albumin (BSA), Folin-Ciocalteu's phenol, gallic acid, 1,1,-diphenyl-2-picrylhydrazyl (DPPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). All other chemicals used were of the highest grade commercially available.

Acetaminophen was dissolved in warm saline (65°C) and cooled to room temperature before intraperitoneal (i.p.) injection. Silymarin were emulsified in a mixture of saline and 10% Cremophor EL before oral use.

The dose of acetaminophen, *Cichoriumintybus* leaves extract, and silymarin were selected based on our preliminary experiments and in accordance with previous published literatures [21-24].

### Sample Preparation

The leaves of *Cichoriumintybus L.* were identified at the Herbarium of the department of Botany, Faculty of Science, Alexandria University. A voucher sample was deposited in the department of Pharmacology and Toxicology, Faculty of Pharmacy, AL-Azhar University, Egypt. The leaves were air-dried in shade, coarsely powdered and kept in air-tied containers. The powder was extracted twice with 70% ethanol at room temperature for 5 days each. The solution was filtered through a cotton wool and re-filtered using a Whatman filter paper. The filtrate was freeze-dried and the dried substance was kept in desiccators (yield 44%). The aqueous

solution of the lyophilized powder of ethanolic extract was prepared immediately by dissolving in distilled water before oral administration to experimental animals.

### Estimation of total phenolic content(TPC)

The phenolic content of the *Cichoriumintybus L.* leaves extract was estimated by the Folin-Ciocalteu assay according to the method of Wong et al (2006) [25]. A standard curve was plotted using various concentrations of Gallic acid and the TPC values were expressed as mg gallic acid equivalents/g of dry extract (mg GAE/100 g dw).

### DPPH Free Radical Scavenging Activity

DPPH scavenging activity was measured using the method described by Cheng et al (2006) [26]. This assay is based on the determination of the concentration of DPPH methanolic solution, after adding the antioxidants. DPPH concentration is reduced by the existence of an antioxidant at 515 nm and the absorption gradually disappears with time. Results were expressed as  $\mu\text{M}$  Trolox equivalent antioxidant capacity/ g dry weight ( $\mu\text{M}$  TEAC/ g dw)

### Animals

Male adult Sprague–Dawley rats weighing  $185 \pm 15$  g were obtained from the animal breeding laboratory (Helwan, Egypt). Animals were caged in sex groups, provided with standard laboratory chow and water ad libitum, and left to acclimatize at the animal house of the Faculty of Pharmacy, Al-Azhar University, Egypt for one week before experiments. The animals were kept in standard conditions, maintained at  $22 \pm 2^\circ\text{C}$  and  $50 \pm 5\%$  relative humidity under a 12 h light–dark cycle. All animal studies were performed in accordance with the ethical guidelines for investigations in laboratory animals and were approved by the ethical committee of Faculty of Pharmacy, Cairo University and comply with the guide for the care and use of laboratory animals [27].

### Experimental design

Forty-eight male adult Sprague-Dawley rats were randomly allocated into sex equal groups (n=8, each); two rats from each group were used for histopathological examination as follows:

Group 1: Received (normal saline, p.o.) and served as normal control.

Group 2: Received silymarin in a dose of (100 mg/kg, p.o.) every 12h for 3 days

Group 3: Received CHE in a dose of (100 mg/kg, p.o.) every 12h for 3 days

Group 4: Received acetaminophen in a single dose of (700 mg/kg, i.p.)

Group 5: Pretreated with silymarin in a dose of (100 mg/kg, p.o.), every 12h for 3 days before and 1 and 12h after a single dose of acetaminophen (700 mg/kg, i.p.).

Group 6: Pretreated with CIE in a dose of (100 mg/kg, p.o.), every 12h for 3 days before and 1 and 12h after a single dose of acetaminophen (700 mg/kg, i.p.).

### Serum and Tissue Preparation

At the end of the experiment, the animals were fasted overnight, blood samples were collected from the retro-orbital venous plexus, 30 h after administration of APAP. Serum was separated from the clotted blood samples by centrifugation at 3000 rpm for 30 min, then aliquoted and stored at -20°C until analysis. After blood collection, all animals were sacrificed then liver was dissected out, rinsed in isotonic sterile saline, blotted dry on a filter paper and weighed. Homogenization was carried out in ice-cold isotonic saline using (heidolphdix 900 homogenizer, Germany) to yield a 10% (w/v) tissue homogenate. The homogenate was then made into aliquots and used for determination of hepatic contents of total protein, malondialdehyde (MDA), reduced glutathione (GSH), nitric oxide, hydroxyproline, caspase-3 and enzymatic activities of catalase (CAT), and superoxide dismutase (SOD).

### Biochemical Analysis

Activities of serum enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total and direct bilirubin, total proteins (TP) and albumin were determined by colorimetric method using commercial assay kits (Biodiagnostics, Cairo, Egypt). In addition to , the serum levels of total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C), urea and creatinine using (Diamond Diagnostics, Egypt) assay kits.

Hepatic hydroxyproline and caspase-3 contents were assessed by employing the quantitative sandwich enzyme immunoassay technique using ELISA kits supplied by (Cusabio®,USA) according to manufacturer's instructions.

Tumor necrosis factor-alpha (TNF-  $\alpha$ ) was estimated in serum using rat TNF-  $\alpha$  ELISA kit provided by (Alpico® diagnostics), this is a solid-phase sandwich ELISA utilizing a monoclonal antibody specific for rat TNF-  $\alpha$  coated on a 96-well plate.

The total protein content of hepatic tissue is determined according to the method of Lowry *et al* (1951) using BSA as standard protein [28].

Reduced glutathione (GSH) is measured according to the method of Beutler *et al*(1963) using Ellman's reagent [5-5' dithiobis(2-nitrobenzoic acid) ] as a substrate [29]. The results were expressed as mg/g wet tissue.

Nitric oxide concentration was determined by indirect measurement of nitriteconcentration, a byproduct of NO transformation in living tissues. The content was estimated in the liver homogenate by measuring the absorbance of the chromophoric azo derivative formed at 540 nm spectrophotometrically according to the method described by Miranda *et al* (2001) [30], the results were expressed as nmol/100 mg wet tissue.

Lipid peroxidation was determined by estimating the level of thiobarbituric acid reactive substances (TBARSs) measured as malondialdehyde (MDA) According to method of Satoh [31], using 1,1,3,3-tetraethoxypropane as a standard. The results were expressed as nmol of MDA/g of wet tissue.

CAT activity was evaluated by the method of Aebi [32], which is based on H<sub>2</sub>O<sub>2</sub> degradation by the action of CAT contained in the examined samples. Catalase activity was expressed as unit/mg protein.

Activity of SOD enzyme is determined according to the method of Nishikimi *et al* (1972) [33]. Activity was expressed as U/mg protein.

### Histopathological Examination

Samples of liver tissue from each animal were fixed in 10% neutral buffered formalin for 24 h, washing was done with tap water and then serial dilutions of ethyl alcohol were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56°C in hot air oven for 24 h. Paraffin wax tissue blocks were prepared for sectioning at 4  $\mu$ m thicknesses by sledge microtome. The obtained tissue sections were collected on glass slides, deparaffinized, stained by hematoxylin and eosin stain, and examined under a light electric microscope by a pathologist unaware of the treatment protocol [34].

### Statistical Analysis

All values were presented as mean  $\pm$  standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism version 6 (GraphPad, San Diego, CA). Comparison between different groups was carried out using one-way analysis of variance (ANOVA), followed by Tukey's test as post hoc. Difference was considered significant at  $p \leq 0.05$ .

## Results

### Effects on cellular integrity, functional and synthetic capacity of hepatocytes

Tables (1,2) show that the serum levels of ALT (193%), AST (323%), ALP (182%), total bilirubin (270%), and direct bilirubin (540%) significantly increased after administration of acetaminophen compared with the control group.

Table (1): Effects of pretreatment with silymarin and CIE on serum liver enzymes, bilirubin, TP, albumin and relative body weight

parameter	Control	APAP	Silymarin	CIE	Silymarin+ APAP	CIE + APAP
ALT (U/L)	27.4 ± 2.1	80.3 ± 3.8 <sup>a</sup>	22.4 ± 1.8	20.9 ± 1.3	42.5 ± 3.2 <sup>a,b</sup>	41.6 ± 2.8 <sup>a,b</sup>
AST(U/L)	34.6 ± 2.2	146.3 ± 4.7 <sup>a</sup>	36.1 ± 2.8	35.2 ± 2.9	58.0 ± 4.0 <sup>a,b</sup>	49.1 ± 3.5 <sup>b</sup>
ALP (U/L)	116.0 ± 8.4	327.0 ± 14.6 <sup>a</sup>	92.8 ± 5.7	117.0 ± 7.3	153.0 ± 8.9 <sup>b</sup>	187.0 ± 9.3 <sup>a,b</sup>
Albumin (g/dl)	5.5 ± 0.34	3.9 ± 0.13 <sup>a</sup>	5.5 ± 0.16	5.1 ± 0.24	4.6 ± 0.10 <sup>b</sup>	4.2 ± 0.13 <sup>b</sup>
Total protein (g/dl)	8.1 ± 0.21	5.3 ± 0.24 <sup>a</sup>	8.3 ± 0.27	9.2 ± 0.49 <sup>b</sup>	6.1 ± 0.33 <sup>b</sup>	6.5 ± 0.21 <sup>b</sup>
Total bilirubin (mg/dl)	0.84 ± 0.06	3.1 ± 0.26 <sup>a</sup>	0.81 ± 0.08	0.74 ± 0.06	1.4 ± 0.11 <sup>a,b</sup>	1.7 ± 0.12 <sup>a,b</sup>
Direct bilirubin (mg/dl)	0.25 ± 0.04	1.6 ± 0.15 <sup>a</sup>	0.33 ± 0.04	0.20 ± 0.02	1.0 ± 0.07 <sup>a,b</sup>	1.1 ± 0.08 <sup>a,b</sup>
Liver/ body weight ratio (100x)	3.0 ± 0.09	3.8 ± 0.06 <sup>a</sup>	3.1 ± 0.06	3.0 ± 0.08	3.4 ± 0.09 <sup>a,b</sup>	3.1 ± 0.06 <sup>b</sup>

Data are expressed as means ± SEM of eight rats per group.

<sup>a</sup>Significantly different from the control group; <sup>b</sup>Significantly different from the acetaminophen-treated group using one-way ANOVA followed by the Tukey test for multiple comparison at  $p \leq 0.05$ .

Table (2): Effects of pretreatment with silymarin and CIE on serum TC, TG, HDL-C, urea and creatinine

parameter	Control	APAP	Silymarin	CIE	Silymarin + APAP	CIE + APAP
Cholesterol (mg/dl)	87.8 ± 5.1	180.0 ± 5.4 <sup>a</sup>	72.7 ± 5.7	92.4 ± 5.4	131.0 ± 6.2 <sup>a,b</sup>	137.0 ± 4.9 <sup>a,b</sup>
Triglycerides (mg/dl)	93.9 ± 6.0	205.0 ± 10.1 <sup>a</sup>	98.1 ± 4.2	102.0 ± 5.3	150.0 ± 5.4 <sup>a,b</sup>	151.0 ± 4.1 <sup>a,b</sup>
HDL-C (mg/dl)	38.7 ± 2.4	24.8 ± 1.5 <sup>a</sup>	45.5 ± 3.6	48.6 ± 2.9	32.2 ± 2.0 <sup>b</sup>	36.1 ± 2.3 <sup>b</sup>
Urea (mg/dl)	35.9 ± 2.4	73.9 ± 5.5 <sup>a</sup>	26.9 ± 1.7	29.6 ± 1.5	35.2 ± 2.0 <sup>b</sup>	36.4 ± 1.9 <sup>b</sup>
Creatinine (mg/dl)	1.3 ± 0.09	3.1 ± 0.25 <sup>a</sup>	1.1 ± 0.11	1.2 ± 0.09	2.0 ± 0.13 <sup>a,b</sup>	2.3 ± 0.15 <sup>a,b</sup>
TNF-α (pg/ml)	35.2 ± 2.1	90.2 ± 3.1 <sup>a</sup>	37.4 ± 2.4	41.2 ± 2.6	53.2 ± 3.9 <sup>b</sup>	56.6 ± 3.0 <sup>b</sup>

Data are expressed as means ± SEM of eight rats per group.

<sup>a</sup>Significantly different from the control group; <sup>b</sup>Significantly different from the acetaminophen-treated group using one-way ANOVA followed by the Tukey test for multiple comparison at  $p \leq 0.05$ .

Table (3): Effects of pretreatment with silymarin and CIE on hepatic content of GSH, MDA, NO, hydroxyproline, caspase-3 and liver enzymes activity

parameter	Control	APAP	Silymarin	CIE	Silymarin + APAP	CIE + APAP
GSH (mg/g. tissue)	6.9 ± 0.63	2.4 ± 0.19 <sup>a</sup>	7.2 ± 0.69	7.6 ± 0.64	6.1 ± 0.66 <sup>b</sup>	6.8 ± 0.59 <sup>b</sup>
CAT (Unit/mg protein)	1.6 ± 0.11	0.7 ± 0.05 <sup>a</sup>	1.4 ± 0.08	2.7 ± 0.2 <sup>a</sup>	1.5 ± 0.10 <sup>b</sup>	2.0 ± 0.18 <sup>b,c</sup>
SOD (Unit/mg protein)	5.3 ± 0.25	1.9 ± 0.11 <sup>a</sup>	4.9 ± 0.29	5.3 ± 0.22	4.1 ± 0.19 <sup>a,b</sup>	5.2 ± 0.38 <sup>b,c</sup>
MDA (nmol/g. tissue)	10.4 ± 0.57	41.1 ± 2.3	8.1 ± 0.61	7.0 ± 0.62	15.5 ± 0.65 <sup>b</sup>	14.0 ± 0.69 <sup>b</sup>
NO (nmol/100 mg tissue)	44.6 ± 2.3	117.7 ± 3.8 <sup>a</sup>	45.8 ± 2.2	43.8 ± 2.4	58.9 ± 2.1 <sup>a,b</sup>	55.7 ± 3.1 <sup>b</sup>
Caspase-3 (ng/g. tissue)	2.7 ± 0.25	9.7 ± 0.35 <sup>a</sup>	2.9 ± 0.25	3.6 ± 0.18	4.1 ± 0.29 <sup>a,b</sup>	5.1 ± 0.23 <sup>a,b</sup>
Hydroxyproline (ng/g. tissue)	2.5 ± 0.12	7.9 ± 0.33 <sup>a</sup>	2.7 ± 0.18	2.9 ± 0.15	3.2 ± 0.16 <sup>b</sup>	3.4 ± 0.14 <sup>b</sup>

Data are expressed as means ± SEM of eight rats per group.

<sup>a</sup>Significantly different from the control group; <sup>b</sup>Significantly different from the acetaminophen-treated group; <sup>c</sup>Significantly different from the silymarin + acetaminophen-treated group using one-way ANOVA followed by the Tukey test for multiple comparison at  $p \leq 0.05$ .

Table (4): Effect of pretreatment with silymarin and CIE on histopathological examination of liver tissues of acetaminophen-treated rats

Histopathological findings	Control	APAP	Silymarin + APAP	CIE+ APAP
Centrolobular necrosis	-	++++	+	+
Ballooning degeneration	-	++++	+	-
Inflammatory cell infiltration	-	+++	+	-
Dilatation and congestion of portal vein	-	-	++	-
Dilation of central vein	-	+	++	+
Kupffer cell proliferation	-	+	++	+++

Scoring key: (-) none, (+) mild, (++) moderate, (+++) severe, (++++) very severe

Foursections from each animal were examined, and were graded as follows: - (0%), + (1-25%), ++ (26-50%), +++ (50%-75%), ++++ (>75%)

Table (5): Total phenolic content and total antioxidant capacity in chicory leaves extract

Parameters	CIE
TPC (mg/g gallic acid)	51 ± 2.4
TAC TEA (µM TEAC/100g dw)	220.6 ± 4.7

Data are expressed as means ± SD of Four determinations

In contrast, treatment with CIE showed remarkable reductions in the activities of ALT, ATS, ALP, and total and direct bilirubin levels amounting to 48%, 66%, 43%, 45%, and 31% respectively with respect to acetaminophen challenged animals. Furthermore, the hepatotoxicity of APAP was evident from increased levels of TC, TG, urea and creatinine by about 195%, 117%, 106% and 138% respectively, accompanied by significant reductions in serum levels of total protein, albumin and HDL-C by about 44%, 38% and 36% respectively when compared to control group. On the other hand, treatment with CIE before and after APAP administration attenuates the elevated levels of TC, TG, urea and creatinine by about 24%, 26%, 51%, and 26% respectively, and significantly increased the serum levels of total protein (30%), albumin (40%) and HDL-C (46%) comparable to APAP-treated group.

### Effects on oxidative stress markers

Data in Table (3) showed that injection of a single dose of acetaminophen (700 mg/kg) produced oxidative stress manifested by significant increases in hepatic MDA and NO contents reaching 295% and 164% respectively, associated with concomitant decreases in hepatic GSH content (66%) and the activity of enzymatic antioxidant parameters; SOD (64%) and CAT (56%) in comparison with the control group. On the other hand, treatment with CIE restored the normal MDA and NO contents to about 66% and 53% respectively; associated with marked increase in hepatic GSH content by about 179%, meanwhile CIE administration restored the normal antioxidant activities of SOD (174%) or even boosted over

the control group for CAT (186%) with respect to APAP alone treatment. Moreover, treatment with CIE alone significantly increased the activity of CAT enzyme by about 69% in comparison with control group. Furthermore, treatment with CIE significantly increased the SOD and CAT activities amounting to 27% and 34%, respectively with respect to the combined regimen of silymarin plus APAP.

### Effect on inflammation biomarker (serum level of the pro-inflammatory mediator; TNF-α)

As clearly demonstrated in figure 1A, treatment with acetaminophen significantly increased the serum level of TNF-α to 156% in comparison with the control group. Such increase was significantly attenuated by CIE treatment to about 37% in comparison with acetaminophen-treated group.

### Effect on apoptosis biomarker (hepatic tissue content of caspase-3)

The ELISA assay technique revealed that administration of acetaminophen alone was accompanied by an increase in caspase-3 content by about 260% compared to the control group. Moreover, administration of CIE before and after acetaminophen showed a remarkable decrease in this level to about 48% as compared to acetaminophen-treated group (figure 1B).

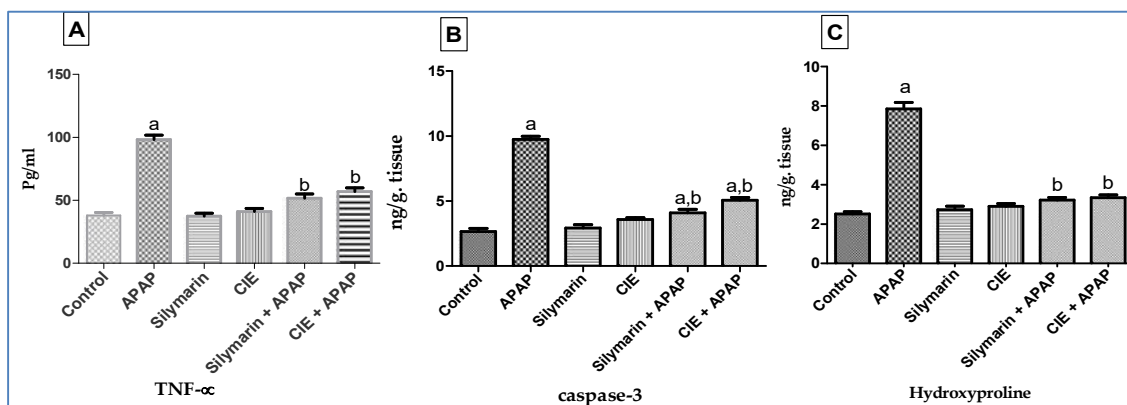
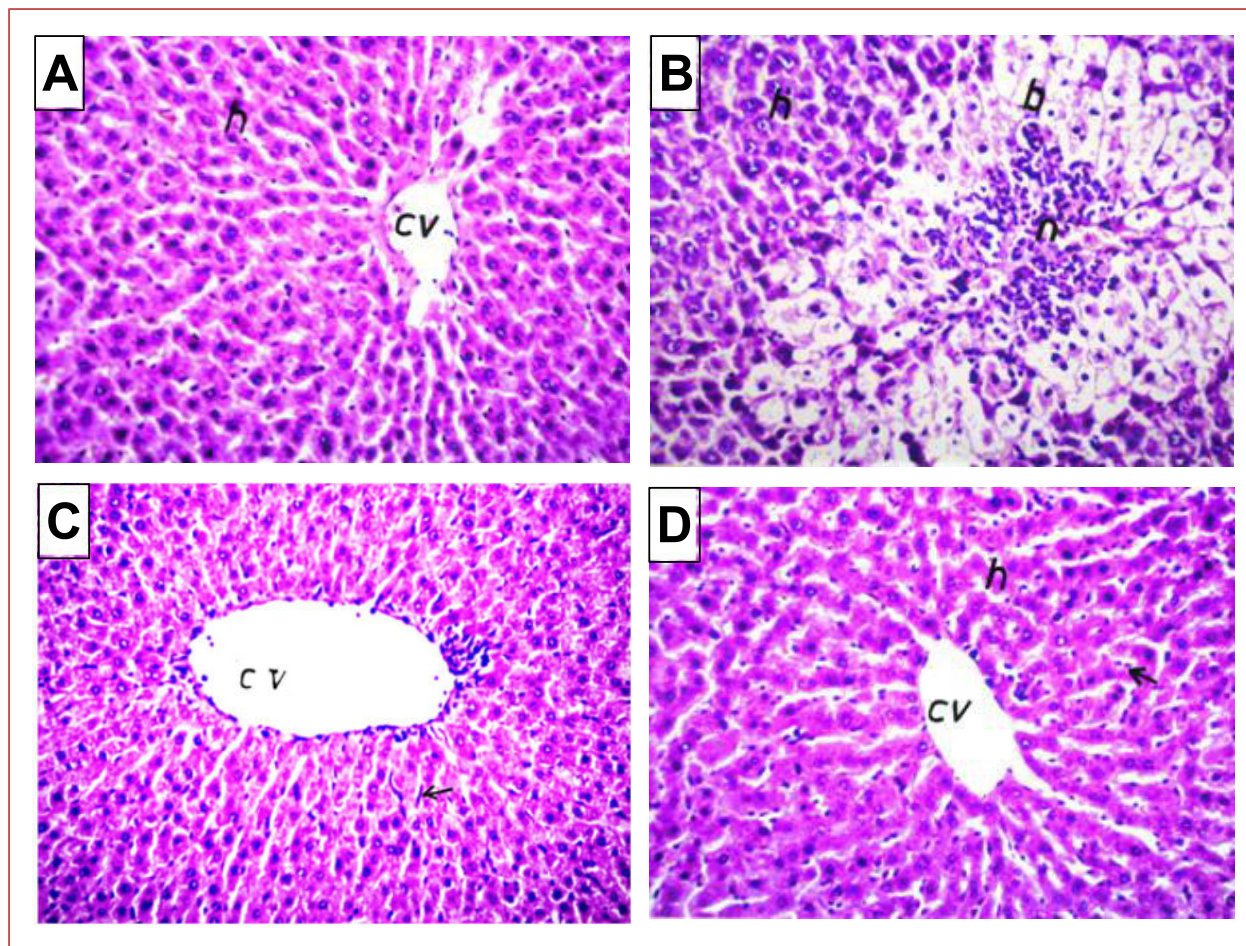


Figure 1: Effects of pretreatment with silymarin and CIE on serum TNF-α level and hepatic caspase-3 and hydroxyproline contents.



**Figure 2: Histology of liver samples of the control, APAP-treated group, silymarin + APAP group and CIE + APAP group.**

(A) Control group: normal histological structure of the central vein (CV) and surrounding hepatocytes (h); (B) Acetaminophen-treated group: severe focal centrilobular necrosis (n) with central pyknotic nuclei surrounded by ballooning degeneration (b) in most of hepatic parenchyma; (C) Silymarin + Acetaminophen-treated group: dilatation in central vein with kuppfer cells proliferation (arrow) between hepatocytes; (D) Chicoricumindicum extract + Acetaminophen-treated group: diffuse kuppfer cells proliferation between hepatocytes. Hematoxylin–eosin staining, magnifications:  $\times 40.m$

### Effect on hydroxyproline content (a marker for collagen accumulation)

Immunoassay technique has shown that, treatment with APAP alone was accompanied by an increase in hepatic hydroxyproline content by about 216% in comparison with the control group. Meanwhile treatment with CIE before and after APAP has shown a normalization effect on this level to about 57% when compared to APAP-treated group (figure 1C).

### Effect on histopathological examination

Examination of liver sections of the control group showed normal architecture of the central vein and the surrounding hepatocytes

(Figure 2A). On the other hand, histopathological analysis of the APAP alone treated rats showed severe focal centrilobular necrosis with central pyknotic nuclei surrounded by ballooning degeneration (Figure 2B). Treatment of the rats with silymarin (Figure 2C) significantly ameliorated the histological parameters induced by APAP. Furthermore, liver sections of the rats pretreated with CIE revealed a preservation of the hepatic integrity against the development of histopathological damage induced by APAP (Figure 2D).

### Discussion

The present study provides evidence of the antihepatotoxic effect of CIE against APAP- induced hepatic injury as compared to the

reference drug silymarin. Increase in serum levels of ALT, AST, ALP, bilirubin, TC, TG, urea and creatinine in addition to, the decrease in serum levels of total protein, albumin and HDL-C as a result of APAP administration are attributable to severe hepatic injury and dysfunction [35-37]. Treatment of rats with CIE before and after acetaminophen alleviated these alterations. These ameliorations can be reported in terms of the ability of CIE to the cell against oxidative insult and indicate preservation of cellular integrity and synthetic capacity of the liver and also suggest an anti-hyperlipidemic effect [38, 39].

The protective effect of CIE can be also attributed to antioxidant activity, whereas the toxic effects of APAP are initiated by (NAPQI) metabolite, which causes GSH depletion, lipid peroxidation, and oxidative stress, causing an imbalance between pro-oxidants and antioxidants in the body [40,41].

GSH is the major non-enzymatic antioxidant present in all cell types and regulates the intracellular redox homeostasis [42]. SOD catalyses the conversion of superoxide anion to O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> which further destroyed by CAT. Thus, the coordinate actions of various cellular antioxidants in cells are critical for effectively detoxifying free radicals. APAP administration declined hepatic antioxidant capacity evidenced by decreased activities of GSH, CAT, and SOD levels, administration of CIE restored their levels and even boosted their activities, preventing oxidative damage to the liver [8].

MDA is a secondary product of oxidative stress formed during lipid peroxidation which may be responsible for tissue injury [43]. In the present study, a significant increase in MDA content was observed with acetaminophen, treatment with CIE significantly decreases its level toward normal, and this finding is in agreement with previous reports [44, 45].

The role of TNF- $\alpha$  and NO in potentiating the liver damage produced by APAP administration has been demonstrated in several studies [46]. Our findings are in accordance with those reports. The present study demonstrated that CIE modulates acetaminophen-induced inflammation by suppressing serum level of TNF- $\alpha$ . In addition, CIE remarkably reduced *in vivo* generation of NO, approaching the control levels.

Apoptosis plays a critical role in acetaminophen-induced hepatic injury, since inhibiting apoptosis prevents the development of acute liver failure [47]. Moreover, hepatic caspase-3 was activated in both wild type and CXCR2 knockout mice within one hour of APAP treatment [48]. Significant increase in caspase-3 activity was found in APAP group, while CIE treatment ameliorates this effect. To the best of our knowledge, this is the first demonstration of a direct effect of CIE on the caspase-dependent apoptotic pathway.

ROS have reported to inactivate collagenase and protease enzymes, this may decrease degradation and increase accumulation of collagen in hepatic tissue [49]. We estimated hepatic collagen content measured as hydroxyproline which used as indicator for tissue repair and healing process [50]. Our data showed for the first time that acetaminophen increased hydroxyproline content in hepatic tissue. Moreover, our results are the first report showed the ameliorated effects of CIE on hydroxyproline content. Pretreatment with CIE also prevented hepatic necrosis; an important histopathological feature of APAP induced hepatotoxicity.

In summary, CIE has proven as potential hepatoprotective agent similar to that of silymarin, which might be attributed to its antioxidant, anti-inflammatory, and caspase-dependent antiapoptotic activities.

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