

ATTENUATION OF CARBON TETRACHLORIDE AND ETHANOL-INDUCED HEPATIC FIBROSIS IN RATS BY *CALLIGONUM COMOSUM* SHOOT EXTRACT

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ABSTRACT

Objective: This study was designed to evaluate the hepatoprotective and antioxidant effects of methanolic extract of *Calligonum comosum* (*C. comosum*) shoots on the hepatic fibrosis induced by carbon tetrachloride and ethanol (CCl₄/ethanol) in rats.

Methods: A liver fibrosis was induced in Sprague Dawley rats by oral administration of CCl₄ (1 ml/kg body weight, twice weekly for 10 weeks) along with ethanol (10% in drinking water 1 week before CCl₄ administration and throughout the experiment). Rats were pretreated with *C. comosum* extract (daily, orally at a dose of 50 mg/kg body weight 1 week before CCl₄ administration). At the end of the experiment, serum, and liver samples were subjected to biochemical investigations. In addition, liver and kidney tissues were evaluated for histopathological changes.

Results: *C. comosum* extract pretreatment significantly reduced CCl₄ - induced elevation in serum levels of aspartate aminotransaminase, alanine aminotransaminase, alkaline phosphatase, total bilirubin, urea, creatinine, and significantly elevated serum contents of total protein and albumin, as well as an improvement in hepatic protein content, albumin/globulin ratio, body weight and relative liver and kidney weights. *C. comosum* extract also significantly increased the hepatic levels of glucose-6-phosphatase, glutathione peroxidase, glutathione-S-transferase, catalase, superoxide dismutase, and glutathione with significant decrease in the contents of malondialdehyde and protein carbonyl. In addition, downregulation in expression of the fibrotic marker matrix metalloproteinase-2 was observed. *C. comosum* extract also ameliorated histopathological changes of CCl₄/ethanol group which further evidenced the biochemical findings.

Conclusion: Our results confirmed that the methanolic extract of *C. comosum* shoots effectively protect against CCl₄/ethanol-induced liver fibrosis, through its antioxidant property.

Keywords: *Calligonum comosum*, Hepatic fibrosis, Carbon tetrachloride, Hepatoprotective effect, Antioxidants, Matrix metalloproteinase-2.

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INTRODUCTION

Hepatic fibrosis is an early finding in most chronic liver diseases and its progression causes liver cirrhosis, and finally, leads to organ failure and death [1]. Cirrhosis is one of the major diseases affecting millions of people worldwide and is the third most common cause of death among people aged 45-65 years [2].

Carbon tetrachloride (CCl₄) is an industrial solvent and one of the most potent hepatotoxins, which is most widely used for experimental induction of liver fibrosis and for screening of hepatoprotective agents [3]. Its hepatotoxic effect is thought to be initiated through its reductive dehalogenation by cytochrome P450 to the highly reactive trichloromethyl radical ([•]CCl₃), which is subsequently converted into a trichloromethyl peroxy radical ([•]OCCl₃) [4,5]. These free radicals initiate the process of lipid peroxidation, which is the most important mechanism in the pathogenesis of hepatocellular damage and enhances production of fibrotic tissue [6].

According to Wang *et al.* [7], reactive oxygen species (ROS) and oxidative stress that have been implicated in the process of liver fibrogenesis, suggested a possible role for antioxidants in preventing or reducing chronic liver damage and fibrosis. There is a resurgence of interest in natural remedies for treatment and/or protection against free radicals causing various ailments including hepatopathy [8,9].

The genus *Calligonum* belongs to the family *Polygonaceae*, which comprises about 80 species and is found in many countries such as Northern Africa, Western Asia, and Southern Europe. *Calligonum*

L'Herit, an Egyptian desert plant, has a reputation in folk medicine as a stimulant, tonic, and astringent [10]. Furthermore, it is used to treat toothache and ophthalmic and stomach problems [11]. Pharmacological and toxicological studies have demonstrated various biological effects of *C. comosum* including antibacterial [12] anti-inflammatory, antinociceptive, antipyretic, gastroprotective [13], and antihypertension [11] effects. Furthermore, *C. comosum* exhibited anticancer activities in mice inoculated with Ehrlich ascites carcinoma cells [14] and dopaminergic effects on haloperidol-induced neuro and hepatotoxicities [15], and finally exhibited significant chemopreventive effects against diethylnitrosamine-induced hepatocarcinogenesis in rats [16]. These biological properties may be attributed to *C. comosum* antioxidative activity, where previous phytochemical analysis of *C. comosum* aerial parts showed that it is rich in polyphenolic compounds, which are responsible for its antioxidant activity [15].

However, little chemical studies were reported on *C. comosum* shoots, and to our knowledge, no reports have recorded the effect of *C. comosum* shoots on hepatic fibrosis. Therefore, the objective of this study is to investigate its hepatoprotective and antioxidant effects on CCl₄/ethanol-induced liver fibrosis.

METHODS**Chemicals**

Streptomycin sulfate was obtained from Pvt. Ltd., (India). 2,4-dinitrophenylhydrazine was obtained from S D Fine-Chem. Ltd. (India). Guanidine hydrochloride, malonaldehydebis (dimethyl acetal), reduced glutathione (GSH), 5,5'-dithiobis-2-nitrobenzoic acid, 1-chloro

2,4-dinitrobenzene, maleic acid, glucose-6-phosphate, gelatin, N,N,N',N'-tetramethylethylenediamine, bisacrylamide, and trizma were purchased from Sigma-Aldrich (St. Louis, MO, USA), Metaphosphoric acid and nitrobluetetrazolium (Fluka, Switzerland) and pyrogallol (Merck, Germany). All other chemicals used were of analytical reagent grade.

Plant material

The shoots of *C. comosum* were collected in April 2012 from international desert road, Dammita, Egypt, authenticated by Dr. Ahmed Mohamed Abd El-Gawad, Science faculty, Mansoura University, Egypt. A voucher sample (No: CP-012-129) was deposited at the herbarium of the National Research Centre.

Extraction and isolation

Shoots of *C. comosum* were shade-dried (800 g), macerated in 70% methanol at room temperature, filtered and dried under vacuum to yield dark black gum (58 g). The dry alcoholic extract was dissolved in distilled water then successively fractionated with n-hexane, methylene chloride, and methanol. The methanol extract (25 g) was subjected to polyamide 6S column chromatography using a water/methanol step gradient afforded 30 fractions that were finally collected to five major fractions C1-C5. Fractions C1-C5 were subjected to different chromatographic techniques afforded 7 pure compounds (1-7). Fraction C5 was subjected to Sephadex LH-20 column eluted with methanol/H₂O (8:2) afforded compounds 1 and 2. Fractions C3 and C4 were separately subjected to preparative paper chromatography eluted by 15% acetic acid then rapid purification on Sephadex LH-20 afforded compounds 3 and 4. Fraction C2 was subjected to Sephadex LH-20 eluted by methanol afforded compounds 5, 6 and 7. The identification of the isolated compounds was occurred using different spectroscopic analysis. Nuclear magnetic resonance spectroscopy was carried out using a JEOL-EX 500 MHz (JEOL Ltd, Japan) in dimethyl sulfoxide-d₆ solvent. Electrospray ionization source spectra were obtained on JEOL JMS-AX 500 (JEOL Ltd, Japan). Ultraviolet (UV) spectra were obtained by OMM 7070E-UV 240 spectrophotometer (Shimadzu, USA).

Phytochemical screening

The methanol extract of *C. comosum* was screened for the presence of various phytoconstituents as described by Dawang and Datup [17] and Mythili and Ravindhran [18].

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay

The antioxidant activity of methanol extract and major (C1-C5) five fractions were measured in terms of radical-scavenging ability, using the stable radical DPPH free radical scavenging activity method with some modification [19]. A volume of 50 µl of a methanolic extract or the major five column fractions C1-C5 solutions of determined concentrations were put into a cuvette. 2 ml of 6×10⁻⁵ mol/L methanolic solution of DPPH was added. The mixtures were well shaken in a vortex for 1 minute and then placed in a dark room. The decrease in absorbance at 517 nm was determined with Jenway 6315 spectrophotometer (Bibby Scientific Ltd, UK) after 1 hr for all samples. The absorbance of the radical without extract was used as control. The amount of sample necessary to decrease the absorbance of DPPH by 50% (IC₅₀) was calculated graphically. The inhibition percentage of the DPPH radical was calculated according to the formula:

$$\text{DPPH inhibition \%} = \frac{(\text{Control absorbance} - \text{Sample absorbance})}{(\text{Control absorbance})} \times 100$$

Acute toxicity study

The mean lethal dose (LD₅₀) of the methanolic extract of *C. comosum* shoots was determined in rats (weighing 120-170 g) using the method described by Chinedu *et al.* [20].

Animals and experimental design

Female Sprague Dawley rats weighing 120-170 g were obtained from the Animal House, National Research Centre, Egypt. Animals were housed under standard laboratory conditions of light (12:12 hrs day-night cycle),

temperature (21±2°C) and relative humidity (55±5%) and were fed with standard laboratory diet and allowed to drink water *ad libitum*. All animal procedures were conducted in accordance with legal requirements appropriate to the species (Guiding Principles for the Care and Use of Laboratory Animals, NIH publication 85-23, revised in 1985) and were approved by the Ethics Committee of the National Research Centre.

Rats were divided randomly into four groups (six rats in each group):

Group I (control group): Rats were orally administrated with olive oil (1 ml/kg body weight) as vehicle twice weekly for 10 weeks.

Group II (*C. comosum* extract group): Rats received *C. comosum* extract as a suspension of water by oral gavage at a dose of 50 mg/kg b. w., daily for 10 weeks.

Group III (CCl₄/ethanol group): Rats were treated with intragastric injections of CCl₄ (diluted 1:1, v/v in olive oil) twice weekly for 10 weeks at dose of 1 ml/kg body weight. They also received 10% ethanol added to their drinking water *ad libitum* before CCl₄ injection by 1 week and continued throughout the experimental period [21].

Group IV (CCl₄/ethanol+*C. comosum* extract group): Rats were treated with *C. comosum* extract preceding CCl₄ administration by 1 week and continued until the end of the experiment. During the experimental period, animals were weighed once weekly to monitor changes in weight and to adjust the dosages of *C. comosum* extract and CCl₄ accordingly.

On the termination day, overnight fasted rats were sacrificed by cervical dislocation under ether anesthesia, blood was collected, and serum was separated by centrifugation at 3000 rpm for 10 minutes at 4°C and stored at -20°C until analysis. Liver and kidney from all animal groups were excised immediately, rinsed with ice-cold saline, dried and weighed. Kidney and part of liver were fixed by immersion in 10% neutral formaldehyde for histopathological investigations. The remaining of the liver was divided into two parts. The two parts were homogenized in normal saline and in 0.1 M phosphate buffer (pH 7.4) to give 10% (w/v) homogenate using TM125 homogenizer (Omni International, USA). The homogenate was centrifuged at 3000 rpm for 15 minutes at 4°C. The supernatant was used for assessment of various biochemical determinations.

Serum biochemical analysis

Serum alkaline phosphatase (ALP), total bilirubin, total protein, albumin, urea, and creatinine were assayed using kits supplied by (Reactivos GPL, Barcelona, Spain). Serum aspartate transaminase (AST) and alanine transaminase (ALT) were assayed using (Biorexfars Diagnostic Limited, UK) kits.

Hepatic biochemical analysis

Saline homogenate was used to estimate glucose-6-phosphatase (G-6-pase) activity according to Swanson assay [22]. Phosphate buffer homogenate was used to estimate protein carbonyl [23] and malondialdehyde (MDA) levels [24] as oxidative stress biomarkers. Furthermore, phosphate buffer was used to estimate antioxidant parameters such as, GSH, glutathione peroxidase (GSH-Px), glutathione-S-transferase (GST), catalase (CAT), and superoxide dismutase (SOD) which were assayed according to the methods of Beutler *et al.* [25], Necheles *et al.* [26], Habig *et al.* [27], Aebi [28], and Minami and Yoshikawa [29], respectively. In addition to this, total protein was estimated according to Lowry *et al.* [30] to calculate the protein content in the hepatic samples.

Matrix metalloproteinase (MMP) zymography

MMP-2 activity was estimated by gelatin zymography as described by Toth *et al.* [31]. 10 µg of liver tissue protein was separated by a 7.5% SDS - polyacrylamide gel electrophoresis using CVS10DSYS-CU mini-protein electrophoresis (Cleaver Scientific Ltd, UK) containing 0.2% gelatin. After electrophoresis, gel was washed twice for 30 minutes in 2.5% Triton X-100 to remove the SDS, then washed in reaction buffer

containing 50 mM Tris-HCl, pH 7.5, 5 mM CaCl₂, 1 μM ZnCl₂. The gels were incubated in a fresh reaction buffer at 37°C overnight. Gelatinolytic activity was visualized by staining the gels with 0.5% Coomassie blue R-250, destained with methanol-acetic acid water (50:10:40 v/v) until clear bands against dark background appeared. The intensity of the bands was assayed by Gel Doc™ XRT molecular imaging system (Bio-Rad, USA).

Histopathological examination

Liver and kidney specimens, fixed in formalin, were processed in paraffin wax then cut into six-micrometer sections. Kidney sections were stained with hematoxylin and eosin (H & E). Liver sections were stained with H & E and Van Gieson's (VG) staining to visualize fibrosis [32]. The hepatic fibrosis was scored according to the Knodell index, as follows: (0) absence of fibrosis; (1) portal fibrous; (2) fibrous portal expansion; (3) bridging fibrosis (portal-portal or portal-central linkage); (4) cirrhosis. Quantitative measurements were achieved using image analyzer (Leica Qwin 500 image) in Image Analyzer Unit, Pathology Department, National Research Centre. To measure the collagen area in slides stained with VG stain, the areas of reactivity were masked and measured in 10 small measuring frames in each specimen.

Statistical analysis

Results are expressed as means±standard error of the mean. Statistical analysis was performed by one-way analysis of variance for multiple comparisons (SPSS, version 14.0) followed by least significant difference test to detect differences between groups. The differences were considered statistically significant at p<0.05.

RESULTS

Phytochemical screening

Phytochemical screening of the *C. comosum* methanolic extract indicated the presence of flavonoids, alkaloids, tannins, steroids, phenols, terpenoids, and essential oil.

Chemical characterization of *C. comosum* extract

The chemical characterization of the methanol extract afforded; quercetin (1), kaempferol (2), kaempferol 3-O-α-l-rhamnopyranoside (3), quercetin 3-O-α-l-rhamnopyranoside (4) in addition to three phenolic acids: Gallic acid (5), methyl gallate (6), and p-comaric acid (7). Compounds 1, 2, 5, 6 and 7 were reported for the first time from *C. comosum*. All compounds were identified according to comparison of their spectral data with those reported previously.

DPPH radical scavenging activity of *C. comosum* extract and column fractions

Methanol extract of *C. comosum* showed the strongest scavenging activity followed by fraction C2 with IC₅₀ 68.2±0.322 and 80.6±0.25, respectively (Fig. 1). The significant antioxidant activity of all tested fractions may be attributed to the presence of flavonoids and phenolic acids.

Effect of *C. comosum* extract on body weight, liver and kidney weights and relative weights of liver and kidney

The average body weight of both control and *C. comosum* extract groups increased regularly till the end of the experiment. However, there was an obvious decrease in the weight of CCl₄/ethanol group after week 6 up to the last week as shown in Fig. 2, with 17% increase in the final mean body weight in CCl₄/ethanol group comparing to 50% increase in the control group as shown in Table 1. On contrary, pretreatment with *C. comosum* extract in Group IV caused gradual increase in the body weight till week 6, then body weight was almost stable till the end of the experiment with 28% increase in the final mean body weight as shown in Table 1.

As shown in Table 1, administration of CCl₄/ethanol significantly elevated liver and kidney weight as compared to control group, producing 64% and 48% increase in relative liver weight (RLW) and relative kidney weight (RKW), respectively. *C. comosum* extract

pretreatment significantly reduced liver and kidney weight and their relative weights (Table 1).

Effect of *C. comosum* extract on hepatic and renal functions

As shown in Table 2, CCl₄/ethanol markedly increased serum levels of AST (3.71-fold), ALT (14.33-fold), ALP (6.5-fold) and total bilirubin (2.3-fold) when compared to the control group. On the other hand, levels of serum protein, albumin and A/G ratio in CCl₄/ethanol group were significantly lower than that in the control group by 21.8%, 40.9% and 32.3%, respectively. Moreover, CCl₄/ethanol treatment significantly increased the serum levels of urea (58.7%) and creatinine (2.4-fold) as compared with the control group. *C. comosum* extract significantly improved all the CCl₄ - induced alterations in both hepatic and renal injury markers. *C. comosum*+CCl₄/ethanol group recorded reduction by 50, 60, 53, 23, 20 and 40% for AST, ALT, ALP, total bilirubin, urea and creatinine, respectively, comparing to CCl₄/ethanol group. Furthermore, serum protein, albumin and A/G ratio levels were increased by 13, 32 and 22%, respectively, relative to CCl₄/ethanol group.

Effect of *C. comosum* extract on G-6-pase and hepatic protein

CCl₄/ethanol-induced rats recorded a significant decrease in G-6-pase by 74% compared to control (Fig. 3). Contradictory, pretreatment with *C. comosum* extract in Group IV recorded increase in G-6-pase activity by 42% compared to CCl₄/ethanol group. Moreover, CCl₄/ethanol treatment in Group III rats resulted in a significant decrease in hepatic protein content by 40% compared to the control group. Pretreatment with *C. comosum* extract attenuated such decrease of hepatic protein by 21.5% (Table 3).

Effect of *C. comosum* extract on oxidative damage biomarkers

The levels of protein carbonyl (marker of protein oxidation) and MDA (marker of lipid peroxidation) in CCl₄/ethanol group have significantly increased by 2.4- and 3.6-fold, respectively, as compared to control level. On the other hand, the treatment of CCl₄/ethanol-induced rats with *C. comosum* extract significantly reduced the level of protein carbonyl (33%) and MDA (46%) in comparing to CCl₄/ethanol group (Table 3).

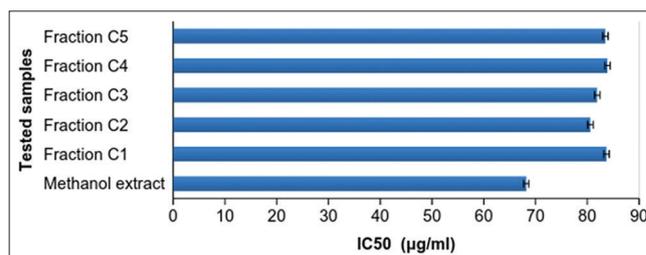


Fig. 1: DPPH radical scavenging activity of *C. comosum* extract and column fractions

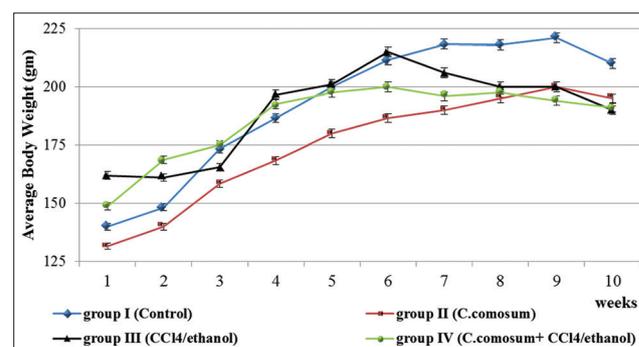


Fig. 2: Comparison of weekly body weight change between groups over the experimental period. Values are expressed as mean±SEM of six rats in each group

Table 1: Effect of *C. comosum* extract on body weight, liver and kidney weights and relative weight of liver and kidney

Groups parameters	(I) Control	(II) <i>C. comosum</i>	(III) CCl ₄ /ethanol	(IV) <i>C. comosum</i> +CCl ₄ /ethanol
Body weight gained (g)	69.54±10.46	63.34±7.14 ^b	27.89±8.02 ^a	41.09±9.78 ^a
Liver weight (g)	5.74±0.22	4.91±0.39 ^b	8.95±0.45 ^a	6.28±0.29 ^b
RLW	2.86±0.11	2.69±0.13 ^b	4.68±0.14 ^a	3.31±0.17 ^{ab}
Kidney weight (g)	1.16±0.04	1.09±0.04 ^b	1.65±0.06 ^a	1.23±0.01 ^b
RKW	0.58±0.02	0.60±0.01 ^b	0.86±0.05 ^a	0.64±0.02 ^b

Values are expressed as mean±SEM of six rats in each group. ^aStatistical significance at p<0.05 as compared with control group. ^bStatistical significance at p<0.05 as compared with CCl₄/ethanol group. *C. comosum*: *Calligonum comosum*, RLW: Relative liver weight, RKW: Relative kidney weight

Table 2: Effect of *C. comosum* extract on hepatic and renal functions

Groups parameters	(I) Control	(II) <i>C. comosum</i>	(III) CCl ₄ /ethanol	(IV) <i>C. comosum</i> +CCl ₄ /ethanol
AST (U/L)	33.16±2.08	27.00±1.57 ^b	123.33±15.20 ^a	61.00±3.77 ^{ab}
ALT (U/L)	16.16±2.18	15.83±1.40 ^b	231.66±19.73 ^a	91.66±5.42 ^{ab}
ALP (U/L)	49.39±3.49	54.40±2.65 ^b	321.38±16.60 ^a	152.03±11.67 ^{ab}
Total bilirubin (mg/dl)	0.32±0.02	0.31±0.02 ^b	0.75±0.04 ^a	0.58±0.03 ^{ab}
Total protein (g/dl)	9.49±0.16	9.37±0.16 ^b	7.42±0.34 ^a	8.40±0.22 ^{ab}
Albumin (g/dl)	3.74±0.21	3.50±0.04 ^b	2.21±0.13 ^a	2.93±0.08 ^{ab}
A/G ratio	0.65±0.05	0.60±0.01 ^b	0.44±0.05 ^a	0.54±0.04
Urea (g/dl)	47.70±2.46	50.92±2.25 ^b	75.70±5.54 ^a	59.85±3.59 ^{ab}
Creatinine (mg/dl)	0.72±0.05	0.68±0.04 ^b	1.76±0.08 ^a	1.05±0.04 ^{ab}

Values are expressed as mean±SEM of six rats in each group. ^aStatistical significance at p<0.05 as compared with control group. ^bStatistical significance at p<0.05 as compared with CCl₄/ethanol group. AST: Aspartate transaminase, ALT: Alanine transaminase, ALP: Alkaline phosphatase. *C. comosum*: *Calligonum comosum*

Table 3: Effect of *C. comosum* extract on oxidative damage biomarkers

Groups	Total protein (mg/g tissue)	Protein carbonyl (nmol/mg protein)	MDA (nmol/mg protein)
(I) Control	280.26±25.44	0.53±0.06	4.35±0.41
(II) <i>C. comosum</i>	284.91±26.03 ^b	0.49±0.47 ^b	4.86±0.38 ^b
(III) CCl ₄ /ethanol	168.41±8.8 ^a	1.28±0.1 ^a	15.88±1.7 ^a
(IV) <i>C. comosum</i> +CCl ₄ /ethanol	220.01±9.82 ^a	0.86±0.09 ^{ab}	8.65±0.82 ^{ab}

Values are expressed as mean±SEM of six rats in each group. ^aStatistical significance at p<0.05 as compared with control group. ^bStatistical significance at p<0.05 as compared with CCl₄/ethanol group. MDA: Malondialdehyde. *C. comosum*: *Calligonum comosum*

Effect of *C. comosum* extract on hepatic antioxidants

CCl₄/ethanol treatment markedly decreased the content of liver GSH as well as antioxidant enzymes. As shown in Fig. 4, CCl₄/ethanol group recorded a significant decrease in the levels of hepatic GSH (78%), GSH-Px (40%), GST (38%), CAT (45%), and SOD (47%) when compared with control group. Treatment with *C. comosum* extract in Group IV significantly improved GSH, GSH-Px, GST and SOD by 34, 21, 20 and 22%, respectively, compared to CCl₄/ethanol group. Interestingly, *C. comosum* extract treatment restored CAT activity to near control level.

Effect of *C. comosum* extract on MMPs activity

As shown in Fig. 5a, gelatin zymography revealed that various rat groups expressed only the latent form of MMP-2 at about 72 kDa. Control rats expressed the least MMP-2 activity. Treatment with CCl₄/ethanol resulted in a clearly increased MMP-2 expression in Group III control, while administration of *C. comosum* extract tended to reduce such expression in *C. comosum* extract+CCl₄/ethanol group. Furthermore, densitometric analysis of zymography bands revealed a significant increase (4.16-fold) in MMP-2 gelatinolytic activity of CCl₄/ethanol group compared to controls. Pretreatment with *C. comosum* extract produced 34% reduction in activity of Group IV in comparison to CCl₄/ethanol group (Fig. 5b).

Effect of *C. comosum* extract on liver morphology

Morphological observations showed that liver of CCl₄/ethanol injured rats lost the normal reddish-brown color and changed to more or less light brown. In addition, the liver was enlarged in size and lost its smoothly surface. Moreover, pale yellow spots and large patches were scattered on the liver surface. These morphological changes were reversed to normal by pretreatment with *C. comosum* extract (Fig. 6).

Histopathological examination of liver

In case of liver H & E staining, microscopic examination of liver sections of control and *C. comosum* extract groups showed normal architecture of hepatic lobules and hepatocytes (Fig. 7a and b). However, rats given CCl₄/ethanol showed dissection nodules and fibrous septa with effacement of the lobular architecture (Fig. 7c) and revealed fatty degeneration which forms signet ring cells (Fig. 7d). On the other hand, liver sections of *C. comosum* extract+CCl₄/ethanol group showed in some cases normal architecture of hepatic lobules and hepatocytes (Fig. 7e) and in the other cases showed necrotic hepatocytes and fatty changes (Fig. 7f).

In case of liver VG staining, the control and *C. comosum* extract groups showed a normal distribution of collagen deposition (Fig. 8a, b and f). However, rats received CCl₄/ethanol the liver exhibited a progress increase in collagen accumulation, with periportal fibrosis characterized by portal-portal fibrous septa surrounding the hepatic lobules which resulted in distorted tissue architecture (Fig. 8c). On the other hand, *C. comosum* extract pretreatment caused septal collagen deposition to disappear or to become thinner (Fig. 8d and e). The quantitative histological analysis presented in Table 4, revealed that the degree of liver fibrosis in CCl₄/ethanol group ranged from moderate fibrosis to cirrhosis and collagen levels were significantly increased as compared with control rats. Alternatively, *C. comosum* extract showed marked improvement in the fibrosis degree and significant decrease in collagen levels as compared to CCl₄/ethanol injured group.

Histopathological examination of kidney

The kidney sections from the control and *C. comosum* extract groups showed normal architecture of glomeruli, tubules, and capillaries

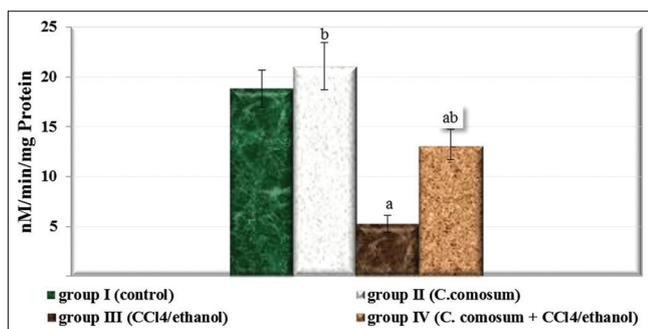


Fig. 3: Effect of *C. cosomum* extract on hepatic G-6-pase enzyme in CCl₄/ethanol-treated rats. Values are expressed as mean±SEM of six rats in each group. ^ap<0.05 is significantly different from control group; ^bp<0.05 is significantly different from CCl₄/ethanol group

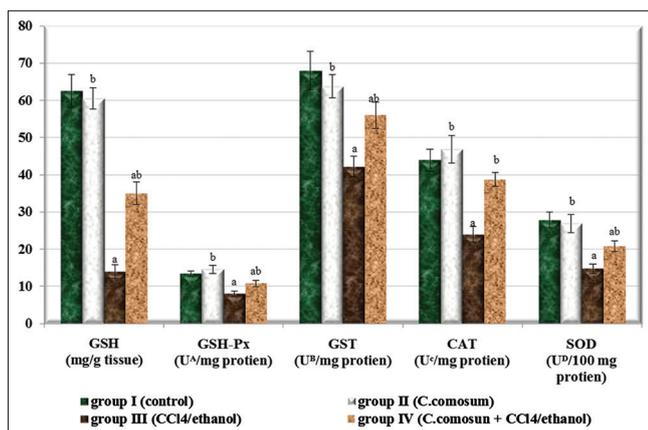


Fig. 4: Effect of *C. cosomum* extract on antioxidant levels in CCl₄/ethanol-treated rats. Values are expressed as mean±SEM of six rats in each group. ^ap<0.05 is significantly different from control group; ^bp<0.05 is significantly different from CCl₄/ethanol group. U^A: µg of GSH oxidized/minute/mg protein, U^B: nmoles of 1-chloro 2,4-dinitrobenzene-GSH conjugate formed/minute/mg protein, U^C: µmole of H₂O₂ decomposed/minute/mg protein, U^D: 50% inhibition of nitroblue tetrazolium reduction for 100 mg protein

(Fig. 9a and b). Conversely, CCl₄ administration caused prominent histopathological damage in the kidney. The CCl₄/ethanol injured rats showed glomerular atrophy, tubular degeneration, epithelium necrosis of the tubules, interstitial edema, and congestion in capillary loops (Fig. 9c). While pretreatment with *C. cosomum* extract preserved normal histological form of the kidney (Fig. 9d). The histopathological changes were graded and summarized in Table 5.

DISCUSSION

Hepatic fibrosis is a pathological progression associated with abnormal proliferation of connective tissue in response to chronically damaged liver tissue. It is often caused by hepatitis, alcoholism and other potentially damaging toxins [33,34]. Hepatic cirrhosis would be developed if hepatic fibrosis treatment was delayed [35]. Chronic alcohol consumption is a major risk factor for development of hepatic fibrosis [36]. Administration of ethanol with repetitive CCl₄ ingestion could be more effective in maintaining the fibrotic pattern because ethanol increases activation of cytochrome P450 [37], resulting in greater liver damage than with either agent alone [38]. Various plant extracts have been widely applied to treat chronic liver diseases, including chronic hepatic inflammation and fibrosis [39].

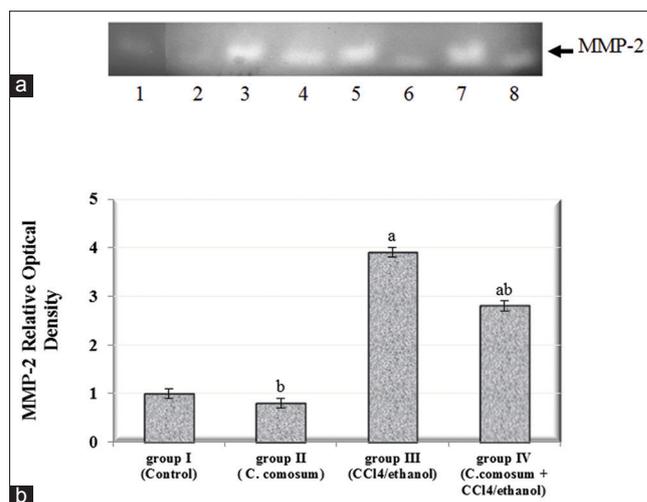


Fig. 5: Gelatin zymogram of liver specimens from different animal groups. (a): Expression of 72 kDa MMP-2 in equal protein quantities of hepatic homogenates from control group (lane 1), *C. cosomum* extract group (lane 2), CCl₄/ethanol group (lane 3-5), and *C. cosomum* extract+CCl₄/ethanol group (lane 6-8). (b): Densitometric analysis of bands detected in gelatin zymograms. The bar graph shows averages of band intensity of MMP-2 (mean±SEM). ^ap<0.05 is significantly different from control group; ^bp<0.05 is significantly different from CCl₄/ethanol group

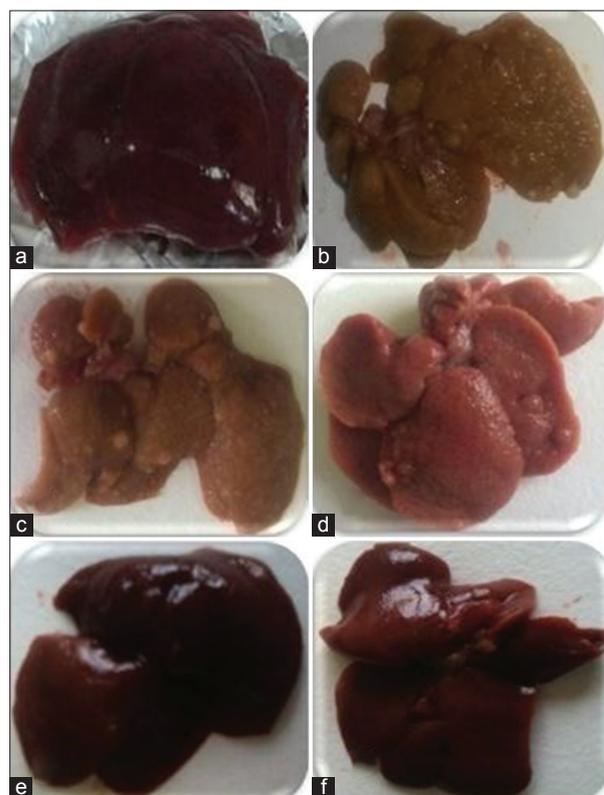


Fig. 6: Morphological structure of normal and CCl₄/ethanol treated livers. (a) Normal liver, (b-d) CCl₄/ethanol-injured livers, (e and f) *C. cosomum* extract+CCl₄/ethanol treated livers

In this study, CCl₄/ethanol-treated rats showed a significant decrease in body weight as well as significant increase in RLW compared to control. These results are in agreement with those reported previously by Soni et al. [40]. Furthermore, a significant increase in RKW was shown. Such

Table 4: Liver fibrosis scoring, collagen % and collagen area in different groups

Groups	Fibrosis grade					Collagen %	Collagen area μm^2
	(0)	(1)	(2)	(3)	(4)		
(I) Control	6	0	0	0	0	0.91	108.84±31.56
(II) <i>C. comosum</i>	6	0	0	0	0	1.44	172.15±53.4
(III) CCl ₄ /ethanol	0	0	1	2	3	11.02	1309.3±537.26 ^a
(IV) <i>C. comosum</i> +CCl ₄ /ethanol	3	2	1	0	0	2.21	263.52±49.44 ^b

Data of collagen area is presented as mean±SEM of six rats in each group. ^aStatistical significance at $p<0.05$ as compared with control group. ^bStatistical significance at $p<0.05$ as compared with CCl₄/ethanol group. *C. comosum*: *Calligonum comosum*

Table 5: Effect of *C. comosum* extract on CCl₄/ethanol-induced histopathological alterations in kidney

Groups	Glomerular atrophy	Tubular degeneration	Epithelium necrosis	Interstitial edema	Capillary congestion
(I) Control	*	*	*	*	*
(II) <i>C. comosum</i>	*	*	*	*	*
(III) CCl ₄ /ethanol	+++	+++	+++	+++	+++
(IV) <i>C. comosum</i> +CCl ₄ /ethanol	*	*	*	*	*

(*): Normal; (+++): Severe changes. *C. comosum*: *Calligonum comosum*

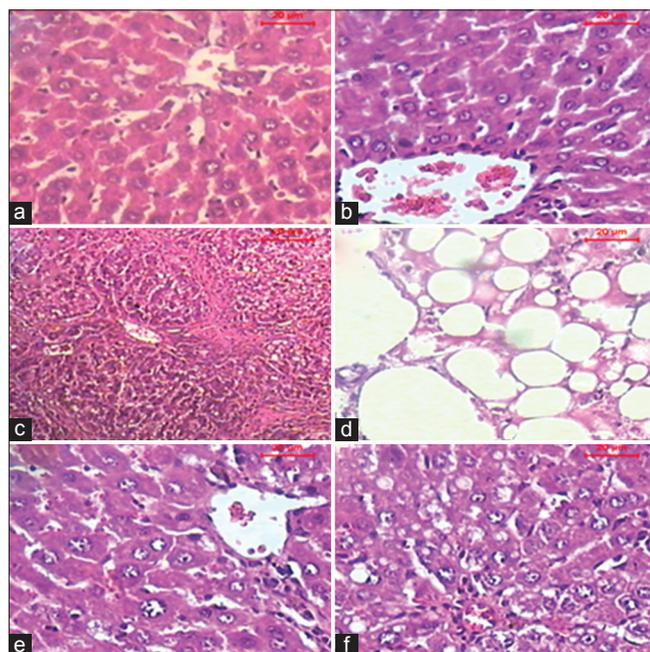


Fig. 7: H & E staining of rat liver sections; (a and b) control and *C. comosum* extract group shows normal architecture of a hepatic lobule, (c): CCl₄/ethanol group shows the presence of dissection nodules and fibrous septa with effacement of the lobular architecture, (d): CCl₄/ethanol group shows fatty degeneration. Notice the large vacuoles within the liver cells which occupy a large proportion of the cell's volume and push the cytoplasm up against the cell membrane, forming "signet ring cells;" (e): (*C. comosum* extract+CCl₄/ethanol) group shows normal architecture of a hepatic lobule and hepatocytes; (f): (*C. comosum* extract+CCl₄/ethanol) group shows some necrotic hepatocytes and fatty changes (Scale Bar: 20 μm)

changes in body weight, RLW and RKW could be attributed to CCl₄ toxic effect. An increase in body weight as well as a decrease in liver weight, kidney weight, RLW and RKW was observed by *C. comosum* extract pretreatment.

Serum AST, ALT, and ALP are the most sensitive markers employed in the diagnosis of hepatic damage [41]. In this study, the hepatic damage produced by CCl₄/ethanol treatment was evident by an elevation in the serum marker enzymes as compared to control. This elevation was

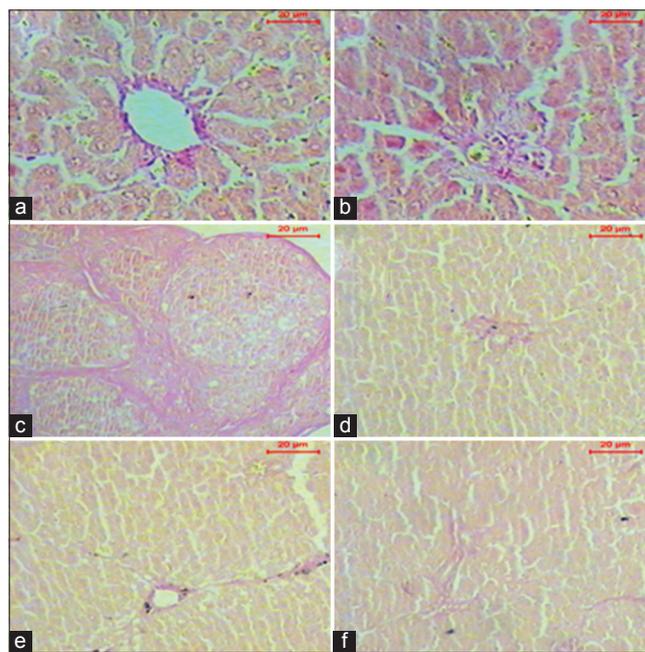


Fig. 8: VG staining of rat liver sections. (a and b) Control rats showing normal distribution of collagen deposition in the hepatic lobule and in the portal area respectively; (c): CCl₄/ethanol group showing a progress increase in collagen accumulation, with portal-portal fibrous septa surrounding the hepatic lobules; (d and e) (*C. comosum* extract+CCl₄/ethanol) group showing complete disappearance or marked reduction in septal collagen deposition respectively; (f): *C. comosum* extract group shows normal distribution of collagen deposition in the hepatic lobule and portal area (Micron bar: 20 μm)

attributed to the leakage of enzymes from cells into the circulatory system due to altered permeability and loss of functional integrity of liver membrane as a result of severe hepatic injury [42,43]. The results obtained in this work are similar to findings of Biswas *et al.* [44]. CCl₄ undergoes reductive metabolism by the hepatic cytochrome P450 into a highly reactive [•]CCl₃, which is converted into peroxy radical ([•]OCCl₃) in the presence of oxygen [45]. These activated radicals induced peroxidative degradation of membrane lipids of endoplasmic reticulum rich in polysaturated fatty acids leads to the formation of lipid peroxides that cause damage to the membrane and alter cellular

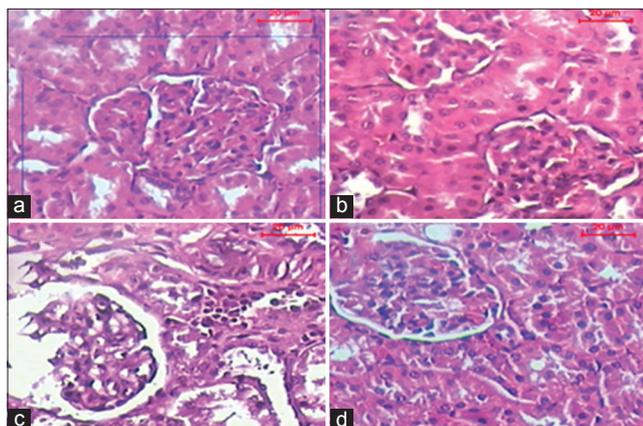


Fig. 9: Photomicrographs of kidney sections. (a and b) Control and *C. comosum* extract groups showing normal architecture of glomeruli, tubules and capillaries; (c) CCl_4 /ethanol injured rat shows glomerular atrophy, tubular degeneration, epithelium necrosis of the tubules, interstitial edema and congestion in capillary loops, (d): (*C. comosum* extract+ CCl_4 /ethanol) group showing normal histological form of the kidney (HE, Scale Bar: 20 μm)

function [46]. Pretreatment with *C. comosum* extract significantly attenuated the elevated activities of serum marker enzymes. This indicates that *C. comosum* extract preserved the structural integrity of the liver cell membrane due to its phytochemical phenolics. Yin *et al.* [47] demonstrated that phytochemical phenolic compounds restore the functional integrity of the hepatocytes membrane by acting against free radical mediated damages, consequently improve liver injury induced by CCl_4 .

Total bilirubin level was elevated in CCl_4 /ethanol treated rats, possibly as a result of increased production, decreased uptake by the liver or decreased conjugation [48]. However, serum bilirubin level was significantly reduced by *C. comosum* extract pretreatment which is indicative of reversal of liver damage by the extract.

Diminution of serum proteins and albumin levels induced by CCl_4 is a further indication of hepatocellular injury [49,50]. The results of this study demonstrated that CCl_4 /ethanol administration significantly decreased serum levels of total protein and albumin. Pattanayak and Priyashree [51] attributed these reductions to the initial damage in the endoplasmic reticulum by CCl_4 activated radicals which impairs the synthetic capacity of the liver that results in decreasing the protein synthesis. On the other hand, serum protein and albumin levels significantly restored near normal by *C. comosum* extract suggesting the hepatoprotective effect of the extract against the deleterious effects of CCl_4 on the liver. This hepatoprotective effect may be attributed to the presence of polyphenol in the extract and their membrane stabilizing activity [52,53]. Thus, *C. comosum* extract improved the decline in liver synthetic function caused by CCl_4 . Stimulation of protein synthesis has been regarded as a contributory hepatoprotective mechanism which enhances the regeneration process and the production of liver cells [54]. There was a significant decline in the A/G ratio in CCl_4 /ethanol group as compared to control. This result is in agreement with Andritoiu *et al.* [55]. An improvement of the A/G ratio revealed in rats pretreated with *C. comosum* extract was due to an increase of serum albumin with a relative decrease of the globulin values.

Administration of CCl_4 causes nephrotoxicity as indicated by a significant elevation in serum level of urea and creatinine. From this study, it is evident that elevation in serum urea and creatinine levels in CCl_4 /ethanol intoxicated rats can be attributed to the damage of nephron structural integrity; this result is in agreement with Khan and Siddique [56]. On other hand, pretreatment with *C. comosum* extract

significantly reduced the elevation in serum urea and creatinine levels, indicating its protective effect.

The depression of liver microsomal G-6-pase activity, which is a marker of endoplasmic reticulum membrane integrity, is one of the early features of CCl_4 intoxication [57]. A significant decline in G-6-pase activity was shown in CCl_4 /ethanol intoxicated rats compared to control group. Loss of G-6-pase activity has been reported during increased lipid peroxidation [58]. Pretreatment with *C. comosum* extract significantly elevated the activity level of G-6-pase by improving the structural integrity of the hepatocyte endoplasmic reticulum membrane.

A significant decrease in hepatic protein content was also observed in CCl_4 /ethanol-treated rats compared to the control group. Such decrease in protein content following administration of CCl_4 may be due to defects in protein biosynthesis as well as disruption and disassociation of polyribosomes from endoplasmic reticulum [59]. *C. comosum* extract pretreatment attenuated the decreased hepatic protein level.

Oxidation of protein's side chains by ROS generates carbonyl moieties that is identified as an early marker of ROS-mediated protein oxidation and is used as a measure of protein damage [60]. The accumulation of oxidized proteins may be an early indication of CCl_4 induced liver damage [61]. The hepatic level of protein carbonyl in CCl_4 /ethanol-treated group was significantly increased compared to that in the control group but was significantly decreased in *C. comosum* extract pretreated group, suggesting an antioxidant protection against protein oxidation.

The major cellular mechanisms involved in CCl_4 hepatotoxicity are the increase in free-radical production and lipid peroxidation [62]. MDA is one of the main lipid peroxidation products, which was assessed as an indicator of lipid peroxidation in oxidative liver damage [63]. Saad *et al.* [62] have reported a close relationship between lipid peroxidation and fibrogenesis in CCl_4 treated rats. CCl_4 /ethanol intoxicated rats showed a significant increase in MDA level in comparison to control group. Our finding revealed that pre-administered *C. comosum* extract significantly inhibited the increase of liver MDA level. The decrease in MDA level suggested the ameliorative effect of *C. comosum* extract on liver fibrosis induced by CCl_4 /ethanol treatment.

Biological systems are supported with various defense mechanisms against environmental and internal stresses [64]. Antioxidant enzymes (such as CAT, SOD, GSH-Px, and GST) are the endogenous first line of defense against damage by oxidative stress. They are actively involved in scavenging of free radicals to maintain the steady state level and consequently integrity and functionality of cells [65,66]. GSH, the main non-enzymatic antioxidant participates directly in the neutralization of free radicals and ROS [67]. In this study, CCl_4 /ethanol treatment significantly reduced the total content of GSH, as well as the hepatic activity levels of antioxidant enzymes, GSH-Px, GST, CAT, and SOD. Our results are in agreement with Al-Dalaen *et al.* [68]. Pretreatment with *C. comosum* extract significantly increased the GSH content, GSH-Px, GST, CAT, and SOD activity levels. Thus, *C. comosum* extract exerts its hepatoprotective effect via increasing activity of endogenous antioxidant defense system possibly due to its free radical scavenging activity. El-Sayed *et al.* [69] reported that during periods of increased oxidative stress, supplementation with exogenous antioxidants increases the antioxidant defense system activity by stimulating synthesis of antioxidant enzymes.

Liver fibrosis also characterized by excess accumulation of collagen-rich extracellular matrix (ECM), which destroys normal liver architecture and finally leads to cirrhosis. At first sight, MMPs, enzymes degrade such fibrillar collagens, might be expected to be under-expressed in fibrosis. However, a number of studies have confirmed that CCl_4 may lead to hepatic fibrosis through upregulating the expressions of some MMPs [70,71]. Arthur and Baranova *et al.* [72,73] reported that MMP-2 increases during liver injury and contribute to liver fibrogenesis. In Agreement with previous data [72,74], we showed that fibrotic rats

had a significant increase in hepatic MMP-2 activity, whereas it is hardly detectable in normal livers. Planagumà *et al.* and Han [75,76] suggested that in response to various hepatic toxins liver starts an immense production of cytokines and growth factors, which induce MMPs expression by hepatic stellate cells (HSCs), including MMP-2. MMP-2 promotes transdifferentiation of inactive HSCs through degrading the normal ECM. Activated HSCs have the ability to synthesize collagen I, which in turn stimulates extra MMP-2 activation, thereby amplifying the transdifferentiation process and finally leading to liver fibrosis. *C. comosum* extract pretreatment significantly reduced hepatic MMP-2 gelatinolytic activity in CCl₄/ethanol-treated rats. Thereby, *C. comosum* extract can reduce liver fibrosis by inhibiting MMP-2 levels.

CONCLUSION

It could be concluded that the phytochemical screening of *C. comosum* extract confirmed the presence of antioxidant compounds such as flavonoids and phenolic acids. The significant antioxidant activity of these compounds may contribute to the protection of the hepatic cells against CCl₄-induced hepatic oxidative damage. Our results suggest that the methanolic extract of *C. comosum* shoots attenuates the fibrogenesis through its ability to stabilize cell membranes by scavenging free radicals, and consequently, avoid the propagation of lipid peroxide. Hence, the mechanism of *C. comosum* extract hepatoprotection may be due to its antioxidant activity. However, further studies are needed to better understand the mechanism of action of the *C. comosum* shoots' methanolic extract.

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