Calligonum comosum extract inhibits diethylnitrosamine-induced hepatocarcinogenesis in rats

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Abstract. *Calligonum comosum* (*C. comosum)* is an Egyptian desert plant that contains polyphenol antioxidants. The present study examined the chemopreventive effect of an extract of *C. comosum* in a rat model of hepatocarcinogenesis. Male Wistar rats (n=40) were administered 100 mg/kg diethylnitrosamine (DEN) by intraperitoneal (i.p.) injection once a week for 3 weeks. Subsequently, depending on whether the rats received further administration of 0.8 mg/kg carbon tetrachloride (CCl_4) i.p. once a week for 7 weeks and 100 mg/kg *C. comosum* extract in their diet for 7 weeks, the rats were divided into four groups as follows: Group 1, treatment with DEN alone; group 2, treatment with DEN and *C. comosum* extract; group 3, treatment with DEN and CCl_4; and group 4, treatment with DEN, CCl_4 and *C. comosum* extract. The supplementation of *C. comosum* extract significantly suppressed the elevation in serum liver enzyme levels, including aspartate aminotransferase, alanine transaminase and γ-glutamyl transferase, and reduced the degree of oval cell proliferation induced by DEN and CCl_4. In addition, *C. comosum* extract significantly decreased the number and area of glutathione S-transferase placental form-positive preneoplastic hepatic foci induced by DEN, with or without CCl_4 treatment. To the best of our knowledge, the present study is the first to provide definitive evidence of the hepatoprotective and chemopreventive effects of *C. comosum*.

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Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CCl_4, carbon tetrachloride; DEN, diethylnitrosamine; DPPH, 1,1-diphenyl-2-picrylhydrazyl; GGT, gamma-glutamyltransferase; GST-P, glutathione S-transferase placental form; HCC, hepatocellular carcinoma; HPLC, high-performance liquid chromatography

Key words: Calligonum comosum, diethylnitrosamine, liver, rat, glutathione S-transferase placental form, oval cell proliferation

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies in humans, with high cancer-associated mortality rates (1). Prominent risk factors associated with the development of HCC include chronic alcohol consumption, the ingestion of aflatoxin B1-contaminated food and hepatitis B and C viral infections (1). The incidence of HCC among geographical populations tends to reflect the local prevalence of its etiological risk factors. For instance, a high rate of HCC is observed in Egypt, where there is a high prevalence of hepatitis B and C viral infections (2). The relatively high exposure to aflatoxin B1 and pesticides in Egypt are also suspected to contribute to the high prevalence of HCC (2). Since it can be challenging to rapidly eliminate risk factors, the application of HCC chemopreventive therapy, particularly in carriers of the hepatitis B and C viruses, has clear benefits (3). Locally available plants may provide accessible and economically feasible sources of herbal HCC preventives.

Calligonum comosum (*C. comosum)* is an Egyptian desert plant that belongs to the Polygonaceae family and is known locally as 'arta'. *C. comosum* is used as a smokeless source of firewood and for tanning leather. In addition, it is used in traditional folkloric medicine to treat abdominal ailments and toothache (4). Several studies have demonstrated a number of biological effects of *C. comosum* extract in animal models, including anti-inflammatory activities, antigastric ulcer properties (5) and antihyperglycemic activities in rats (6). Furthermore, *C. comosum* exhibited anticancer activities in mice inoculated with Ehrlich ascites carcinoma cells (7). The biological properties of *C. comosum* may be associated with its antioxidative activity, which has been consistently demonstrated *in vitro* (7,8). In accordance with this hypothesis, several antioxidative flavonoids have been isolated from the ethyl acetate fraction of *C. comosum* methanolic extract, including (+)-catechin, dehydrodicatechin A, kaempferol-3-*O*-rhamnopyranoside,

quercetin (quercetin-3-*O*-rhamnopyranoside), isoquercitrin (quercetin-3-*O*-glucopyranoside),kaempferol-3-*O*-glucuronide and mequilianin (quercetin-3-*O*-glucuronide) (7). The antioxidative activity of the *C. comosum* extract has also been demonstrated *in vivo*. In particular, the extract has been identified to suppress the accumulation of malondialdehyde, one of the final decomposition products of lipid peroxidation, in brain and liver tissues of haloperidol-treated rats (8). On the basis of these findings, the present study aimed to investigate whether the *C. comosum* extract may reduce the formation of glutathione S-transferase placental form-positive (GST-P) preneoplastic hepatic foci in rats exposed to diethylnitrosamine (DEN), with or without administration of carbon tetrachloride (CCl_4). In addition, the current study evaluated whether the extract may be a potential chemopreventive agent in hepatocarcinogenesis.

Materials and methods

Plant materials and extract preparation. Aerial parts of *C. comosum* were obtained from Cairo-Suez Road (Cairo, Egypt) in April 2012. A herbarium specimen of the plant was identified by Dr. Mohamed Abdel-Aleem (Department of Botany, Faculty of Science, Cairo University, Cairo, Egypt), and a sample was kept in the Herbarium of the Department of Pharmacognosy (Faculty of Pharmacy, Cairo University; specimen number, CC-01204). The plant materials were air-dried, ground to a powder and then kept in dark, airtight, closed containers prior to extraction. In order to prepare the extract, a 750-g sample was extracted with three 2-liter volumes of absolute methanol in an Ultra Turrax T25 homogenizer (IKA®-Werke GmbH & Co. KG, Staufen, Germany). The solvent was then distilled under reduced pressure and the extracts were lyophilized, yielding a 69-g dry extract that was stored at 4˚C.

Phytochemical analysis. The phenolic content of the *C. comosum* extract was measured using the Folin-Ciocalteu method (9) with the modifications proposed by Abdel-Sattar *et al* (8). The total phenolic content was determined by measuring the absorbance at 685 nm using gallic acid as a reference standard (0.02-0.30 mg/ml) and the result was expressed in milligram equivalents of gallic acid per gram of dry extract. In addition, the total flavonoid content in the extract was determined using a previously described aluminum chloride colorimetric assay, which relies on quantifying the formation of flavonoid-aluminum complexes by measuring the absorbance at 430 nm (10). Quercetin was used as a reference standard (0.03-0.30 mg/ml). The total flavonoid content was expressed in milligram equivalents of quercetin per gram of dry extract.

Determination of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity. The free radical-scavenging activity of the methanolic extract was measured based on its ability to quench the free radical-producing DPPH using a previously reported method (8,11). Briefly, the concentration of DPPH was determined by measuring the absorbance at 517 nm prior to and subsequent to the addition of a specified quantity of the extract. Next, the % inhibition of radical formation was calculated using the following equation: % inhibition = $(A_{control} - A_{sample}) / A_{control} \times 100$, where $A_{control}$ is the absorbance of DPPH alone and A_{sample} is the absorbance of DPPH with *C. comosum* extract.

High-performance liquid chromatography (HPLC) analysis of C. comosum extract. In total, 100 mg dried methanolic extract was hydrolyzed in 30 ml 1.2 M HCl in 50% aqueous methanol at 80˚C for 2 h under reflux conditions, as previously described (12), but with slight modifications. Subsequent to cooling, the mixture was diluted to 50 ml with absolute methanol, sonicated (37 kHz) for 2 min and then passed through a 0.45-μm filter. Next, the hydrolyzed and non-hydrolyzed extract samples were analyzed by HPLC fractionation on a 250x4.6-mm octadecylsilane column with a 5-mm particle size (LiChrospher® 100 RP-18; Merck KGaA, Darmstadt, Germany), which was attached to an Agilent HP1200 series HPLC system equipped with a G1322A quaternary pump, degasser and G1314A variable wavelength UV detector (Agilent Technologies, Inc., Santa Clara, CA, USA). Chromatographic separation was achieved with a linear gradient from 95% eluent A [0.1% (v/v) formic acid in water] and 5% eluent B [0.1% (v/v) formic acid in methanol] to a final mobile phase composed of 5% eluent A and 95% eluent B over 40 min at a flow rate of 0.8 ml/min, with UV detection at 280 and 340 nm. (+)-Catechin was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and was used as the reference standard.

Chemicals. DEN (CAS no. 55-18-5; purity, >99.0%) and CCl_4 (CAS no. 56-23-5; purity, >99.9%) were purchased from Sigma-Aldrich Chemie GmbH (Hamburg, Germany).

Animals. All the animal procedures were performed in accordance with the guide for the care and use of laboratory animals established by the Committee for the Purpose of Control and Supervision of Experiments on Animals and the National Institutes of Health (13). In total, 40 male Wistar rats (age, 6 weeks) were obtained from the Animal House Colony of the National Research Center (Cairo, Egypt). The animals were fed a standard diet (Al Wadi Co., Giza, Egypt) and had access to water *ad libitum*. The rats were housed in plastic cages in an air-conditioned room at 21˚C with a 12 h light/dark cycle. All the animals were allowed to acclimatize to the conditions for one week prior to the initiation of the experiments. The experimental protocol was approved by the Animal Ethics Committee of Kafrelsheikh University (Kafr el-Sheikh, Egypt).

Hepatocarcinogenesis model. The experimental protocol is summarized in Fig. 1. All the rats received an intraperitoneal (i.p.) injection of 100 mg/kg of body weight DEN dissolved in 0.9% NaCl solution once per week for three weeks in order to induce hepatocarcinogenesis, as previously reported (14). The rats were then divided into four groups (n=10 each) one week after the final dose of DEN. The rats in group 1 received no additional treatment during the remaining 7 weeks of the study. The rats in group 2 were fed a diet that included 100 mg *C. comosum* extract per 1 kg of food for 7 weeks. The rats in group 3 received an i.p. injection of 0.8 ml/kg CCl_4 once per week for 7 weeks in order to promote the development of DEN-induced hepatic foci and were fed a normal diet during

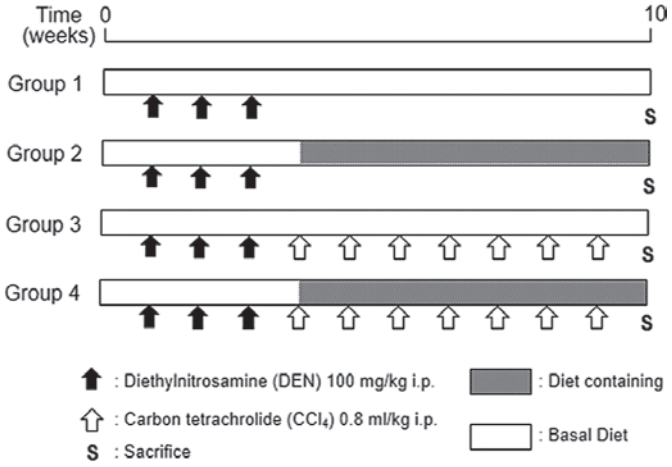

Figure 1. Experimental protocol for studying the protective activity of *Calligonum comosum* methanolic extract against hepatocarcinogenesis in rats.

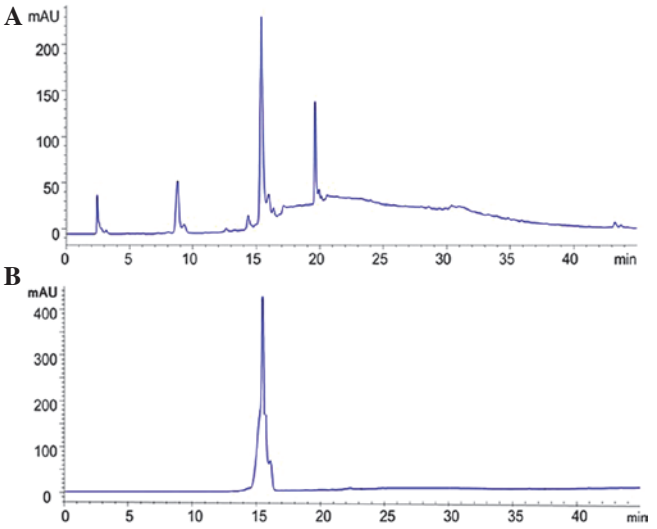

Figure 2. High-performance liquid chromatography chromatograms of (A) the hydrolyzed methanol extract of *Calliogonum comosum* and (B) a standard 0.8 μg/ml (+)-catechin solution. The chromatograms were obtained under the same conditions as described in the Materials and methods section. The UV absorbance was monitored at 280 nm.

this time period. The rats in group 4 received the same CCl_4 treatment as group 3, but were fed a diet that contained 100 mg of *C. comosum* extract per 1 kg of food for the remaining 7 weeks of the study. At the completion of the 7-week treatments, the rats were sacrificed under anesthesia, at which time the total food intake and body weights were recorded, blood samples were drawn from the caudal vena cava, and the livers were resected and weighed. The blood samples were then centrifuged at 3,000 x g for 15 min, and the isolated serum was stored at -20˚C until analysis.

Serum biochemical assay. The serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and γ-glutamyl transferase (GGT) were determined using AST/SGOT Liqui-UV®, ALT/SGPT Liqui-UV® and Gamma-GT LiquiColor® kits, respectively (Stanbio Laboratory, Boerne, TX, USA).

Histological and immunohistological analyses. Liver slices obtained from the right and median lobes were fixed in 10% neutral-buffered formalin. The specimens were then dehydrated in a graded series of ethanol, cleared in xylene and embedded in paraffin wax. Next, the paraffin-embedded specimens were sectioned into 4-μm samples for hematoxylin and eosin staining (Sigma-Aldrich Chemie GmbH). and immunostaining. The number of oval cells was counted at high power (magnification, x400) in three area fields for each rat. Immunohistochemical staining for placental glutathione S-transferase was performed as follows. The serial sections were dewaxed, rehydrated, immersed in 0.05 M citrate buffer (pH 6.8) and heated at 100˚C for 8 min in an autoclave. The slides were then gently rinsed with distilled water or phosphate-buffered saline (PBS; pH 7.2) and treated with hydrogen peroxide 0.3% in absolute methanol for 30 min at room temperature to inactivate the endogenous peroxidase. The sections were subsequently incubated for 30 min with Protein Block Serum-Free (Dako, California, USA), followed by overnight at 4˚C with a polyclonal rabbit anti-rat GST-P antibody (cat. no. 311, Medical & Biological Laboratories Co., Ltd., Nagoya, Japan; dilution, 1:500) The sections were then incubated with a goat anti-rabbit secondary antibody (cat. no. K4003, EnVision+™ System Horseradish Peroxidase Labelled Polymer; Dako) for 30 min at room temperature. The sections were rinsed with PBS containing 0.1% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) between each step. Following signal detection by using a freshly prepared solution of 3,3'-diaminobenzidine tetrahydrochloride (Liquid DAB+ Substrate Chromogen System; Dako), the sections were washed in distilled water, counterstained with Mayer's hematoxylin, and dehydrated. As a negative control procedure, the primary antibody was replaced with normal rabbit serum. The analysis of GST-P-positive foci was then performed as previously described (15,16).

Statistical analysis. For comparisons among different treatment groups, the homogeneity of the variance was tested using Bartlett's test that was set at a significance of 0.05. When the samples demonstrated homogeneous variance, comparisons among treatment groups were analyzed using one-way analysis of variance, followed by Fisher's least significant difference tests. In the case that the variance was not homogeneous, the Kruskal-Wallis test was performed. The Steel-Dwass test was applied when the Kruskal-Wallis test indicated a significant difference between the groups. Data are expressed as the mean ± standard deviation. A value of $P<0.05$ was used to indicate a statistically significant difference.

Results

Phytochemical analysis

Total phenolic and flavonoid contents. The total phenolic and flavonoid contents of the methanolic *C. comosum* extract were estimated to have an activity equivalent to 335 mg of gallic acid and 11.1 mg of quercetin, respectively, per gram of dry extract.

DPPH radical scavenging activity. The *C. comosum* extract exhibited a dose-dependent free radical-scavenging activity in the standard DPPH assay. The concentration at which 50% inhibition of DPPH was observed (IC_{50}) was 29.2 μg/ml.

Table I. Body and liver weights after the 7 weeks of treatment.

Group	Treatment	No. of rats	Body weight, g	Liver weight	
				Absolute, g	Relative, %
1	DEN	10	223.4±39.3	9.1±0.76	4.14±0.55
2	DEN + *C. comosum* extract	10	216.3±23.7	7.67±0.57[b]	3.57±0.32[a]
3	DEN + CCl_4	10	194.7±13.1	11.01±1.92[a]	5.68±1.07[a]
4	DEN + CCl_4 + *C. comosum* extract	10	218.5±17.4[c]	8.64±1.12[c]	3.97±0.56[c]

[a]P<0.05 and [b]P<0.005, vs. group 1; and [c]P<0.05 vs. group 3. Statistically significant differences were determined using Kruskal-Wallis test, followed by Steel-Dwass test. Data are expressed as the mean ± standard deviation. CCl_4, carbon tetrachloride; DEN, diethylnitrosamine; *C. comosum, Calliogonum comosum*.

Table II. Serum levels of AST, ALT and GGT.

Group	Treatment	AST, IU/l	ALT, IU/l	GGT, IU/l
1	DEN	85.36±3.57	98.73±3.37	20.28±3.03
2	DEN + *C. comosum* extract	55.23±5.43[a]	44.75±1.72[a]	13.65±2.06[a]
3	DEN + CCl_4	102.71±4.79[a]	133.08±2.99[a]	35.85±2.88[a]
4	DEN + CCl_4 + *C. comosum* extract	64.59±3.58[a,b]	68.60±4.56[a,b]	17.91±1.23[a,b]

[a]P<0.005 vs. group 1; and [b]P<0.005 vs. group 3. Statistically significant differences were determined using analysis of variance, followed by Fisher's least significant difference test. Data are expressed as the mean ± standard deviation. AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, γ-glutamyl transferase; CCl_4, carbon tetrachloride; DEN, diethylnitrosamine; *C. comosum, Calligonum comosum*.

HPLC profile of the methanolic extract. Whereas the HPLC profile of the non-hydrolyzed methanolic extract exhibited a number of peaks with an absorbance at 280 nm (data not shown), the hydrolyzed extract demonstrated only three major peaks (Fig. 1A). The most prominent peak eluted with a retention time of 15.1±0.1 min, and was subsequently identified to be (+)-catechin by comparing with the retention time of a (+)-catechin standard solution (0.5 mg/ml). Using standard curve analysis, the amount of (+)-catechin in the extract was determined to be 113 mg/g of dry extract (Fig. 2).

Biological assay

Body and liver weights. In all the treatment groups, the rats survived throughout the entire study period with no observed severe adverse clinical effects. In addition, no significant differences in food consumption were observed among the treatment groups (data not shown). The final body and liver weights of the rats from the different treatment groups are presented in Table I. The rats treated with DEN and CCl_4 (group 3) had lower body weights over the course of the study, whereas dietary supplementation with the *C. comosum* extract (group 4) was found to significantly suppress weight loss (P<0.05). Furthermore, the DEN-treated rats that had received CCl_4 demonstrated significantly increased absolute and relative liver weights compared with the corresponding weights of the rats treated with DEN alone (P<0.05 for absolute and relative values). Notably, supplementation with *C. comosum* extract significantly inhibited the increase in the absolute and relative liver weights that was induced by the DEN and CCl_4 treatment (P<0.05; groups 3 and 4 for absolute and relative values).

C. comosum extract protects against the induction of liver enzymes. The serum levels of AST, ALT and GGT in the rats from the different treatment groups are presented in Table II. The enzyme levels were consistently and significantly lower in the rats that were fed the diet containing *C. comosum* extract (group 2) compared with the rats that were fed the standard diet (group 1; P<0.005 for AST, ALT and GGT). Compared with treatment with DEN alone (group 1), treatment with CCl_4 and DEN also increased the enzyme levels (group 3; P<0.005 for all enzymes). Furthermore, the co-administration of *C. comosum* extract (group 4) significantly suppressed the increase in liver enzyme levels compared with the levels in the rats receiving treatment with CCl_4 and DEN (group 3; P<0.005 for all enzymes).

C. comosum extract suppresses the induction of oval cell proliferation. Representative photomicrographs of the rat liver sections and the number of oval cells detected in these sections are shown in Fig. 3. Moderate oval cell proliferation was observed in the periportal region of liver sections from rats treated with DEN alone (group 1). By contrast, the proliferation was significantly reduced by supplementation with *C. comosum* extract (group 2; P<0.001). Similarly, CCl_4 treatment induced a 3.3-fold increase in the proliferation of oval cells that infiltrated from the periportal region into the intralobular region (group 3; P<0.001). However, this

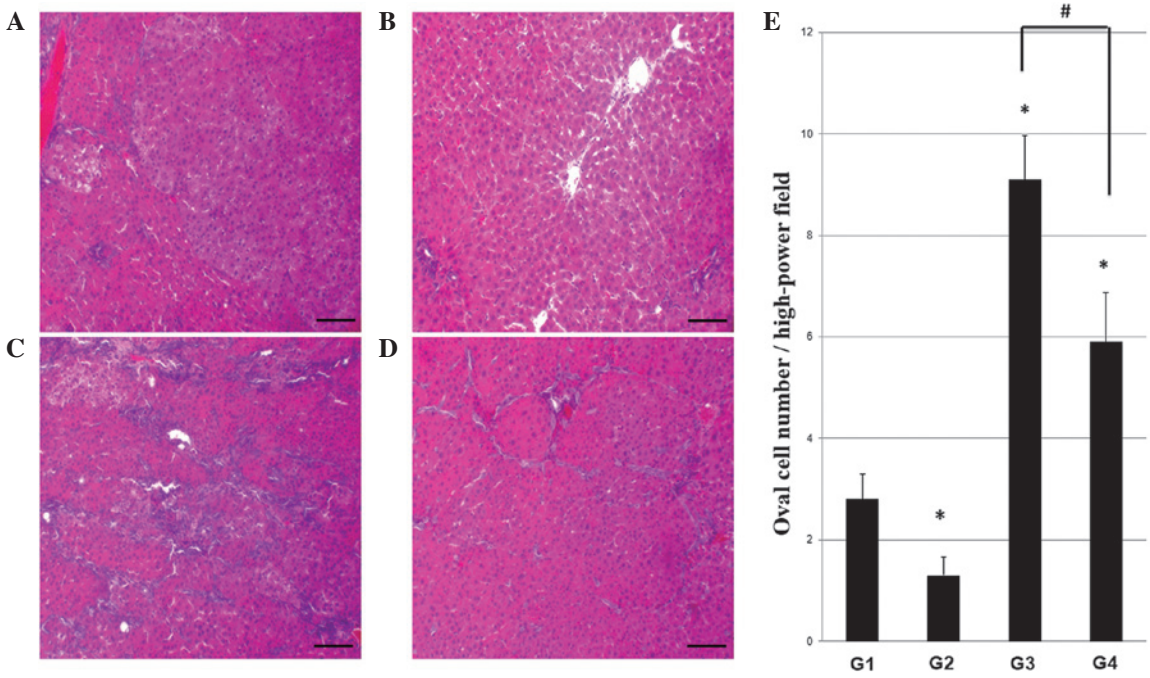

Figure 3. Histology of liver sections obtained from the rats of the different treatment groups: (A) Group 1, DEN alone; (B) group 2, DEN + *C. comosum* extract; (C) group 3, DEN + CCl_4; and (D) group 4, DEN + CCl_4 + *C. comosum* extract. Note the presence of oval cell proliferation and altered hepatic foci in panel (A), and marked proliferation of the oval cells that infiltrate from the periportal region to the intralobular region in panel (C). (E) Number of oval cells detected in the liver sections. Data are expressed as the mean ± standard deviation. Statistically significant differences were determined using Kruskal-Wallis test, followed by Steel-Dwass test. *P<0.001 vs. group 1; and #P<0.001 vs. group 3. DEN, diethylnitrosamine; *C. comosum*, *Calligonum comosum*; CCl_4, carbon tetrachloride.

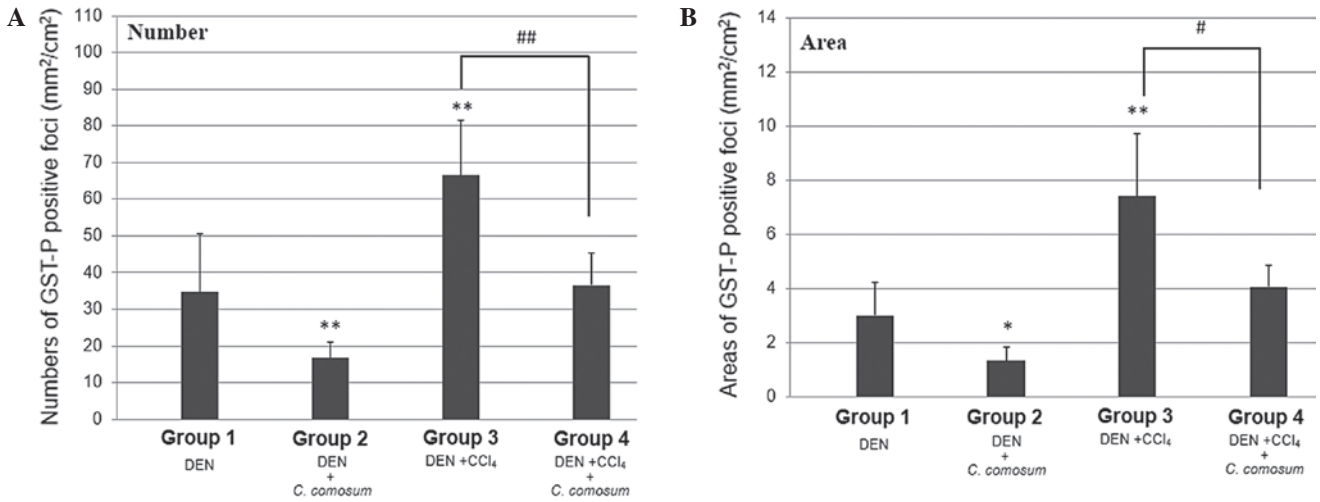

Figure 4. **(A)** Numbers and (B) mean areas of GST-P-positive foci (>0.2 mm in diameter). Data are expressed as the mean ± standard deviation for the 10 rats in each group. Statistically significant differences were determined using Kruskal-Wallis test, followed by Steel-Dwass test. *P<0.05 and **P<0.005, vs. group 1; #P<0.01 and ##P<0.005, vs. groups 3 and 4. DEN, diethylnitrosamine; *C. comosum*, *Calliogonum comosum*; CCl_4, carbon tetrachloride; GST-P, glutathione S-transferase placental form.

induction in proliferation was significantly suppressed by the co-administration of the *C. comosum* extract (P<0.001; groups 2 and 4).

C. comosum extract suppresses the formation of GST-P-positive foci. The quantitative data obtained for the GST-P-positive foci are shown in Fig. 4. In addition, representative photomicrographs of the immunohistochemically-stained sections are shown in Fig. 5. As expected, the repeated administration of CCl_4 following DEN treatment (group 3) significantly increased the number and mean area of GST-P-positive foci (P<0.005 for number and area). By contrast, *C. comosum* extract significantly decreased the number and mean area of GST-P-positive foci when co-administered with DEN alone (group 2; P<0.005 and 0.01 for number and area, respectively), as well as when DEN was combined with CCl_4 (group 4; P<0.005 and P<0.01 for number and area, respectively).

Discussion

At present, the majority of treatments for HCC are not sufficiently effective, making this malignancy the second most lethal

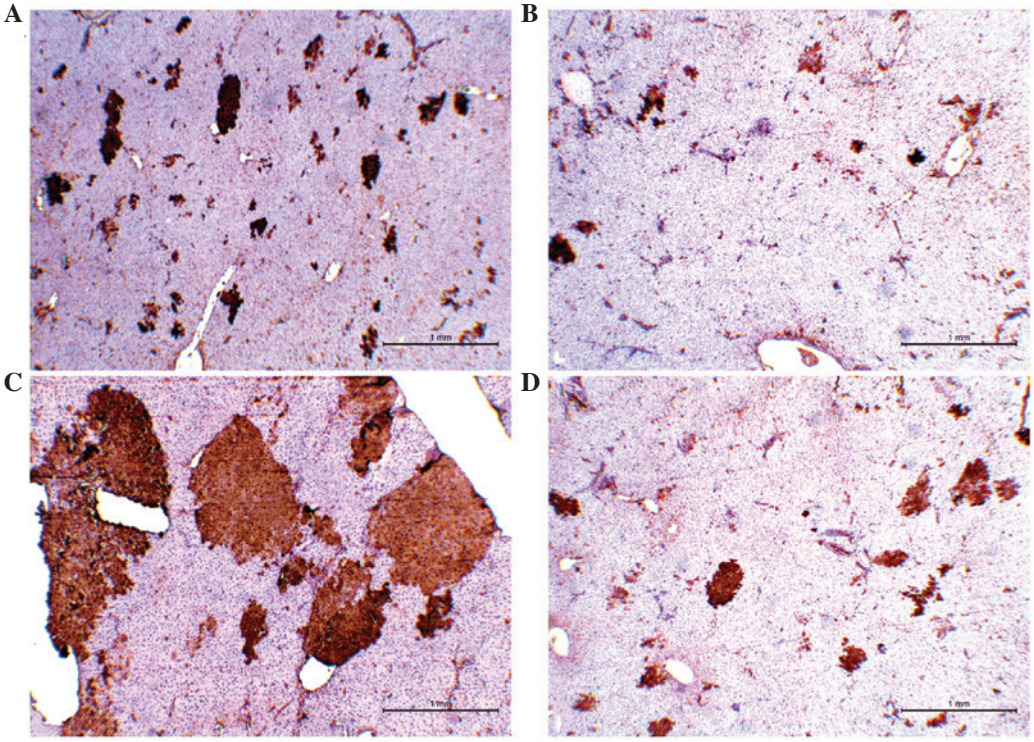

Figure 5. Immunostaining for GST-P in (A) group 1, (B) group 2, (C) group 3 and (D) group 4. Panel (A) presented GST-P positive foci. Scale bar, 1 mm. GST-P, glutathione S-transferase placental form.

type of cancer and the third leading cause of cancer-associated mortalities (1). Since the current treatment strategies are not particularly effective, chemoprevention is an important tool that can be used in order to prevent HCC (3). Chemopreventive agents isolated from plants local to areas of high HCC incidence may be particularly valuable, given their potential practical and economic benefits. A number of plant phenols, including flavonoids, are known to have HCC chemopreventive properties (3). *C. comosum* is a readily available Egyptian desert plant that has been reported to contain a diverse array of antioxidant flavonoids (7). The present study is the first to examine the hepatoprotective activity of *C. comosum*.

The phytochemical analysis of the methanolic *C. comosum* extract in the present study indicated that its phenolic content, flavonoid content and radical-scavenging activity were comparable to those of a previously reported extract obtained from plants in a different area (7). In addition, the HPLC analysis positively identified (+)-catechin in the hydrolyzed *C. comosum* extract. This result was also consistent with those previously reported for the methanolic extract and its fractions obtained from plants in a different area (7). These data suggest that *C. comosum* has a consistent phytochemical content, irrespective of the area or environment in which it is grown.

The present study also revealed that the *C. comosum* extract exhibited a chemopreventive activity against hepatocarcinogenesis induced by treatment with DEN alone or DEN in combination with CCl_4. This protective effect was most notable in the suppression of proliferation of GST-P positive preneoplastic foci. The reduced serum levels of AST, ALT and GGT produced by the extract suggested hepatoprotective properties, which was confirmed by the histological evaluations of the liver sections. The protective activity of the *C. comosum* extract most likely originates from its ability to reduce damage to the liver by carcinogens, which suppress the promotion of hepatic foci induced by hepatocyte regeneration. Oval cells are not normally involved in liver regeneration, due to the regenerative capacity of hepatocytes. However, in certain conditions that cause severe liver damage, oval cells have been observed and are, therefore, considered to replenish damaged hepatocytes and bile duct cells (17). In a previous study, the proliferation of oval cells was decreased in the livers of hepatocarcinogenesis-resistant DRH strain rats (18). The presence of oval cells following DEN and CCl_4 treatment is therefore indicative of severe liver damage. Thus, it is reasonable to conclude that a significant decrease in the number of oval cells in the current study indicates that the *C. comosum* extract reduced the severity of liver damage caused by DEN and CCl_4.

The basis for the hepatoprotective effects of *C. comosum* extract may be associated with its antioxidant activity. A previous study reported that the (+)-catechin in a *C. comosum* extract exhibited a high antioxidant activity (7). HPLC analysis of the extract prepared in the present study also identified (+)-catechin. In addition, the extract exhibited a strong radical-scavenging activity. Catechin is a flavan-3-ol (or simply flavanol), which is a type of natural phenol and antioxidant. It is part of the chemical family of flavonoids. Epigallocatechin-3-gallate is the most abundant and potent catechin, acting as a selective inhibitor of the tyrosine phosphorylation of platelet derived growth factor receptor β (19). In addition, it has been identified to induce apoptosis in prostate cancer cells through agonist-dependent androgen receptor (AR) activation and AR-regulated signaling pathways (20). Furthermore, catechin hydrate has been demonstrated to suppress the proliferation of different cancer

cells, including MCF-7 human breast cancer cells through the induction of apoptosis (21) and lung cancer cell lines by the upregulation of the let-7 tumor-suppressor microRNAs (22). In addition to antioxidative properties, catechins affect the molecular mechanisms involved in carcinogenesis, including extracellular matrix degradation, angiogenesis, multidrug resistance and regulation of cell death (23). Notably, (+)-catechin can also suppress the CCl_4-induced lipid peroxidation of rat livers, which is considered an important component of the mechanism by which CCl_4 induces hepatocellular necrosis (24). In addition, a previous study revealed that catechins inhibited collagen production in hepatic stellate cells despite enhanced collagen transcription and suppressed collagenase activity, which indicated an antifibrogenic effect of these substances (25).

C. comosum extracts contain a number of other antioxidant flavonoids that may protect against liver damage (7). In fact, a previous study suggested that the unique combination of flavonoids in *C. comosum* may synergistically enhance its antioxidant activity (7). Since oxidative stress is considered to be an important component of hepatitis- and alcohol-induced hepatocarcinogenesis in humans (1,26,27), the antioxidant activity of *C. comosum* extracts is a particularly promising and potentially chemoprotective agent for at-risk populations.

In conclusion, the present study revealed that a methanolic extract of *C. comosum* exhibited significant chemopreventive effects against DEN-induced hepatocarcinogenesis in rats. Therefore, *C. comosum*-derived formulations are promising candidates for further evaluation and the development of practical and economical chemopreventative agents in regions with high HCC prevalence.

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