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ORIGINAL ARTICLE

Proanthocyanidins Rich Extract of *Calligonum comosum* Ameliorates Doxorubicin-Induced Immunosuppression and Hepatorenal Toxicity

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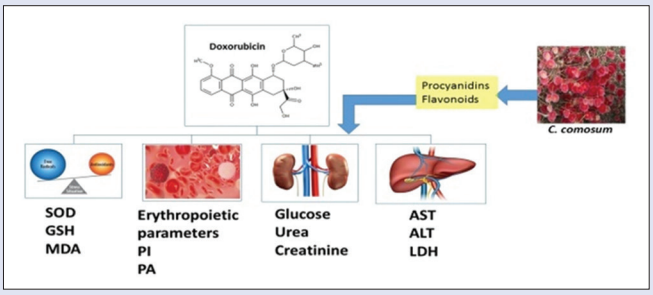
ABSTRACT

Background: Doxorubicin (DOX) is a highly effective chemotherapeutic agent which use has been restricted due to its multi-organ toxicity. **Objective:** We investigated the possible protective effect of *Calligonum comosum* extract (CCE) against DOX toxicity while identifying its major phytoconstituents. **Materials and Methods:** CCE was administered at 100 mg/kg b.w for 2 weeks to rats which have previously received an intraperitoneal injection of DOX (20 mg/kg). Major phytoconstituents of CCE were assessed using high-performance liquid chromatography and UPLC/MS/MS. **Results:** CCE was rich in phenolic constituents, especially proanthocyanidins, corresponding to a concentration of 167 mg catechin per gram of the extract after hydrolysis. Other constituents identified were procyanidin B1-gallate, procyanidin B2-gallate, quercetin, and kaempferol. Animals that received CCE following DOX injection showed less signs of oxidative stress (indicated by levels of malondialdehyde, superoxide dismutase, and reduced glutathione), DNA fragmentation, and hepato-renal genotoxicity (Comet's assay). When compared to animals that received DOX only, administration of CCE following DOX maintained normal tissue architecture and restored liver and kidney functions to near their normal levels (measured as creatinine, glucose, urea serum concentration and aspartate aminotransferase, lactate dehydrogenase, and alanine transaminase activities). Interestingly, healthy animals receiving CCE showed an increase in total lymphocyte count and phagocytic activity, whereas those receiving CCE following DOX intoxication showed no signs of immunosuppression that was observed in animals receiving DOX only. **Conclusion:** *C. comosum* is a promising candidate as a supportive treatment for those receiving DOX as a chemotherapeutic agent due to its ability to ameliorate signs of DOX toxicities.

Key words: *Calligonum comosum*, doxorubicin, hepato-renal toxicity, immunostimulant, oxidative stress, proanthocyandins

SUMMARY

- Ethanolic extract of *Calligonum comosum* (CCE) is rich in phenolic compounds, especially (epi) catechin and its derivatives procyanidin B1-gallate, procyanidin B2 and 3-galloyl-(epi) catechin. We investigated the effect of CCE when administered for 2 weeks to animals that previously received single intraperitoneal injection of doxorubicin (DOX). Oral administration of CCE relieved signs of oxidative stress induced by DOX (malondialdehyde, superoxide dismutase, and reduced glutathione). In addition, both liver and kidney functions were negatively affected after the administration of DOX while in animals that received CCE, biochemical markers for liver and kidney functions were observed at their normal values. Genotoxicity and histopathological signs were observed in liver and kidney tissue from DOX treated animals but were mostly absent from animals that received CCE. Meanwhile, healthy animals that received CCE showed immunostimulant effect as indicated by an increase in leukocyte count, phagocytic activity, and phagocytic index, while those receiving DOX showed clear immunosuppression that was reversed after administration of CCE. We propose that the use of CCE with DOX can reduce its multi-organ toxicities and maximize the benefit of this chemotherapeutic agent.



Abbreviations used: Alb: Albumin; ALT: Alanine transaminase; AST: Aspartate aminotransferase; CCE: *Calligonum comosum* extract; DOX: Doxorubicin; GSH: Reduced glutathione; Hb: Hemoglubin; LC/MS: Liquid chromatography/mass spectrometry; LDH: Lactate dehydrogenase; MDA: Malondialdehyde; PA: Phagocytic activity; PCV: Packed cell volume; PI: Phagocytic index; ROS: Reactive oxygen species; SOD: Superoxide dismutase; TLC: Total leukocyte volume; TP: Total protein.

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INTRODUCTION

Doxorubicin (DOX), also known as adriamycin, is an anthracycline antibiotic that has been clinically successful as a first-line anticancer drug for human neoplasmas, including leukemias, lymphomas, and solid tumors.[1] DOX exerts its anticancer action through preferentially intercalating DNA of rapidly dividing tumor cells, causing cell cycle arrest in the G2 phase.[2] However, the therapeutic use of DOX is hampered by its dose-related toxicities such as hematopoietic suppression, hepatotoxicity, nephrotoxicity, and cardiomyopathy.[3,4] Multifactorial mechanisms are believed to be involved in the pathogenesis of DOX-induced toxicity, with oxidative stress identified as the main initiator by triggering several forms of cell death, including apoptosis, necrosis, and autophagy.[5,6] A great therapeutic benefit can be achieved by developing treatments that can prevent or decrease DOX toxicity without affecting its antineoplastic properties. Possible approaches to reduce DOX toxicity is to neutralize free radicals generated by its anthracycline moiety and/or to introduce exogenous antioxidant molecules as additional supplements.[7,8]

In previous studies, *Calligonum comosum* L' Herlit (family Polygonaceae) was found to possess strong anti-oxidant activities and showed a promising cardioprotective effect against DOX-induced cardiomyopathy when screened among other plants.[9] In addition, a standardized extract of *C. comosum* (CCE) prepared from its aerial parts protected against haloperidol-induced oxidative stress in brain and liver tissues of rats and ameliorated testicular toxicity induced by Aroclor1260.[10,11]

The current study was designed to evaluate the protective effect of *C. comosum* against DOX-induced toxicity in other organs. To achieve this goal, DOX was given intraperitoneal (i.p.) to rats, and several hematological parameters, immune response, lipid peroxidation biomarker, antioxidant biomarkers, kidney and liver functions, and genotoxicity were assessed and compared to those observed in DOX-intoxicated rats that received a daily dose of CCE. In addition, liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) fingerprinting of CCE was carried out to identify the major chemical constituents of CCE that may be responsible for its effects.

MATERIALS AND METHODS

Chemicals

DOX (Adriblastina® produced by Carlo Erba) was purchased from a local pharmacy in the form of 10 mg/ampoule. Acetonitrile and formic acid used for high-performance liquid chromatography/MS (HPLC/MS) analysis were of HPLC grade and were purchased from Sigma-Aldrich Co., Germany. All other chemicals were of analytical grade and purchased from local vendors.

Plant materials and extraction

Aerial parts of *C. comosum* were collected from Cairo-Suez desert Road (Cairo, Egypt) in April 2012. A herbarium specimen was kept in the Herbarium of the Pharmacognosy Department (Faculty of Pharmacy, Cairo University; CC01204C). The methanolic CCE was prepared as described previously with a percentage yield of 9.2%.[12]

High-performance liquid chromatography-electrospray ionization/mass spectrometry–mass spectrometry of *Calligonum comosum* extract

HPLC was performed using a Dionex Ultimate 3000 RS liquid chromatography system (Thermo Fisher Scientific Inc., MA, USA) equipped with a Dionex Acclaim RSLC-120, C18 column (2.1 × 100 mm, 2.2 μm) applying the following gradient (solvent A: H_2O with 0.1% formic acid; solvent B: CH_3CN with 0.1% formic acid) at flow rate of 0.4 mL/min: 0–5 min: 5% B; 5–37 min: linear gradient to 100% B; 37–47 min: isocratic 100% B. The sample was prepared by dissolving CCE in methanol (MeOH) by ultrasonication for 10 min to a final concentration of 5 mg/mL, 2 μL of the solution was injected into the system after centrifugation. Eluted compounds were detected using a Dionex Ultimate DAD-3000 RS and a Bruker Daltonics microTOF-QII mass spectrometer equipped with an Apollo electrospray ionization (ESI) source in positive mode using the following instrument settings: nebulizer gas N_2, 4 bar; dry gas N_2, 9 L/min, 200°C; capillary voltage – 4500 V; endplate offset – 500 V; transfer time 100 μs; prepulse storage 6 μs; collision gas N_2; collision energy 8 eV (Full MS) or 40 eV (MS/MS); collision RF 130 Vpp. MS/MS scans were triggered using auto MS2 settings that select up to two ions within an *m/z* range of 200–1500 and a minimum intensity of 2000 counts as precursor ions for two successive MS/MS scans. Internal dataset calibration was performed for each analysis using 10 mM solution of sodium formate in 50% isopropanol infused during LC re-equilibration using a divert valve.

LC-MS data were processed using Data Analysis 4.1 SR1 (Bruker Daltonics). The monoisotopic molecular weight of each compound was determined through an in-house VA script from the most intense adduct ion found in its full MS spectrum. Compounds were identified on the basis of their accurate *m/z* values of their corresponding adduct ions, fragment ions, or neutral losses observed in full MS or preferably MS/MS spectra and whenever possible, ultraviolet (UV) spectra. Metlin and KNApSAcK databases were used as references in the identification of the eluted compounds.

Animals

Thirty-two male Wistar albino rats weighing 150–180 g were obtained from the animal research center, Tanta University, Egypt. Rats were kept in plastic cages and acclimatized for 1 week. The animals were given a commercial balanced diet (Al Wadi Co., Giza, Egypt) and provided with water *ad libitum* throughout the experiment. The study was conducted according to our institutional animal care guidelines (Kafr-Elsheikh University) and following "Guide for the Care and Use of Laboratory Animals," United States National Research Council, 2011.

Experimental protocol

Animals were divided into four groups (n = 8). Group 1 (control) received only normal saline. Animals in Group 2 (CCE) were treated daily with CCE (100 mg/kg b.wt.) for 2 weeks by intragastric gavage. Animals in Group 3 (DXR) and Group 4 (DXR + CCE) were injected with DOX (20 mg/kg b.wt.) i.p. once at the 1st day of the experiment, and only animals in Group 4 received a daily dose of CCE (100 mg/kg b.wt.) orally by stomach tube for 2 weeks. The dose of CCE (100 mg/kg b.wt.) was selected based on a previous study performed using an extract of the same plant.[12]

Blood sampling

At the end of the experiment (24 h after the last treatment with CCE), blood samples were collected through retro-orbital venous plexus under light anesthesia (2.0% isoflurane) and immediately divided into divided into three aliquots: one containing EDTA, for the measurement of haematological parameters and estimation of reduced glutathione (GSH), superoxide dismutase (SOD), and malondialdehyde (MDA) that was immediately stored at −20°C. The second aliquot was left to clot then centrifuged at 3000 rpm for 15 min then sera were collected and stored at −20°C. The third aliquot containing heparin for estimation of

the phagocytic activity of neuterophils. Animals were then sacrificed by decapitation and livers, kidneys were excised, cleaned, perfused with cold saline and stored at −80°C for DNA fragmentation and Comet assay.

Immunological parameters

Phagocytic activity of polymorphic nuclear cells and phagocytic index was performed using *Candida albicans* according to methods described by Khan *et al.*[13]

Hematological analysis

Hematological parameters, including red blood cells (RBCs) count, hemoglobin (Hb), packed cell volume (PCV), total leukocytic count (TLC), differential leukocyte count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and the MCH concentration were immediately evaluated in the fresh EDTA treated blood.[14]

Serum biochemical assay

Commercial kits (Quimica Clinica Aplicada, Spain) were used for measuring the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the fresh sera. Lactate dehydrogenase (LDH) was determined according to the method of Buhl and Jackson, 1978, total proteins (TP) was evaluated according to Lowry *et al.* 1951.[15,16] Albumin (Alb) was measured according to Henry *et al.* 1974.[17] Urea and creatinine serum concentration were measured using commercial kits (Bio-merieux Co., France). Glucose was determined according to Trinder, 1969.[18]

Estimation of oxidative stress and antioxidant biomarkers

Measurement of MDA was performed according to Mihara and Uchiyama, 1978.[19] GSH and SOD were estimated according to Weydert and Cullen, 2009.[20]

Quantification of DNA damage, data scoring, and photomicrographs

Genomic DNA was extracted from preserved liver and kidney according to the method proposed by Manaitis *et al.* 2012.[21] Briefly, high-molecular-weight genomic DNA was precipitated by absolute ethanol after 10–20 mg of liver or kidney tissues were homogenized in 400 ml hypotonic lysis buffer (10 mM Tris base, 1 mM EDTA, and 0.2% Triton X-10). The cell lysate was centrifuged at 11,000 rpm for 15 min at 4°C and the supernatant containing small DNA fragments was reconstituted in 12 ml of Tris-EDTA buffer and 3 ml loading buffer, incubated at 37°C for 20 min, and then electrophoresed on 1% agarose gel with 0.71 mg/ml ethidium bromide. DNA was visualized and photographed under UV transillumination.

DNA damage was measured using Comet assay under alkaline conditions as previously described.[22] The specimens were homogenized in the chilled buffer, pH 7.5 containing 75 mMb NaCl and 24 mM Na_2EDTA, pH 13, to obtain a 10% tissue homogenate. Samples were kept on ice during and after homogenization and 6 µl of kidney or liver homogenate were suspended in 0.5% low-melting agarose and sandwiched between a layer of 0.6% normal-melting agarose and a top layer of 0.5% low-melting agarose on fully frosted slides. The slides were kept on ice during the polymerization of each gel layer. After solidification of the agarose layer, the slides were immersed in a lysis solution (1% sodium sarcosinate, 2.5 m NaCl, 100 mM Na_2EDTA, 10 mm Tris-HCl, 1% TritonX-100, and 10% DMSO) at 4°C for 1 h, then the slides were placed in electrophoresis buffer (0.3 M NaOH, 1 mM Na2EDTA, pH 13) for 10 mins at 0°C to allow DNA to unwind. Electrophoresis was performed for 10 min at 300 mA and 1 V/cm. The slides were then neutralized with Tris–HCl buffer, pH 7.5 and stained with 20 µg/ml ethidium bromide. Each slide was analyzed using an automated fluorescence microscope (Carl Zeiss, Germany). Images were analyzed using Comet Score software (Komet IV). One hundred cells were analyzed on each slide using the Comet assay II automatic digital analysis system. Three parameters were used as indicators of DNA damage: Tail length (TL in µm), % DNA in Comet tail (% DNA in the tail), and tail moment (TM), (TM = TL X% DNA in tail). Both tail length and tail intensity were measured automatically.[23]

Histopathology

Liver and kidney tissues were fixed in 10% neutral buffered formaldehyde and prepared by dehydration and routine paraffin embedding. Tissue sections (5 µm) were stained with hematoxylin and eosin and examined under a light microscope on a blind basis for the different treatment groups. A five-point scale for both the liver and kidney tissues were assessed on the basis of the summation of the vascular changes (congestion and hemorrhage), vacuolation, cytoplasmic eosinophilia, (nuclear pyknosis and apoptosis) and evidence of necrosis. Injury score for each parameter was varied from 0.0, 0.25, 0.50, 0.75, and 1 consistent with no detected lesions, mild, moderate, marked, and severe lesions, respectively.

Statistical analysis

Data were analyzed using the one-way analysis of variance with Duncan's *post hoc* test LSR multiple comparison tests, to determine the significant differences among means in this study with the General Linear Model using SPSS Statistics 17.0 (Statistical Packages for the Social Sciences, released 23 August 2008 New York, USA). These differences were analyzed at the 5% probability level ($P \leq 0.05$ was considered statistically significant). All data were expressed as means ± standard error of the mean.

RESULTS

Phytochemical analysis

The total phenolic and flavonoid contents of the CCE were determined to be equivalent to 335 mg of gallic acid and 11.1 mg of quercetin, respectively, per gram of dry extract. Catechin was determined as the major component in the acid hydrolysate of CCE (167 mg/g of dry extract) using HPLC/UV assay.[12]

Chemical constituents of *C. comosum* were analyzed using HPLC-ESI/MS–MS and PDA and 22 metabolites were tentatively identified among 30 metabolites detected [Table 1]. The major peaks in the LC/MS chromatogram were that of flavonoids and proanthocyanidins. Four proanthocyandins ((epi) catechin, procyanidin B1 gallate, procyanidin B2, 3-galloyl-(epi) catechin) were identified based on their characteristic UV absorbance at 279 and the mass of (epi) catechin fragment at 289 m/z as well as other fragments [Supplementary Figure 1]. Flavonol glycosides of quercetin and kaempferol were identified based on their characteristic UV absorbance and the characteristic fragment of their aglycons at 301 and 285, respectively. In addition, one flavanonol (taxifolin), three fatty acids (linolenic, linoleic and palmitic acid), two monoterpenes: (dihydroactinidiode and dihydronepetalactone), three amino acids, one amino acid conjugate: Ferloyltyramine, one saponin glycoside: Diurnoside 1 and four porphyrins derivatives were tentatively identified based on *m/z* of their molecular ion and the produced fragments.

Table 1: Compounds identified in *Calligonum comosum* extract LC/MS/MS/mass spectrometry analysis

Comp	RT	MW	UV (nm)	Formula	Quasi-Ion	Error (mDa)	MS2 product ions (%)	Tentative identity
1	1.28	115.0634		$C_5H_9NNaO_2$	[M+Na]	0.1		L-proline
2	1.58	165.0789	260	$C_9H_{12}NO_2$	[M+H]	−0.1	120	L-phenylalanine
3	3.12	204.0918	224, 280	$C_{11}H_{13}N_2O_2$	[M+H]	2.0	146, 188	tryptophan
4	5.87	290.0817	279, 310	$C_{15}H_{15}O_6$	[M+H]	−2.7	119,123, 139,147,161, 179, 189	(Epi) catchin
5	11.09	730.1579	279	$C_{37}H_{31}O_{16}$	[M+H]	4.6	123, 127, 139, 163, 247, 259, 409	monogalloylatedepicatechin-[4β→8]-catechin (procyanidin B1 3-o-gallate)
6	11.14	578.1470	279	$C_{30}H_{27}O_{12}$	[M+H], [M+Na]	4.6	425, 407, 289	(−)-Epicatechin-(4β→8)-(−)-epicatechin (Procyanidin B2)
7	12.13	304.0605	280	$C_{15}H_{13}O_7$	[M+H]	−2.0	123, 149, 153, 213, 231,241	taxifolin
8	12.18	442.0926	280	$C_{22}H_{19}O_{10}$	[M+H]	2.4	123, 139, 153, 165, 273	3-galloyl-(epi) catechin
9	12.36	478.0790	256, 356	$C_{21}H_{19}O_{13}$	[M+H]	4.0	303, 285, 113	Quercetin-hexuronide
10	13.17	462.0825	264, 348	$C_{21}H_{19}O_{12}$	[M+H]	2.4	463, 287	Kaempferol-hexuronide
11	13.25	448.1047		$C_{21}H_{21}O_{11}$	[M+H]	4.1	409, 303	quercetin-3-*O*-rhamnoside
12	14.88	313.1344	220, 299, 320	$C_{18}H_{20}NO_4$	[M+H]	−2.7	117, 121, 145,149,177	Feruloyltyramine
13	15.50	1196.5959	280	$C_{56}H_{93}O_{27}$	[M+H]	−13.4	273, 435, 741	Diurnoside 1
14	18.26	180.1154		$C_{11}H_{16}NaO_2$	[M+Na]	−0.4	55, 101, 103, 137, 153, 163	Dihydroactinidiolide
15	20.16	168.1158		$C_{10}H_{16}NaO_2$	[M+Na]	0.8	123, 169	Dihydronepetalactone
16	31.25	278.2262		$C_{18}H_{30}NaO_2$	[M+Na]	−1.6		Linolenic acid
17	32.33	608.2671		$C_{35}H_{37}N_4O_6$	[M+H]	3.7		Porphyrin: Harderoporphyrin
18	33.02	280.2411		$C_{18}H_{32}NaO_2$	[M+Na]	−0.9		Linoleic acid
19	33.09	608.2673		$C_{35}H_{37}N_4O_6$	[M+H]	3.8		Porphyrin: Harderoporphyrin
20	33.80	592.2716		$C_{35}H_{37}N_4O_5$	[M+H]	−3.1		Porphyrin: Pheophorbide a
21	34.42	592.2724		$C_{35}H_{36}N_4O_5$	[M+H]	3.8		Porphyrin: Pheophorbide a
22	34.62	256.2419		$C_{16}H_{33}O_2$	[M+H]	−1.7		Palmitic acid

mDa: milliDalton; MW: Molecular weight; RT: retention time; UV: ultraviolet

Effect of doxorubicin and *Calligonum comosum* extract treatment

The bodyweight of animals receiving CCE was not significantly different from that of the control group. On the other hand, the DOX-treated group revealed a significant decline in the final weights by about 20.1% compared to the control group ($P \leq 0.05$). Treatment of DOX intoxicated animals with CCE resulted in 14.7% increase in body weight when compared to DOX intoxicated animals in Group 3 [Supplementary Table 1].

Immune response

Effects of CCE and/or DOX on phagocytic activity of neutrophils (PA) and the phagocytic index (PI) are represented in Figure 1. Animals treated with CCE only showed a significant enhancement in PA and PI ($P < 0.05$) by about 24.8% and 40.4%, respectively compared to the control group indicating an immunostimulant effect. Meanwhile, DOX-treated group showed a significant reduction ($P < 0.05$) in PA and PI by about 63.6 and 66.6%, respectively, compared to control group. However, oral administration of CCE following DOX injection caused remarkable improvement in PA and PI ($P < 0.05$) by about 99.3% and 165.5%, confirming the immunostimulant effect of the extract.

Effect on Hematological parameters parameters

No significant change in Hematological parameters was observed in animals supplemented with CCE only [Supplementary Table 2]; however, these animals showed a significant increase in lymphocyte count ($P < 0.05$) by 30.8% compared to the normal control group. On the other hand, DOX-intoxicated animals suffered from a significant reduction in erythron parameters, including RBCs counts, Hb concentration, and PCV% by about 27.6%, 28.5%, and 29.8%, respectively, revealing a picture of normocytic normochromic anemia. In addition, the same animals showed significant reduction in TLC, neutrophils, lymphocytes, and monocytes counts when compared to the normal control group by about 37.4%, 57.6%, 11.6%, and 39.8%, respectively. Administration of CCE to DOX treated animals reduced all changes in the hematological parameters, as it significantly increased RBCs counts, Hb concentration, and PCV% by 20.4%, 24.9%, and 23.2%, respectively. In addition, total TLC, neutrophil, lymphocytes, and monocyte counts were also improved by about 38.9%, 84.6%, 9.5%, and 51.6%, respectively.

Effect on liver and kidney functions

Animals receiving CCE only showed no significant changes in parameters related to liver and kidney functions [Table 2] compared to the normal control group. On the other hand, the DOX-treated group exhibited a significant increase ($P \leq 0.05$) in serum enzyme activities of ALT, AST, and LDH (210.5%, 189.9%, and 219.3%, respectively) and a significant reduction in serum TP and Alb concentrations ($P \leq 0.05$) by 18.5%, and 23.2%, respectively, compared to control group. DOX treatment significantly elevated serum concentrations of glucose and renal injury markers (urea and creatinine) ($P \leq 0.05$) by 46.1%, 37.9%, and 106.1%, respectively, compared to the control group. However, administration of CCE at dose of 100 mg/kg ameliorated these changes, causing a significant reduction ($P \leq 0.05$) in the elevated hepatic marker enzymes (ALT, AST and LDH) by 50.3%, 39.4%, and 57%, respectively, while restoring TP and Alb concentrations close to normal values ($P \leq 0.05$). Moreover, reduction in the elevated glucose level, urea and creatinine by 27.4%, 21.4%, and 35.6%, respectively, were observed in DXR + CCE animals compared to animals treated with DOX only.

Effect on oxidative stress

No significant change in MDA, GSH levels or SOD activity was observed in animals receiving CCE only compared to normal control group, whereas the DOX-treated group exhibited a significant increase ($P \leq 0.05$) in MDA level by 353.7% together with diminished

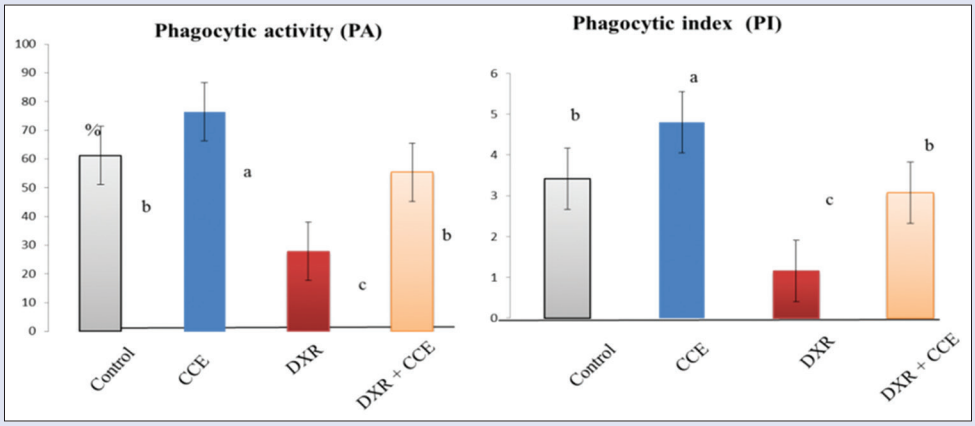

Figure 1: Effect of the administration of *Calligonum comosum* extract and doxorubicin on phagocytic activity and phagocytic index. Immune responses: Phagocytic activity and phagocytic index of different experimental animal groups. Results are given as mean ± standard error. Different superscripts (a, b, c… etc.,) indicate significantly different values ($P < 0.05$)

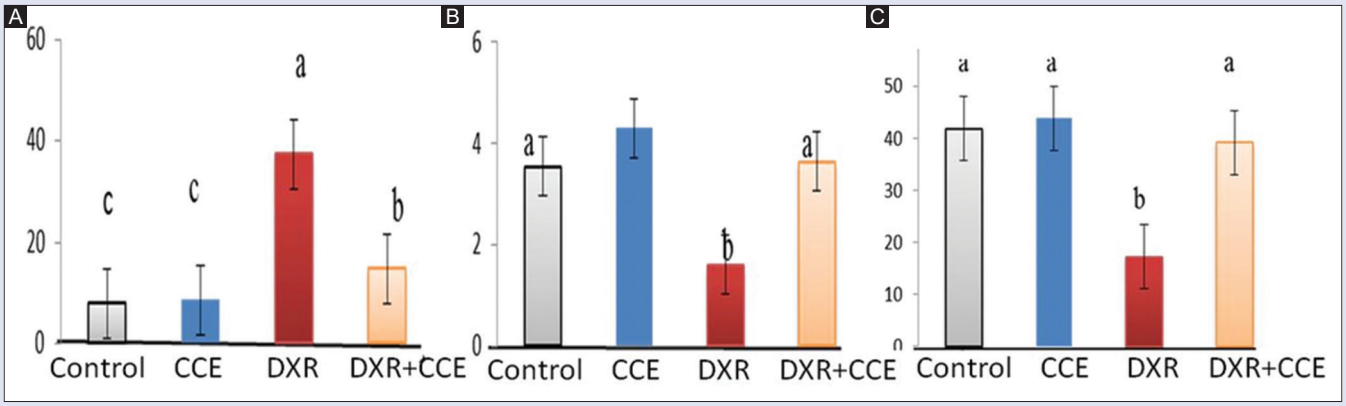

Figure 2: Effect of administration of *Calligonum comosum* extract and doxorubicin on oxidative status. Levels of serum malondialdehyde: (a) reduced glutathione (Reduced glutathione: [b] and super oxide dismutase: [c] in different experimental animal groups). Data are presented as mean ± standard error. [a, b]Superscripts indicate significant difference ($P < 0.05$)

Table 2: The effect of *Calligonum comosum* extract and/or doxorubicin on serum biochemical parameters

Parameter	Control (Group I)	CCE (Group II)	DXR (Group III)	DXR + CCE (Group IV)
ALT (IU/L)	24.60±1.25^{c}	23.60±2.6^{c}	76.40±2.9^{a}	38.00±1.8^{b}
AST (IU/L)	37.60±1.16^{c}	38.40±2.7^{c}	109.00±4.9^{a}	66.00±2.14^{b}
LDH (IU/L)	278.40±15.7^{c}	280.40±18.7^{c}	889.00±39.4^{a}	382.60±25.4^{b}
TP (g/dL)	4.98±0.07^{ab}	5.30±0.31^{a}	4.06±0.21^{c}	4.70±0.27^{b}
Albumin (g/dL)	2.2±0.09^{a}	2.10±0.09^{a}	1.69±0.05^{b}	1.90±0.14^{a}
Globulin (g/dL)	2.78±0.08	3.20±0.33	2.37±0.19	2.80±0.186
Albumin/globulin	0.79±0.05	0.66±0.13	0.71±0.05	0.68±0.10
Glucose (mg/dL)	86.40±3.9^{b}	80.00±1.1^{b}	126.20±2.6^{a}	91.60±2.2^{b}
Urea (mg/dL)	44.80±0.86^{b}	44.20±2.13^{b}	61.8±4.02^{a}	48.6±1.91^{b}
Creatinine (mg/dL)	0.98±0.06^{b}	0.90±0.09^{b}	2.02±0.09^{a}	1.3±0.2^{b}

Statistical analysis was performed using the one-way ANOVA-test followed by Duncan's multiple range test; n=8 ($P \leq 0.05$). Different superscripts (a, b, c) within the same row indicate statistical significance ($P \leq 0.05$). ANOVA: Analysis of variance; CCE: *Calligonum comosum* extract; DXR: Doxorubicin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; LDH: Lactate dehydrogenase; TP: Total proteins

antioxidant capacity as indicated by decline in SOD activity and GSH level 58.9% and 54.8%, respectively, compared to normal control group [Figure 2]. The animals receiving both DOX and CCE showed restoration of the antioxidant capacity as indicated by normal values of SOD and GSH and reduction of MDA levels by 60.2% compared to the DOX treated group.

DNA fragmentation and comet assay

Random DNA fragmentation was scored at (3+) for DOX-intoxicated animals and at (+1) in animals receiving CCE after DOX treatment indicating a less genotoxic effect [Supplementary Figure 2]. DOX renal and hepatic genotoxic potentials were further assessed using Comet assay [Supplementary Figure 3] where no significant change in the

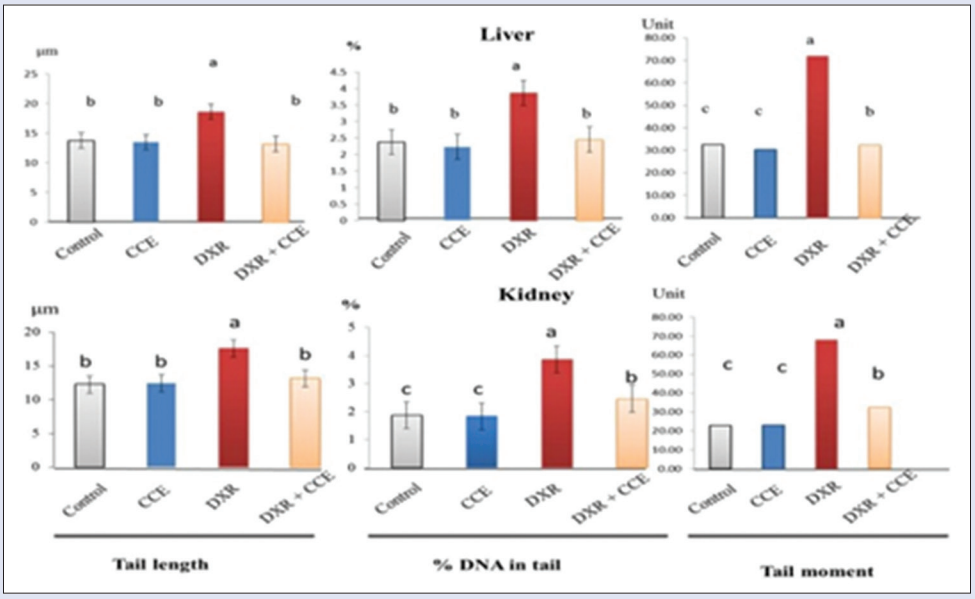

Figure 3: Genotoxic effects in the liver and kidney of animals receiving *Calligonum comosum* extract, doxorubicin or both. Comet assay parameters, including tail length, % DNA in tail (tail intensity), and tail moment in the liver tissue (upper panel) and kidney tissue (lower panel) in rats belonging to different experimental groups. Results are given as mean ± standard error. [a,b]Superscripts indicate significant difference ($P < 0.05$)

tail length of DNA, tail intensity (DNA %) or TM in renal and hepatic tissues of CCE supplemented rats was observed compared to the normal control group. Meanwhile, an increase by 43.4%, 106.3%, and 195.9% was observed in the tail length of DNA, tail intensity (DNA%), and TM in renal tissue of the DOX treated rats, respectively, compared to normal control group [Figure 3]. A similar increase in the tail length, tail intensity (DNA%), and TM by 35.5%, 62.8%, and 120.5% in the hepatic tissue of DOX-treated rats was observed. On the other hand, animals treated with DOX + CCE showed significantly reduced renal DNA tail length, intensity and moment by 25.1%, 36.4%, and 52.3%, respectively, when compared to animals treated with DOX only with similar reduction in hepatic DNA tail length, intensity, and moment by 29.1%, 36.4%, and 54.9%, respectively [Figure 3].

In addition, histopathological examination of the liver of DOX-treated animal showed hepatic vacuolation mostly beginning perinuclear and extending to the outer cytoplasmic membrane with diffusion extent of nuclear pyknosis and apoptosis [Figure 4]. Treatment with CCE resulted in a marked decrease in cellular vacuolation and apoptosis. Similarly, DOX-treated animals revealed severe congestion within the glomerular tufts, diffuse nuclear pyknosis, and apoptosis of the tubular lining epithelial cells of the kidney tissue and vacuolation of the collecting renal tubules. A marked decrease in the extent of tissue damage was observed in kidneys excised from animals treated with both DOX and CCE [Figure 4 and Supplementary Figure 4].

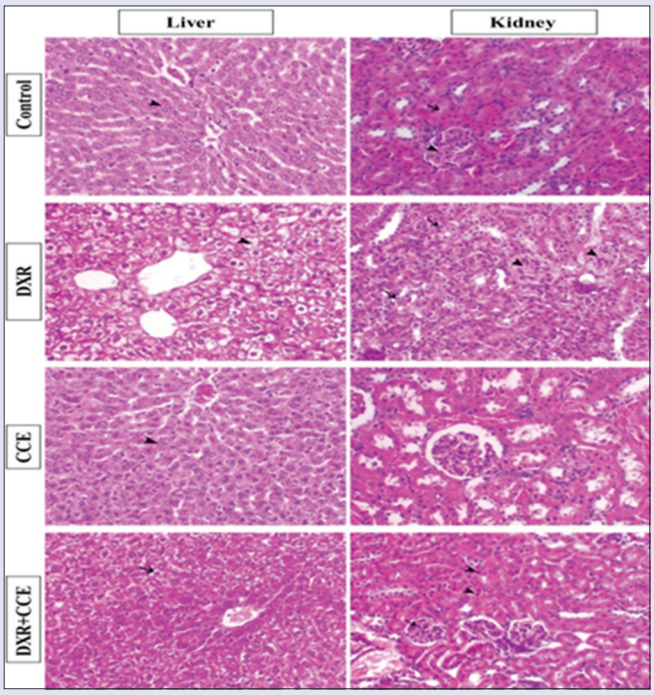

Figure 4: Histopathological effects in the kidney and liver tissues of animals receiving doxorubicin, *Calligonum comosum* extract or both. Photomicrographs of kidney and liver sections from rats of different experimental groups

DISCUSSION

Several studies hypothesized that the co-administration of chemotherapeutic agents such as DOX with a potent antioxidant is an appropriate approach for reducing the toxicity of these anticlastogenic drugs.[24,25] *C. comosum* is a desert plant that is distributed in North Africa, Arab peninsula, and Southwest Asia and was previously reported to demonstrate a cardioprotective effect in DOX-treated rats.[9] The purpose of this study was to investigate the protective effect of *C. comosum* against other organ toxicities induced by DOX and to evaluate how its phytoconstituents may play a role in its organ protective effects.

Using the model described here, animals treated with DOX showed multiple signs of organ toxicity as indicated by a significant decrease in body weight, decreased food intake, hematopoietic and immune-suppression, decline in kidney and liver functions that were associated with DNA and tissue damage. These results are consistent with previous findings detailing signs of hematopoietic suppression and hepatorenal toxicity after DOX treatment.[26,27]

The undesired toxicity of DOX is thought to largely come from its stimulation of intracellular production of reactive oxygen species, that damage cell membranes and induce drastic apoptosis and tissue damage in vital organs such as the liver and kidney which was confirmed by our finding that showed clear signs of oxidative stress after administration of DOX including increased MDA levels and impaired activity of SOD and level of GSH in DOX-treated animals.[8] Therefore, a strong antioxidant drug/plant extract can reverse signs of DOX multi-organ toxicities. Our results here indicated that the treatment of DOX intoxicated animals with CCE eliminated signs of oxidative stress and reduced all biochemical and histological signs of DOX-induced renal and hepatotoxicity as can be inferred from the values of the corresponding biochemical parameters [Figure 2 and Table 2]. The beneficial effect of CCE on the oxidative status can be attributed to a number of natural antioxidant molecules such as catechin, 3-galloyl-(epi) catechin, procyanidin B2, procyanidin B1-3-*O*-gallate, quercetin, and kaempferol derivatives that were identified in CCE using LC/MS/MS analysis. Catechins and their dimeric compounds (procyanidin B1, B2) are known to protect the integrity of cell membranes by interacting with lipid membrane bilayer and preventing free radical-induced tissue damage.[28] Furthermore, catechin and its gallate derivatives were shown by other investigators to prevent DOX-induced damage in malignant hepatocytes (HCT-8) without affecting DOX-anti-proliferative effect.[29] Other phytoconstituent of *C. comosum* such as kaempferol and quercetin are known for their potent antioxidant and anti-inflammatory effects.[30,31] In previous studies, quercetin decreased signs of cardiomyopathy associated with DXR through its antioxidant and metal chelating effect.[32,33]

Furthermore, administration of CCE reduced tissue injury score and cellular apoptosis and maintained normal tissue architecture in both kidney and liver of animals receiving both DOX and CCE [Figure 4 and Table 2] in comparison to animals receiving DOX only. Similar hepatorenal protective effect has been reported for other preparations rich in procyanidins such as grape seeds extract.[34,35]

Interestingly, restoring the balance in the oxidative status was not the only mechanism by which CCE counteracts DOX toxicity. Animals that received CCE only showed an increase in phagocytic activities and an increase in lymphocyte count, indicating an immunostimulant effect of CCE. Hence when DOX treated animals received a daily dose of CCE, signs of DOX-immunosuppression were completely reversed [Figure 1 and Table 1]. This immunostimulant effect may also be related to the high phenolic content of CCE, especially catechin derivatives such as procyanidin B2 which has been previously shown to enhance the phagocytic response of macrophages.[36] The net result of the diverse biological effects of the different constituents of CCE is an apparent reversal of most signs of DOX toxicity. Although, *in vitro* studies concluded that CCE does not undermine the antiproliferative effect DOX, further studies are needed to investigate this effect in animal models.

CONCLUSION

Overall, CCE is a very promising drug to be further investigated for use in chemotherapy regimen with DOX and possibly other chemotherapeutic agents that exert their antineoplastic effect through a similar mechanism to reduce their organ toxicities. In this study, we showed that CCE eliminated DOX hepatorenal toxicity, hemopietic and immune suppression and in a previous report by Ashour *et al*. CCE was shown to reduce DOX-induced cardiotoxicity. It is worth mentioning that CCE was shown to possess some antitumor activity of its own which may add to its therapeutic benefit in a chemotherapy regimen.[12] The phenolic constituents of CCE as revealed in this study were shown to interact efficiently to protect the different vital organs and enhance body defense mechanism as seen by the increase in lymphocyte count and enhancement of phagocytic activity. However, the effect of CCE on the anticancer activity of DOX should be thoroughly studied before incorporating CCE in any chemotherapeutic regimen. Therefore, this study should encourage further research of CCE and other plants with similar phytochemical profile.

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Conflicts of interest

There are no conflicts of interest.

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