

Ceratonia siliqua pods (Carob) methanol extract alleviates doxorubicin – induced nephrotoxicity via antioxidant, anti-inflammatory and anti-apoptotic pathways

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Abstract

Doxorubicin (DOX) is an effective antitumor therapy but its use is limited by its deleterious toxic effects including nephrotoxicity and cardiotoxicity. The aim of this work was to assess the potential protective effect of *Ceratonia siliqua* methanol extract (CME) on DOX-induced nephrotoxicity in 5 groups of rats. Rats in groups 1 and 2 were given normal saline while groups 3–5 were given Vitamin C (reference antioxidant, 250mg/kg), CME (500mg/kg) and CME (1000 mg/kg) for 5 days. On the 5th day, 1 hour after the last treatment dose, rats of groups 2–5 were given DOX in a dose of 15 mg/kg IP. DOX increased serum creatinine, urea, sodium and potassium and decreased GSH concentration, GST, CAT, SOD and MPO activities but increased MDA. It increased the inflammatory mediators (COX-2, IL-6, TNF- α , and NF- $\kappa\beta$) but decreased the anti-inflammatory cytokine (IL-10) and the Transforming growth factor- β (TGF- β). DOX has up-regulated COX-2, Caspase-3, Caspase-9, Bax and NF- $\kappa\beta$ transcripts and down-regulated the anti-apoptotic Bcl-2 as assessed by immunohistochemistry and gene expression analysis. CME significantly improved the levels of kidney function parameters and restored the levels of the oxidative stress markers. It also decreased the level of COX-2, IL-6, TNF- α , and NF- $\kappa\beta$ and stimulated the production of IL-10 and TGF- β . CME down-regulated the expression levels of the Bax, Cox-2 and caspases and up-regulated the anti-apoptotic Bcl-2. Microscopically, CME alleviated the DOX-induced renal damage in dose dependent manner. Phytochemical analysis revealed the presence of 26 compounds among which 4 major compounds (over 5%) in the CME. Acute toxicity test revealed that CME is not toxic up to 5 g/kg orally into rats. In conclusion, CME could effectively alleviate the deleterious effects of DOX on the kidney. The safety of carob extract encourages its use in the preparation of valuable therapeutic agents.

Introduction

Doxorubicin is a very effective therapeutic agent used for the management of different types of malignancies (Xiao et al. 2012). However, its use is associated with many toxic effects such as nephrotoxicity (El-Sheikh et al. 2012), cardiotoxicity (Reis-Mendes et al. 2021) and oxidative damage to various biological macromolecules (Sun et al. 2016). Moreover, DOX triggers apoptosis through the mitochondrial pathway, the chief signaling pathway in DOX-induced toxicities (Xiao et al. 2012) leading to mitochondrial dysfunction and release of mitochondrial cytochrome C, Apaf-1, and caspase-9 enzyme into the cytoplasm leading cell death (Beere et al. 2000). Moreover, DOX stimulates the release of various pro-inflammatory mediators such as cyclooxygenase-2 (COX-2) (Abd El-Aziz et al. 2012) which are mediated mainly by upregulation the expression of the nuclear factor kappa-B (NF- $\kappa\beta$). It was reported that this inflammatory pathway plays an essential role in DOX-induced nephrotoxicity (Rashid et al. 2013). *Ceratonia siliqua* fruit (carob) is used in food industry as flavoring, thickener and stabilizer in food (Dakia et al. 2007). Carob's pulp (pods) is the most important edible part of the carob fruit. Recently it has been reported that carob induces a variety of pharmacological actions including antimicrobial, anti-inflammatory, anti-diarrheal, anti-ulcer, antioxidant, anti-constipation effects (Rtibi et al. 2017). It has antidiabetic (Qasem et al. 2018), hepatoprotective (Souli et al. 2015) and antioxidant and cytotoxic activities effects (Custodio et al. 2011, Ayache et al. 2020). It has also been found to be effective in

neurodegenerative diseases (Lakkab et al. 2018) and has a protective effect on colon adenoma cells from genotoxic impact of H₂O₂ (Klenow et al. 2009).

Ceratonia siliqua contains a large number of phytochemicals in all parts such as phenolics, tannins, flavonoids, anthocyanins, glycosides, proteins, alkaloids and minerals (Goulas and Georgiou, 2019). The concentration of phenolic constituents in carob fruit, which are mainly phenolic acids and tannins (Stavrou et al. 2018), depends on genomic and environmental factors (Goulas et al. 2016) and the type of the solvent used for extraction (Goulas and Georgiou, 2019). According to the best of our knowledge, there is no study concerned on the evaluation of the protective effect of *Ceratonia siliqua* methanol extract (CME) on DOX-induced nephrotoxicity. The objective of this work was to assess the protective effect of CME on the DOX-induced nephrotoxicity and to elucidate its mechanism of action by testing the associated antioxidant, anti-inflammatory, immunomodulatory and apoptotic effects in the rat model.

Material And Methods

Plant material

Ceratonia siliqua pods (carob) were purchased from a local herbal market and were identified by the staff members of the Department of Flora, Ministry of Agriculture, Giza, Egypt.. A voucher sample was kept in the Pharmacology Department, Faculty of Veterinary Medicine, Cairo University. The seeds of the dried pods were removed and the carob pulps were powdered in an electric blender. Two hundred grams of the dried powder were extracted with methanol 95% for 24 hours, followed by percolation 5–7 times till complete extraction. The methanol extract was concentrated under reduced pressure at low temperature and reserved at – 4°C until subsequent use. The extract was freshly suspended in sterile phosphate buffer saline (pH 7.2) to a final concentration of 200 mg/ml.

Acute Toxicity Testing

Twenty mice were allocated randomly into 4 groups of 5 mice each. Before testing, the animals were fasted for 12 hrs (Atta et al. 2017) but allowed free access to drinking water. Rats in groups A, B, C and D were orally administered 0.5, 1, 2 and 4g/kg b. wt. of the carob pulps methanol extract, respectively. Mortality and symptoms of toxicity such as jerks and writhes were observed over 24 hours and daily up to 5 days.

Phytochemistry

Gas Chromatography (Agilent Technologies 7890A) interfaced with a mass selective detector (Agilent 7000 Triple Quad) and Agilent HP-5ms capillary column (30 m×0.25 mm ID and 0.25 µm film thickness) were used. The temperatures of the injector and the detector were adjusted to 200°C and 250°C, respectively. The flow rate was 1 mL/min. The acquisition mass range was 50–600. The formulae of the

components as identified by comparing their mass spectra and RT with those of NIST and WILEY library were recorded.

Animals, treatments and sampling

Thirty-five Wistar rats of 200–250 g body weight were obtained from Animal Breeding House, national Research centre, Giza, Egypt. Animals were reared under strict hygienic conditions for 7 days for acclimatization. Animals were randomly allocated into 5 equal groups. Rats of the first and second group were given normal saline (1mL/rat) orally by gastric gavage for 5 days. Rats of the third group were given Vitamin C (reference antioxidant drug) in a dose of 250 mg/kg b. wt. by oral route. Rats in the fourth and fifth groups were given CME at a dose 500 and 1000 mg/kg b. wt. respectively by the same route for the same period. In the fifth day, rats of groups 2–5 were given DOX at a dose of 15 mg/kg intraperitoneal (IP), 1 hour after the last treatment dose (Ibrahim et al. 2019). After 48 hrs, body weight was recorded and samples of blood were taken from retro orbital plexus of veins under light anaesthesia. Blood samples were left to clot to obtain clear serum after centrifugation (4000 rpm for 10 min). Serum was maintained at -20°C for estimation of kidney function parameters (creatinine, urea, calcium, potassium, sodium and chloride). Rats were then euthanized with pentobarbital sodium (150 mg/kg b. wt. IP). Both kidneys were removed from all rats, weighted and were used for preparation of tissue homogenate, immunohistochemistry and histopathology.

The kidneys were dissected and washed with PBS (10 mM (10 mM KH₂PO₄, 8 mM NaH₂PO₄–, 137 mM NaCl, 2.7 mM KCl; pH = 7.4). The kidney tissues were then blended in PBS and centrifuged at 10,000×g for 20 min at 4°C. The supernatant was kept at -80°C till used for assessment of oxidative stress, inflammatory and immune responses and apoptotic markers.

Assessment Of Nephrotoxicity Indices In Serum

Serum creatinine and urea levels were estimated using kits purchased from Erba, Germany. Serum sodium, potassium, chloride, and calcium levels were determined using kits purchased from Centronic Company, Germany.

Assessment Of Oxidative Stress Markers In Kidney Homogenate

Reduced glutathione (GSH) concentration (Ellman 1959) and the activities of superoxide dismutase (SOD) (Marklund & Marklund 1974), glutathione-S-transferase (GST) (Habig et al. 1974), and Lipid peroxide by-product as malondialdehyde (MDA) contents (Ohkawa et al. 1979) were estimated in the supernatant of kidney homogenate. All Analytical chemicals were purchased from Sigma Alderish, USA. The concentration of MDA was estimated using commercial test kit obtained from Biodiagnostic Co, Egypt. These parameters were estimated using a spectrophotometer (T80 UV/VIS PG instrument Ltd, UK),

Catalase (CAT) and myeloperoxidase (MPO) activities were assessed by ELISA using the commercially available kit (SUNLONG, China).

Assessment Of Inflammatory, Immune Response And Apoptotic Markers In Kidney Tissue Homogenate

The pro-inflammatory cytokines; the interleukin-6 (IL-6) and the tumor necrosis factor alpha (TNF- α) were estimated by ELISA using commercially available kits (SUNLONG, China) and (Cloud Clone Crop, China), respectively. The levels of the anti-inflammatory mediators; interleukin-10 (IL-10) and transforming growth factor- β (TGF- β) were also determined by ELISA using the commercially available kits (SUNLONG, China). The apoptotic marker; Caspase-3 (Cas-3) was assessed by ELISA using the commercial kits from SUNRED, China. In brief, kidney tissue homogenates were incubated with the immobilized specific antibodies and visualized using HRP-TMB reaction.

Real-time Pcr For Assessment Of Gene Expression

The transcript level of COX-2, Cas-9, Bax and Bcl-2 was assessed by the R-T PCR. Total RNA of the kidney tissue was extracted using EasyRNA™ Cell/Tissue RNA Mini Kit (Biovision #K1337). The synthesis of the first-strand cDNA was carried out using SuperScript Reverse Transcriptases (Thermoscientific) according to the instructions of the manufacturer. Quantitative PCR was performed using Power Track™ SYBR Green Master Mix Applied Biosystems™ on an ABI Prism Step OnePlus Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. The primer sets of the assessed genes were listed in Table 1. The relative mRNA expression of the target genes was calculated as fold change of the normal control after normalization to *actb*, reference transcript, using $2^{-\Delta\Delta CT}$ methods.

Table 1
The primer sets of the assessed genes

Gene	Forward primer	Reverse primer	Product	Accession No.
<i>cas-9</i>	CTGTGTTCCAAGGTCTCGGC	CCAGGCTCACTTAGCAAGGAA	149	NM_031632.2
<i>bax</i>	CACGTCTGCGGGGAGTCAC	TTCTTGGTGGATGCGTCCTG	248	NM_017059.2
<i>Bcl-2</i>	TCGCGACTTTGCAGAGATGT	CAATCCTCCCCAGTTCACC	116	NM_016993.2
<i>cox-2</i>	CTCAGCCATGCAGCAAATCC	GGGTGGGCTTCAGCAGTAAT	172	NM_017232.3
<i>actb</i>	CCGCGAGTACAACCTTCTTG	CAGTTGGTGACAATGCCGTG	297	NM_031144.3

Immunohistochemistry And Histopathology

B-cell lymphoma-2 protein-associated X protein (Bax), B-cell lymphoma-2 protein (Bcl-2), cyclooxygenase-2 (COX-2), and nuclear factor kappa (NF- κ B) were immunohistochemically stained in paraffin-embedded

tissue sections after antigen retrieval using citrate buffer (pH 6). Primary antibodies against Bax (InVitrogen PA5-78857, USA), Bcl-2 (InVitrogen PA5-27094, USA), COX-2 (InVitrogen PA1-37505, USA), and NF- κ B P65 (sc-8008, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were applied to the slides, followed by endogenous peroxidase blocking by hydrogen peroxide. Secondary horseradish peroxidase (HRP)-labeled antibody was applied according to the protocol of the manufacturer (Universal PolyHRP DAB kit for mouse and rabbit, Genemed, Sakura Torrance, CA, USA). Secondary antibodies were used without primary antibodies in the negative control slides.

Kidney specimens were fixed in 10% neutral buffered formalin. Fixed tissue was dehydrated in ascending concentrations of ethanol, cleared in xylene and embedded in paraffin. The tissue was sectioned by microtome (Lieca 2135, Germany) into 4 μ m thickness sections and stained by hematoxylin and eosin stain. Light microscope (Olympus BX43, Japan) equipped by digital camera (Olympus DP27 camera) was used for examination.

Statistics

Data were presented as mean $\pm$ SD. Difference between means of different groups were tested for significance using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test via IBM SPSS (IBM Corporation, Armonk, NY, USA). The difference was considered significant at $P < 0.05$.

Results

Acute Toxicity

No symptoms of illness or discomfort appeared for a 5 days observation period in rats' receiving graded doses of *Ceratonia siliqua* methanol extract up to 4g/kg b. wt. administered orally. These results indicate that *Ceratonia siliqua* methanol extract is nontoxic.

Effect Of Cme On Serum Biochemical Constituents

Serum creatinine, urea, sodium and potassium concentrations were significantly ($P < 0.01$) increased in DOX-intoxicated rats, while serum calcium level was decreased significantly as compared to normal control rats. Treatment of DOX-intoxicated rats with CME (500, and 1000 mg/kg b.wt.), as well as Vit C, significantly ($P < 0.01$) decreased serum creatinine, urea, sodium, and potassium while serum calcium level markedly ($P = 0.01$) increased in comparison with DOX-intoxicated group. However, chloride concentration was not affected. (Table 2)

Table 2

Effect of Carob methanol *extract (CME)* and *Vitamin C* on kidney function parameters in the serum of Doxorubicin -induced nephrotoxicity in rats (Mean $\pm$ SE, n = 6)

Groups	Creatinine (mg/dl)	Urea (mg/dl)	Sodium (mEq/l)	Potassium (mEq/l)	Chloride (mEq/l)	Calcium (mg/dl)
Control	0.27 $\pm$ 0.02 ^c	38.69 $\pm$ 6.4 ^c	134.06 $\pm$ 3.4 ^c	4.38 $\pm$ 0.47 ^b	100.66 $\pm$ 3.3	10.89 $\pm$ 0.2 ^a
Doxorubicin	0.68 $\pm$ 0.13 ^a	55.56 $\pm$ 7.0 ^a	143.13 $\pm$ 2.8 ^a	5.45 $\pm$ 0.78 ^a	97.76 $\pm$ 3.8	9.87 $\pm$ 0.2 ^c
Vit C (250 mg/kg b.wt.)	0.45 $\pm$ 0.06 ^b	46.46 $\pm$ 7.2 ^b	135.75 $\pm$ 3.1 ^{bc}	4.54 $\pm$ 0.34 ^b	98.10 $\pm$ 2.2	10.37 $\pm$ 0.1 ^b
CME500 mg/kg b.wt.	0.52 $\pm$ 0.07 ^b	46.69 $\pm$ 4.3 ^b	136.54 $\pm$ 1.7 ^{bc}	4.62 $\pm$ 0.25 ^b	98.40 $\pm$ 1.3	10.42 $\pm$ 0.2 ^b
CME1000 mg/kg b.wt	0.53 $\pm$ 0.05 ^b	47.10 $\pm$ 4.7 ^b	138.89 $\pm$ 2.2 ^b	4.92 $\pm$ 0.21 ^{ab}	99.74 $\pm$ 1.8	10.57 $\pm$ 0.3 ^b
Means with different superscripts in the same column are significantly different at $P < 0.05$.						

Effect Of Cme On Oxidative Stress Markers In Kidney Tissue Homogenate

GSH and GST concentration and CAT and SOD activities were significantly ($P < 0.05$) decreased and MDA was significantly increased in the kidney tissue homogenate of rats with Doxorubicin -induced nephrotoxicity. Treatment of DOX-intoxicated rats with CME 500, and 1000 mg/kg b.wt., as well as Vit C, significantly ($P < 0.05$) restored the values of oxidative stress markers (GSH, CAT, SOD, and MDA) to the normal levels (Table 3).

Table 3

Effect of Carob methanol extract (CME) and Vitamin C on oxidative stress markers in the kidney tissue homogenate of rats with Doxorubicin-induced nephrotoxicity (Mean $\pm$ SE, n = 6)

Groups	GSH (nMol/100mg protein)	GST (nMol/min/mg protein)	Catalase (pg/ml)	SOD (U/ml tissue)	MDA (mMol/g tissue)
Control	3.00 $\pm$ 0.80 ^c	0.32 $\pm$ 0.02 ^a	83.33 $\pm$ 9.44 ^b	73.80 $\pm$ 2.71 ^b	8.48 $\pm$ 0.36 ^b
Doxorubicin	0.70 $\pm$ 0.10 ^d	0.26 $\pm$ 0.01 ^b	45.00 $\pm$ 2.98 ^d	66.60 $\pm$ 6.56 ^c	10.18 $\pm$ 1.54 ^a
Vit. C (250 mg/kg b.wt.)	9.20 $\pm$ 1.10 ^a	0.27 $\pm$ 0.042 ^b	62.50 $\pm$ 1.77 ^c	83.80 $\pm$ 1.60 ^b	7.72 $\pm$ 0.33 ^b
CME500 mg/kg b.wt.	7.80 $\pm$ 0.70 ^b	0.32 $\pm$ 0.05 ^a	91.50 $\pm$ 8.60 ^a	79.20 $\pm$ 9.52 ^b	7.87 $\pm$ 0.05 ^b
CME1000 mg/kg b.wt.	8.90 $\pm$ 1.40 ^a	0.25 $\pm$ 0.01 ^b	60.00 $\pm$ 7.07 ^c	90.80 $\pm$ 3.87 ^a	6.55 $\pm$ 0.43 ^c
Means with different superscripts in the same column are significantly different at $P < 0.05$. GSH: Reduced Glutathione, GST: Glutathione-s-transferase, SOD: Superoxide dismutase, MDA: malondialdehyde.					

Effect of CME on the inflammatory, immune responses, and apoptotic markers in the kidney tissue homogenate

The level of IL-10 was decreased in the kidney tissue homogenate of DOX-intoxicated rats as compared to normal one (87.08 $\pm$ 6.21 vs 125.00 $\pm$ 14.14 pg/ml, respectively). TGF- β level was markedly decreased in the kidney tissue homogenate of DOX- intoxicated group as compared to normal one (105.83 $\pm$ 27.09 vs 646.66 $\pm$ 3.02 pg/ml). Treatment of rats with DOX-induced nephrotoxicity with either low or high doses of the CME as well as with Vit C significantly increased the IL-10 (Fig. 1A) and TGF- β levels (Fig. 1B). On the other hand, the pro-inflammatory marker; IL6 and the TNF- α were significantly increased in the DOX group (26.25 $\pm$ 2.62 pg/ml) as compared to normal rats (17.16 $\pm$ 0.81 pg/ml). Treatment of DOX-intoxicated rats with either low or high dose of CME as well as Vit C significantly decreased the levels of IL6 (Fig. 1C) and the TNF- α (Fig. 1D) as compared to DOX-intoxicated one. MPO was decreased in the kidney tissue of the DOX group as compared to the normal control (11.98 $\pm$ 0.36 vs 9.32 $\pm$ 1.19 mU/mg). Administration of CME at the small or the large dose restored significantly the level of MPO (10.71 $\pm$ 0.86 and 10.66 $\pm$ 0.81 mU/mg, respectively). The standard drug (Vit C) has normalized the level of MPO (Fig. 2A). On the other hand, the proapoptotic marker, Cas-3 was significantly increased in the renal tissue of DOX-nephrotoxic rats as compared to the normal level (3.47 $\pm$ 0.19 vs 2.96 $\pm$ 0.20 U/ml). Treatment of DOX-intoxicated rats with CME either the low or high dose as well as with Vit. C has decreased the level of Cas-3 (Fig. 2B).

Effect on the transcript level of COX-2, Cas-9, Bax and Bcl-2

The DOX-intoxicated group showed significant up-regulation of COX-2, Cas-9, Bax transcripts as well as a significant down-regulation of the anti-apoptotic marker, Bcl-2. Administration of CME alleviated the injurious effects induced by DOX as it suppressed the over-expression of COX-2, Cas-9, and Bax transcripts in dose dependent manner and significantly up-regulated the Bcl-2 gene (Fig. 3A, B, C and D).

Immunohistochemistry Of Bax, Bcl-2, Cox-2, And Nf- κ B

Immunolabeling of tubular epithelium in control against Bax, Bcl-2, NF- κ B and COX-2 was weak to moderate in control rats. Immunolabeling of tubular epithelium in DOX-intoxicated rats was moderate to strong against Bax, Bcl-2, NF- κ B and COX-2. Immunolabeling of tubular epithelium in Vit C (m), CME500 (n) and CME1000-treated rats was mostly weak against Bax, Bcl-2, NF- κ B and COX-2 (Fig. 4a - t). The area percent of Bax, **NF- κ B** and Cox-2 expression was markedly increased while the area percent of Bcl-2 was markedly decreased in DOX-induced nephrotoxic rats compared to normal one. Treatment of DOX-intoxicated rats with CME at small and large doses as well as Vit C has markedly decreased the area percent of Bax, **NF- κ B** and Cox-2 expression and increased the area percent of Bcl-2 expression (Fig. 5a, b, c, d).

Histopathological Findings

Microscopical examination of renal tissue showed normal histological structure in control rats (Fig. 6a). In DOX-intoxicated group, the glomeruli were atrophied and showed dilated Bowman's capsules. Moreover, the renal tubules were dilated and the tubular epithelium was vacuolated and sometimes exfoliated in the lumen. Moderate multifocal mononuclear leukocytes infiltration was observed in the interstitial tissue (Fig. 6b). In Vit C treated group, the histopathological lesions were less severe compared to the DOX- intoxicated group (Fig. 6c). The renal lesions were alleviated partially in CME 500 (Fig. 6d) and were alleviated almost completely in CME1000 compared to DOX- intoxicated group (Fig. 6e).

Phytochemical Constituents

Gas Chromatography (GC/MS) analysis of the methanol extract of carob pods revealed the presence of 26 compounds (Fig. 7, Table 4). The major components were Linolenic acid (28.86%), Malic Acid (15.28%), n-Hexadecanoic acid (13.29%) and 2,3-Butanediol (10.24%).

Table 4
Phytochemical analysis of *Ceratonia siliqua* methanol extract

No.	RT (min)	Name	Area Sum%
1	4.83	2,3-Butanediol	10.24
2	5.70	Butanoic acid, 2-ethyl-	4.73
3	7.13	Malic Acid	15.28
4	9.27	Ascaridiol	1.43
5	10.30	Dodecanoic acid, ethyl ester	0.50
6	10.80	Dodecanoic acid, ethyl ester	2.53
7	12.06	Myristic acid	0.35
8	12.27	D-Gluconic acid, δ -lactone	1.79
9	12.70	Pentadecanoic acid	2.99
10	13.13	Flavonols 3',4',5,7-OH, 3-O araglucoside	0.56
11	13.70	n-Hexadecanoic acid	13.29
12	14.04	Heptadecanoic acid	2.15
13	14.50	Silybin B	0.55
14	14.80	Linolenic acid	28.86
15	15.59	Eicosanoic acid	1.07
16	16.10	D-Pinitol	1.99
17	17.05	17-Octadecynoic acid	3.00
18	17.49	Octadecanedioic acid	0.72
19	17.62	2-Hexadecanol	0.29
20	18.20	Scutellareintetramethyl ether	0.30
21	19.79	1-Heptatriacotanol	1.53
22	20.70	Quercetin 3-glucoside	2.63
23	21.06	Phythol	0.70
24	21.44	Thunbergol	0.82
25	22.75	δ -Tocopherol	0.93

No.	RT (min)	Name	Area Sum%
26	22.90	Colchifoleine	0.79

Discussion

The results of the acute toxicity of *Ceratonia siliqua* (carob) showed that the carob methanol extract caused no symptoms of illness or discomfort indicating that the oral administration of the methanol extract in doses up to 4000 mg/kg was safe. It has been reported (Buck et al. 1976) that plant extracts with LD50 of more than 5000 mg/kg are considered nontoxic. Moreover, it has been reported that oral administration of the methanol extract of carob during 14 days experimental course showed no symptoms of toxicity (Qasem et al. 2018). The present results suggest that the use of carob extract as a potential source for therapeutic agents to treat various types of ailments is safe and without acute toxic effects.

Nephrotoxicity induced by drugs is a major health problem associated with severe renal tubular impairments and may lead to acute renal failure and a high rate of morbidity and mortality (Hussain et al. 2021). The rat model was successfully used to evaluate drug-induced amelioration on kidney function since their intra-renal enzyme distribution is similar to that in humans (Saad et al. 2009). DOX is a very effective chemotherapeutic agent and has useful application for the treatment of several types of malignancies (Xiao et al. 2012, Zhu and Lin 2021), however, its use is limited by the deleterious effects on the kidney and various biological membranes (Sabapathy et al. 2021, Mahzari et al., 2021). In the this work, we aimed to explore the potential preventive effect of methanol extract of *Ceratonia siliqua* (carob) against Doxorubicin -induced nephrotoxicity by testing its effects on kidney function, inflammatory/anti-inflammatory cytokines, oxidant/antioxidant markers, apoptosis cascade and confirm our results by histopathological, and immunohistochemical findings and by gene expression markers analysis.

In this study, further evidence of DOX-induced deleterious effects on renal function as indicated by elevation of serum creatinine, urea, serum sodium, potassium and calcium levels. The elevated level of serum creatinine, urea, and electrolytes are general indices of kidney injury impelled by drug treatment (Hussain et al. 2021; Xing et al. 2022). The decreased activity of renal SOD, GST and CAT accompanied by GSH depletion in the Dox-intoxicated rats is probably due to the increased generation of reactive free radicals (Nagai et al. 2018; Soltani Hekmat et al. 2021). The increased generation of reactive free radicals (hydroxyl radicals and superoxide radical) by DOX and hence peroxidation of lipid membrane is the cause of increased production of lipid peroxide by-product, MDA in DOX-treated animals (Mahzari et al. 2021). The deleterious effects of DOX were also confirmed by histopathology and immunohistochemical analysis in the present results as well as in previous studies (Soltani Hekmat et al. 2021). Pre-treatment with CME has down-regulated these parameters to a level comparable to normal control and maintained normal histopathological picture. Similar protective effects of *Ceratonia siliqua* have been reported against 6-Hydroxydopamine in Zebrafish (Abidar et al., 2020)

The cell cytokines such as IL-1 β , IL-6 and tumor necrosis factor alpha TNF- α , TGF- β , and NF- κ B play a central role in inflammation (Turner et al. 2014, Zhou et al. 2020). The enhanced production of ROS, decreased antioxidant enzymes apoptosis and oxidative stress in the kidney altering the permeability of glomerular capillary and inducing tubular degeneration might be the underlying causes of DOX-induced renal injury (Carvalho et al. 2009; Sun et al. 2016). On the other hand, the Nuclear factor E2-related factor 2 (Nrf2) is the main regulator of anti-oxidative responses (Vomund et al. 2017). DOX administration has been shown to downstream the antioxidant genes and proteins, leading to kidney toxicity. This is probably due to the reduction of both mRNA and protein expressions of Nrf2 (Zhang et al. 2017; Kamble and Patil 2018).

The observed increased generation of various inflammatory mediators including COX-2, IL-6 and TNF- α triggers apoptotic cascades and their gene expression (Xiao et al. 2012, Wu et al. 2021). Moreover, the increased level of these pro-inflammatory mediators in sequence leads to more tissue injury and further stimulation of NF- κ B which amplifies inflammatory signals and exacerbates tissue injury (Natarajan et al. 2018) which is considered a vital pathway in DOX-induced nephrotoxic effect (Rashid et al. 2013). Recently, a significant increase in the cardiorenal pro-inflammatory cytokines viz TNF- α , IL-6 in rats in DOX-intoxicated rats were previously reported (Xing et al. 2022). The increased expression of the NF- κ B mediates the decreased level of the IL-10 and TGF- β in kidney tissue as it has been reported in this study as well as others (Abd El-Aziz et al. 2012; Rashid et al. 2013). In DOX-treated rats, MPO was decreased as compared to normal rats. The decrease in MPO in DOX-treated animals may be due to early impairment to the enzyme leading to less subsequent oxidative stress (Reis-Mendes et al. 2021). The oxidative damage pathway has been thought to be a key anticancer mechanism of doxorubicin (Gupta and Srivastava 2012; Das et al. 2022). Treatment of DOX-intoxicated rats with CME has restored the level of the pro-inflammatory cytokine (TNF- α , IL-6). Interleukin-10 is an anti-inflammatory cytokine, which significantly contributes to the maintenance and reestablishment of immune homeostasis (Bedke et al. 2019). Enhanced production of anti-inflammatory cytokine (IL-10) mediates protection against several inflammatory conditions by preventing tissue pathology (Kalkal et al. 2022, Short et al. 2022). Similar to IL-10, TGF- β is another regulatory cytokines with important roles in the immune system (Li and Flavell, 2008). Treatment of DOX-intoxicated rats with CME has enhanced the production of the anti-inflammatory role of the cytokine IL-10 and the proteins TGF- β , an effect that can counteract the DOX-induced stimulation of the release of the pro-inflammatory cytokines; COX-2, IL-6 and TNF- α . The present results may be underlying the mechanism of the anti-inflammatory effects exhibited by *Ceratonia siliqua* leaves ethanol extracts (Alqudah et al., 2022)

Apoptosis is a process of controlled cell death in multicellular organism and its management is critical for normal growth, development, homeostasis and cancer treatment. Changes in normal apoptosis can result in aberrant cell growth, excessive cell division and mutation accumulation. Several molecular factors such as Bcl-2 and Bax play a crucial role in the implementation of apoptosis (Youle and Strasser 2008). Bax is an important executioner of mitochondrial regulated cell death through its lethal activity of permeabilizing the mitochondrial outer membrane. The physiological function of Bax is to ensures tissue homeostasis and its dysregulation leads to aberrant cell death (Spitz and Gavathiotis 2021). Caspases

are a large family of cysteine proteases that are essential for the initiation and execution of apoptosis (Fan et al. 2005, Sakamaki and Satou 2009). Apoptosis induced by DOX was evaluated by analysis of Bax, Bcl-2 and caspase-9 gene expression as well as by immunohistochemical analysis of Bax, Bcl2, NF-kB and COX-2. DOX decreased anti-apoptotic Bcl-2 gene expression and up-regulated both Bax and Cas-9 genes. DOX induced apoptosis is linked to the generation of ROS (Simon et al. 2000; Ozben 2007).

Bcl-2 protein is a crucial regulator in the apoptotic pathway which triggers and accelerates cell death. Bax inhibits the expression of Bcl-2 antiapoptotic protein by forming Bcl-2/Bax heterodimers (Morgan et al. 2021). The caspase family is a group of cysteine proteases that trigger apoptosis. The activation of caspases leads to the induction of apoptotic pathways (Ebedyet al. 2022). Our results indicated that Bax, Cas-9 and COX-2 transcriptions were up-regulated by DOX as well as the level of the pro-apoptotic marker, Cas-3.

Carob methanol extract down-regulated significantly the expression levels of the Bax, COX-2 and caspases an effect which might be the underlying mechanism by which carob extracts suppressed the DOX-induced nephrotoxicity. Bax, member of Bcl-2 family proteins, undergoes conformational changes and become translocated to the mitochondria to initiate apoptosis when activated. In the present study, immunohistochemical studies as well as gene expression showed increased Bax positivity in renal tissues of DOX -intoxicated rats. This result is in accordance with other research which indicated that DOX mediates apoptosis through the activation of Bax (Mostafa et al. 2021). The treatment with Vit C, CME 500 and CME 1000 mg/kg b.wt., decreased Bax expression as compared to the DOX-intoxicated group. On the other side, DOX was reported to decrease the expression of Bcl-2 and therefore induce apoptosis (Vu et al. 2020). In the same line, the present study showed that Bcl-2 expression was decreased in DOX group. This detrimental effect of DOX was however relieved in the Vit C and carob1000 mg/kg -treated rats.

Nuclear factor kappa-B (NF-kB) is believed to contribute significantly in controlling the transcription of various pro-inflammatory cytokines (Baldwin 1996). The results of this study confirm that DOX-induced nephrotoxicity is through stimulation of the NF- κ B signaling pathway (Wang et al. 2002; Mantawy et al. 2014) which was significantly decreased by Vit C and carob methanol extract treatment. The protective effect of carob methanol extract could be attributed to its active constituents. One of the major constituents is the Linolenic acid (28.86%) which has presented cardioprotective and radioprotective effects (Poorani et al.2020). Moreover, it decreased the levels of inflammatory cytokines and chemokines in an *in Vitro* study (Morin et al.2022). *Ceratonia siliqua* contains diverse bioactive phytoconstituents with high antioxidant activity such as phenolic compounds, flavonoids, alkaloids and tannins (Lakkab et al. 2018). The antioxidant activity of *Ceratonia siliqua* has been reported to be strongly related to the high level of its phenolic compounds (El Hajaji et al. 2011). Moreover, carob methanol extract was found to exhibit protective effect against lipid peroxidation caused by ROS in tissue and prevent depletion of the antioxidant enzyme; SOD, CAT and GPx. In addition, *Ceratonia siliqua* aqueous pods extract was reported to protect the gastric mucosa via its anti-inflammatory and antioxidant activities maintaining normal macroscopic and histological picture (Lachkar et al. 2016). Plant avonoids have also been reported to

alleviate gingival inflammation via reduction of nuclear NF- κ B translocation and myeloperoxidase activity (Gugliandolo et al. 2019). The phytochemical analysis of *Ceratonia siliqua* methanol extract revealed the presence of α -linolenic acid as one of the major constituents. α -linolenic acid; is an omega-3-fatty acid known to possess anti-inflammatory activity (Otuechere & Farombi, 2020) which may explain the reported inhibition of the pro-inflammatory mediators. .

Conclusions

The methanol extract of carob showed positive and dose-dependent effects with promising anti-inflammatory and antioxidant actions, and antiapoptotic effects as evidenced by preserving the histopathological features of kidney tissue.

Declarations

The authors declare that they have no competing interests.

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Data availability: The data used in the present study are available from the corresponding author on reasonable request

Ethics approval: This study was approved by the Institutional Animal Care and Use Committee, Cairo University, Faculty of Veterinary Medicine, Giza, Egypt. The protocol approval reference No: Vet CU 2009 2022496

Consent to participate: All individual participants gave their oral and written informed consent in this work.

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Author contribution Conceptualization: A.H.A., S.M.N., S.M.M., Reviewing and Methodolgy: S.M.M., S.R. Formal analysis S.A.A., M.K., T.H.A., M.I., Writing-original draft: A.H.A., S.A.A., M.K., T.H.A., M.I., Writing-review and editing: A.H.A., S.M.N.

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Figures

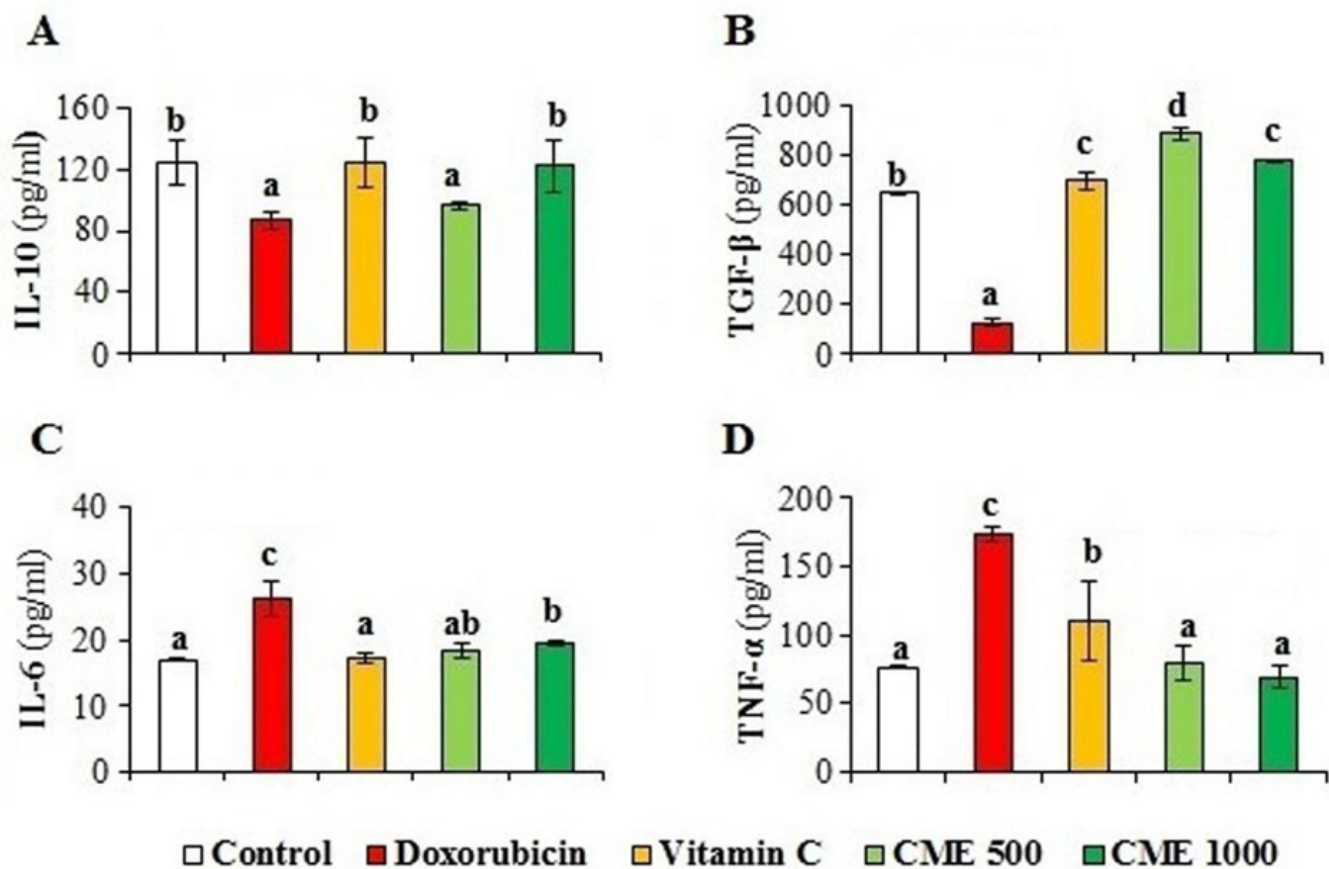


Figure 1

Effect of Carob methanol extract (CME 500 and 1000 mg/kg) on cytokines levels in kidney tissue homogenate of Doxorubicin-induced nephrotoxicity in rats. (A): Interleukin-10 (IL-10), (B): Transforming growth factor β (TGF- β), (C): Interleukin-6 (IL-6), and (D): Tumour necrosis factor alpha (TNF- α). Data are expressed as Mean $\pm$ SD, n = 6. Means with different letters in the same parameter are significantly different at $P < 0.05$.

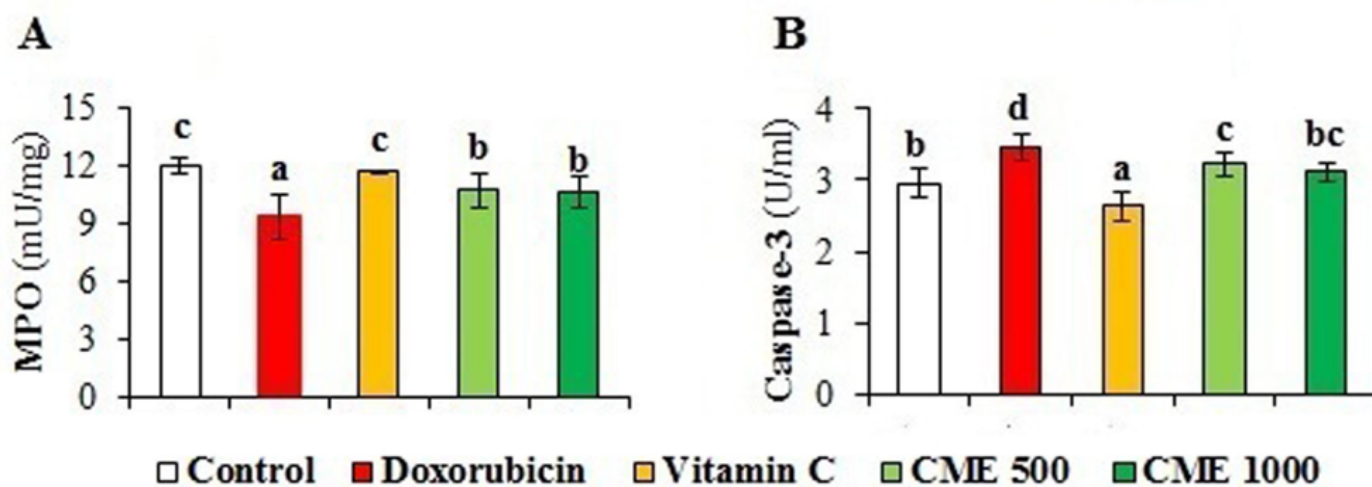


Figure 2

Effect of Carob methanol extract (CME 500 and 1000 mg/kg b.wt.) on the level of myeloperoxidase (MPO) (A) and Caspase-3 (B) in kidney tissue homogenate of Doxorubicin-induced nephrotoxicity in rats. Data are expressed as Mean $\pm$ SD, n = 6. Means with different letters in the same parameter are significantly different at $P < 0.05$.

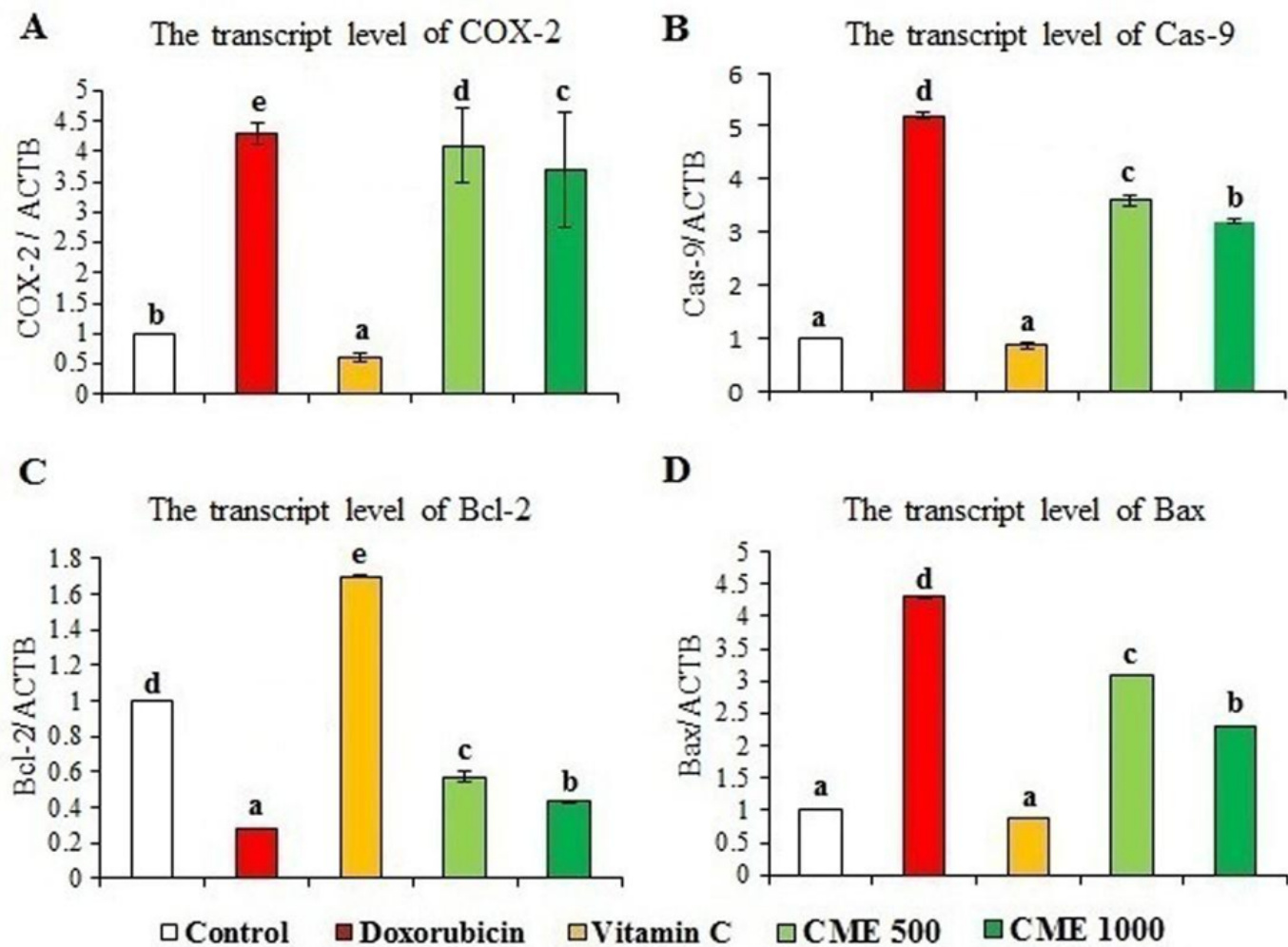


Figure 3

Effect of Carob methanol extract (CME 500 and 1000 mg/kg b.wt.) on the transcript level of: (A) COX-2 (cyclooxygenase-2), (B) Cas-9 (Caspase-9), (C) Bcl-2, and (d) Bax in the kidney tissue of rats with Doxorubicin-induced nephrotoxicity. Data are expressed as Mean $\pm$ SD, n = 6. Means with different letters in the same parameter are significantly different at $P < 0.05$.

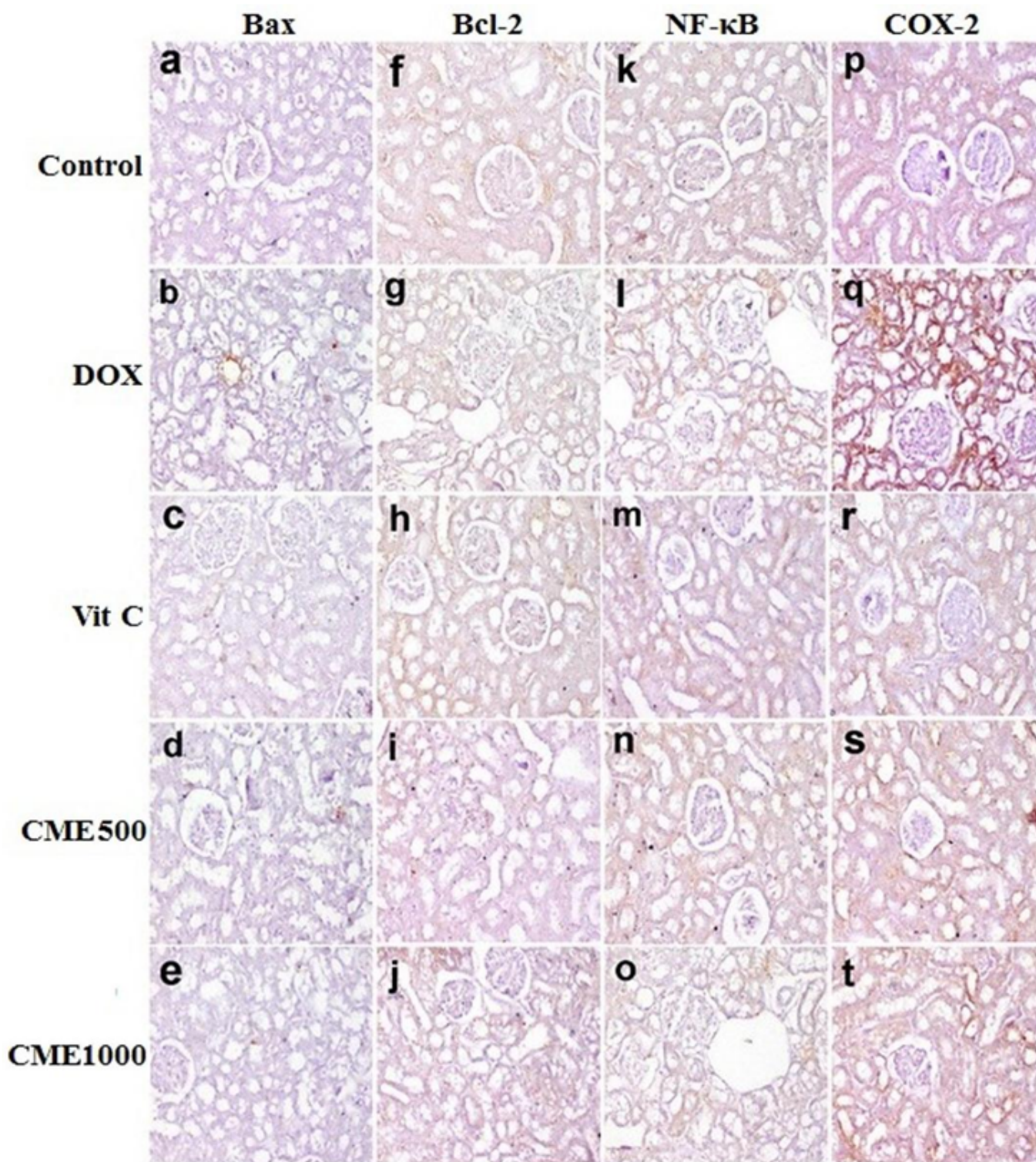


Figure 4

Immunohistochemistry of renal tissue in different groups showing Bax immunolabeling of tubular epithelium which was weak in control group (a), moderate in Dox group (b), weak in Vit C (c), Carob methanol extract (CME)500 (d) and CME1000 (e) groups, Bcl-2 immunolabelling of tubular epithelium which was moderate in control group (f), weak in DOX group (g), moderate in Vit C (h), CME500 (i) and CME1000 (j) groups, NF- κ B immunolabelling in tubular epithelium which was weak in control group (k),

moderate in DOX group (l), weak in Vit C (m), CME500 (n) and CME1000 (o) groups, COX-2 immunolabelling in tubular epithelium which was moderate in control group (p), strong in DOX group (q), and weak in Vit C (r), CME500 (s) and CME1000 (t) groups. (Immunoperoxidase and hematoxylin counterstain, X200). CME: Carob methanol extract.

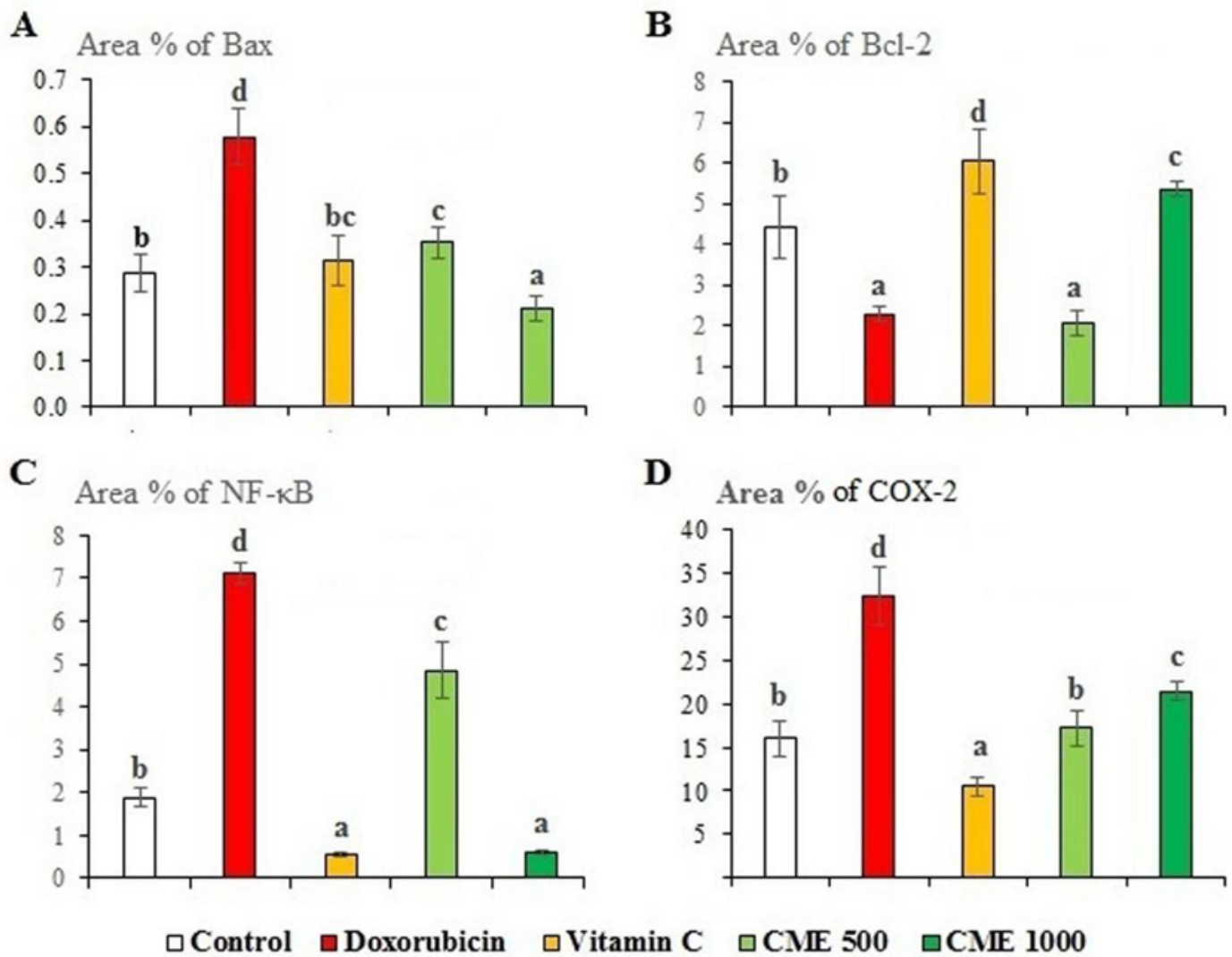


Figure 5

The area percent of **(A)**B-cell lymphoma-2 protein -associated X protein (Bax), **(B)**B-cell lymphoma-2 protein (Bcl-2), **(C)** NF-κB (Cyclooxygenase-2), and **(D)** COX-2 immunohistochemistry in different groups. Columns bearing different lowercase letter indicate significance at P value < 0.05 . (Mean $\pm$ SD, $n = 6$). CME: Carob methanol extract.

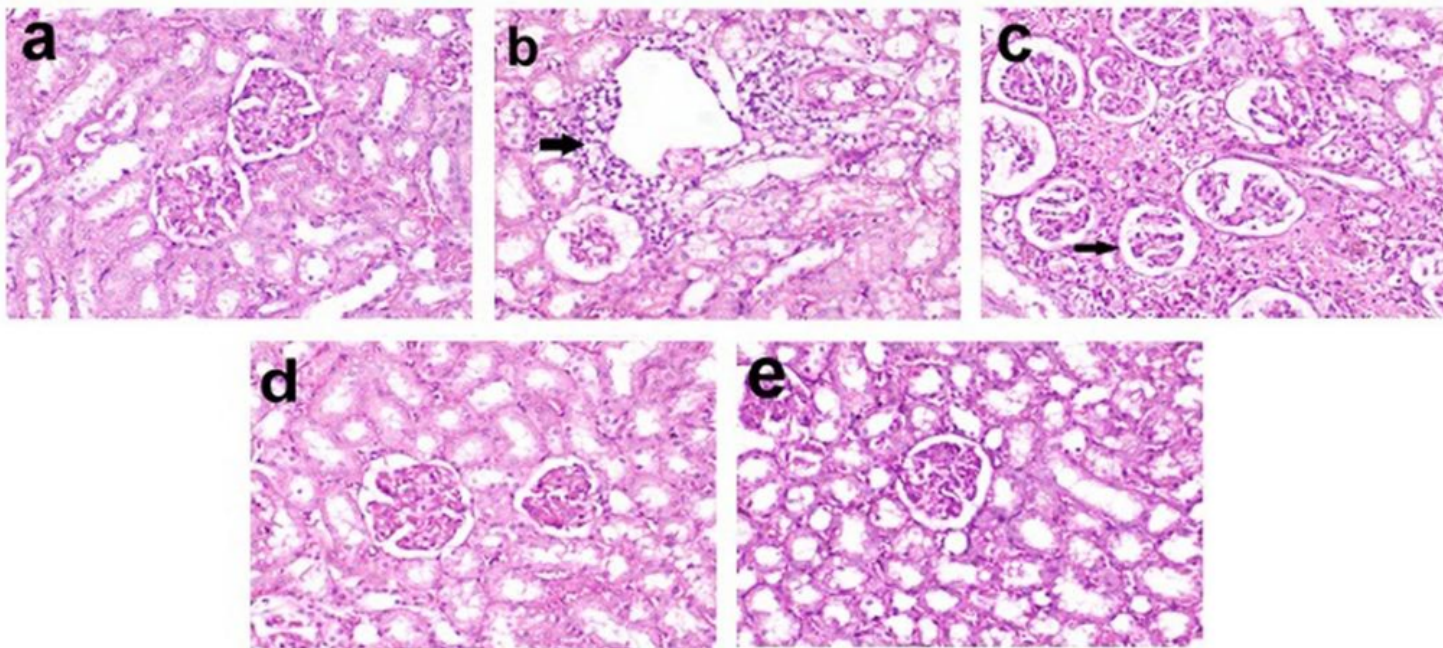


Figure 6

Histopathology of kidney from different groups: (a) Normal histological structure of kidney in the normal control group, (b) Glomerular atrophy, tubular dilatation and degeneration with interstitial leukocytes infiltration (arrow) in doxorubicin-intoxicated group, (c) Glomerular atrophy (arrow) and tubular degeneration in Vit C-treated group, (d) Moderate tubular degeneration in CME 500 and (e) Mild tubular degeneration in CME 1000 (H&E, X200).

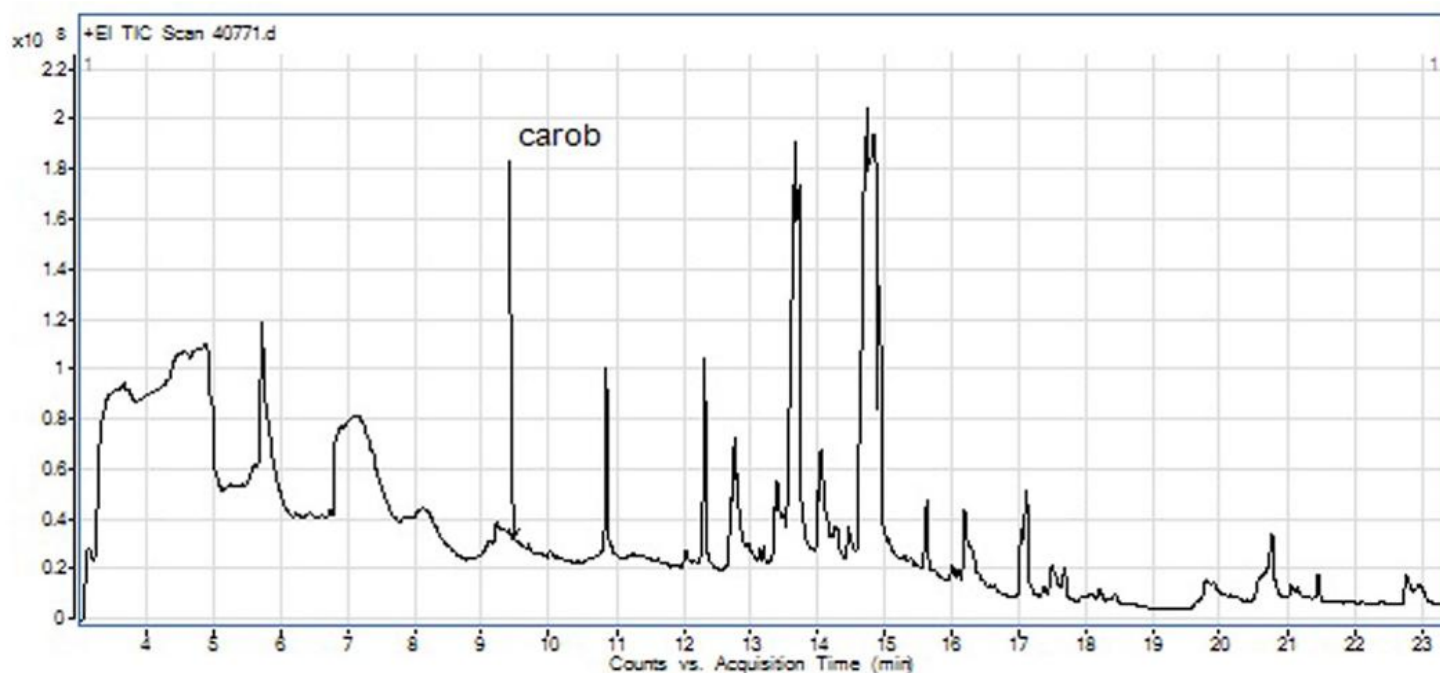


Figure 7

Phytochemical constituents of Carob methanol extract.