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Research Article
Camel Urine Prevents Cisplatin-induced Nephrotoxicity in Rats by Attenuating Oxidative Stress and Apoptosis

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Abstract

Background and Objectives: Cisplatin (CP) is a potent chemotherapeutic agent widely used for cancer treatment; however, the adverse side effects limit its use. Camel Urine (CU) contains several active metabolites and essential inorganic elements which exhibited several biological activities. This study aimed to identify the constituents and Radical Scavenging Activity (RSA) of CU and to explore its ameliorative effect of CU on CP nephrotoxicity in rats. **Materials and Methods:** The GC and ICP-MS analyses were applied to identify the constituents of CU and the RSA was determined using DPPH assay and cyclic voltammetry. To evaluate the nephroprotective effect of CU, rats received CU for 8 weeks and a single injection of CP at week 7. **Results:** Ten active metabolites and 7 inorganic essential elements were identified in CU which showed a potent *in vitro* RSA. The CU prevented histological alterations and ameliorated serum creatinine and urea, urinary albumin and creatinine clearance in CP-intoxicated rats. In addition, CU suppressed renal lipid peroxidation, oxidative DNA damage, cytochrome c oxidase and increased Bcl-2. **Conclusion:** The CU is rich in different active metabolites and inorganic elements and showed potent *in vitro* RSA. Camel urine (CU) attenuated CP-induced nephrotoxicity by suppressing tissue injury, oxidative stress and apoptosis.

Key words: Urotherapy, chemotherapy, lipid peroxidation, cell death, radical scavenging activity, oxidative stress, cisplatin

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cisplatin (CP) has been widely used for chemotherapy over four decades and showed a remarkable successes in cancer therapy[1]. Research studies showed that cisplatin and related platinum-based therapeutics are now being used for the treatment of testicular, head and neck, ovarian, cervical, non-small cell lung carcinoma and many other types of cancer[1,2]. This is attributed to its strong potency which demonstrate higher cure rates reaching 90% in some cases[1,2]. The CP provides more promising results in cancer therapy; however, its use is mainly limited by the acquired molecular resistance mediated via defective apoptotic signaling in response to DNA damage, increased DNA repair and neutralization by sulfur-containing molecules, such as; glutathione as well as severe side effects in normal tissues[3,4]. Although various approaches to synthesize and screen for novel CP analogues that have lower toxicity in normal tissues have been achieved, the side effects of CP in normal tissues were the most major limiting factor which minimize its use in cancer therapy. The adverse effects of CP include neurotoxicity, ototoxicity, nausea, vomiting and nephrotoxicity[5,6]. The later remains a major factor that limits the use and efficacy of CP in cancer therapy[5,6].

CP-induced cellular oxidative stress and inflammatory disorders which significantly are responsible for cellular kidney dysfunction[7]. These harmful effects could be partially prevented by several chemical and natural compounds, such as; antioxidants[8]. Thus, treatment with drugs and also drug discovery should be focused more than before on natural source of antioxidants. Medicinal plants are a well-known source for a variety of natural antioxidants[9]. They are used for the treatment of diseases all over the world. Previous studies showed that therapeutic strategies containing sources of antioxidant agents such as selenium, vitamin E and camel milk are effective in reducing oxidative toxicity of CP[9,10]. Pharmacological roles of camel milk and urine have been reported in many research studies showing their antioxidant, anti-cancer and anti-diabetic activities[11-15]. Camel Urine (CU) showed an anti-metastatic effect on breast cancer cells via down-regulation of proteins that promote cancer, including surviving, β-catenin and cyclin-D1 [11,15]. Many studies showed that CU contains several active metabolites and essential inorganic elements which exhibited several biological activities against many diseases and infections[16,17]. Besides, several studies in experimental animals showed no side effects of using CU and milk as alternative treatment strategy[18,19]. Although, CU is traditionally regarded as a high-quality alternative remedy for curing several diseases, studies regarding its protective effect against CP nephrotoxicity are few. In this study, the constituents of CU were identified by using GC-MS and ICP-MS and its Radical Scavenging Activity (RSA) was determined *in vitro* by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and electrochemical measurements. In addition, the protective effect of CU against CP-induced oxidative stress, apoptosis and kidney injury in rats were studied.

MATERIALS AND METHODS

Collection of CU samples: The CU samples were collected from adult female lactating camels (1-6 year old) at Marsa Matrouh Desert (Egypt) during September 2018 and freely voided daily in the early morning in clean containers. During normal urination, approximately 250-300 mL of the urine sample was collected directly from each animal into a stainless-steel container and then transferred to suitable glass vials. Urine samples were then carried out to the laboratory and stored at -80°C until further use.

Assessments of active metabolites and essential elements in CU: In this experiment, GC-MS and ICP-MS analyses were used to estimate both active metabolites and essential elements in CU as previously reported by Ahamad *et al.*[16]. The active metabolites in CU were extracted with dichloromethane (DCM) with a low boiling point and completely derivatized within 20 min. The samples were transferred to GC vials in an appropriate aprotic solvent such as DCM. Then, about 80 µL of BSTFA and 50 µL of pyridine were added to the sample. For the completion of the reaction efficiently, the GC vials were capped tightly and heated at 65°C for 20 min. Finally, the samples were allowed to cool down at room temperature and injected into the GC/MS (Perkin Elmer Clarus 600 gas chromatograph linked to a mass spectrometer, Turbomass). An aliquot of 2 mL of the extract was injected into Elite-5MS column of 30 m, 0.25 µm film thickness and 0.25 mm internal diameter.

For the elemental analysis, 1 g of freeze-dried urine sample and 50 mL of 20% nitric acid were added to an Erlenmeyer flask. For complete digestion, the mixture was heated to 70-85°C for 48 h. Then, the contents of the Erlenmeyer flask were filtered using a Nalgene filter unit (Thermo scientific). The filtrate was collected in a 100 mL volumetric flask and allowed to cool. Finally, the filtrate sample was made up to 100 mL using Milli Q water and analyzed with ICP-MS by using ELAN-DRC-II (Perkin Elmer, USA)[16].

Assessments of Total Antioxidant Capacity (TAC) of CU

DPPH assay: A solution of 0.06 mmol L^{-1} DPPH was prepared in 100 mL methanol. A 100 µL aliquot of CU was added to 3.9 mL DPPH solution. The decay in absorbance at 515 nm was monitored every 10 min until the reaction reached its equilibrium. The absorbance was measured in triplicate and TAC was expressed as the percentage of scavenged DPPH or the equivalent concentration of antioxidant which is required to reduce the original amount of the radical in 50% (EC_{50})[20].

Electrochemical measurements: The CU samples were prepared for the analysis by DMSO and TBAPF in 6 0.1 mol L^{-1} as supporting electrolyte. Ascorbic acid solutions dissolved in DMSO with TBAPF6 0.1 mol L^{-1} were used to construct the calibration curve. The TAC obtained by electrochemical methods was expressed in terms of equivalent of ascorbic acid as previously reported by Barros *et al.*[21]. The cyclic voltammograms were obtained by using; scan rate = 100 mV sec^{-1} differential pulse voltammograms used pulse width = 5 mV, pulse amplitude = 60 mV and scan rate = 20 V sec^{-1}. The total charge under the anodic waves and the peak currents of the background signal (solvent + supporting electrode) were subtracted from the total charges under the anodic waves. Peak currents of each sample were measured within the range from 0.0-1.5 V.

Experimental animals and treatments: The *in vivo* experiment and assays were carried out during the period from October 2018 to June 2019 in Mansoura University, Egypt. A total of 24 male Sprague Dawley rats with a mean weight of 250 g were obtained from Mansoura University's Animal house. The animals were familiarized for 15 days under optimum environmental conditions (12 h light-dark cycles, temperature (22±2°C) and moderate humidity (60±5%)). All procedures of the current work were ethically approved according to the guidelines of the Institutional Research Board (IRB) of the Faculty of Medicine, Mansoura University (Permit Number: PT1020).

The rats were allocated randomly to three groups (n = 8) as follows:

- **Group I:** Rats received a single intraperitoneal (i.p.) injection of 0.9% NaCl at the end of week 7
- **Group II:** Rats received 7 mg kg^{-1} CP (Sigma, USA)[22] dissolved in 0.9% NaCl via i.p., injection at the end of week 7
- **Group III:** Rats received 20 mL kg^{-1} CU orally twice a week for 8 weeks[19,23] and a single[22] i.p. 7 mg kg^{-1} CP at the end of week 7

At the end of the experiment, blood samples were collected via direct cardiac puncture to measure kidney function markers. Blood samples were allowed to clot for 30 min and then centrifuged at 3000 rpm for 15 min. Clear sera were collected and stored at -20°C. For histopathological examination, animals were sacrificed after 8 weeks and kidneys were removed, cut longitudinally into two halves, washed and fixed in 4% paraformaldehyde.

Assessment of kidney function: Creatinine[24] and urea[25] assessment were performed according the instruction of reagent kits supplied by Biolabo (Maizy, France). Urinary albumin was quantified by immunoassay technique using ELISA kit (Cell Biolabs, USA). Creatinine clearance as an index of Glomerular Filtration Rate (GFR) was calculated from serum creatinine and a 24 h urine sample creatinine levels.

Assessment of malondialdehyde (MDA), 8-Oxo-2'-deoxyguanosine (8-Oxo-dG) and TAC: MDA as a marker of lipid peroxidation was quantitatively estimated in kidney homogenate using high performance liquid chromatography as reported previously by Grotto *et al.*[26]. 8-Oxo-dG, a marker of DNA damage was assayed in kidney homogenate using ELISA kit (MyBioSource, USA). Also, colorimetric Assay Kit (BioVision, USA) was used to estimate serum TAC in all rats. The antioxidant equivalent concentrations were measured at 570 nm as a function of Trolox concentration according to the manufacturer's instructions.

Assessment of cytochrome c oxidase (COX) and Bcl-2: COX and Bcl-2 were measured in kidney samples using ELISA kits supplied by Chemicon (USA) and Merck Millipore (Germany), respectively.

Kidney histology and histopathology: Kidney tissues were fixed in 4% paraformaldehyde for 16 h at 4°C and 4 µm sections of paraffin embedded kidney tissues were stained with Hematoxylin and Eosin (H and E). Kidney histology was examined and photographed by a histopathologist blinded to the experimental protocols.

Statistical analysis: Graphpad Prism 7 was employed to conduct the statistical analysis. Data were expressed as Mean±Standard Deviations (SD) and then, the one-way ANOVA test was employed for comparing means. This followed by Tukey's *post hoc* analysis to determine the significance between-groups. The statistical significance was assigned at p-value <0.05.

RESULTS

Active metabolites and essential elements of CU: GC-MS and ICP-MS analyses were used to estimate both active metabolites and essential elements in the camel urine as shown in Fig. 1a-b. In this experiment, 10 different active metabolites were identified in the studied CU samples. The components present in higher quantity were 2-deoxygalactopyranose, D-galactose, pseudouridine, hexadecanoic acid and trans-9-octadecenoic acid (Fig. 1a). Melibiose, D-glucuronic acid, benzene propanoic acid, azelaic acid and prostaglandin F1A were estimated as minor compounds in the samples of camel urine (Fig. 1a). In addition, a total of 7 inorganic elements were evaluated in CU by using ICP MS analysis. Na, K, Mg and Mn were the major components found in CU in higher quantities than Cr, Fe and P (Fig. 1b).

***In vitro* radical scavenging activity of CU:** In this experiment, the *in vitro* TAC of CU was estimated with DPPH radical scavenging assay along with the cyclic voltammetry assay, one of the most recommended electrochemical measurements (Table 1). In both assays, sequential increase in free radical scavenging activity with increased concentration of CU was reported. The CU at 100 μg mL^{-1} concentration showed the

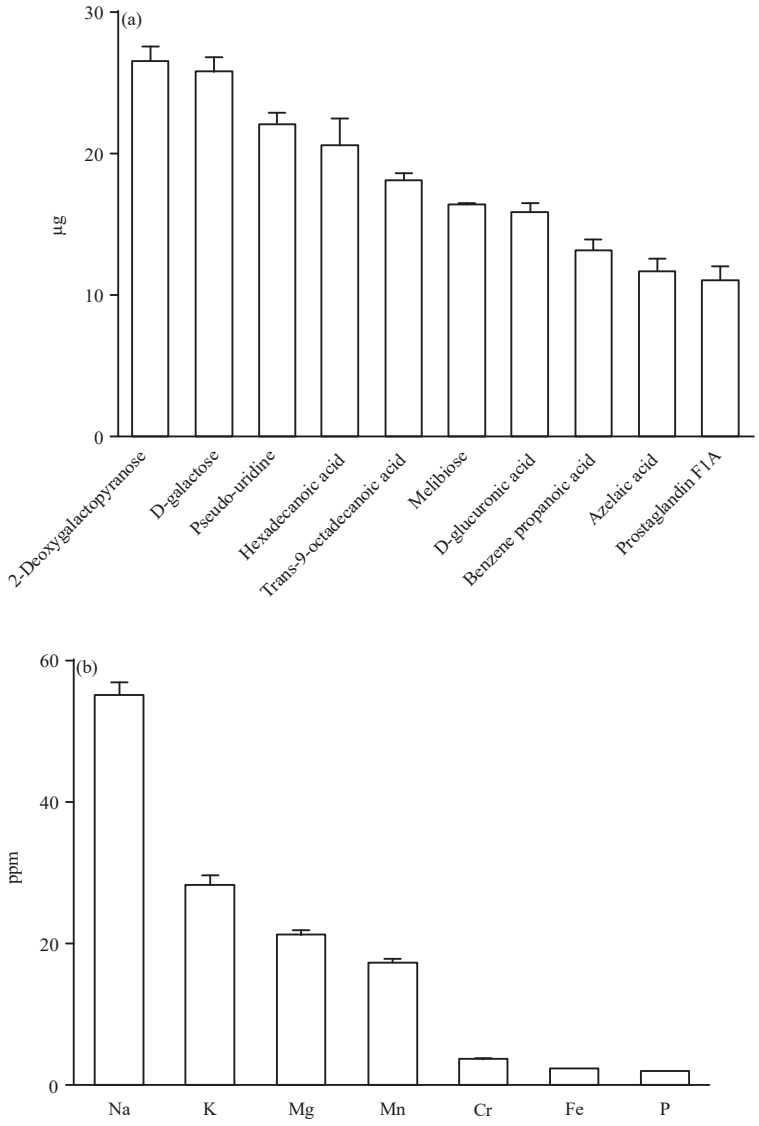

Fig. 1(a-b): Estimated urinary, (a) Active metabolites and (b) Inorganic elements in freeze-dried CU by GC-MS and ICP-MS
The assays were performed in triplicates and the data expressed in Mean±SD

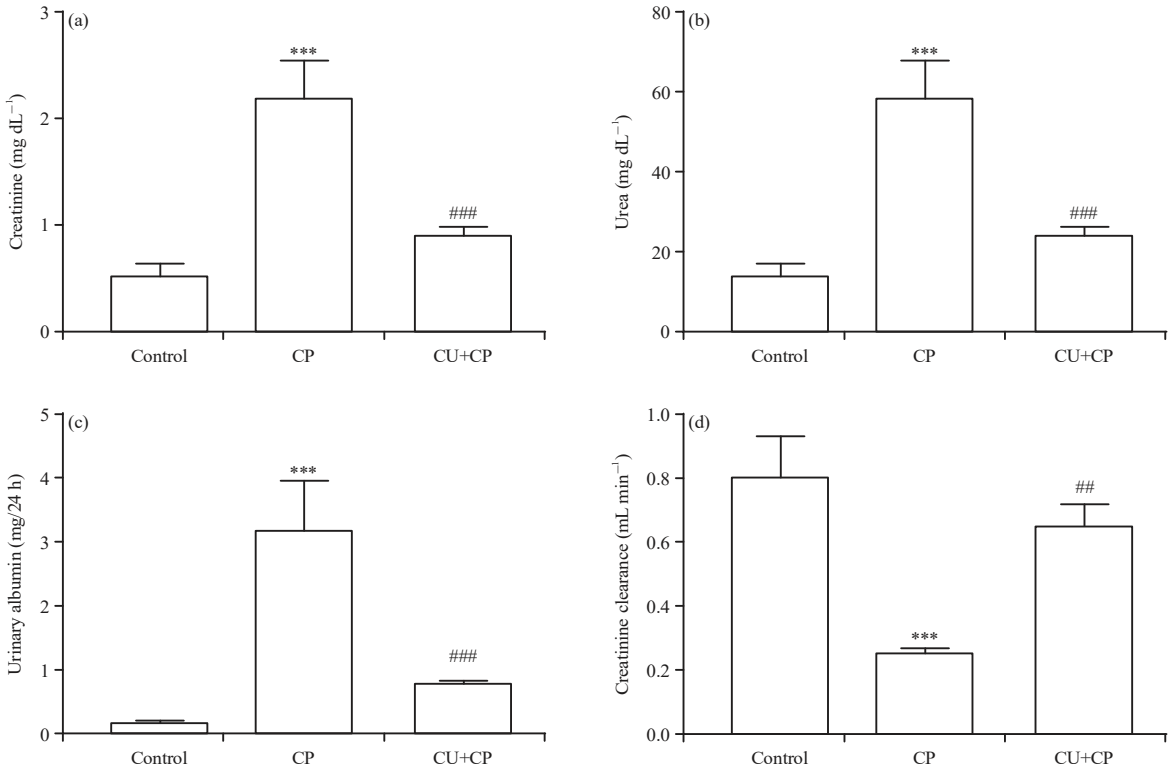

Fig. 2(a-d): Effect of CU treatment on (a) Serum creatinine, (b) Urea, (c) Urinary albumin excretion and (d) Creatinine clearance in CP-intoxicated rats

Data are Mean±SD (n = 8), ***p<0.001 vs. Control and ##p<0.01 and ###p<0.001 vs. CP

Table 1: Total antioxidant capacity (TAC) of CU determined by DPPH and cyclic voltammetry assays

	Total antioxidant capacity		
		Cyclic voltammetry assay	
CU (µg mL^{-1})	DPPH assay (EC_{50}, µg mL^{-1})	CV-Q (mg ascorbic acid/g)	CV-Ipa (mg ascorbic acid/g)
25	280±18.9	125±11	215±18
50	798±86	390±38	545±96
100	1850±115***	456±68***	869±75***

Cyclic voltammetry assay was expressed with the total charge under the curve (CV-Q) and the sum of the anodic peak currents (CV-Ipa) of CU samples. Each value is expressed as mean of three values±SD (n = 3), In cyclic voltammetry assay, TAC was expressed as mg ascorbic acid/g and in DPPH assay expressed as EC_{50} (µg mL^{-1}), ***p<0.001, Sequential increase in free radical scavenging activity of CU was significantly (p<0.001) reported with higher concentrations of CU values

strongest free radical scavenging activity against DPPH radical and an increase in electron transfer which led to the oxidation of ascorbic acid at the surface of the working electrode resulting in an increase of the total charge from the antioxidant molecules and subsequently provide with higher TAC.

CU prevents CP-induced kidney injury in rats: Biochemical kidney function markers were determined to assess the renoprotective effect of CU in CP-induced rats. A significant increase (p<0.001) in the levels of serum creatinine (Fig. 2a) and urea (Fig. 2b) and urinary albumin (Fig. 2c) along with a decrease in creatinine clearance (Fig. 2d) were reported in CP-intoxicated rats. In contrast, treatment of the CP-induced rats with CU ameliorated all determined kidney function markers.

These biochemical findings were confirmed by the histological investigation (Fig. 3). The renal cortex of the control group showed normal histological structure with even distributed glomeruli surrounded by narrow Bowman's spaces. The renal tubules occupied the bulk of parenchyma between the glomeruli with a predominance of the proximal convoluted tubules lined by eosinophilic epithelial cells and smaller numbers of distal convoluted tubules and collecting

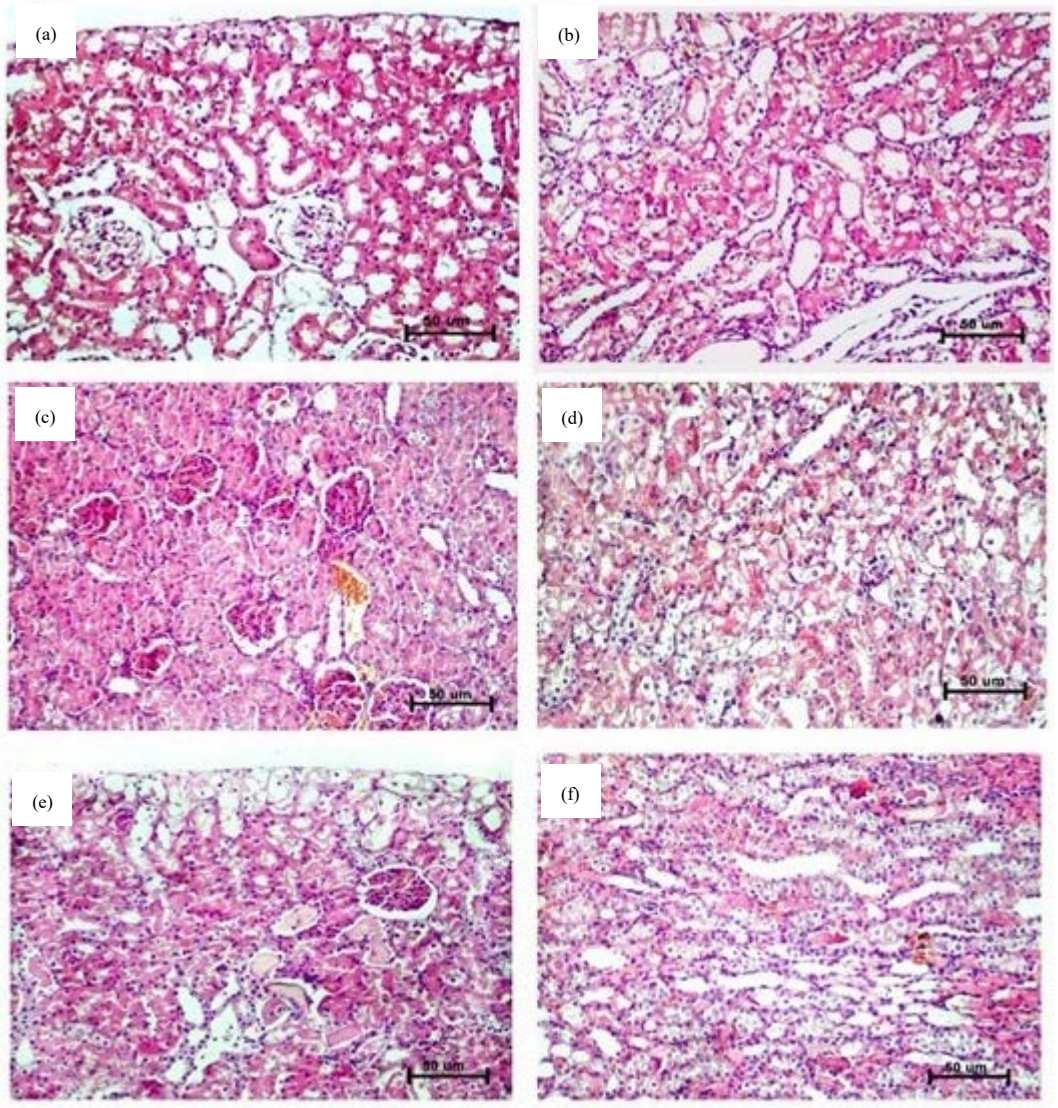

Fig. 3(a-f): Photomicrographs of H and E-stained sections from the kidney of (a-b) Control rats, (c-d) CP-intoxicated rats and (e-f) CP-intoxicated rats treated with CU

tubules (Fig. 3a). The renal medulla had closely packed proximal and distal tubules with intervening collecting tubules (Fig. 3b). CP-treated group showed distorted corpuscular structure with congested vasculature, collapsed glomeruli and widened Bowman's spaces. The proximal convoluted tubules of the cortex and medulla had less eosinophilic staining and wide lumens. The distal and the collecting tubular cell lining showed widely distributed apoptotic figures in the form of darkly stained nuclei and vacuolated cytoplasm (Fig. 3c-d). The CU-treated groups exhibited apparently normal corpuscles and Bowman's spaces. The cortical vessels were less congested and the proximal convoluted tubules in both cortex and medulla kept the apical brush border. Most of the epithelial lining of the proximal convoluted tubules appeared normal with minimal cytoplasmic vacuolations. The distal convoluted tubules and the collecting tubules appeared almost normal (Fig. 3e-f).

CU suppresses oxidative stress, DNA damage and apoptosis in CP-induced rats: The effects of CU on oxidative stress and apoptosis markers in the kidney of CP-induced rats are represented in Fig. 4. The CP-intoxicated rats exhibited a significant increase in kidney MDA (Fig. 4a) and 8-Oxo-dG (Fig. 4b) and decreased TAC (Fig. 4c), when compared with the control group. Treatment with CU significantly reduced MDA and 8-Oxo-dG whereas increased TAC.

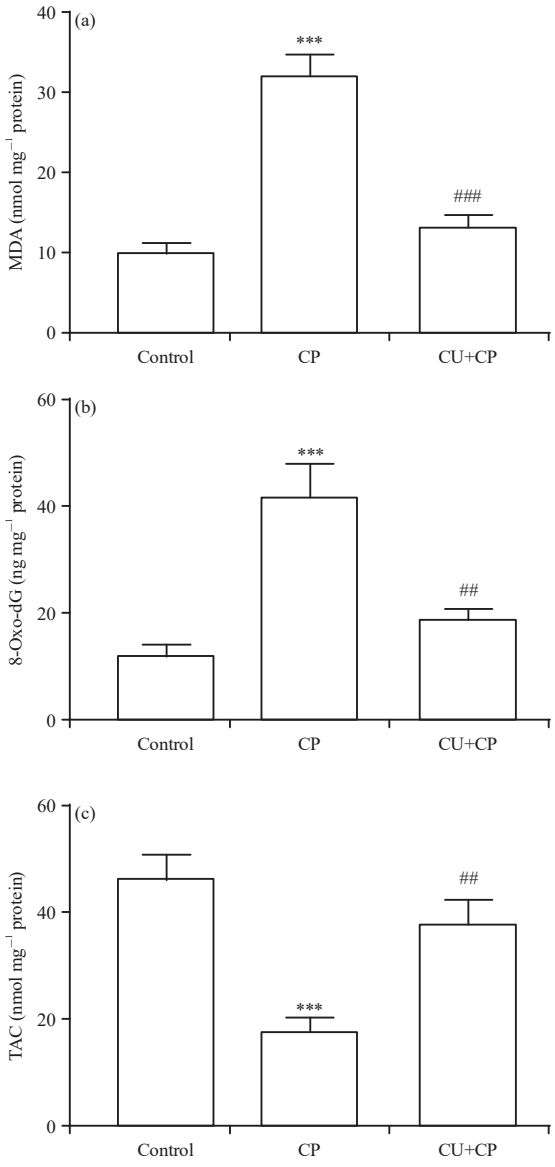

Fig. 4(a-c): Camel Urine (CU) effect on (a) Renal lipid peroxidation (MDA), (b) Oxidative DNA damage (8-Oxo-dG) and (c) TAC in CP-intoxicated rats

Data are Mean±SD (n = 8), ***p<0.001 vs. Control and ##p<0.01 and ###p<0.001 vs. CP, TAC: Total antioxidant capacity, MDA: Malondialdehyde

Besides the apoptotic figures observed in the kidney sections, apoptosis in the kidney of CP-induced rats was confirmed by the significantly increased COX (Fig. 5a) and decreased Bcl-2 (Fig. 5b) when compared with the control rats. In contrast, CP-induced rats treated with CU exhibited a significant decrease in COX accompanied with increased Bcl-2 expression.

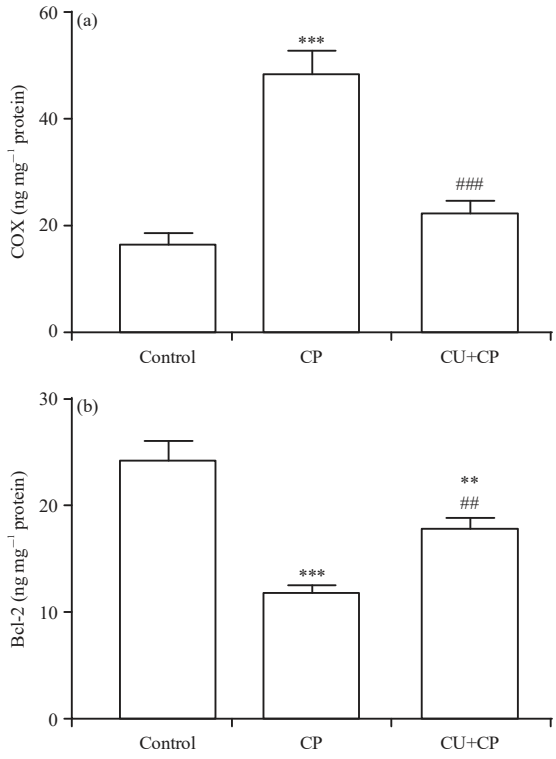

Fig. 5(a-b): CU attenuates (a) Renal COX and (b) Bcl-2 of CP-intoxicated rats

Data are Mean±SD (n = 8), **p<0.01 and ***p<0.001 vs. Control and ##p<0.01 and ###p<0.001 vs. CP

DISCUSSION

In this study, the constituents of CU were identified and its potential to protect rats against CP-induced nephrotoxicity was evaluated. The CU ameliorated kidney injury, improved kidney function markers and suppressed oxidative stress and apoptosis in CP-intoxicated rats. The metabolites of the collected CU samples were determined using GC-MS and ICP-MS analyses which revealed the presence of 10 different active metabolites and 7 essential inorganic elements. The active components present in higher quantities were 2-deoxygalactopyranose, D-galactose, pseudouridine, hexadecanoic acid and trans-9-octadecenoic acid, whereas, melibiose, D-glucuronic acid, benzene propanoic acid, azelaic acid and prostaglandin F1A were found in lesser amounts. In addition, CU was found to contain Na, K, Mg, Mn, Cr, Fe and P. In this context, previous studies reported the presence of different active metabolites in CU, such as canavanine, erythritol, benzenepropanoic acid, melibiose, D-galactose, 2-deoxy-galactopyranose and benzenepropanoic acid[15,16,23,27-29].

The variability in the active metabolites content of CU occurs as a result of different climate, food and metabolic pathways. It has been reported that most of the volatile components identified in CU are chemically related to glycolysis, fatty acid synthesis, nucleoside androgens, mineralocorticoids, tyrosine inhibitor and the arachidonic acid pathway[11,16,23]. The active metabolites of CU have been proposed to be responsible for its beneficial effects, including anticancer, antiplatelet, antibacterial, antifungal, anti-parasitic and hepatoprotective activities[16,23,27-29]. Besides the metabolites, inorganic essential elements in CU have been demonstrated to play a pivotal role in the biological systems via promoting the cellular biological activities against toxicological and abnormal disorders[15,23]. It was reported previously that Na, K, Mg, Ca, Fe, Zn, Cu, Mn, Cr, Mo and Se were essentially required for normal biological functions such as biosynthesis of ATP, synthesis of DNA and RNA and as cofactors in more than 300 enzymatic reactions necessary for the structural function of proteins, nucleic acids and mitochondria[30-33].

Here, the antioxidant activity of CU was investigated *in vitro* using DPPH radical scavenging and cyclic voltammetry assays. The results revealed a concentration-dependent antioxidant effect of CU. In cyclic voltammetry, an increase in electron transfer was reported from CU samples. This electron transfer led to the oxidation of ascorbic acid at the surface of the working electrode, resulting in an increase of the total charge from the antioxidant molecules and subsequently high TAC. The present results supported the role of both volatile metabolites and inorganic elements present CU as free radical scavengers and would protect against oxidative stress occurs a result of toxicants in the biological systems and produce severe cellular damage[14,16,23].

The antioxidant and anti-apoptotic properties of CU in the kidney of CP-induced rats were evaluated. Interestingly, CU prevented histological alterations in the kidney and ameliorated serum creatinine and urea as well as urinary albumin and creatinine clearance in CP-intoxicated rats, demonstrating a potent nephroprotective efficacy. Cisplatin CP has been shown to produce severe toxicity mediated via excessive generation of free radicals, resulting in nephrotoxic effects, such as lipid peroxidation, inflammation, DNA damage and cell death[5,6]. Therefore, counteracting oxidative stress represents an effective strategy to protect against CP nephrotoxicity. In agreement with the *in vitro* data, CU suppressed lipid peroxidation and DNA damage in the kidney of CP-intoxicated rats. The antioxidant efficacy of CU could be directly connected to its active metabolites and essential elements, most of which have been reported to possess antioxidant activity and to overcome oxidative stress and cell death[19,23,34]. Besides its antioxidant activity, the nephroprotective effect of CU might be directly connected to the presence of inorganic elements. Previous studies showed that treating electrolyte abnormalities associated with acute or chronic nephrotoxicity can ameliorate kidney diseases[35-38]. Depletion in cellular Ca, K, Na and Mg is associated with severe kidney disorders. For instance, depletion of Mg is usually associated with additional ion abnormalities such as hypocalcemia, hypokalemia and metabolic alkalosis[36,38].

Previous studies reported that CP undergoes metabolic activation to produce highly reactive metabolites which significantly affect cellular redox balance by diminishing GSH and antioxidant enzymes[6,34]. In addition, CP interacts with different cellular components and macromolecules causing functional and structural damage to lipids, DNA and proteins[6,34,39]. This has been confirmed by the significant increase in renal MDA and 8-Oxo-dG in CP-induced rats. Many molecular pathways of CP nephrotoxicity are triggered in the tubular epithelial cells. THe CP can alter the number and size of lysosomes and mitochondria as well as accelerate cellular apoptosis via oxidative pathways[6,34,39]. It was reported that p53 along with COX from mitochondria are known as major mediators of CP-induced cell death. In the kidney, CP induces the activation of p53 which in turn provokes the release of mitochondria cytochrome c and consequent activation of caspases, resulting in cell death[40]. Interestingly, treatment with CU for 8 weeks suppressed CP-induced apoptosis in rat kidney as evidence by decreased COX and increased expression of the anti-apoptotic protein Bcl-2. These findings point to the potent protective effect of CU against CP-induced nephrotoxicity. The deleterious effects of CP on the renal cortex and medulla were reversed by the protective effect of CU, confirming its nephroprotective role. The data of this study suggested that the active metabolites and inorganic essential elements present in CU are responsible for its protective activity against CP nephrotoxicity.

CONCLUSION

These findings demonstrate the ability of CU to attenuate CP-induced nephrotoxicity via its dual antioxidant and anti-apoptotic properties. The results revealed the presence of different active metabolites and inorganic elements in CU. By using an *in vitro* assay and cyclic voltammetry, CU demonstrated potent and concentration-dependent radical scavenging activity. The antioxidant efficacy of CU was confirmed *in vivo* where it suppressed lipid peroxidation and

DNA damage in the kidney of CP-intoxicated rats. Moreover, CU ameliorated kidney function and prevented histological alterations and renal apoptosis provoked by CP in rats. These beneficial effects of CU could be directly attributed to its rich content of active metabolites and inorganic elements.

SIGNIFICANCE STATEMENT

This study identified the constituents and radical scavenging activity of Camel Urine (CU) and explored its ameliorative effect against cisplatin (CP) nephrotoxicity in rats. The results revealed the presence of different active metabolites and inorganic elements in CU. DPPH and cyclic voltammetry assays showed the potent and concentration-dependent radical scavenging activity of CU. Additionally, CU effectively attenuated CP-induced oxidative stress, DNA damage and kidney injury via its dual antioxidant and anti-apoptotic properties. Given these beneficial effects, this study will be beneficial for the researchers for possible use of CU metabolites and elements as an adjuvant to prevent nephrotoxicity in patients on CP therapy, pending further studies to explore the exact mechanisms.

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