PROTECTIVE EFFECT OF *CITRUS MEDICA* 'OTROJ' EXTRACT ON GENTAMICIN-INDUCED NEPHROTOXICITY AND OXIDATIVE DAMAGE IN RAT KIDNEY

M. A. AL-YAHYA[a], R. A. MOTHANA[a,b,*], M. S. AL-SAID[a,b], M. AL-DOSARI[a], M. AL-SOHAIBANI[c], M. K. PARVEZ[a], S. RAFATULLAH[b]
[a]Department of Pharmacognosy, College of Pharmacy, King Saud University, P.O. Box 2457,Riyadh 11451, Saudi Arabia
[b]Medicinal, Aromatic and Poisonous Plants Research Center (MAPPRC), College of Pharmacy,   King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia
[c]Department of Pathology, King Khalid University Hospital, King Saud University, P.O. Box 2925, Riyadh 11461, Saudi Arabia

The present study was conducted to determine whether an ethanolic extract of *Citrus medica* 'Otroj' (EEOT) can possibly exert nephroprotective and antioxidant activity against gentamicin-induced renal toxicity in rats. Wistar albino rats of either sex were divided into four groups. Group-1 received normal saline. Group-2 received gentamicin (GM) (80 mg/kg/d, for 8 days intraperitoneally). Group-3 and 4 received EEOT (250 and 500 mg/kg/d, for 21 days orally) plus gentamicin simultaneously. The rats were sacrificed on the twenty first day and kidneys were collected for histopathological assessment. Renal activities were determined in serum for gamma glutamyl transferase (GGT), creatinine, urea and uric acid. Moreover, non-protein sulfhydryl (NP-SH) malondialdehyde (MDA) and total protein (TP) were determined in renal tissues. Additionally, *in vitro* protection against 2′,7′-dichlorofluorescein-induced human nephrotoxicity was carried out with HEK293 cells using MTT assay. GM caused an elevation in serum GGT, creatinine, urea and uric acid, a reduction of the NP-SH and TP levels and an increase the MDA concentration in the kidney tissue. Administration of EEOT significantly protected kidney tissues against nephrotoxic effect of gentamicin as evident from amelioration of the marker enzymes and lipid peroxidation and elevated NP-SH and TP levels, besides some indices of histopathological alterations. The MTT-test showed a 48% protection at a concentration of 1 mg/ml. It is concluded that *C. medica* could protect the kidney against oxidative stress induced by gentamicin probably through its antioxidant and/or free radical scavenging properties caused by flavonoids and limonene.

1. Introduction

Citrus medica L. (Brain citron), locally known as Otroj in Saudi Arabia, is a fruit with pleasant fragrance. It is an important member in the genus *citrus*, belonging to the Rutaceae family. There are about 16 species and many horticulture varieties of citrus found in Arabian peninsula [1]. Almost all species and varieties of citrus are known to possess therapeutic benefits and used in traditional medicine of many countries. Various parts of citrus plant not only used for medicinal purposes but for their chemical constituents are utilized in cosmetic, perfumery and in beverage industries.

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*Corresponding author: rmothana@ksu.edu.sa

Citrus medica fruit is known since ancient times in Europe, Mediterranean countries for its edible and medicinal properties [2]. A number of ethnomedicinal uses are attributed to otroj fruit including carminative, stomachic, heart tonic, appetite stimulant and as refrigerant [3]. The chewing of dried fruit peel is used to cure dysentery and the fruit of otroj is consumed to improve digestion in folklore medicine [4]. The peel of *Citrus medica* being used in Indian, Arab and Chinese traditional medicine for the treatment of inflammation, to control increase urinary output and to treat kidney stones [5,6]. Various parts of the otroj plant including fruit, leaves, twigs, flowers, seeds are useful therapeutically [7]. In an earlier study *Citrus medica* seed extract showed an antidiabetic and hypolipidemic activity in diabetic rats [8]. The root of this plant has been reported to have anticancer activity [9]. Lin *et al*. (2011) [10] has reported that citrus flavonoids, has suppressing effect on blood cholesterol and triglycerides. In different *in-vitro* assays *Citrus medica* peel extract has shown to possess antioxidant activity and reported to contain limonene and gamma-terpinene among others [11]. In a recent study, it was demonstrated that citrus pulp and juice containing hesperidin and β-cryptoxanthin possesses chemoprentative effects in various tissues [12]. An essential oil from epicarp of *Citrus medica* showed fungi toxic effects against *A. flavus* and *A. vesicolor* fungi [13]. This study is thus an attempt to explore the effect of otroj '*Citrus medica*' ethanol extract (EEOT) on its possible nephroprotective and antioxidant potential in rats to validate its use in Arab traditional medicine.

2. Experimental

Plant material T
The fresh fruits of *Citrus medica* (otroj) purchased from a local vegetable market of Riyadh. They were identified and authenticated by Dr. Mohammed Yusuf. A voucher specimen was deposited at the Herbarium of the College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

Preparation of the extract
Shade dried coarsely pulverized whole fruit of otroj were placed in glass percolator with 96% ethanol and allowed to stand at room temperature for about 72 h. The percolate was collected and dried under reduced pressure in vacuum. The obtained extract was later used and dissolved in distilled water for evaluation of nephroprotective activity.

Animals
Healthy Wistar albino rats, of either sex and approximately of same age (8-10 weeks), weighing 180-200 g were obtained from the Animal Care Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. Animals were kept at a constant temperature (22±2°C), humidity (55%) and light-dark conditions (12/12 h light/dark ratio). Animals were provided with Purina chow diet and drinking water *ad libitum*. The protocol of the current study was approved by the Ethics Committee of the Experimental Animal Care Society, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

Experimental design
Rats were divided into four groups (1, 2, 3 and 4) (N = 6 animals/group). Group 1 was kept as a control (no treatment). Group 2, 3 and 4 received Gentamicin (GM) 80 mg/kg body weight intraperitoneally (i.p.) for 8 days [14]. Only gentamicin was administered to Group 2. Group 3 and 4 were treated with EEOT at the doses of 250 and 500 mg/kg body weight (orally), respectively for 14 days before gentamicin treatment and thereafter concurrently with gentamicin (100 mg/kg) for 8 days. The blood samples were collected after 24 h of last dose. The blood was allowed to clot and the serum was separated for biochemical estimations. After blood collection, the animals were sacrificed using ether anaesthesia. The kidney was dissected out and used for biochemical and histological examination studies.

Serum analysis
Creatinine,[15] uric acid,[16] Urea,[17] gamma glutamyl transferase (GGT) [18] and total protein levels were estimated in serum using Reflotron® Plus Analyzer and Roche kits.

Determination of malondialdehyde (MDA)
The method reported by Utley *et al*. (1967) [19] was followed. The kidney tissues were removed and each tissue was homogenized in 0.15 M KCl (at 4°C; Potter-Elvehjem type C

homogenizer) to give a 10% w/v homogenate. Aliquots of homogenate (1 ml) were incubated at 37°C for 3 h in a metabolic shaker.   Then 1 ml of 10% aqueous trichloroacetic acid was added and mixed. The mixture was then centrifuged at 800 g for 10 min. 1 ml of the supernatant was removed and mixed with 1 ml of 0.67% thiobarbituric acid in water and placed in a boiling water bath for 10 min. The mixture was cooled and diluted with 1 ml distilled water. The absorbance of the solution was then read at 535 nm. The content of malondialdehyde (nmole/g wet tissue) was then calculated by reference to a standard curve of malondialdehyde solution.

Estimation of non-protein sulfhydryls (NP-SH)

Renal non-protein sulfhydryls was measured according to the following method [20]. The kidney was homogenized in ice-cold 0.02 mmol/L ethylenediaminetetraacetic acid (EDTA). Aliquots of 5 ml of the homogenates were mixed in 15 ml test tubes with 4 ml of distilled water and 1 ml of 50% trichloroacetic acid (TCA). The tubes were shaken intermittently for 10 min and centrifuged at 3000 rpm. Two milliliters of supernatant was mixed with 4 ml of 0.4 mol/L Tris buffer (pH 8.9). 0.1 ml of 5, 5'-dithio-bis (2-nitrobenzoic acid) (DTNB) was added and the sample was shaken. The absorbance was measured within 5 min of addition of DTNB at 412 nm against a reagent blank.

Determination of total proteins (TP)

The TP was estimated by the kit method, supplied by Crescent Diagnostics, Jeddah, Saudi Arabia [21].

Histopathological evaluation

The kidney tissue samples were fixed in neutral buffered formalin for 24 h. Sections of the kidney tissue were histopathologically examined. These sections were stained with haematoxylin and eosin using routine procedures.

Ex vivo assay of 'Otraj' on cultured nephrocytes.

Cells and Reagents

Human kidney cell lines, HEK293 was grown in DMEM-GlutaMax growth medium (Gibco), supplemented with 10% bovine serum and 1x penicillin-streptomycin (HyClone Laboratories) at 37^0C in a humified chamber with 5% CO_2 supply. 2′,7′-dichlorofluorescein (DCFH) (Sigma-Aldrich) was used as the *ex vivo* cytotoxin.

Nephrotoxicity and treatment

HEK293 cells were seeded (10^5 cells/well in triplicate) in a 96-well flat-bottom plate (Becton-Dickinson Labware) a day before treatment and grown. DCFH commonly used to measure oxidative stress, *in vitro*, [22] was used as a cytotoxic agent (IC50: 120 μg/ml, personal observation), prepared in DMSO (Sigma). Stock of Otraj extract (10 mg/ml) was prepared in serum-free growth media, followed by diluting in growth media to prepare five doses of 'Otraj' (50, 250, 500, 1000, and 2500 μg/ml). The cells (in triplicate) were replenished with growth media containing 120 μg/ml DCFH plus a dose of 'Otraj, including untreated as well as DCFH-treated controls, and further incubated for 48 hours.

Microscopy

A direct visual observation was made under an inverted microscope (Optica, 40x and 100x) to see any morphological changes in the cells cultured with 'Otraj'and/or DCF on day1 and 2.

Cell proliferation and viability Test

On day2 of treatment, nephrocyte proliferation and viability test was performed using TACS-MTT Cell Proliferation and Viability Assay Kit (TACS).

Phytochemical screening

Preliminary phytochemical screening for terpenoids, alkaloids, flavonoids, anthraquinons, saponins, carbohydrates, tannins and coumarins was performed with the extract by using chemical methods and thin-layer chromatography (TLC) according to the methodology described by Wagner and Bladt (1996) [23].

Statistical analysis

Values are given as arithmetic means ± standard error of the mean (S.E.M.). Data was statistically analyzed by using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests.

3. Results

Effect of EEOT on biochemical parameters in serum.
The effect of ethanolic extract of Otroj treatment on the gentamicin-induced nephrotoxicity on creatinine, uric acid, urea, GGT and LDH levels in serum and urine are shown in Table 1. Administration of gentamicin significantly elevated the levels of creatinine, uric acid, urea and GGT in serum. Treatment of rats with the extract (500 mg/kg b.w.) significantly prevented the elevation of creatinine, uric acid, urea, GGT and LDH levels in serum and urine. However, the treatment of rats with lower dose (250 mg/kg b.w.) has shown to insignificantly decrease the creatinine, uric acid and urea level in serum.

Table 1: Effect of EEOT on gentamicin-induced nephrotoxicity in serum.

Treatments (n=6)	Dose (mg/kg)	Creatinine (mg/l)	GGT (U/l))	LDH (U/l)	Urea (nmol/l)	Uric acid (mg/dl)
Control	-	3.98± 0.33	5.58± 0.43	99.50± 1.37	26.63± 2.10	1.33± 0.09
Gentamicin only	80	7.96± 0.69***[a]	11.28± 0.59***[a]	161.23± 4.47***[a]	131.83± 4.90***[a]	4.94± 0.28***[a]
EEOT + Gentamicin	250	6.57± 0.52 [b]	9.58± 0.60 [b]	161.89± 4.61[b]	112.83± 3.14**[b]	4.72± 0.29 [b]
EEOT+ Gentamicin	500	5.74± 0.37*[b]	7.68± 0.37***[b]	134.12± 3.05***[b]	79.60± 2.93***[b]	3.41± 0.21**[b]

All values represent mean ± SEM. *p<0.05; **p<0.01; ***p<0.001; ANOVA, followed by Dunnett's multiple comparison test. [a] As compared with control group, [b] As compared with Gentamicin only group.

Effect of EEOT on renal MDA.
As depicted in Figure 1, the MDA, an end product of lipid peroxidation, in the rats' kidney tissue, treated with gentamicin was increased significantly when compared with the normal control rats. Treatment of rats with EEOT resulted in a significant and dose dependent decrease in the concentration of MDA.
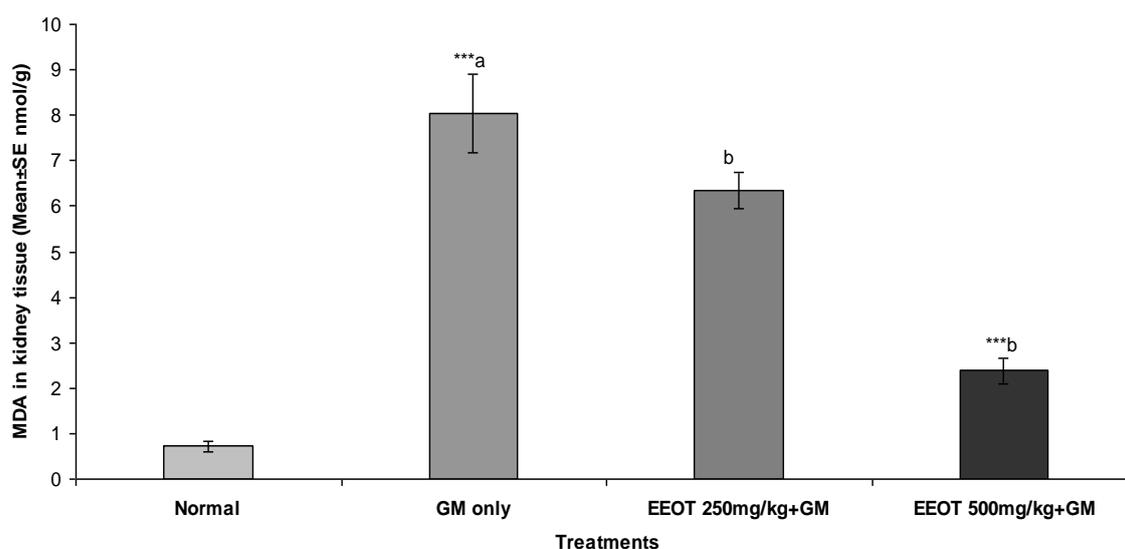
Fig. 1. Effect of EEOT on MDA concentration changes in kidney tissue induced by gentamicin in rat. All values represent mean ± SEM. ***p<0.001; ANOVA, followed by Dunnett's multiple comparison test. [a]: As compared with normal group, [b]: As compared with only GM only group.

Effect of EEOT on renal NP-SH

As shown in Figure 2, the reduced level of NP-SH caused by gentamicin treatment was significantly elevated by EEOT treatment at the high dose (500 mg/kg b.w.) in the kidney tissue.
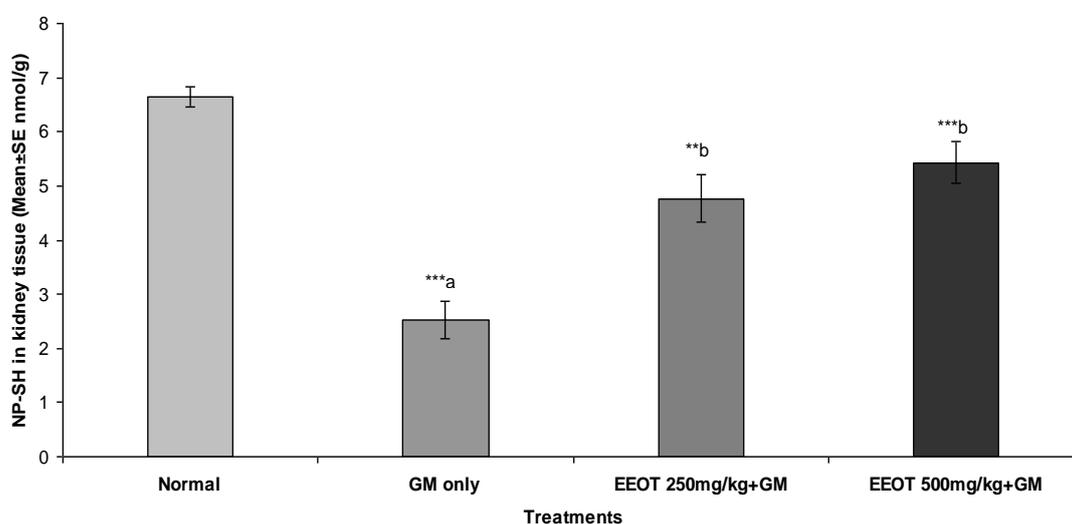

Fig. 2. Effect of EEOT on NP-SH concentration changes in kidney tissue induced by gentamicin in rats. All values represent mean ± SEM. ** P<0.01, ***p<0.001; ANOVA, followed by Dunnett's multiple comparison test. [a]: As compared with normal group, [b]: As compared with only GM only group.

Effect of EEOT on renal TP

Figure 3 demonstrated that the total protein level was significantly decreased in gentamicin only treated group. Otroj at higher dose significantly elevated the protein concentration in the kidney tissue.
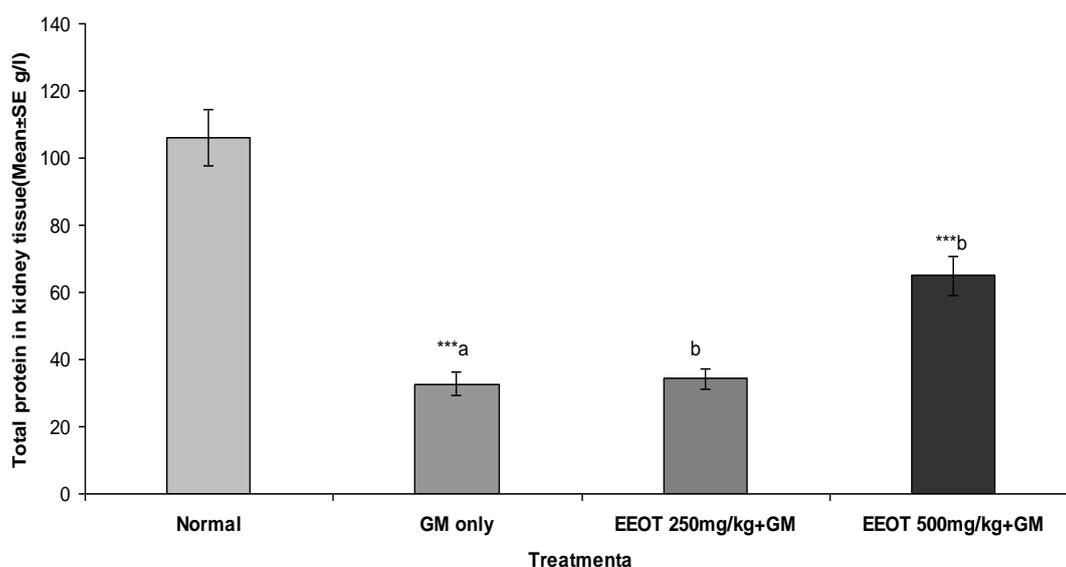
Fig. 3. Effect of EEOT on the level of total protein concentration changes in kidney tissue induced by gentamicin in rat. All values represent mean ± SEM. ***p<0.001; ANOVA, followed by Dunnett's multiple comparison test.[a]: As compared with normal group,[b]: As compared with   only GM only group.

Histopathological studies

Histopathological changes such as tubular atrophy, necrosis, vasculization and peritubular blood vessel congestion were observed in the gentamicin administered group. Pretreatment with EEOT (500 mg/kg) significantly prevented histopathological changes towards normal.

Ex vivo assay of 'otroj' on cultured nephrocytes.

DCFH-treated cells exhibited severe cytotoxic effect on the kidney cells as reflected by altered morphology compared to untreated cells. Interestingly, the DCFH-treated cells supplemented with 1000 and 2500 μg/ml of 'Otroj' were different from the DCFH- or/and other doses of 'Otroj'-treated cells morphology but close to that of untreated cells (data not shown).

Nephroprotection and viability restoration by EEOT

Our MTT test showed a protective effect of 'Otroj', at the best dose of 2500 μg/ml against DCFH-induced nephrotoxicity at 48 hours post-treatment (Figure 5) that was in line with our microscopic observation. DCFH-toxicated kidney cells were recovered to about 48% and 65% of untreated cells, with 1000 μg/ml and 2500 μg/ml of 'Otroj', respectively, compared to of DCFH. The further lower doses did not attenuate the DCFH-toxicity. The 'Otroj' supplementation therefore, restored the nephrocytes viability by 2-fold (50%) against DCFH-toxicity.
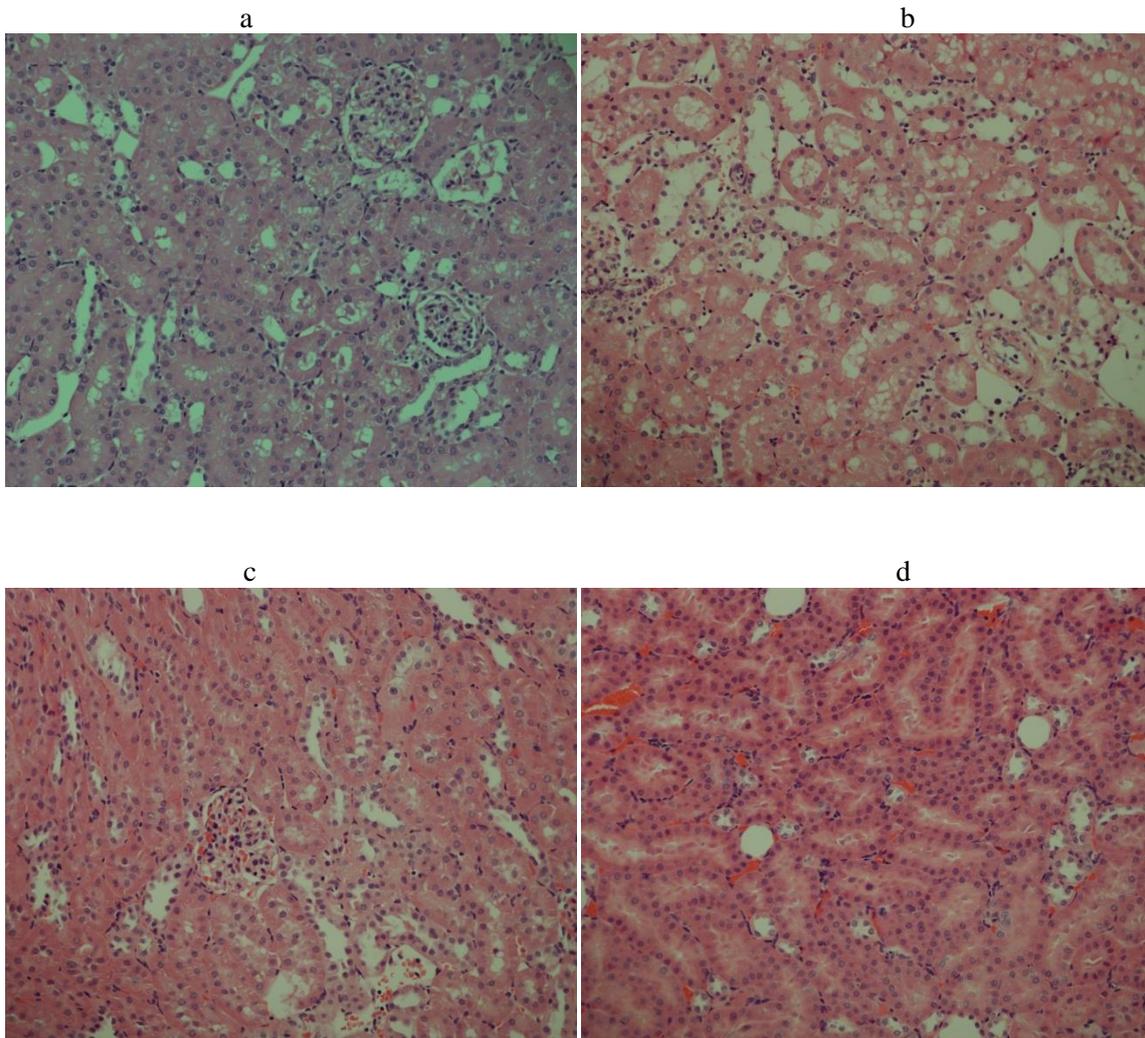
Fig. 4. Light micrographs showing the effect of EEOT extract on kidney tissues. a: Normal kidney. Normal tubule interstitial and glomeruli, H.&E. 100x; b: Gentamicin only: It shows tubular atrophy, necrosis and vasculization. H.&E. 100x; c: EEOT 250 mg/kg + GM. Interstitial nephritis – this can be incidental or secondary – little improvement in renal tubules with presence of vasculization. H.&E. 100x; d: EEOT 500 mg/kg + GM. Better tubules with absence of necrosis and no interstitial nephritis, residual vasculization and atrophy is minimal. H.&E. 100x.
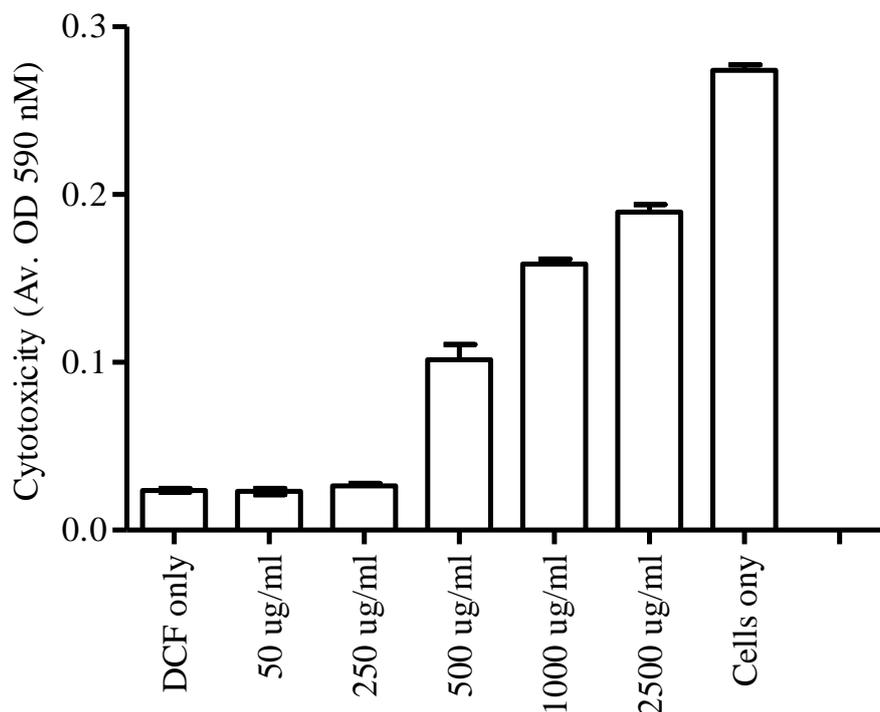
Fig. 5. Effect of EEOT against DCF-induced nephrotoxicity at 48 hours post-treatment.

Phytochemical screening

The phytochemcial screening of the EEOT showed the presence of flavonoids e.g. apigenin, quercetin, rutin and hesperidin as major constituents. Moreover, some monoterpenoids were found such as limonene and γ-terpinene. No alkaloids, anthraquinons, saponins or steroids were detected in the extract.

4. Discussion

Aminoglycoside antibiotics, particularly gentamicin-induced renal toxicity is one of the most concerned side effects even in therapeutic doses [24] for the treatment of severe infections of Gram-negative bacteria [25]. Various mechanisms occur in gentamicin-induced nephrotoxicity as evident by a mounting number of studies [26]. Gentamicin-induced renal toxicity is functionally characterized an elevation in marker enzyme; serum creatinine, urea and uric acid and renal malfunction, while structurally is associated with glomerular atrophy, tubular necrosis and fibrosis and perinascular edema, inflammation and glomerular congestion [27,28]. It has been reported that GM causes significant nephrotoxicity due to oxidative stress, GM usually accumulates is renal proximal tubules and triggers hydrogen peroxide generation by the mitochondria, which in turn accelerates oxidative stress [29]. The prevalent strategies to reduce GM-induced nephrotoxicity, the use of some known antioxidants like Vitamin E and C among others [30,31]. A large number of medicinal plants, crude drugs, vegetables, herbs and their derived phytoconstituents have been reported to possess preventative effects against various diseases including liver, kidney disorders and other chronic diseases [21,32]. Sohn et. al 2009 [32] evaluated the recovery effects of 251 herbal medicines on acetaminophen-induced nephrotoxicity. It was shown that among these extracts, 8 herbal medicines (*Ledebouriella divaricata, Sparganium simplex, Panax ginseng, Aster tataricus, Citrus aurantium, Sanguisorba officianlis, Arisaema consanguineum, and Polygonum aviculare*) had a strong recovery effect on acetaminophen-induced damage in HEK 293 cells [32]. In recent years efforts are focused on the development of antioxidants from natural sources

including, herbs, vegetables and fruits which are able to minimize the toxic effects of GM on kidney [33,34].

The present study demonstrate that gentamicin induce renal injury as evident from the elevated serum creatinine, urea and uric acid levels and also from the elevated concentrations of malondialdehyde (MDA) and decreased contents of NP-SH and protein in rats' kidney tissue. These changes show that GM induced kidney dysfunction which in accordance with previous studies [35]. Elevation of creatinine, urea and uric acid levels are taken as the index of nephrotoxicity [36]. Estimation of urea and uric acid has been thought to be the most important biomarkers to assess renal injury in laboratory animals [37]. As the serum creatinine, urea and uric acid are the ultimate metabolites of purine which may alter the glomerular filtration rate and lead to enhance their levels in serum and associated with renal damage [38].

Co-administration of ethanolic extract of *Citrus medica* 'Otroj' (EEOT) in the present investigation for 21 consecutive days successfully prevented renal damage associated with GM assessed by renal functioning biomarkers and histopathological examination. *Citrus medica* extract significantly reduced the GM induced elevated levels of creatinine, urea and uric acid in serum, besides the extract also showed a marked improvement in the decreased concentration of NP-SH and protein in kidney tissue, whereas a significantly lowered level of MDA was also observed in the kidney which was elevated by GM treatment, as the GM causing oxidative damage to the renal cortex might be antagonized by the EEOT. Some earlier reports have been shown to demonstrate the similar ameliorating action of antioxidants upon GM related nephropathy [33,39,40]. The GM-induced lipid peroxidation was evident in the present study by elevated content of MDA, a marker of lipid peroxidation. GM-induced increment in kidney MDA content significantly prevented by EEOT in the current study, which indicates the diminishing lipid peroxidation process in the kidney tissue [41].

On the other hand, in this study, the higher dose (500 mg/kg, b.w.) of EEOT could restore the kidneys antioxidant status and prevent renal damage. Non-Protein thiol in the cell is known to contribute in the xenobiotic metabolism. Although GSH is present in minute or less amounts in the kidney, this is reason the kidney become more sensitive to toxic encounters [42]. The elevated NP-SH level in EEOT treated animals partially mediate its protective mechanism(s) and could provide possible GSH mediated detoxification reaction in the kidney [43]. The obtained results suggests that EEOT may stimulate enzymatic systems responsible for the synthesis of free-SH, and/or inhibit those that are involved in its deterioration [44]. The current study revealed that nephrotoxic effects of GM are pronouncedly diminished by the administration of EEOT by exerting a significant decrease in serum creatinine concentration, urea and uric acid and kidney tissue MDA levels. Similarly, the co-administration of gentamicin with ethanol extract of *Citrus medica* improved histological indices of kidney damage characterized by a marked reduction in tubular atrophy, necrosis and vasculization. Of note, DCFH is generally used to measure *in vitro* oxidative stress generated by free radicals through the principle of oxidation of DCFH to the fluorescent DCF. However, we used this agent because of its highly toxic effects on cultured cells. In this study, our *ex vivo* "Otroj" protection against DCFH-induced human nephrotoxicity further supported the *in vivo* effects of 'Otroj' in gentamicin-induced kidney injury in rats. Thus, we have confirmed our results by using two different toxins in two different systems, at least for nephroprotection.

The results of our phytochemical screening are in agreement with several published reports on *C. medica* which showed the presence of several flavonoids e.g. apigenin, quercetin, rutin and hesperidin as well as some monoterpenoids e.g. limonene [45,46]. Recently, we reported interesting high antioxidant and free radical scavenging activity of EEOT as well as high phenolic and flavonoidal contents [47]. In view of the obtained results, it is suggested that the renal protective activity of *Citrus medica* extract might partially be due to its antioxidant property. The presence of the flavonoids might be responsible for the exhibited antioxidant and nephroprotective activity. Our findings are in agreement with recent studies for some flavonoids e.g. heperidin, rutin, naringenin and luteolin showing nephroprotective activity [48-51]. Furthermore, other studies indicated that limonene has interesting antioxidant activity and exert its chemopreventive activity through the inhibition of inflammation, oxidative stress and radicals-signaling [52,53].

5. Conclusions

In conclusion, the results of the present study indicate that EEOT provides adequate protection against gentamicin-induced nephrotoxicity. The nephroprotective effect may be due to its antioxidant properties. This provides persuasive evidence and supports its use in Arab traditional medicine for the adjunct treatment of renal disorders. Nevertheless, further studies are needed to confirm its clear mechanism of action in nephroprotection.

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