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RESEARCH ARTICLE

Standardized aqueous stem bark extract of *Gmelina arborea* roxb. possesses nephroprotection against adriamycin-induced nephrotoxicity in Wistar rats

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ABSTRACT

Nephrotoxicity is a major limitation of adriamycin (ADR) chemotherapy. We hypothesized that administration of standardized aqueous bark extract of *Gmelina arborea Roxb*. (GA) (Family; Verbenaceae), a traditional therapeutic agent, may reduce the nephrotoxicity caused by ADR in Wistar rats. The dose-dependent nephroprotective activity of the standardized GA extract was investigated in ADR-induced (20 mg/kg, ip) nephrotoxicity in male Wistar rats ($n = 6$/group). The lyophilized powder of the aqueous refluxed (4 h) GA extract was administered at 100, 300 and 500 mg/kg doses orally for three consecutive days. Fosinopril sodium (0.09 mg/kg) was used as the positive control. Assessment of biochemical parameters on serum, urine and histopathology on H and E stained kidney sections were done at the end of the intervention. The treatment with GA and fosinopril decreased the elevation of serum creatinine, blood urea nitrogen, cystatin C, β_2-microglobulin and loss of total protein in urine in nephrotoxic rats in a dose-dependent manner ($p < 0.05$). In contrast, serum concentrations of albumin and total protein were increased significantly ($p < 0.05$). H and E stained kidney sections showed an attenuation of renal parenchymal injury following the treatment. The aqueous extract of GA demonstrated antioxidant potential *in vitro*. Present findings conclude that the standardized aqueous extract of GA stem bark exerted a dose-dependent protection against ADR-induced nephrotoxicity *in vivo* and may be a promising adjunct in ADR chemotherapy.

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Introduction

Adriamycin (ADR) is an anthracycline antibiotic with a broad spectrum of anti-neoplastic potential. It is commonly used in the therapy of variety of human solid tumors and hematological malignancies (El-Sheikh *et al*. 2012, Benzer *et al*. 2018, Heravi *et al*. 2018). In spite of the high antitumor efficacy, the use of ADR in chemotherapy has been largely limited by the occurrence of dose related renal, cardiac, pulmonary, hematological, and testicular toxicities which intensify the clinical conditions of cancer patients even endangering their lives. However, nephrotoxicity has become one of the most common ADR-induced side effects, which can cause temporary or permanent kidney injury in patients who are on ADR treatment (Ayla *et al*. 2011, El-Sheikh *et al*. 2012, Heidari-Soreshjani *et al*. 2017, Benzer *et al*. 2018). In fact, it is associated with increased morbidity and mortality, prolonged hospital stays and high healthcare costs of cancer patients.

The exact mechanism of ADR-induced nephrotoxicity remains unclear, despite numerous studies performed over several decades. However, the oxidative stress caused by increased generation of reactive oxygen species is the main stay in explaining the ADR nephrotoxicity (Principal *et al*. 2010). Prevention of ADR nephrotoxicity without decreasing the efficacy is a highly desirable therapeutic aim in treatment regimes. Apparently, the antioxidant therapy has been identified as an effective approach to reduce nephrotoxicity induced by ADR (Heidari-Soreshjani *et al*. 2017, Molehin 2020).

Administration of a number of antioxidant compounds has demonstrated protective effects against ADR-induced nephrotoxicity in preclinical studies. Lycopene; a carotenoid occurring in tomatoes, nicotinamide; a derivative of vitamin B_3, the antioxidant coenzyme Q_{10}, vitamin A and E, selenium are few such examples (Ajith *et al*. 2008, Ayla *et al*. 2011, El-Sheikh *et al*. 2012). Most of them augment the therapeutic efficacy of ADR while preserving kidney functions (Principal *et al*. 2010). The ethnomedicinal plants from traditional system of medicine which are acclaimed by Ayurveda physicians for renal diseases have nephroprotective properties. Several medicinal plants with promising antioxidant effects were able to improve kidney functions against ADR-induced nephrotoxicity *in vivo* (Khajavi-Rad et al. 2017, Amarasiri *et al*. 2018, Molehin 2020). Hence, co-administration of herbal extracts would be beneficial for patients who are on ADR treatment to reduce the incidence of nephrotoxicity induced by ADR

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(Khajavi-Rad et al. 2017, Insaf and Raju 2019). Therefore, we postulate that the nephroprotecitve extracts with strong antioxidant properties that have been used in Sri Lankan traditional medicine might be promising in declining nephrotoxicity associated with ADR.

Gmelina arborea Roxb. (GA) (family; Verbenaceae), commonly known as Et-demata (Figure 1) is widely used in the treatment of variety of diseases in Sri Lankan traditional medicine (Jayaweera 1982, Rohit et al. 2012, Lawrence *et al.* 2016). Different parts of the plant are used for the management of kidney-related diseases by the Ayurvedic physicians. The roots of the plant are used for the treatment of kidney stones (Dhande 2016). It is used in the Ayurvedic preparation, 'brihat panchamoola' for the treatment of urinary tract infections and anuria (Lawrence *et al.* 2016). The stem bark has been recommended for the treatment of burning sensations and urinary discharge (Rohit et al. 2012). It forms one of the ingredients of 'Dashamoolarishta', a reputed restorative tonic, traditionally used for the treatment of urinary disorders (Ayurveda Pharmacopoeia 1985, Pathala *et al.* 2015, Lawrence *et al.* 2016).

Extensive research has been conducted on the stem bark of the plant for the investigation of bioactivities including anti-inflammatory, antioxidant, antimicrobial, antipyretic, gastroprotective, anti-diabetic, diuretic etc. (Attanayake et al. 2015, Lawrence *et al.* 2016, Kaur *et al.* 2018). No signs of toxicity have been reported for the methanol and aqueous extracts of GA stem bark in animal models (Kaur *et al.* 2018). Further, tyrosol, balanophonin, gmelinol, phenylethanoid glycoside, 2,6-dimethoxy-p-benzoquinone and 3,4,5-trimethoxyphenol were identified as bioactive compounds in the stem bark of GA (Lawrence *et al.* 2016). However, nephroprotective effect of GA stem bark has not been scientifically investigated to date. Herein, an attempt was taken to evaluate the nephroprotective activity of the aqueous extract of standardized GA stem bark against ADR induced nephrotoxicity in Wistar rats.

Materials and methods

Chemicals

All chemicals used in the study were of analytical grade. ADR (Doxorubicin hydrochloride) was purchased as 'Doxutec' (50 mg/25 mL injectable form) from United Biotech, India. Fosinopril sodium and all other chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

Plant material

The stem bark of GA was collected from natural habitat in Western province, Sri Lanka and the botanical identity was confirmed by comparing with the authenticated samples at the National Herbarium, Royal Botanical Gardens, Peradeniya. A voucher specimen was deposited at the mini herbarium at the Department of Biochemistry, Faculty of Medicine, University of Ruhuna; the voucher number; PG/2016/55/04 was assigned.

Preparation of plant extracts

The pieces of stem bark were washed under running tap water, cut into small pieces, dried at 40 °C to a constant weight and coarsely ground. Powdered plant material (12 g) was refluxed with 240 mL of distilled water for four hours. The extract was strained through a cheese cloth, concentrated and freeze dried at −40 °C to yield 2.6 g of lyophilized powder (percentage yield 21.8%). The lyophilized powder was reconstituted in distilled water to desired concentrations and used in the evaluation of antioxidant potential *in vitro* and nephroprotective activity *in vivo*.

Standardization of Gmelina arborea *stem bark and determination of its' antioxidant activity*

Physicochemical analysis

Determination of total ash content, water-soluble ash content and acid-insoluble ash content was carried out for the crude powder form of the plant material. Further, the powdered plant material was subjected to cold extraction and hot extraction with ethanol and distilled water respectively for the determination of extractive values. The moisture content was determined on the basis of loss of drying (WHO 2007).

Microbial limits and heavy metal analysis

Microbial limits and heavy metal analysis were carried out using the powder of GA stem bark. Microbial limits were determined as per the Sri Lanka Standards (SLS 1982, SLS 1992). Heavy metal analysis was carried out by inductively coupled plasma mass spectrometry (Agilent 7900 ICP-MS, California) following standard protocols (SLS 1973, AOAC 2000). The limits of quantification were 0.2, 5.0, 0.3 and 10.0 ppm for mercury, arsenic, cadmium and lead, respectively, according to the WHO guidelines (WHO 2007).

Thin-layer chromatography (TLC) fingerprint

GA stem bark (10 g) was added to a round bottom flask containing 50 mL of methanol and refluxed for 1 h. Then filtered and filtrate was concentrated using a rotary evaporator (Buchi, B-480, UK) until the volume reduced up to 5 mL. Silica gel aluminum-coated plate was used as the adsorbent. A mixture of ethyl acetate and dichloromethane was used in a ratio of 3:2 was used as the mobile phase.

Preliminary phytochemical analysis

The powdered plant material was refluxed for 2 h with distilled water and filtrate was used in the preliminary phytochemical analysis (Farnsworth 1966).

Quantification of total polyphenol content and total flavonoid content

Total polyphenol content of GA stem bark extract was estimated by Folin–Ciocalteu method (Singleton *et al.* 1999) using gallic acid as the standard. Quantification was done with respect to the standard curve of gallic acid

($y = 3.2256x + 0.0121$) and the results were expressed in terms of gallic acid equivalents of the dry weight (mg GAE/g).

Total flavanoid content was estimated using aluminum chloride method according to the method described by Siddhuraju and Becker (2003). The flavonoid content was calculated using the calibration curve of quercetin ($y = 13.619x + 0.0024$). The results were expressed in terms of mg quercetin equivalent/g of extract.

In vitro antioxidant studies

The antioxidant activity of the lyophilized powder of GA stem bark extract was determined by 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay (Blois 1958), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{+}$) radical scavenging assay (Re *et al*. 1999) and oxygen radical absorbance capacity (ORAC) assay (Ou *et al*. 2001). The absorbance readings were measured using a microplate reader (Spectra max, USA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used as the reference compound and the results were expressed in terms of Trolox equivalent antioxidant capacity (TEAC; mg Trolox/g of extract).

Evaluation of nephroprotective activity against ADR-induced nephrotoxic rats

Experimental animals

Adult male Wistar rats weighing 270–320 g were purchased from Medical Research Institute, Colombo, Sri Lanka. The animals were maintained under standard environmental conditions (12 h light/12 h dark) in the Animal House, Department of Biochemistry, Faculty of Medicine, University of Ruhuna. The animals were fed with a standard laboratory diet of pellets and water *ad libitum*. Ethical clearance was obtained from the Ethical Review Committee, Faculty of Medicine, University of Ruhuna (Reference No. −14.12.2015:3.1). Experiments were carried out within the approved ethical framework minimizing potential pain, suffering or distress to the experimental animals.

Experimental design

Overnight fasted (eight hours) healthy male Wistar rats were randomly divided into six groups with six rats in each. Nephrotoxicity was induced in experimental rats of group 2–6 with a single intraperitoneal dose of ADR at 20 mg/kg. Rats in group 1 (normal control group) and group 2 (nephrotoxic control group) received an equivalent amount of distilled water. Nephrotoxic rats of group 3, 4 and 5 were administered with the freeze dried form of the aqueous extract of GA orally, at 100, 300 and 500 mg/kg doses, respectively. The nephrotoxic rats administered with the standard drug; fosinopril sodium (0.09 mg/kg) was considered as the positive control. The treatment regimens were initiated 24 hours following the induction of nephrotoxicity and continued for three consecutive days. The body weight of experimental animals was measured on day one and five of the experiments. Consumption of food and intake of water were measured daily during the intervention.

The experimental animals of each group were individually housed in metabolic cages and 24 hour urine samples were collected following treatment with the final dose of the plant extracts/standard drug. The animals were sacrificed on the fifth day of the study by mild ether anesthesia. Blood samples were collected by cardiac puncture into serum collecting gel tubes. Kidney tissues were excised from the sacrificed animals for histopathological studies.

Evaluation of kidney functions using biochemical parameters

The blood samples were allowed to coagulate for one hour at room temperature (27 °C). Serum was separated by centrifugation at 3500 rpm for 15 minutes and used for evaluating biochemical parameters. Biochemical parameters including serum creatinine (Bartels *et al*. 1972), blood urea nitrogen (BUN; Sampson *et al*. 1980), serum total protein (Weichselbaum 1946), serum albumin (Bartholomew and Delaney 1964) and urine total protein (Watanabe *et al*. 1986) were estimated using spectrophotometric assay kits (UV-1800, SHIMADZU, USA). The serum concentrations of β_2-microglobulin and cystatin C were estimated using enzyme-linked immunosorbent assay (ELISA) kits (BIO TEK, USA).

Histopathology of H and E stained sections of the kidney

Paraffin sections (5 µm) of formalin-fixed kidney samples were prepared and stained with hematoxylin and eosin (H and E) for light microscopic examinations (Olympus CX 21, Japan). Histopathological assessment was carried out according to a semiquantitative score system developed by the investigators. The tissue sections were examined for the presence of indicators of cellular damage by two independent investigators. The investigators were blinded to the sample groups. Ten high power fields (x400) in the inner part of the kidney cortex were examined in each kidney section for the presence of cytoplasmic vacuolization and pyknosis of tubular epithelial cells, loss of brush border in the tubular epithelium, cast formation, intertubular hemorrhage and glomerular congestion. A value of ‘1’ was assigned to a field present with above-mentioned features, whereas ‘0’ value was assigned in the absence of damage. The score for each feature was calculated per section (10 high power fields) and sum up all together to give the total histological score out of the maximum score of 60 (six features). Consequently, the mean histological scores per rat and the experimental groups were calculated respectively.

Statistical analysis

The biochemical data were statistically analyzed using one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) multiple comparison test. Kruskal–Wallis test was used to analyze semi-quantitative data of histopathological examination. All values were expressed as mean ± standard error (±SEM), while the results at $p < 0.05$ were considered statistically significant. The

Table 1. Physicochemical parameters and heavy metal analysis of *Gmelina arborea* stem bark.

Specification	Result
Ash values	
Total ash (% w/w)	7.5 ± 0.2
Acid-insoluble ash (% w/w)	<0.01
Water soluble ash (%w/w)	2.1 ± 0.2
Extractive values	
Cold water extractive (% w/w)	4.1 ± 0.2
Cold ethanol extractive (% w/w)	2.7 ± 0.1
Hot water extractive (% w/w)	6.1 ± 0.1
Hot ethanol extractive(% w/w)	3.6 ± 0.0
Moisture content (% w/w)	10.1 ± 0.0
Heavy metal analysis	
Mercury (Not more than 0.2 ppm)	<0.05
Arsenic (Not more than 5.0 ppm)	<0.05
Lead (Not more than 10 ppm)	<0.05
Cadmium (Not more than 0.3 ppm)	<0.05

Values are expressed as mean ± SD, $n = 3$.

triplicates of each sample were used for statistical analysis in *in vitro* studies.

Results

Standardization and antioxidant activity of Gmelina arborea *stem bark*

The findings on physicochemical parameters and heavy metal analysis are shown in Table 1. The hot water extract showed the highest extractive value, whereas the lowest value was obtained with the cold ethanol extract. The heavy metals such as arsenic, lead, cadmium and mercury were present within the WHO acceptable limits in the GA stem bark. There were no microorganisms including *Escherichia coli*, *Staphylococcus aureus*, coliforms, yeast and mold in the selected plant material of GA.

The TLC fingerprint of the methanol extract of GA stem bark is shown in Figure 2. The methanol extract showed fourteen spots in the TLC fingerprint at R_f 0.08, 0.11, 0.15, 0.18, 0.26, 0.35, 0.49, 0.57, 0.66, 0.75, 0.80, 0.89, 0.94, and 0.98.

The phenolics, tannins, flavonoids, steroid glycosides, saponins and terpenoids were present whereas alkaloids and coumarins were absent in the aqueous extract of GA stem bark. The total polyphenol content was 38.01 ± 2.65 mg gallic acid equivalents/g of extract as determined from the regression equation of calibration curve. The total flavonoid content was 9.65 ± 0.18 mg quercetin equivalent/g of extract according to the calibration curve of quercetin as the reference compound. The total antioxidant activity of the aqueous extract of GA stem bark was estimated by DPPH, ABTS and ORAC assays and the values in terms of trolox equivalent antioxidant capacity (mg Trolox/g of extract) are shown in Table 2.

Evaluation of nephroprotective activity against ADR-induced nephrotoxicity in rats

The induction of nephrotoxicity by ADR resulted in a significant loss of body weight (10%) in the nephrotoxic control group at the end of the study period of five days ($p < 0.05$). Accordingly, a reduction in food consumption and intake of water was noted following an induction of nephrotoxicity with ADR. The administration of the GA extract resulted in significant dose-dependent attenuation of the percentage reduction in body weight in the group of rats treated with 500 mg/kg dose of GA with compared to the nephrotoxic control rats ($p < 0.05$). The average consumption of food was improved upon the administration of all three selected doses of GA whereas; average intake of water was significantly improved upon the treatment with the 300 and 500 mg/kg doses of GA only ($p < 0.05$). The results are shown in Table 3.

Evaluation of kidney functions

The evaluation of kidney functions with the treatment of GA extracts is shown in Table 4. There was a significant increase in the concentrations of serum creatinine and BUN by 60% and 43% respectively, in rats of nephrotoxic control group compared to the normal healthy animals ($p < 0.05$). The treatment with the GA extract at the doses 100, 300 and 500 mg/kg markedly improved the kidney functions which was manifested as a significant reduction in creatinine and BUN by 18% 32%, 44% and 19%, 25%, 26% respectively for the three doses compared to the rats of ADR induced nephrotoxic control group ($p < 0.05$). Proteinuria is one of the early signs of kidney damage. The elevation of total protein concentration in urine over 70% in the nephrotoxic control group compared to the healthy control group signifies the kidney damage induced by ADR in experimental animals. The elevation of urinary protein due to glomerular and tubular injury resulted in subsequent reduction in serum concentrations of total protein (36%) and albumin (50%) in ADR induced nephrotoxic control rats. However, the standard drug, fosinopril was able to reduce proteinuria by 86% whereas the aqueous extract of GA stem bark at the three selected doses; 100, 300, 500 mg/kg reduced proteinuria by 48%, 74%, 73%, respectively. Further, serum total protein and albumin levels showed a dose dependent increase in GA-treated experimental animals. However, none of the experimental groups was able to restore the level of serum albumin within the short experimental period.

The serum concentration of β_2-microglobulin showed an increase (63%) in the nephrotoxic control group compared to the healthy control group ($p < 0.05$). As shown in Table 4, the elevation of serum β_2-microglobulin caused by ADR decreased significantly ($p < 0.05$) following the treatment with GA extracts. The highest dose of GA extract used in the present study was able to restore the level of β_2-microglobulin to normal. However, changes in the concentrations of cystatin C were not statistically significant compared to ADR control group rats ($p < 0.05$), even though the values varied in a dose dependent manner. Moreover, the selected doses of GA were more effective in reducing the elevated levels of β_2 -microglobulin and cystatin C compared to the standard drug, fosinopril.

The median effective dose (ED_{50}) for the aqueous extract of GA was exceeded the highest selected dose (500 mg/kg) with reference to the biochemical parameters; BUN, serum creatinine, serum albumin, serum total protein and serum cystatin C. However, the calculated ED_{50} values for serum β_2-

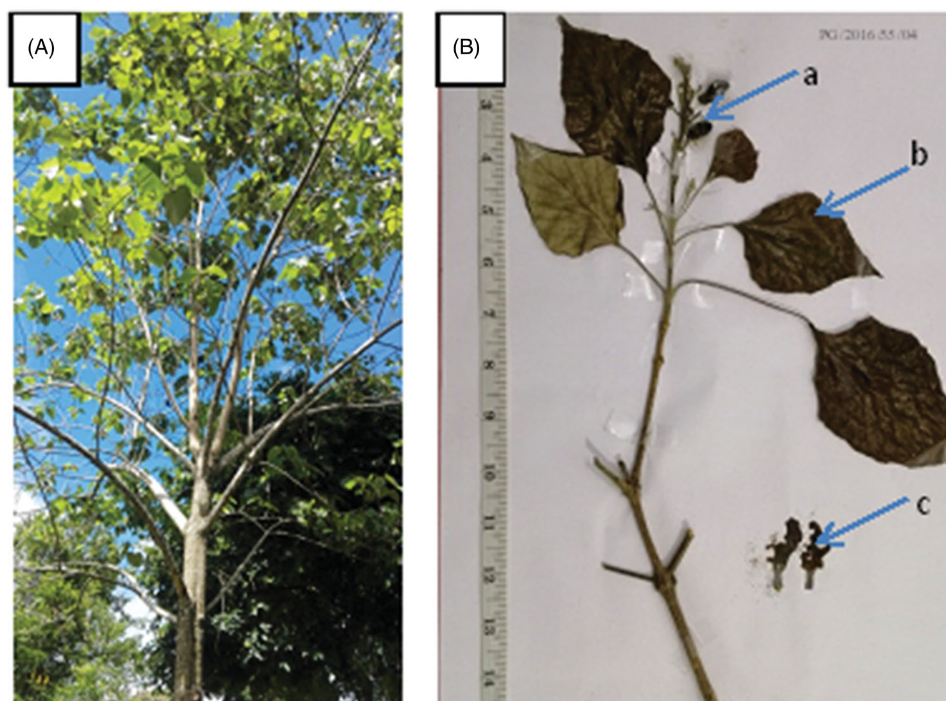

Figure 1. Plant of *Gmelina arborea* Roxb. at natural habitat (A) and the voucher specimen deposited at the mini herbarium, Department of Biochemistry, Faculty of Medicine, University of Ruhuna (B). The voucher specimen includes fruits (a), leaves (b) and flowers (c) of the plant.

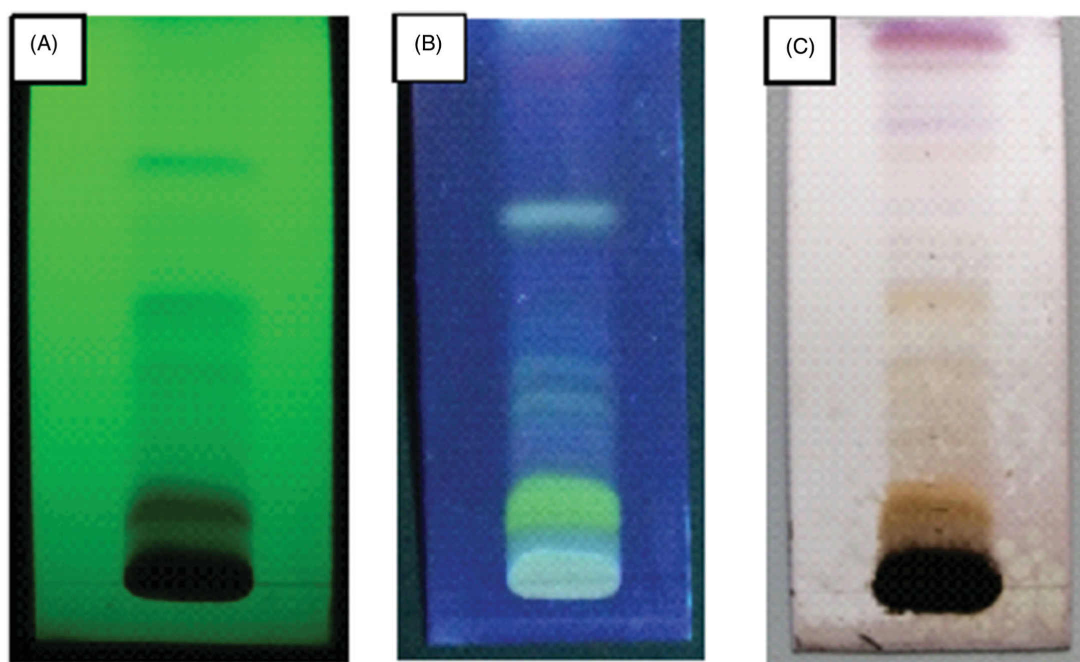

Figure 2. TLC fingerprint for methanol extract of *Gmelina arborea* stem bark at UV 254 nm (A), UV 366 nm (B) and after spraying vanillin sulfuric acid (C).

Table 2. Total antioxidant activity of *Gmelina arborea* stem bark.

Antioxidant assay	Total antioxidant activity
DPPH assay (mg trolox/g of extract)	32.90 ± 0.14
ABTS assay (mg trolox/g of extract)	106.87 ± 1.28
ORAC assay (mg trolox/g of extract)	60.31 ± 2.49

Values are expressed as mean ± SD, $n = 3$. The antioxidant capacity is expressed in terms of trolox equivalent antioxidant capacity (TEAC: mg trolox/g of extract). Higher TEAC values exhibited by the plant extract represent higher level of antioxidant activity of *Gmelina arborea*. DPPH: 2,2-diphenyl-2-picrylhydrazyl, ABTS: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, ORAC: oxygen radical absorbance capacity.

microglobulin and urine total protein were 241.23 mg/kg and 286.60 mg/kg respectively.

Assessment of histopathology

The photomicrographs of the H and E stained kidney sections of experimental groups are shown in Figure 3. Renal cortex showed more pronounced changes in nephrotoxic animals compared to the normal control. Therefore, the areas in renal cortex containing renal corpuscles and tubules were selected

Table 3. Effect of the aqueous extract of *Gmelina arborea* stem bark on body weight, average consumption of food and average intake of water.

Group	Body weight (g)				
	Initial weight	Weight on 5th day	Mean percentage change	Average consumption of food (g)	Average intake of water (mL)
Normal control	296.00 ± 5.10	290.00 ± 4.47	2%[c]	16.97 ± 0.70[c]	32.50 ± 1.25[a]
ADR alone (20 mg/kg)	276.00 ± 9.27	250.00 ± 6.32	10%	0.46 ± 0.46	19.79 ± 5.09
ADR (20 mg/kg) + *G. arborea* (100 mg/kg)	282.50 ± 8.54	255.00 ± 6.45	10%	0.71 ± 0.41	17.92 ± 3.86
ADR (20 mg/kg) + *G. arborea* (300 mg/kg)	277.50 ± 10.31	255.00 ± 9.57	8%	0.50 ± 0.50	23.28 ± 6.07
ADR (20 mg/kg) + *G. arborea* (500 mg/kg)	292.50 ± 4.79	275.00 ± 6.45	6%[a]	0.89 ± 0.59	20.10 ± 2.42
ADR (20 mg/kg) + fosinopril (0.09 mg/kg)	275.00 ± 8.66	242.50 ± 8.54	11%	0.56 ± 0.48	32.03 ± 3.91

Values are expressed as mean ± SEM of six animals in each group. Results are significant compared to the adriyamycin (ADR) induced nephrotoxic control group at: $^{a}p < 0.05$, $^{b}p < 0.01$, and $^{c}p < 0.001$.

Table 4. Effect of the aqueous extract of *Gmelina arborea* stem bark on biochemical parameters of kidney function.

Treatment Group	BUN (mmol/L)	Serum creatinine (μmol/L)	Serum total protein (g/L)	Serum albumin (g/L)	Serum β_2-microglobulin (μg/ mL)	Serum cystatin C (ng/mL)	Urine total protein (g/dL)
Normal control	5.06 ± 0.38[c]	39.78 ± 9.29[c]	64.07 ± 1.83[c]	34.85 ± 0.47[c]	0.10 ± 0.01[b]	7.94 ± 0.99	83.86 ± 6.33[c]
ADR alone (20 mg/kg)	8.87 ± 0.98	100.48 ± 15.62	47.23 ± 1.19	23.18 ± 0.43	0.27 ± 0.05	8.13 ± 0.36	304.99 ± 18.59
ADR (20 mg/kg) + *G. arborea* (100 mg/kg)	7.18 ± 0.29	82.43 ± 13.07	44.78 ± 3.16	23.60 ± 0.56	0.12 ± 0.02[b]	11.59 ± 2.60	159.66 ± 15.48[c]
ADR (20 mg/kg) + *G. arborea* (300 mg/kg)	6.65 ± 0.47[a]	68.51 ± 1.88[b]	55.05 ± 1.58[b]	24.38 ± 0.25	0.12 ± 0.04[b]	9.34 ± 1.60	78.86 ± 17.12[c]
ADR (20 mg/kg) + *G. arborea* (500 mg/kg)	6.55 ± 0.21[a]	56.28 ± 2.62[c]	61.77 ± 2.63[c]	25.43 ± 0.35[a]	0.10 ± 0.01[b]	5.94 ± 1.09	81.61 ± 18.51[c]
ADR (20 mg/kg) + fosinopril (0.09 mg/kg)	5.50 ± 0.22[c]	60.29 ± 2.52[c]	54.47 ± 0.86[a]	27.83 ± 0.59[c]	0.20 ± 0.07	9.87 ± 1.93	41.99 ± 7.59[c]

Values are expressed as mean ± SEM of six animals in each group. Results are significant compared to the adriyamycin (ADR) induced nephrotoxic control group at: $^{a}p < 0.05$, $^{b}p < 0.01$, and $^{c}p < 0.001$. BUN; blood urea nitrogen concentration.

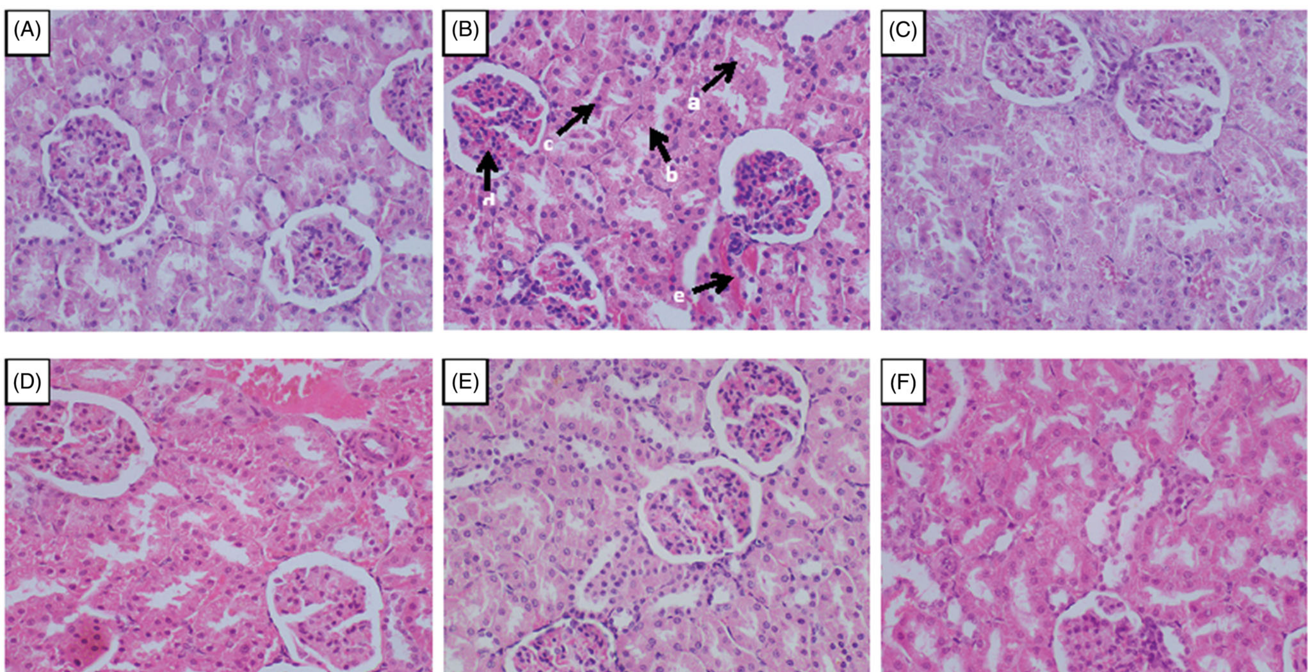

Figure 3. Photomicrographs of kidney tissues of ADR induced nephrotoxic rats treated with the aqueous extract of *Gmelina arborea* stem bark (x400). Normal control group (A) ADR-induced nephrotoxic control group (B) fosinopril-treated/positive control group (C) groups of animals treated with the aqueous extract of *Gmelina arborea* stem bark at three selected doses; 100, 300 and 500 mg/kg (D,E,F) are shown. The features of acute tubular necrosis as shown in Plate 3 (B) are as follow; a: tubular vacuolization, b: loss of brush border, c: pyknosis in tubular epithelium, d: glomerular congestion and e: hemorrhage.

for the examination of histopathological changes. Microscopic examination of the H and E stained kidney sections of the healthy control group showed normal tissue architecture with the lowest score according to the semi quantitative score system followed as shown in Table 5. The kidney sections of the nephrotoxic control group showed the early features of acute tubular necrosis involving most of the renal cortex with significant increase in the average score value of 36% ($p < 0.05$). Most of the proximal convoluted tubules showed morphological evidence of cellular damage with pale cytoplasm due to vacuolization (Figure 3(B)) and disappearance of the brush borders (Figure 3(B)). The epithelial cell lining of the tubules had hypereosinophilic cytoplasm with pyknotic nuclei (Figure 3(B)). Numerous glomeruli showed glomerular congestion (Figure 3(B)). Intertubular hemorrhages (Figure 3(B)) were predominant and hyaline casts were occasional. However, no large areas of necrosis or glomerular sclerosis were observed in the H and E stained kidney sections of the nephrotoxic control group. The concurrent administration of the selected doses of the aqueous extract of GA stem bark ameliorated ADR induced renal tubular and glomerular alterations as observed in the improved mean histological score values of the groups (Figure 3(D–F)). The histological changes with the most significant reduction in the score included pyknosis and loss of brush border in the tubular epithelium and glomerular congestion. A similar attenuation of morphological destruction was observed with the fosinopril-treated rats in the present study.

Discussion

Nephrotoxicity of chemotherapeutic drugs remains a significant drawback in the treatment of cancers. It has been accounted for a substantial number of patients undergoing cancer chemotherapy leading to significant mortality worldwide (Heidari-Soreshjani *et al*. 2017, Insaf and Raju 2019, Pršа *et al*. 2020). ADR is a key chemotherapeutic drug of anthracycline family which is used for the treatment of a variety of cancers (Principal *et al*. 2010, Raza and Naureen 2020). ADR is metabolized via multiple pathways *in vivo*. However, oxidative damage mediated through the generation of reactive oxygen species has been identified as a major pathway of causing and amplifying nephrotoxicity (Principal *et al*. 2010). This is illustrated in Figure 4. The toxic intermediates of ADR are generated primarily in the liver and transported to the kidney in engendering ADR nephrotoxicity (Mohammadi and Ahmadizadeh 2018). The large volume of renal blood flow which accounts for 25% of the cardiac output may also cause kidneys to expose much higher level of toxic compounds than the other organs leading to nephrotoxicity (Al-Attar *et al*. 2017, Mohammadi and Ahmadizadeh 2018). However, it is assumed that ADR induced nephrotoxicity is initiated with the action of several oxidoreductases, including cytochrome P_{450}, NADPH-dependent reductase and NADH dehydrogenase which facilitate the biotransformation of ADR to reactive intermediates (Heravi *et al.* 2018, Mohammadi and Ahmadizadeh 2018). Electron transformation from NADPH or NADH and its derivation with ADR by these oxidoreductases generates a semiquinone radical of ADR. The semiquinone form of ADR is a toxic short-life metabolite which initiates a cascade of reactions producing reactive oxygen species (ROS) such as superoxide anion radicals ($O_2^{-\circ}$), hydroxyl radicals (OH°) and hydrogen peroxide (H_2O_2) in aerobic conditions (Ajith *et al*. 2008, Benzer *et al.* 2018, Heravi *et al*. 2018). The increased production of ROS and reduced activity of antioxidant enzymes may lead to a state of oxidative stress resulting tissue injury which links with protein oxidation and membrane lipid peroxidation in kidney tissues (Ayla *et al*. 2011, El-Sheikh *et al*. 2012). Moreover, ADR may induce acute kidney damage via its direct toxic effects by accumulation of the parent molecule as well as its metabolites in kidney tissues (Al-Attar *et al*. 2017, Benzer *et al*. 2018).

In our study, the nephrotoxicity was induced with a single intraperitoneal dose of ADR at 20 mg/kg body weight. The dose of ADR was selected based on the reported literature (Ayla *et al*. 2011, Yagmurca *et al*. 2015, Shahbazi *et al*. 2020). Two other doses of ADR, 17 mg/kg and 23 mg/kg were trialed. However, the dose of 17 mg/kg was not able to induce kidney damage satisfactorily and the dose of 23 mg/kg, caused premature mortality in experimental animals (data not shown). The results are corroborated with published reports (El-Sheikh *et al*. 2012, Molehin 2020). Induction of ADR nephrotoxicity was detected in the present study by significant changes in biochemical parameters which were confirmed by histopathological evidence of cell injury compared to the healthy control group. The elevation of both serum creatinine and BUN were more than 40%, in the rats of nephrotoxic control group. The findings are in agreement with the previous studies which describe a reduction in the glomerular filtration rate with a rise in the serum concentrations of creatinine and BUN (Allam *et al*. 2015, Mohebbati *et al*. 2016, Ibrahim *et al*. 2020). The significant increase in proteinuria and subsequent reduction in serum total protein and albumin of the nephrotoxic control group further corroborates the nephrotoxicity induced by ADR ($p < 0.05$). Moreover, a significant increase in the serum concentration of β_2-microglobulin was noted in the nephrotoxic control group compared to the healthy control indicating impaired renal functions ($p < 0.05$). Our findings are in agreement with the findings of Oddoze *et al*. (2001) indicating that serum β_2-microglobulin is a better marker of glomerular filtration than serum cystatin C (Aksun *et al*. 2004). It is mentioned that serum β_2-microglobulin is a more sensitive and specific biomarker than cystatin C in kidney damage (Behairy *et al.* 2017). The higher individual variance was reported for cystatin C which is an additional disadvantage of using it in experiments (Aksun *et al.* 2004). These reports explain the discrepancies observed in cystatin C values compared to β_2-microglobulin in the present study.

The early features of acute tubular necrosis characterize the nephrotoxicity on H and E stained kidney sections of the nephrotoxic control group. The nephrotoxic control group reported the highest histological score according to the semi quantitative score system and these findings further substantiate the results of biochemical parameters.

Treatment with the standardized aqueous extract of GA stem bark at the three selected doses provoked a marked

Table 5. Effect of the aqueous extract of *Gmelina arborea* stem bark on histopathology of kidney tissues.

Treatment Group	Histological score for the individual features observed						
	Loss of brush border	glomerular congestion	Intertubular hemorrhage	cytoplasmic vacuolization	Pyknosis	Cast formation	Mean histological score
Normal control	7.00 ± 0.58[b]	3.86 ± 0.99[b]	7.71 ± 0.89[b]	8.14 ± 0.74	1.00 ± 0.31[b]	0.29 ± 0.18	28.00 ± 2.45[c]
ADR alone (20 mg/kg)	9.86 ± 0.14	8.86 ± 0.40	9.57 ± 0.43	9.57 ± 0.20	6.86 ± 0.40	0.00 ± 0.00	44.71 ± 1.19
ADR (20 mg/kg) + *G. arborea* (100 mg/kg)	4.67 ± 0.21	9.83 ± 0.17	10.00 ± 0.00	9.83 ± 0.17	4.50 ± 0.34	0.00 ± 0.00	38.83 ± 0.54[a]
ADR (20 mg/kg) + *G. arborea* (300 mg/kg)	9.25 ± 0.18	8.33 ± 0.28	9.92 ± 0.08	9.75 ± 0.18	1.75 ± 0.33[b]	0.00 ± 0.00	39.00 ± 0.63[a]
ADR (20 mg/kg) + *G. arborea* (500 mg/kg)	2.83 ± 0.87[c]	8.33 ± 0.61	10.00 ± 0.00	10.00 ± 0.00	2.00 ± 0.52[a]	0.33 ± 0.21	33.50 ± 0.96[b]
ADR (20 mg/kg) + fosinopril (0.09 mg/kg)	8.83 ± 0.24	6.83 ± 0.77	8.67 ± 0.49[a]	9.41 ± 0.12	0.17 ± 0.11[c]	0.17 ± 0.17	34.50 ± 1.19[b]

Mean score was given out of the total score of 60. Values are expressed as mean ± SEM of six animals in each group. Results are significant compared to the adriyamycin (ADR) induced nephrotoxic control group at: [a]$p < 0.05$, [b]$p < 0.01$, and [c]$p < 0.001$.

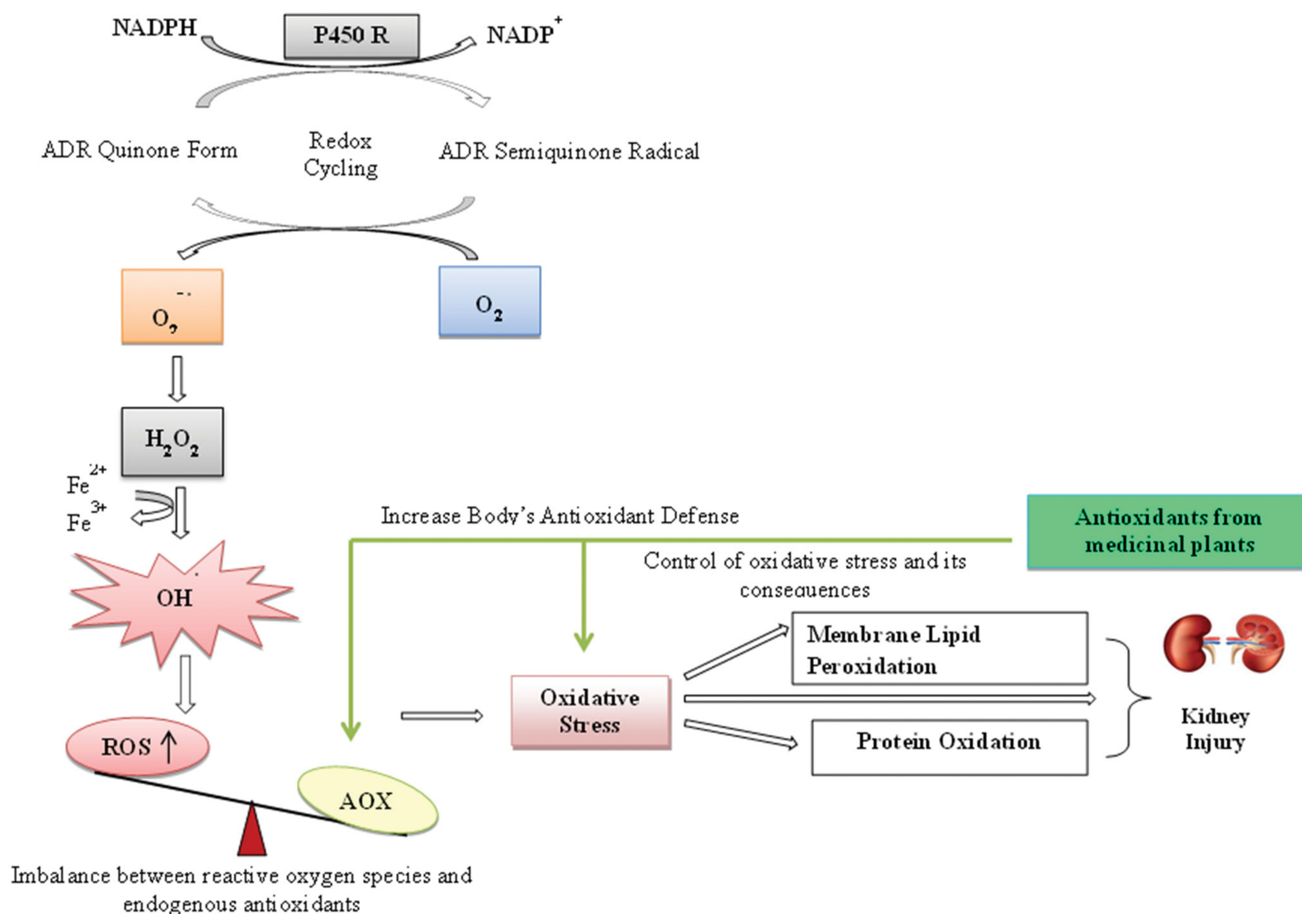

Figure 4. Suggested mechanisms of ADR-induced nephrotoxicity. NADPH: Nicotinamide adenine dinucleotide phosphate, $NADP^+$: oxidized form of NADPH, P450R: NADPH-dependent reductase, O_2: oxygen, $O_2^{-\circ}$: superoxide anion radicals, H_2O_2: hydrogen peroxide, OH°: hydroxyl radicals, ROS: reactive oxygen species, AOX: antioxidants.

nephroprotection in experimental rats as evidenced by biochemical parameters and by detailed histopathological assessment. A significant dose-dependent reduction was observed in serum concentration of creatinine and BUN in the treatment groups compared to the nephrotoxic control group indicating a progress in kidney function. Urine total protein is considered a potential biomarker of acute kidney damage induced by nephrotoxic drugs. Under normal circumstances, the glomeruli restrict the transport and migration of high molecular weight proteins from the blood

stream to the lumen of nephrons. However, in pathological conditions, high molecular weight proteins can be detected in urine due to nephron dysfunction (Al-Naimi *et al*. 2019). Therefore, high molecular weight proteins such as albumin are regarded as more sensitive proteins in the early detection of glomerular filtration dysfunction. Treatment of GA extract at the 100, 300 and 500 mg/kg doses were able to reverse the ADR induced increase in urine total protein in the present study. Interestingly, the equivalent human therapeutic dose (300 mg/kg) and the selected highest dose (500 mg/kg) resulted in significant reduction of proteinuria which is comparable to the results of healthy control group. The standard drug, fosinopril showed a remarkable reduction in proteinuria exceeding the level of normal control as well as the plant extracts. Fosinopril is an angiotensin converting enzyme inhibitor used in the treatment of chronic kidney disease. Angiotensin converting enzyme inhibitors demonstrate hypoalbuminuria by inhibition of renin-angiotensin system. Therefore, angiotensin converting enzyme inhibitors have become the standard of care for patients with albuminuria due to its action by reducing urinary albumin excretion and ameliorating proteinuria (Xiao *et al*. 2013). The subsequent elevation observed in serum total protein and albumin with the reduction of proteinuria in the nephrotoxic rats treated with plant extracts further corroborates the results of other kidney function tests substantiating the protective effects of GA against ADR induced nephrotoxicity. β_2-microglobulin and cystatin C are low molecular weight proteins that reflect the underlying renal glomerular and/or tubular damage during nephrotoxicity (Izzedine and Perazella 2017, Al-Naimi *et al*. 2019). Generally, low-molecular-weight proteins are reabsorbed at the renal proximal tubules. However, the presence of an excess amount of low molecular weight proteins leads to nephron overload, exceeding the reabsorbing capacity of proximal renal tubules. Therefore, damage to the proximal renal tubules may result in low molecular weight proteinuria (Al-Naimi *et al*. 2019). Thus, the concentrations of those low molecular weight proteins in serum would be directly proportional to the degree of nephrotoxicity. However, both parameters showed a dose dependent improvement in the results following treatment with GA even though the changes were not significant for serum cystatin C. However, the selected doses of GA were more effective in reducing the elevated levels of both β_2-microglobulin and cystatin C compared to the standard drug, fosinopril. Moreover, in the present study, the calculated ED_{50} values of GA extract with reference to serum β_2-microglobulin and urine total protein were closer to the human equivalent therapeutic dose in rats.

The histopathological findings corroborated the results of biochemical parameters. Concurrent administration of the plant extracts and the standard drug appeared to mitigate the severity of ADR induced renal injury, reducing the histological score compared to the nephrotoxic control group. These findings reconfirm the protective effects of the aqueous extract of GA stem bark against ADR-induced nephrotoxicity.

The aqueous extract of GA stem bark exhibited relatively a high free radical scavenging potential as expressed by high trolox equivalent antioxidant capacity values by the three assays. In fact antioxidant capacity of the medicinal plants is closely correlated with their phenolic content (Sreejith *et al*. 2014). According to the reported literature, polyphenols and flavonoids are the two main phytoconstituents responsible for the antioxidant capacity (Sharma *et al*. 2015). The relatively high polyphenol and flavonoid contents reported in the GA extract substantiate the antioxidant potential of the GA stem bark. Tyrosol and phenylethanoid glycoside; the phenolic compounds isolated from the stem bark of GA, and 3,4,5-trimethoxyphenol, a flavonoid isolated from the ethyl acetate soluble extraction of GA have been reported with antioxidant activity *in vitro* (Falah *et al*. 2008). These phytochemicals may impart nephroprotection via antioxidant pathways neutralizing the chain reactions, scavenging ROS and increasing antioxidant enzymes as illustrated in Figure 4. Further, these may accelerate the elimination of free radicals controlling oxidative stress and its consequences toward the nephroprotection. Similarly, medicinal plants abounding antioxidant capacity such as *Curcuma longa* L., *Zingiber officinale* Roscore, *Solanum torvum* Sw, *Plectranthus amboinicus* L. and *Nigella sativa* L. have shown protective effects against ADR induced nephrotoxicity (Principal *et al*. 2010, Khajavi-Rad et al. 2017, Amarasiri *et al*. 2018, Molehin 2020).

Conclusion

The administration of standardized aqueous stem bark extract of GA (300 mg; equivalent therapeutic dose and 500 mg/kg) showed significant nephroprotective activity by normalizing the commonly used renal biochemical parameters in experimental rats of ADR-induced nephrotoxicity. In addition, GA extract reduced the severity of ADR-induced acute renal damage. The nephroprotective activity of the GA extract most probably is due to the antioxidant potential of the plant extract governed by polyphenols and flavonoids. Based on the pre-clinical data, we conclude that the administration of the GA extract would sufficiently reduce the ADR nephrotoxicity, thereby it would be a promising adjunct for cancer patients who are on ADR chemotherapy. However, clinical trials of GA extract are warranted to determine the expediency of the above results to be extrapolated against ADR induced nephrotoxicity in cancer patients.

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Disclosure statement

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