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Research Article

Flavonoids-rich Extract of *Beta vulgaris* Subsp. cicla L. var. Flavescens Leaf, a Promising Protector Against Gentamicin-induced Nephrotoxicity and Hepatotoxicity in Rats

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Abstract

Background and Objective: The major complication of gentamicin antibiotic is nephrotoxicity which limited its clinical use. The current study was conducted to evaluate the possible protective effect of *Beta vulgaris* L. subsp. cicla var. flavescens (Swiss chard) against gentamicin-induced nephrotoxicity and hepatotoxicity in rats. **Methodology:** Twenty four rats were divided into 4 groups, group 1 served as control. Group 2 to group 4 were intraperitoneally (i.p.) injected with gentamicin at a dose of 80 mg kg^{-1} body weight daily for 15 days. Swiss chard extract was orally administrated 1 week before and along with gentamicin treatment to groups 3 and 4 at doses of 300 and 600 mg kg^{-1} body weight daily, respectively. **Results:** The results revealed that, gentamicin significantly altered serum levels of kidney and liver markers as well as tumor necrosis factor alpha (TNF-α). These results were associated with significant changes in urinary urea, creatinine, micro-total protein (Micro TP) and vascular epithelial growth factor (VEGF) levels. A significant decrease in renal and hepatic reduced glutathione (GSH) levels, glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide dismutase (SOD) enzymatic activities with a significant decrease in the expression levels of SOD1 and GSH-Px genes were also observed. In contrary, a significant increase in malondialdehyde (MDA), protein carbonyl levels and caspase-3 gene expression levels were also detected in gentamicin treated rats. Pretreatment with Swiss chard, dose dependently, ameliorated such altered changes. In harmony with this line, Swiss chard greatly decreased the severity of renal tubular and hepatic necrosis induced by gentamicin. **Conclusion:** Swiss chard leaf extract can attenuate gentamicin-induced nephrotoxicity and hepatotoxicity possibly by its antioxidant, anti-inflammatory and anti-apoptotic properties.

Key words: Gentamicin, nephrotoxicity, hepatotoxicity, swiss chard, phenolics, antioxidant activity, anti-inflammatory effect

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

INTRODUCTION

Gentamicin is an aminoglycoside antibiotic obtained from *Micromonospora purpurea* fungus and other related species. It has a broad-spectrum action against life threatening Gram-negative bacterial infections and some strains of Gram-positive bacteria[1,2]. Inspite of its beneficial importance as a potent bactericidal agent, its use has been restricted owing to its adverse effects causing ototoxicity, nephrotoxicity, hepatotoxicity and neuromuscular problems[3,4].

Nearly 10-20% of patients under gentamicin therapy showed the signs of nephrotoxicity, particularly with high and extended dose[4,5]. Renal toxicity occurred due to selective accumulation of gentamicin in renal tubular cells which induces necrosis, apoptosis and destruction of cells which finally results in renal failure with sudden and sustained decrease in glomerular filtration rate[2,6]. Renal damage induced by gentamicin can be accompanied by liver injury which leads to transient hepatomegaly and increases in serum bilirubin, lactate dehydrogenase and transaminases[7,8]. So there is an urgent need to search for agents that can protect kidney and liver against such harmful actions.

For decades, the use of natural and dietary products as potent chemoprotective agents increases day after day for the continuous discoveries of novel therapies. Swiss chard (*Chenopodiaceae*, *Beta vulgaris* subspecies cicla L.var. flavescens), a herbaceous plant cultivated around the world, is one of these magnificent plants as it is an excellent renewable source of various nutrients as well as phytochemicals[9]. Chemical screening for Swiss chard leaves showed that they have impressive amount of minerals as potassium, iron, phosphorus, calcium, magnesium and rich in vitamins as vitamin A, B3, B5, B9, E, also they are enriched with phenolic acids and flavonoids[10,11]. Syringic and caffeic acids are the most abundant phenolic acids[10]. Apigenin glycosides, namely vitexin, vitexin-2-O-α-rhamnosyl and vitexin-2-O-β-xylosyl are the most abundant flavonoids in Swiss chard leaves[12].

Recently, in prior study six flavonoids from the leaves of Swiss chard, two of which are novel natural products namely herbacetin 3-O-β-xylopyranosyl-(1"'→2")-O-β-glucopyranoside and 2'',2"'-di-O-α-rhamnopyranosyl vicenin II have been identified[13].

Modern pharmacology indicated the importance of such bioactive flavonoids contained in Swiss chard and demonstrated their hypoglycemic effect[14,15], anti-inflammatory, antihypertension and anticancer effects[12,16], hypolipidemic and hepatoprotective activities[13] as well as high antioxidant activity[11]. Folk medicine also showed that *Beta vulgaris* L. species were used for treating liver and kidney diseases by stimulating the hematopoietic and immune systems[17].

However, no study has been reported in the available current literature concerning the protective effect of Swiss chard extract on gentamicin induced nephrotoxicity and hepatotoxicity. Therefore, the present study aimed to investigate the nephroprotective and hepatoprotective effects of that extract against gentamicin-induced toxicity in Sprague Dawley rats.

MATERIALS AND METHODS

Plant material and preparation of extract: Leaves of Swiss chard were collected from plants cultivated in the Nile Delta near Cairo. The plant was identified and authenticated by Dr. Mohamed El Gebali, National Research Centre, Cairo, Egypt. A voucher specimen (B 201) was deposited at the Herbarium of the National Research Centre. Fresh leaves collected (3 kg) were exhaustively extracted with 75% MeOH in water (v/v). The extract obtained was dried under vacuum to afford a sticky dark brown aqueous methanolic extract (350 g) that was used in the present study.

Experimental animals: Male Sprague-Dawley rats, weighing 180-220 g, were obtained from the animal house of National Research Centre, Egypt. They were kept in polycarbonate cages under standard laboratory conditions at temperature 23±2°C with relative humidity of 50-60% and on a 12 h light/dark cycle, with free access to food and tap water *ad libitum*. All experimental procedures were approved by the Medical Ethical Committee of the National Research Centre and the animals were handled in accordance with "Principles of Laboratory Animal Care" (NIH publication No. 85-23, revised 1985).

Experimental design: Experimental animals were randomly divided into 4 equal groups of 6 animals each. The treatment schedule was as follows:

Control group	Rats i.p., injected with 1 mL/day physiological saline solution for 15 days
Gentamicin group	Rats i.p., injected with gentamicin sulphate, supplied by Memphis company for pharmaceutical and chemical experiment industries, Cairo, Egypt. At a dose of 80 mg kg^{-1}/day for 15 days as mentioned previously[18]
Extract (300 mg kg^{-1})+ gentamicin group	Rats received Swiss chard extract (300 mg kg^{-1}/day, orally) started 1 week before gentamicin injection and continued with gentamicin for 15 days
Extract (600 mg kg^{-1})+ gentamicin group	Rats received Swiss chard extract (600 mg kg^{-1}/day, orally) started 1 week before gentamicin injection and continued with gentamicin for 15 days

Samples preparation: After the last dose, animals were immediately kept in individual metabolic cages for 24 h urine collection. Urine samples were centrifuged at 1500 rpm for 10 min to remove debris and supernatant were stored at -20°C until analyzed. Blood samples were collected from Retro-orbital venous plexus of overnight fasted rats under ether anesthesia. Serum samples were separated by centrifugation at 3000 rpm for 15 min and stored at -20°C until assayed. The animals were then sacrificed by cervical dislocation, kidneys and livers were removed, rinsed with ice-cold normal saline, blotted with a piece of filter paper and weighed. A part of the kidney and the liver were immediately kept in 10% formalin solution for histopathological assay. Another part of the kidney and the liver were homogenized in 0.1 mol L^{-1} potassium phosphate buffer (pH 7.4) using tissue master TM125 (Omni International, USA) to obtain 1:10 (w/v) homogenate. After centrifugation at 3000 rpm for 10 min, the clear supernatant was stored at -80°C to be used for biochemical analysis. The remaining part of kidney and liver were stored at -80°C to be used for molecular analysis.

Biochemical analysis

Determination of kidney function tests: Serum and urinary levels of urea and creatinine were estimated with commercial kits developed by Spectrum Diagnostics Company (Egypt) based on the methods described previously[19]. Blood urea nitrogen was calculated using urea kit and creatinine clearance was calculated by the standard formula. Also, urinary micro TP and serum electrolytes, such as potassium, sodium and chloride were estimated using reagent kits purchased from Spectrum Diagnostics Company (Egypt) according to methods of Watanabe *et al.*[20] and Mitchell *et al.*[21], respectively.

Determination of liver function tests: Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total protein (TP) and albumin (Alb) were assayed using reagent kits purchased from Spectrum Diagnostics Company, (Egypt) according to the methods of Reitman and Frankel[22], Moss *et al.*[23] Tietz[24,25], respectively.

Determination of VEGF and TNF-α: Urinary VEGF and serum TNF-α were investigated by the enzyme-linked immunosorbent assay using kits obtained from Koma Biotec Inc, (Korea) according to the manufacturer's instructions.

Estimation of oxidative stress biomarkers in renal and hepatic homogenates: The GSH was estimated according to the method of Beutler *et al.*[26] after precipitating kidney and liver proteins with 10% metaphosphoric acid. GSH-Px, CAT and SOD activities were investigated according to the methods of Necheles *et al.*[27], Aebi[28] and Masayasu and Hiroshi[29], respectively. Lipid peroxidation was assayed as MDA level in renal and hepatic tissues according to method of Lefevre *et al.*[30]. Protein carbonylation, a type of protein oxidation, was calculated by assay of Evans *et al.*[31].

Estimation of total protein in renal and hepatic homogenates: The level of total protein in kidney and liver homogenates was estimated according to Lowry *et al.*[32].

Gene expression analysis

Isolation of total RNA: TRIzol® Reagent (Invitrogen, Germany) was used to extract total RNA from liver tissues of rats according to the manufacturer's instructions with minor modifications. Isolated total RNA was treated with one unit of RQ1 RNAse-free DNAse (Invitrogen, Germany) to digest DNA residues, re-suspended in DEPC-treated water and quantified photospectrometrically at 260 nm. Purity of total RNA was assessed by the 260/280 nm ratio which was between 1.8 and 2.1. Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis (data not shown). Aliquots were used immediately for reverse transcription (RT), otherwise they were stored at -80°C.

Reverse transcription (RT) reaction: Complete Poly $(A)^+$ RNA isolated from liver tissues was reverse transcribed into cDNA in a total volume of 20 µL using Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas, Germany). An amount of total RNA (5 µg) was used with a master mix. The master mix was consisted of 50 mM $MgCl_2$,10×RT buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 10 mM of each dNTP, 50 µM oligo-dT primer, 20 IU ribonuclease inhibitor (50 kDa recombinant enzyme to inhibit RNase activity) and 50 IU MuLV reverse transcriptase. The mixture of each sample was centrifuged for 30 sec at 1000 rpm and transferred to the thermocycler. The RT reaction was carried out at 25°C for 10 min, followed by 1 h at 42°C and finished with a denaturation step at 99°C for 5 min. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for cDNA amplification through quantitative real time-polymerase chain reaction (qRT-PCR).

Quantitative real time- PCR (QRT-PCR): QIAGEN's real-time PCR cycler (Rotor-Gene Q, USA) was used to determine the

Table 1: Primer sequences used for qRT-PCR amplification

Gene	Primer sequence (5'–3')	References/ NCBI
SOD1	F: CAG GAC CTC ATT TTA ATC CTC AC R: TGC CCA GGT CTC CAA CAT	Kobori *et al.*[33]
GSH-Px	F: TTT CCC GTG CAA TCA GTT C R: TCG GAC GTA CTT GAG GGA AT	Kobori *et al.*[33]
Caspase 3	F: GGA CCT GTG GAC CTG AAA AA R: GCA TGC CAT ATC ATC GTC AG	NM- 012922.2
β-actin	F: CAC GTG GGC CGC TCT AGG CAC CAA	Khalil and Booles[34]

Table 2: Effect of Swiss chard extract on body weight, relative kidney and liver weights in gentamicin administered rats

Groups	Body weight (g)	RKW (%)	RLW (%)
Control	201.85±5.78	0.79±0.01	3.26±0.11
Gentamicin	130.48±2.55^{a}	1.14±0.06^{a}	3.60±0.05^{a}
Extract (300)+gentamicin	184.33±6.58^{ab}	0.95±0.03^{ab}	3.35±0.08
Extract (600)+gentamicin	199.60±6.31^{b}	0.88±0.03^{b}	3.01±0.07^{b}

Data are expressed as Mean±SEM of 6 rats in each group. Values with superscripts are statistically significant at p<0.05. a: Indicates comparison versus control group, and b: Indicates comparison versus gentamicin treated group

Table 3: Effect of Swiss chard extract on serum kidney markers in gentamicin administered rats

	Control	Gentamicin	Extract (300 mg kg^{-1}) +gentamicin	Extract (600 mg kg^{-1}) +gentamicin
Urea (mg dL^{-1})	41.64±3.42	81.27±4.09^{a}	61.17±4.69^{ab}	51.05±2.91^{b}
BUN (mg dL^{-1})	19.44±1.59	37.95±1.91^{a}	26.39±1.54^{ab}	23.84±1.36^{b}
Creatinine (mg dL^{-1})	0.52±0.07	1.37±0.12^{a}	0.85±0.06^{ab}	0.77±0.05^{b}
Potassium (mM)	4.04±0.16	5.89±0.23^{a}	5.46±0.15^{a}	5.06±0.27^{ab}
Sodium (Meq L^{-1})	199.17±9.19	135.09±3.20^{a}	149.18±3.04^{a}	166.10±4.34^{ab}
Chloride(mM)	97.96±2.84	91.62±1.90	93.40±2.70	96.93±2.78

Data are expressed as Mean±SEM of 6 rats in each group. Values with superscripts are statistically significant at p<0.05. a: Indicates comparison versus control group, and b: Indicates comparison versus gentamicin treated group

cortex cDNA copy number. The PCR reactions were set up in 25 mL reaction mixtures containing 12.5 mL 1×SYBR® Premix Ex Taq TM (TaKaRa, Biotech. Co. Ltd.), 0.5 mL 0.2 mM sense primer, 0.5 mL 0.2 mM antisense primer, 6.5 mL distilled water and 5 mL of cDNA template. The reaction program was allocated to 3 steps. First step was at 95°C for 3 min. Second step consisted of 40 cycles in which each cycle divided to 3 steps: (a) At 95°C for 15 sec, (b) At 55°C for 30 sec and (c) At 72°C for 30 sec. The third step consisted of 71 cycles which started at 60°C and then increased about 0.5°C every 10 sec up to 95°C. At the end of each qRT-PCR a melting curve analysis was performed at 95°C to check the quality of the used primers. Each experiment included a distilled water control. The sequences of specific primer used for SOD1, GSH-Px and caspase 3 genes are listed in Table 1. The RT-PCR quantitative values of genes were normalized on the bases of β-actin expression. At the end of each qRT-PCR a melting curve analysis was performed at 95°C to check the quality of the used primers. The relative quantification of the target to the reference was determined by using the $2^{-\Delta\Delta CT}$ method as follows:

- Relative expression was calculated by $2^{-\Delta\Delta CT}$

Histopathological investigation: The histopathologic examination was performed by light microscopy on kidney and liver tissue specimens that were fixed in 10% formalin. After fixation, the samples were processed to obtain 5 μm thick paraffin sections. Kidney and liver sections were stained with hematoxylin and eosin (H and E). Then the slides were examined by Olympus photomicroscope.

Statistical analysis: Results were expressed as Means±SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) for multiple comparisons (SPSS, Version 19.0) followed by LSD test to detect differences between groups. The differences were considered statistically significant at p<0.05.

RESULTS

Effect of Swiss chard extract on body weight, relative kidney weight (RKW) and relative liver weight (RLW): Gentamicin administration caused a significant weight loss of the whole body and significant increase in RKW and RLW as compared with control group. Treatment with Swiss chard extract improved these changes, particularly with 600 mg kg^{-1} dose as compared with gentamicin intoxicated rats (Table 2).

Effect of Swiss chard extract on renal function biomarkers: The serum levels of kidney markers in control and experimental groups are shown in Table 3. The levels of serum

Table 4: Effect of Swiss chard extract on urinary kidney markers in gentamicin administered rats

	Control	Gentamicin	Extract (300 mg kg^{-1}) +gentamicin	Extract (600 mg kg^{-1}) +gentamicin
Urea (mg dL^{-1})	130.27±17.40	59.49±6.11^{a}	96.14±6.75^{ab}	116.26±4.05^{b}
Creatinine (mg dL^{-1})	108.44±2.57	44.33±1.66^{a}	62.64±3.09^{ab}	82.71±3.45^{ab}
Creatinine clearance (mg min^{-1})	0.60±0.03	0.37±0.03^{a}	0.45±0.06^{a}	0.51±0.03^{b}
Micro TP (mg/24 h)	13.60±1.09	43.57±4.66^{a}	21.06±1.90^{b}	17.38±1.87^{b}

Data are expressed as Mean±SEM of 6 rats in each group. Values with superscripts are statistically significant at p<0.05. a: Indicates comparison versus control group, and b: Indicates comparison versus gentamicin treated group

Table 5: Effect of Swiss chard extract on serum liver markers in gentamicin administered rats

	Control	Gentamicin	Extract (300 mg kg^{-1}) +gentamicin	Extract (600 mg kg^{-1}) +gentamicin
AST (U L^{-1})	73.83±4.52	127.67±7.43^{a}	89.33±3.37^{b}	77.67±5.80^{b}
ALT (U L^{-1})	15.83±1.44	26.50±1.38^{a}	22.00±1.15^{ab}	17.17±0.83^{b}
ALP (U L^{-1})	79.49±6.21	128.49±7.42^{a}	107.00±4.56^{ab}	86.29±5.00^{b}
TP (g dL^{-1})	7.72±0.14	6.09±0.10^{a}	6.90±0.27^{ab}	7.32±0.34^{b}
Alb (g dL^{-1})	4.01±0.09	3.02±0.16^{a}	3.88±0.19^{b}	3.85±0.30^{b}

Data are expressed as Mean±SEM of 6 rats in each group. Values with superscripts are statistically significant at p<0.05. a: Indicates comparison versus control group, and b: Indicates comparison versus gentamicin treated group

urea, blood urea nitrogen (BUN), creatinine and potassium were significantly increased in gentamicin induced rats as compared to control animals, while sodium levels were significantly decreased. Oral administration of Swiss chard extract to the gentamicin treated rats significantly improved the levels of these markers in a dose related manner. Chloride level was almost similar among the whole groups.

On the other hand, urinary urea, creatinine and creatinine clearance showed significantly decreased levels in gentamicin group as compared to control rats, while urinary Micro TP level showed a significant increase. Administration of Swiss chard extract significantly improved excretion of urea, creatinine and Micro TP levels compared to gentamicin treated rats. While, creatinine clearance was significantly improved only at a dose of 600 mg kg^{-1} of the extract (Table 4). Furthermore, nephrotoxicity induced by gentamicin was associated with a significant increase in urinary VEGF level, whereas, treatment with Swiss chard extract significantly lowered its level as compared to gentamicin group (Fig. 1).

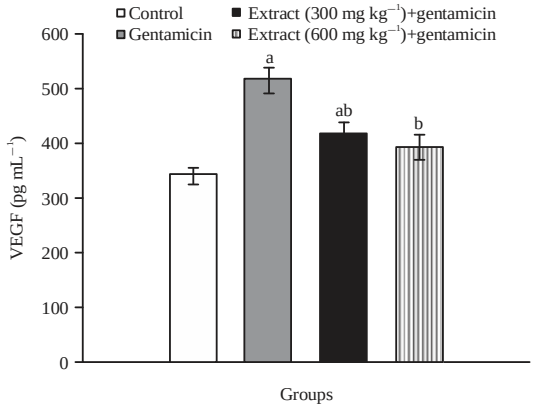

Fig. 1: Effect of Swiss chard extract on urinary vascular epithelial growth factor in gentamicin administered rats

Values are expressed as Mean±SEM of 6 rats in each group. [a]Statistically significant as compared to control group, p<0.05, [b]Statistically significant as compared to gentamicin group, p<0.05

Effect of Swiss chard extract on liver function markers: Serum AST, ALT and ALP activities in gentamicin group showed a significant elevation as compared to the control group. In contrast, TP and Alb concentrations were significantly decreased as a result of gentamicin administration. Treatment with Swiss chard extract (300, 600 mg kg^{-1}) significantly reversed the serum level of AST, ALT, ALP, TP and Alb when compared with the gentamicin treated group (Table 5).

Effect of Swiss chard extract on tumor necrosis factor-α: Gentamicin intoxicated rats showed significant elevation in the serum TNF-α level as compared to control rats. However, administration of Swiss chard extract at a dose of 600 mg kg^{-1} significantly decreased the level of TNF-α when compared with the gentamicin group. A minor improvement was also observed in TNF-α level with Swiss chard treatment at 300 mg kg^{-1} dose but without statistical significance in comparing to gentamicin group (Fig. 2).

Effect of Swiss chard extract on renal and hepatic oxidative stress biomarkers: In the present study, there was a significant decline in GSH levels and GSH-Px, CAT and SOD activities in the kidney and liver tissues of gentamicin group as compared to normal rats. The treatment with Swiss chard extract dose dependently increased the renal and hepatic

Table 6: Effect of Swiss chard extract on renal and hepatic oxidative stress biomarkers in gentamicin administered rats

	Control	Gentamicin	Extract (300 mg kg^{-1}) +gentamicin	Extract (600 mg kg^{-1}) +gentamicin
Kidney				
GSH (mg g^{-1} tissue)	81.53±4.52	55.86±4.75^{a}	68.99±1.21^{ab}	71.75±3.08^{b}
GSH-Px (U^{A} mg^{-1} tissue)	3.79±0.23	2.86±0.08^{a}	3.19±0.03^{ab}	3.40±0.11^{b}
CAT (U^{B} mg^{-1} tissue)	81.83±3.74	24.40±0.61^{a}	67.15±5.17^{ab}	72.66±4.25^{b}
SOD (U^{C} g^{-1} tissue)	49.20±4.82	16.40±1.61^{a}	21.83±1.51^{a}	31.17±1.88^{ab}
MDA (µmol g^{-1} tissue)	2.04±0.09	3.16±0.11^{a}	2.60±0.15^{ab}	2.26±0.09^{b}
Protein carbonyl (nmol mg^{-1} protein)	0.23±0.02	1.14±0.08^{a}	0.54±0.04^{ab}	0.40±0.02^{ab}
Liver				
GSH (mg g^{-1} tissue)	138.39±9.55	84.69±6.75^{a}	118.78±10.62^{b}	121.83±12.20^{b}
GSH-Px (U^{A} mg^{-1} tissue)	3.82±0.02	2.95±0.06^{a}	3.43±0.04^{ab}	3.59±0.06^{ab}
CAT (U^{B} mg^{-1} tissue)	375.78±42.58	81.10±8.72^{a}	278.90±18.70^{ab}	320.00±23.04^{b}
SOD (U^{C} g^{-1} tissue)	66.79±5.65	26.30±1.81^{a}	43.90±3.51^{b}	59.73±5.86^{b}
MDA (µmol g^{-1} tissue)	2.18±0.16	2.75±0.16^{a}	2.31±0.10^{b}	2.19±0.11^{b}
Protein carbonyl (nmol mg^{-1} protein)	0.36±0.06	1.021±0.24^{a}	0.72±0.06^{ab}	0.60±0.07^{ab}

Data are expressed as Mean±SEM of 6 rats in each group. Values with superscripts are statistically significant at p<0.05. a: Indicates comparison versus control group, and b: Indicates comparison versus gentamicin treated group. U^{A}: µg of GSH consumed min^{-1} mg^{-1} tissue, U^{B}: µmol of H_2O_2 decomposed min^{-1} mg^{-1} tissue, U^{C}: 50% inhibition of nitroblue tetrazolium reduction g^{-1} tissue

antioxidant parameters in the treated rats compared to the gentamicin group (Table 6). On the other hand, MDA and protein carbonyl levels were significantly higher in the kidney and liver tissues of gentamicin group as compared to control rats. Swiss chard extract pretreatment caused a dose-dependent decrease in both renal and hepatic MDA and protein carbonyl levels when compared to the gentamicin treated rats. The increase in MDA was nearly prevented by administration of 600 mg kg^{-1} Swiss chard extract (Table 6).

Effect of Swiss chard extract on renal and hepatic total protein: Administration of gentamicin resulted in a significant decrease in TP contents in the kidney and liver tissues as compared to normal rats. Pretreatment with Swiss chard extract significantly increased renal TP in renal tissues in a dose dependent manner as compared to gentamicin group, while in hepatic tissues both doses of Swiss chard extract had almost the same improving effect on hepatic TP (Fig. 3).

Effect of Swiss chard extract on the expression alterations of renal and hepatic genes encoding antioxidant and apoptotic related enzymes: Expression of SOD1, GSH-Px and caspase-3 genes quantified by real time-polymerase chain reaction (RT-PCR) is summarized in Fig. 4-6. The results showed that gentamicin treatment significantly decreased the expression levels of SOD1 and GSH-Px genes in liver and kidney tissues compared with those in control rats. In contrary, gentamicin administration significantly increased the expression levels of caspase-3 gene in liver and kidney tissues compared with the control group. Pretreatment with Swiss chard extract significantly increased the expression

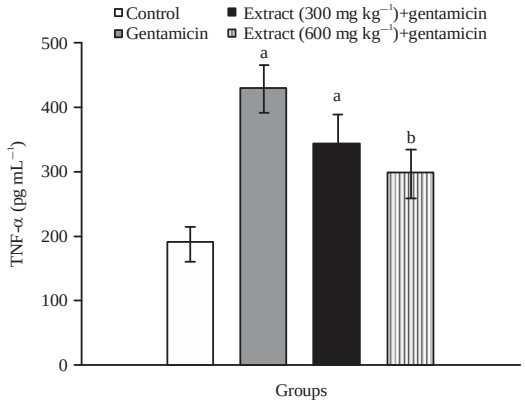

Fig. 2: Effect of Swiss chard extract on serum tumor necrosis factor-α in gentamicin administered rats
Values are expressed as Mean±SEM of 6 rats in each group. [a]Statistically significant as compared to control group, p<0.05, [b]Statistically significant as compared to gentamicin group, p<0.05

levels of renal and hepatic SOD1 and GPx genes with significant decrease in the expression levels of renal and hepatic caspase-3 gene in a dose dependent manner compared with the gentamicin group.

Effect of Swiss chard extract on renal histopathology: Pathologically, there is a positive correlation between oxidative stress of gentamicin and nephrotoxicity. Drug induced nephrotoxicity is associated with their accumulation in renal cortex, depending upon their affinity to kidneys and on kinetics of gentamicin trapping process. In the present study, histopathological examination of sections from kidney treated with gentamicin showed tubular, glomerular and epithelial changes. Tubules showed evident variability in tubular size, generalized and extensive vacuolar degeneration

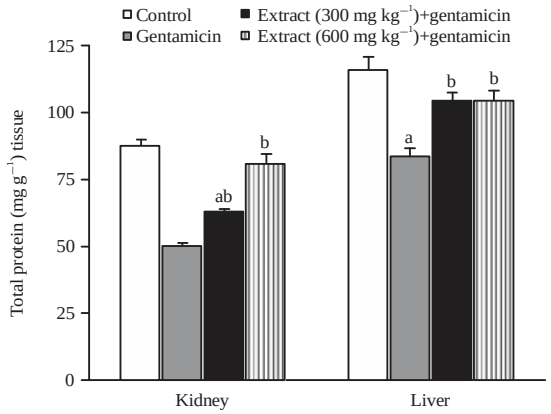

Fig. 3: Effect of Swiss chard extract on renal and hepatic total protein in gentamicin administered rats

Values are expressed as Mean±SEM of 6 rats in each group. [a]Statistically significant as compared to control group, p<0.05, [b]Statistically significant as compared to gentamicin group, p<0.05

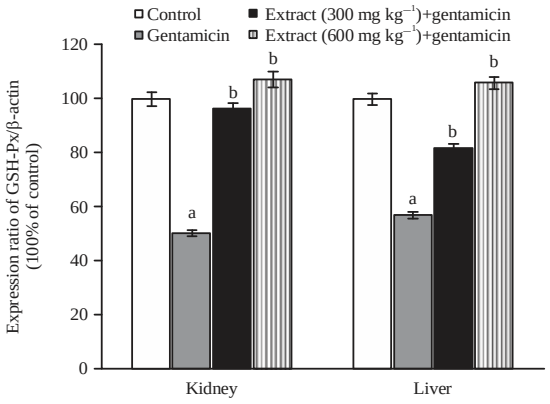

Fig. 5: Effect of Swiss chard extract on expression of renal and hepatic GSH-Px gene in gentamicin administered rats

Data are presented as Mean±SEM of 6 rats in each group, [a]Statistically significant as compared to control group, p<0.05, [b]Statistically significant as compared to gentamicin group, p<0.05

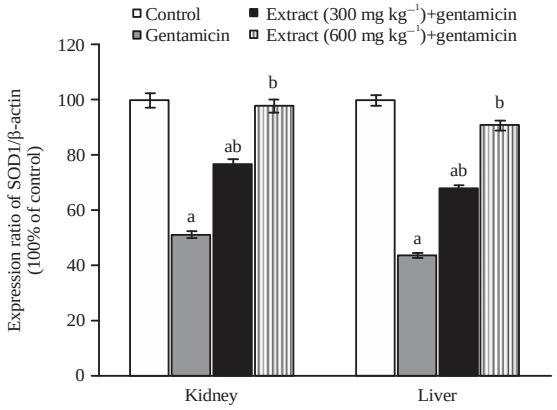

Fig. 4: Effect of Swiss chard extract on expression of renal and hepatic SOD1 gene in gentamicin administered rats

Data are presented as Mean±SEM of 6 rats in each group, [a]Statistically significant as compared to control group, p<0.05, [b]Statistically significant as compared to gentamicin group, p<0.05

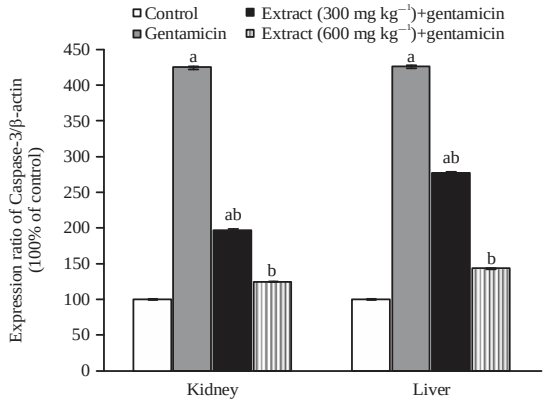

Fig. 6: Effect of Swiss chard extract on expression of renal and hepatic caspase-3 gene in gentamicin administered rats

Data are presented as Mean±SEM of 6 rats in each group. [a]Statistically significant as compared to control group, p<0.05, [b]Statistically significant as compared to gentamicin group, p<0.05

in 60% of epithelial cell lining of proximal convoluted tubules (hydropic degeneration). Coagulative necrosis, focal sloughing in luminal aspects, detached apical brush borders and evident hyaline casts were observed in 40% of tubular population. Also, there was tubular dilation in 30% of tubules. Glomeruli showed atrophy in 30% of their population. One fourth of renal glomeruli showed deposition of eosinophilic proteinaceous material. Bowman`s capsule was intact all around. Intersitium was edematous and vacuolar showing mild inflammatory exudates formed mainly of lymphocytes and some plasma cells (Fig. 7b-e).

On the other hand, the histopathological sections of kidney in animals treated with 300 mg kg^{-1} Swiss chard extract and gentamicin showed mild renal tubular impairment expressed in decreased deposition of tubular eosinophilic proteinaceous hyaline casts in the tubular lumina. However, tubular epithelial cell size, tubular vaculation and tubular sloughing showed the same pathological changes as that in the gentamicin group. Glomerular changes considering glomerular atrophy and hyaline deposits were the same as gentamicin group. Interstitium was cleared up with no edema, no vascularity and no inflammatory cell exudates (Fig. 7f). Moreover, treatment with 600 mg kg^{-1} Swiss chard extract plus gentamicin showed abrupt and very evident improvement of all tubular changes. There was great improvement of vacuolar degeneration of tubular cell lining, no coagulative necrosis, no variability in tubular size and no

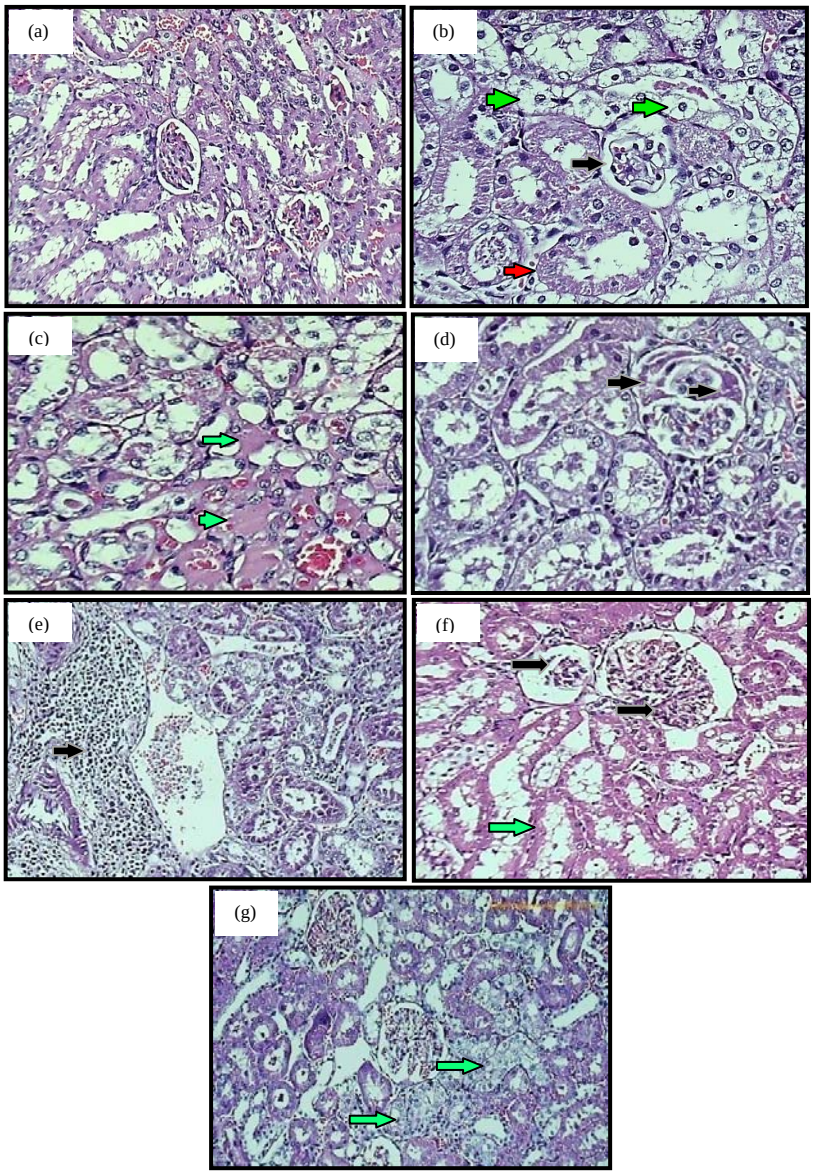

Fig. 7(a-g): Photomicrographs (H and E stain) of kidney sections from control and experimental rats, (a) Control group shows normal kidney histomorphology, (X100), (b) Gentamicin group shows glomerular atrophy (black arrow), variable size proximal tubules with cloudy swelling (green arrows) and focal shedded epithelial lining in some convoluted tubules (red arrow), (X400), (c) Gentamicin group shows tubular casts (green arrows), (X400), (d) Gentamicin group shows deposition of proteinaceous material within glomeruli (black arrows), (X400), (e) Gentamicin group shows diffuse interstitial inflammatory exudate formed mainly of lymphocytes and few plasma cells (black arrow), (X100), (f) Group treated with 300 mg kg^{-1} Swiss chard extract and gentamicin shows small and large glomeruli (black arrows) as well as diffuse and cloudy swelling in proximal convoluted tubules (green arrow), (X100) and (g) Group treated with 600 mg kg^{-1} Swiss chard extract and gentamicin shows almost regular and uniform glomerular size. However, some proximal convoluted tubules have cloudy swelling (green arrows) (X100)

hyaline casts. Glomeruli and interstitium appeared as normal (Fig. 7g). Control rats showed normal architecture of glomeruli, proximal convoluted tubules, distal convoluted tubules and interstitium, (Fig. 7a). These histopathological findings were summarized in Table 7.

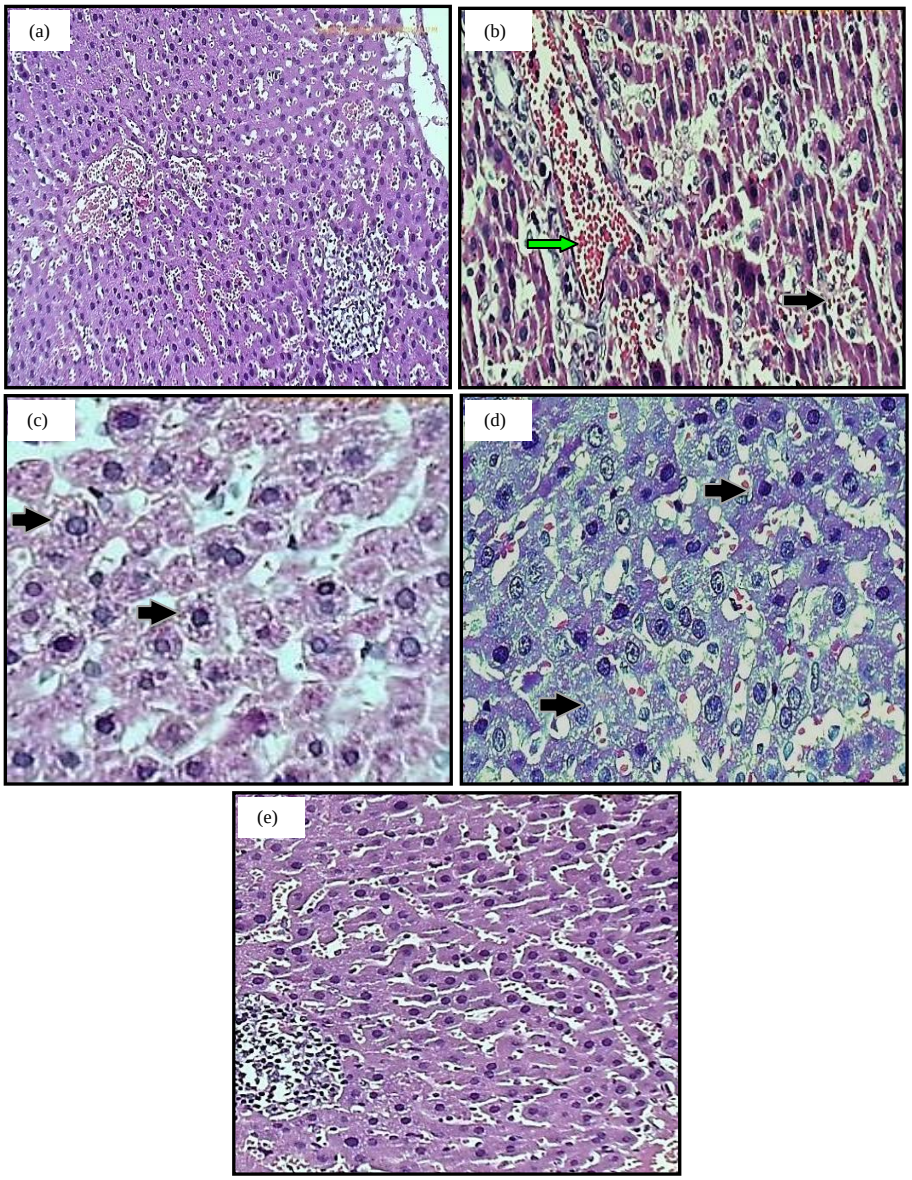

Fig. 8(a-e): Photomicrographs (H and E stain) of liver sections from control and experimental rats, (a) Control group shows normal histological architecture of hepatic tissues, (X100), (b) Gentamicin group shows disturbed lobular pattern with focal necrosis in hepatocytes (black arrow), intervening hepatocytes exhibiting cloudy swelling and dilated congested sinusoids in between (green arrow), (X200), (c) Gentamicin group shows very evident hydropic degeneration within hepatocytes (black arrows), (X400), (d) Group treated with 300 mg kg^{-1} Swiss chard extract plus gentamicin shows evident cloudy swelling within hepatocytes (black arrow), (X400) and (e) Group treated with 600 mg kg^{-1} Swiss chard extract plus gentamicin shows almost normal lobular pattern, (X200)

Effect of Swiss chard extract on hepatic histopathology: Liver sections of rats treated with gentamicin revealed periportal hydropic degeneration characterized by cytoplasmic vacuoles. These vacuoles were small and large in size, clear and random in shape with sharp boundaries. There was focal necrosis dispersed in some hepatocytes. In addition, there were dilated congested sinusoids as well as inflammatory exudates in portal areas, (Fig. 8b and c). On the other hand, gentamicin group pretreated with 300 mg kg^{-1} extract revealed the same pathologic changes as gentamicin group but to a lesser extent, (Fig. 8d). While rats pretreated with 600 mg kg^{-1} extract revealed totally normal architecture, (Fig. 8e). These histopathological findings were summarized in Table 8.

Table 7: Histopathological features of gentamicin induced nephrotoxicity in different treatment groups

Histopathological features	Control	Gentamicin	Extract (300 mg kg^{-1}) +gentamicin	Extract (600 mg kg^{-1}) +gentamicin
Tubules				
Cloudy swelling	-	+++	+++	+
Luminal epithelial sloughing	-	+++	+++	+
Hyaline casts	-	+++	+	-
Glomeruli				
Atrophy	-	++	+	-
Hyaline deposits	-	++	+	-
Interstitium				
Inflammation	-	++	-	-
Edema	-	+	-	-
Increased vascularity	-	+	-	-

Scoring was done as follows: None (-), mild (+), moderate (++) and severe (+++)

Table 8: Histopathological features of gentamicin induced hepatotoxicity in different treatment groups

Histopathological features	Control	Gentamicin	Extract (300 mg kg^{-1}) +gentamicin	Extract (600 mg kg^{-1}) +gentamicin
Hepatocytes				
Cloudy swelling	-	+++	+	-
Focal necrosis	-	++	+	-
Portal area				
Congestion	-	++	+	-
Inflammation	-	++	+	-

Scoring was done as follows: None (-), mild (+), moderate (++) and severe (+++)

DISCUSSION

In the current study, intraperitoneal administration of gentamicin at a dose of 80 mg kg^{-1} for 14 days caused a reduction in body weight and an increase in relative kidney and liver weights. These results are in agreement with those of previous study[1]. Body weight loss could be attributed to the increased catabolism occurring in acute renal failure which is accompanied by anorexia[35]. Moreover, gentamicin is deleterious to renal epithelial cells and may decrease water reabsorption causing dehydration and decrease in body weight[36,2]. However, gentamicin induced toxicity causes edema and inflammation in liver and kidneys which may be responsible for the increase in relative liver and kidney weights[37,38]. On the other hand, pretreatment with Swiss chard extract at 300 and 600 mg kg^{-1} provided a protective effect through increasing the body weight and decreasing relative kidney and liver weights.

The nephrotoxicity of gentamicin was characterized by a marked elevation in serum creatinine, urea and BUN levels as well as marked reduction in creatinine clearance. This impairment in glomerular function was accompanied by a decreased excretion of creatinine and urea as compared with the normal control. These findings were further confirmed by renal histological examination, which revealed tubular necrosis. The findings of the present study are in accordance with those described previously[38,39]. The intracellular accumulation of gentamicin in the renal proximal convoluted tubules causes severe proximal renal tubular necrosis[40], which leads to diminished creatinine clearance and renal dysfunction[417]. Also, the increased serum creatinine level in gentamicin group was found to be an indicative of decrease in glomerular filtration rate, whereas the increased serum urea and BUN levels were found to be an indicative of parenchyma tissue injury after tubular necrosis[4]. Serum electrolytes were also disturbed significantly in gentamicin treated rats. These results are in agreement with other investigators[42,43]. The decreased level of sodium indicates kidney inability to conserve sodium and chloride ions[44], while the increased level of potassium may be due to renal tubular epithelium lesions[45].

Urine analysis showed significant excretion of urinary protein along with significant elevation in VEGF level in gentamicin intoxicated rats as compared to control. These results are in accordance with those obtained previously[39,46]. Also, it has been reported that gentamicin administration impaired the capacity of renal tubule to reabsorb low-weight proteins[47]. The novel urinary protein biomarker, VEGF, together with the more classical urinary parameter, total protein, may be useful biomarkers that are more sensitive than BUN and serurm creatinine in detecting minimal to mild renal damage and dysfunction[48]. However, pretreatment with Swiss chard extract, dose dependently, ameliorated the

gentamicin-induced nephrotoxicity by decreasing the elevated levels of serum kidney markers (urea, creatinine and BUN) and increasing the level of creatinine clearance as well as restoring the levels of the serum electrolytes to near normal. In addition, Swiss chard treatment elevated urinary urea and creatinine levels and reduced urinary Micro TP and VEGF levels. These results revealed that the methanolic extract of Swiss chard exerted a functional protection against gentamicin-induced nephrotoxicity, thereby improving renal function.

Gentamicin induced hepatotoxicity was evidenced by increased serum levels of hepatic marker enzymes ALT, AST and ALP as well as decreased levels of serum total protein and albumin, the major serum protein synthesized by the liver. These findings are in accordance with those described previously[3,49]. The elevation in levels of hepatic enzymes in gentamicin treated rats could be attributed to damage in hepatocytes plasma membrane that leads to loss of the functional integrity and increase in cell permeability, which results in release of enzymes from the damaged hepatocytes into serum[50]. Leakage of AST and ALT from hepatic cell can occur as a secondary change to cellular necrosis[51]. Whereas, decreased levels of serum protein and albumin are indicative of damage in hepatic protein synthesizing subcellular structures[52]. Present results revealed that Swiss chard pretreatment significantly lowered serum levels of ALT, AST and ALP which suggested that Swiss chard extract improved the functional status of the hepatic cells by preventing leakage of intracellular enzymes through restoring cell membrane integrity. These effects could be attributed to the presence of the flavonoid glycosides in the extract[13]. In addition, the normalization of serum protein and albumin levels due to Swiss chard administration may be due to the well-functioning capacity of hepatocytes in protein synthesis.

In this study, gentamicin administered rats showed a significant elevation in serum TNF-α, one of cytokines regulating inflammation, which is in parallel with that described previously[37]. Cellular damage and necrosis stimulate the generation of inflammatory mediators by the injured cells as well as by immune cells, which induce migration and infiltration of leukocytes into the injured organs[53]. Inflammatory response is one of characteristics of gentamicin-nephrotoxicity[52].

On the other hand, pretreatment with Swiss chard reduced the elevated level of serum TNF-α. This reduction in TNF-α level in Swiss chard treated rats (600 mg) was prominent than (300 mg) treated group. The TNF-α reduction might be attributed to anti-inflammatory property of Swiss chard extract which could be due to its flavonoids. The ability of polyphenolic compounds to inhibit TNF-production was previously demonstrated[54]. It has been declared that blockade of TNF-α is a valuable approach for managing nephrotoxicity[55].

In the current study, gentamicin administration induced oxidative stress in rat liver and kidneys as evidenced by a significant decrease in the activities of renal and hepatic antioxidant enzymes (GSH-Px, CAT and SOD) as well as GSH levels coupled with significant increase in MDA level (marker of lipid peroxidation) as compared to normal control. These results are consistent with those reported previously [56,57]. The expression levels of antioxidant related genes (SOD1 and GSH-Px) were also significantly decreased in gentamicin treated rats. Gentamicin administration increases the generation of ROS that is followed by reducing the activities of antioxidant enzymes[58,59] and by depleting intracellular concentrations of GSH during the process of combating oxidative stress, which enhances lipid peroxidation[60]. On the other hand, pretreatment with Swiss chard significantly restored the activities of these antioxidant enzymes and GSH content as well as decreased the level of MDA in renal and hepatic tissues. Moreover, a significant increase in the expression levels of antioxidant related genes (SOD1 and GSH-Px) were also observed. These results could be attributable to the antioxidant activity of Swiss chard, which markedly attenuated the oxidative stress induced by gentamicin and led to the inhibition of lipid peroxidation, the maintaining of GSH levels at near normal values and the enhancement of the gene expression and activities of antioxidant enzymes. In the same line with our findings, it has been reported that Swiss chard exhibited high antioxidant activity that could be due to its phenolic compounds[10]. Moreover, polyphenolic compounds promote the anti-free-radical activity of several herbal extracts[61]. The capacity of Swiss chard extracts for radical scavenging could be attributed to their concentration of hydroxyl group in the phenolic compounds which regulate the expression of antioxidant's mRNA. Therefore, the anti-free-radical capacity of Swiss chard extracts depends on the concentration of the phenolic compounds and their molecular structure. Thus, that explain why the high concentration of the Swiss chard extract (600 mg) is more effective than its low concentration (300 mg) on the antioxidant enzymes activities and the expression levels of related genes (SOD1 and GSH-Px).

Gentamicin treatment induced a significant decrease in total protein contents and significant increase in protein carbonyl contents (marker of protein oxidation) in renal and hepatic tissues compared to the control group. Our results are in agreement with those reported that the formation of

protein carbonyl as a consequence of oxidative stress, may be an early marker for protein oxidation[62,63]. Gentamicin administration causes abnormal production of ROS which induces cellular injury and necrosis through membrane phospholipids peroxidation and protein denaturation[64]. Among ROS, hydroxyl radical is thought to be the most damaging species and the one mainly responsible for lipid and protein oxidation[65]. Pretreatment with Swiss chard afforded significant protection against the oxidative modification of proteins induced by gentamicin treatment. A lot of investigations reported that natural phenolic compounds, particularly flavonoids, have strong antioxidant potency and protective activity as well as free radicals generation prevention[10,66].

The expression levels of the apoptotic enzyme gene (caspase-3) were significantly increased in the kidney and hepatic tissues of gentamicin-treated group. Present results are consistent with earlier reports which mentioned that gentamicin treatment elevated the expression level of caspase-3 through increasing the production of ROS which finally lead to apoptosis[67,68]. Apoptosis mediated by ROS is an important mechanism in gentamicin-induced cytotoxicity[67,69]. Caspases are endoproteases that have important roles in controlling apoptosis pathways in mammalian cells[70]. Pretreatment with Swiss chard extract suppressed gentamicin-induced apoptosis by reducing the expression levels of caspase-3. It has been indicated that Swiss chard extract, rich in flavonol glycosyls are responsible for the anti-mitotic and anti-apoptotic activities[71].

CONCLUSION

In conclusion, that the results of the present study indicated that the methanolic extract of Swiss chard possesses significant protective effect in a dose dependent manner against gentamicin-induced hepato-renal toxicity. The high antioxidant capacity of Swiss chard together with the anti-inflammatory and anti-apoptotic properties are supposed to be related to its flavonoid contents. Hence, Swiss chard could be a promising therapeutic agent in alleviating gentamicin-induced nephrotoxicity and hepatotoxicity in clinical trials.

SIGNIFICANT STATEMENT

This study discovers the possible protective effect of Swiss chard extract that can be beneficial in treatment of gentamicin toxicity in rats. This study will help the researcher to uncover the role of Swiss chard extract in reducing the side effects of gentamicin nephrotoxicity on long-term therapy that many researchers were not able to explore. Thus, a new theory on combination of gentamicin and Swiss chard extract may be arrived at.

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