

Protective Effect of *Enicostemma littorale* Blume Methanolic Extract on Gentamicin-Induced Nephrotoxicity in Rats

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Abstract: Problem statement: The Amino glycoside antibiotics cause drug induced nephrotoxicity at therapeutic doses and thus limits the use clinically. Oxidative stress is one of the main reasons for the development of nephrotoxicity. **Approach:** In light of this the aim of the present study was to investigate the role of mitochondrial as well as post-mitochondrial oxidative stress in the development of Gentamicin (GM) induced nephrotoxicity and to elucidate the role of *Enicostemma littorale* (EL) extract, as a promising antioxidant therapy. Rats were divided into four groups, 1) (Control) 2) GM (i.p., 80 mg kg⁻¹ day⁻¹ for 8 days) (treated) 3) GM+EL treated (i.g, 2.5 gm kg⁻¹ day⁻¹) and (4) GM+ Vitamin C (VC) treated (i.g, 600 mg kg⁻¹ day⁻¹). GM treated animals showed high oxidative stress in mitochondrial as well as post-mitochondrial fractions of renal tissues as evidenced by increased lipid per oxidation levels, decrease in GSH content and activities of antioxidant enzymes, SOD and GPx. **Results:** Oxidative stress was more pronounced in mitochondrial fraction as compared to post-mitochondrial fraction. GM-induced nephrotoxicity was further corroborated by an increase in serum creatinine and Blood Urea Nitrogen (BUN) levels and altered kidney histopathological observations. Treatment with EL ameliorates antioxidant defense system of mitochondrial as well as post-mitochondrial fraction, with better improvement seen in mitochondrial fraction. **Conclusion:** The present study explored beneficial effect of EL extract as an antioxidant therapy to counteract mitochondrial and post-mitochondrial oxidative stress generated in kidney upon GM-treatment, thus prevented nephrotoxicity.

Key words: *Enicostemma littorale* blame, oxidative stress, mitochondrial fraction, post-mitochondrial, Amino glycoside antibiotics, antioxidant therapy, Vitamin C (VC), lipid per oxidation

INTRODUCTION

Amino glycoside antibiotics, especially Gentamicin (GM) are widely applied in veterinary and human clinical practices for treatment of life-threatening gram negative infections. These Aminoglycoside antibiotics also cause drug induced

nephrotoxicity in 10-20% of therapeutic courses. Therefore, the clinical use of these drugs is limited by the development of nephrotoxicity. GM induced nephrotoxicity is characterized by tubular necrosis, without any morphological change in glomerular structures (Eisenberg *et al.*, 1987; Cuzzocrea *et al.*, 2002; Pedraza-Chaverri *et al.*, 2003). Rats with GM-

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induced nephrotoxicity provides an excellent model of acute renal failure and to test the protective effects of chemical compounds, plant extracts or drugs GM-induced nephrotoxicity is an ideal model (Ali and Bashir, 1996). The detailed mechanism by which GM induces nephrotoxicity is not well understood; however, reactive oxygen species have been shown to be involved in pathogenesis (Cuzzocrea *et al.*, 2002; Walker and Shah, 1988; Mazzon *et al.*, 2001).

GM has been shown to generate superoxide anion (O₂⁻), Hydrogen Peroxide (H₂O₂) and Hydroxyl radical (HO) production from renal mitochondria (Yang *et al.*, 1995; Walker *et al.*, 1999). In addition to generation of free radicals it also increases (Guidet and Shah, 1989) lipoperoxidation (Pedraza-Chaverri *et al.*, 2000; Ali, 2002), protein nitrosylation and carbonylation (Maldonado *et al.*, 2003; Mazzon *et al.*, 2001) and reduces glutathione content (Sener *et al.*, 2002) in renal cortex from GM-treated rats. Moreover, the administration of several compounds with antioxidant properties, Reactive Oxygen Species (ROS) scavengers and/or antioxidant enzymes were able to ameliorate the severity of GM-induced renal damage by reducing oxidative stress (Pedraza-Chaverri *et al.*, 2000; Ali, 2002; Maldonado *et al.*, 2003; Sener *et al.*, 2002). In addition, kidney is deficient in the antioxidant enzymes Mn-Superoxide Dismutase (Mn-SOD) (Pedraza-Chaverri *et al.*, 2000; Al-Majed *et al.*, 2002) Glutathione Peroxidase (GPx) (Pedraza-Chaverri *et al.*, 2000), Glutathione Reductase (GR) (Maldonado *et al.*, 2003) and catalase (Pedraza-Chaverri *et al.*, 2000) and thus more vulnerable to ROS in GM-treated rats.

Plants with potent antioxidant activity, has been shown to protect against gentamicin-induced nephrotoxicity. The *Enicostemma littorale* Blume is a small perennial herb of family Gentianaceae. The whole plant is used for medicinal purposes. It is commonly known as “chota chirayita” in Hindi or “mamejua” in Gujarati. We have shown hypoglycemic and antioxidant effect of aqueous as well as metabolic extract in alloxan-induced diabetic rats (Vijayvargia *et al.*, 2000; Maroo *et al.*, 2002; 2003a; 2003b) and also elucidated mechanism of its action (Maroo *et al.*, 2002; 2003a). We are the first one to report the hypoglycemic, antioxidant and hypolipidemic properties of the aqueous extract of EL in newly diagnosed NIDDM patients (Vasu *et al.*, 2003) as well as in cholesterol fed rats (Vasu *et al.*, 2005). We have also demonstrated preventive effect of EL on nerve function and oxidative stress in animal model of diabetic neuropathy Bhatt *et al.* (2009) and islet neogenetic potential in vitro by

purified compound from EL (Gupta *et al.*, 2010). Apart from antidiabetic potential, we also exhibited vitro anti-malarial activity of methanolic extract of plant and swertiamarin isolated from it (Soni and Gupta, 2009).

This herb is also known for its anti-inflammatory (Sadique *et al.*, 1987; Roy *et al.*, 2010), antiulcer (Roy *et al.*, 2010) and anticancer property (Kavimani and Manisenthilkumar, 2000). The hepatoprotective effect of EL extract was examined in liver injury by carbon tetrachloride (CCl₄) in male Wistar rats (Gupta and Singh, 2007) as well as in ethanol-induced liver injury rat model (Baranisrinivasan *et al.*, 2009). Hypolipidemic and hepatoprotective effect of aerial part of EL has been also evaluated in p-dimethylaminobenzene (p-DAB) induced hepatotoxic animals (Gopal *et al.*, 2004). Aqueous extract of EL has also shown effect on key carbohydrate metabolic enzymes in alloxan-induced diabetic rats (Srinivasan *et al.*, 2005) and in vitro antioxidant activity (Vaijanathappa and Badami, 2009). C-glycosidic flavonoid derived from *Enicostemma hyssopifolium* showed aldose reductase inhibitory activity (Patel and Mishra, 2009). It is also used as an antidiabetic herbomineral preparation (Babu and Prince, 2004).

Swertiamarin is a secoiridoid glycoside present in EL, has been reported for its number of activity namely, hepatoprotective, antiedematogenic, free radical scavenging activity, antispastic activity (Vaijanathappa and Badami, 2009) and antinociceptive activity (Jaishree *et al.*, 2008). Swertiamarin also showed antihyperlipidaemic effects in P-407-induced hyperlipidaemic rats (Vaidya *et al.*, 2009a; 2009b) and as well as in cholesterol fed rats. Sonawane *et al.* (2010) reported ameliorative effect of swertiamarin and aqueous extract on nephropathic condition on type 1 diabetic animals. In the light of this, we have explored the possible protective role of *Enicostemma littorale* Blume, having good antioxidant potential (Maroo *et al.*, 2003b) on gentamicin-induced oxidative stress in kidney, causing nephrotoxicity.

MATERIALS AND METHODS

Chemicals: All chemicals used were of analytical grade.

Extract preparation: Authentic plant material was purchased from local market and identified at the Botany Department, M.S. University, Baroda, Voucher specimen (Oza 51,51 (a)) is present at the Herbarium of Botany Department, M.S. University, Baroda. Whole dried plant was used for extract preparation. The plant

was powdered in grinding meal. The powder was extracted with petroleum ether for 12 h in a Soxhlet apparatus. Residues were again extracted with methanol for 24 h. After the extraction, methanol was recovered by distillation and remaining traces of methanol were completely removed by keeping methanol extract at 60°C for 4 days. The yield was found to be 40% and carboxymethylcellulose solution was used as vehicle (Maroo *et al.*, 2003a).

Animals and treatment: Male Charles Foster rats (body weight 200-250 g) were used for the study. They were allowed ad libitum access to water and food. During the study, rats were maintained in stainless steel metabolic cages with a 12-h light/dark cycle to collect 24 h urine at the end of the study. All the animal studies were approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Rats were divided into four groups of six rats each as follows:

- Group 1: Control (C), injected intraperitoneally (i.p) with saline and intragastrically (i.g.) with 1% Carboxy Methyl Cellulose (CMC).
- Group 2: Gentamicin Treated (GM) treated i.p with GM and i.g. with 1% CMC.
- Group 3: GM + EL treated with EL and with GM. EL were given i.g. at a dose of 2.5 g of methanol extract/kg body wt day⁻¹ (Maroo *et al.*, 2003b).
- Group 4: GM + Vit C treated with Vit C and with GM. Vitamin C was given i.g at a dose of 600 mg kg⁻¹ b.wt day⁻¹.

The onset of damaging renal function induced by GM occurs after 5-8 days' treatments between of 80 and 150 mg kg⁻¹. In this study, GM was injected intraperitoneally at the dose of 80 mg kg⁻¹, for eight successive days, which is well known to cause significant nephrotoxicity in rats (Abdel-Gayoum *et al.*, 1995; Kumar *et al.*, 2000; Ali *et al.*, 2004). The *Enicostemma littorale* extract was given at a dose of 2.5 g kg⁻¹ b. wt day⁻¹ via gastric intubation for 11 days (Maroo *et al.*, 2003a). The Vit C, potent antioxidant, was used as reference drug at a dose of 600 mg kg⁻¹ b.wt day⁻¹ (Kavutcu *et al.*, 1996) for 11 days. EL and Vit C treatment was started three days prior to the 8 days gentamicin treatment and continued till the end of gentamicin treatment. At the end of experiment final weight of all animals were taken and urine collection has been done for 24 h with the help of metabolic cages. Blood samples were collected at the end of treatment on 9th day. The animals were sacrificed, kidney dissected out for estimation of various

parameters as mentioned below. Change in body weight was monitored before and after the treatment.

Blood/serum creatinine and urea determination: Blood/serum creatinine (Reckon diagnostics ltd. CRK.008) and urea (Reckon diagnostics ltd. UAG.19N) were measured as the marker of glomerular damage at the end of the experiment. Blood Urea Nitrogen (BUN) was obtained by correcting the urea value by a 0.467 factor.

Antioxidant parameters: The tissues were minced and washed repeatedly with the sucrose buffer pH 7.4 to remove adhering blood and 10% (w/v) homogenates were prepared using a Potter-Elvehjem type glass-Teflon homogenizer. Then the mitochondrial and post-mitochondrial fractions were prepared (Kaushal *et al.*, 1999).

Lipid peroxides reacts with Thiobarbituric Acid (TBA) and forms Thiobarbituric Acid Reacting Substances) (TBARS) giving a characteristic pink colour which can be measured colorimetrically at 532 nm indicating the levels of LPO (Beuge and Aust, 1978). GSH levels were estimated by monitoring the reduction of DTNB (dithiobis-2-nitrobenzoic acid) forming a yellow coloured anion at 412 nm (Beutler *et al.*, 1963). Measurements were carried out to determine the activities of Super Oxide Dismutase (SOD) (Marklund and Marklund, 1974; Patel and Katyare, 2006), Catalase (CAT) (Satav *et al.*, 2000; Abei, 1984), Glutathione Peroxidase (GPx) (Paglia and Valentine, 1967). Protein estimation was according to the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Histological analysis: Thin slices of kidney tissue with cortex and medulla were fixed by immersion in buffered formalin (pH 7.4), dehydrated and embedded in paraffin. Sections (5 µm) were stained with hematoxylin and eosin (H and E). Light microscopy was used to evaluate tubular necrosis, which was graded as follows (Karahan *et al.*, 2005).

Statistical analyses: Statistical analyses of data was done by Student's test, one-way analysis of variance and all groups were compared by means of Bonferroni, with significance set at p<0.05. All values represent the mean ± S.E.M. Data were analyzed using PRISM Graph Pad software (version 3).

RESULTS

Body weight and urinary volume: Body weight among different treated group did not change significantly from the control group (Table 1). Urinary volume increased significantly in the GM group (Table

1) as compared to control group. In EL and VC treated rats, urine volume was significantly reduced as compared to GM treated rats (GM+EL and GM+VC groups). In fact, EL and VC treated rats showed decrease in the urine volume to the level of the control.

Serum creatinine and urea: Serum creatinine level increased by 368% and Blood Urea Nitrogen level (BUN) increased by 176% in the GM group compared to the control (C) group (Table 2). EL attenuated the increase in creatinine by 60 and 63% fold in BUN level. Similarly, VC attenuated the increase in creatinine and BUN level by 55%.

LPO, GSH and Antioxidant activity in mitochondrial and post-mitochondrial fraction of kidney; MDA is a marker of oxidative stress. GM treated group showed 159 and 123% increase in MDA levels of mitochondrial and post-mitochondrial fraction respectively. Thus the content of LPO is higher in mitochondrial fraction than in post-mitochondrial fraction (Fig. 1). The EL treated group showed 80 and 63% decrease in mitochondrial and post-mitochondrial MDA levels respectively. However, the VC treated group decreases MDA levels by 77 and 64% in mitochondrial and post-mitochondrial respectively. Decrease in MDA levels were more in rats treated with EL compared to VC treated rats.

The GSH content in the post-mitochondrial fraction was relatively higher as compared to mitochondrial fraction in control group. GM treated rats showed decrease in GSH by 50% in mitochondrial fraction and 33% in post-mitochondrial fraction (Fig. 2). The EL treated group showed 75 and 88% improvement in GSH content of mitochondrial and post-mitochondrial fractions respectively. Similarly, VC treated group showed 62 and 89% increase in mitochondrial and post-mitochondrial GSH content.

Table 1: Effect of EL treatment on GM-induced changes body weight and urine volume

Groups	Body weight (gm sec)	Urine volume (mL 24h ⁻¹)
C	272±8.33	5.8±0.24
GM	267±6.63	15.6±1.18 ^{aaa}
GM+EL	270±8.22	7.3±0.39 ^{bbb}
GM+VC	272±7.66	6.9±0.25 ^{bbb}

Values are expressed as mean ± SEM (n = 6 in each group). aaa, p<0.001 Vs C, bbb, p<0.001 Vs GM. C = Control: GM = Gentamicin treated GM+EL = Getamicin +EL methanolic extract treated GM+VC = Getamicin + Vitamin C treated

Table 2: Effect of EL treatment on GM-induced changes in creatinine, BUN and tubular necrosis

	Cretonne (mg/dL)	BUN (mg/dL)	Tubular necrosis
C	0.76±0.04	21.5±1.08	----
GM	3.57±0.14 ^{aaa}	59.3±2.31 ^{aaa}	+++
GM+EL	1.88±0.11 ^{bbb}	35.3±2.06 ^{bbb}	++
GM+VC	2.02±0.08 ^{bbb}	38.7±1.31 ^{bbb}	++

Values are expressed as mean ± SEM (n = 6 in each group). aaa, p<0.001 Vs. C, bbb, p<0.001 Vs GM. C = Control GM = Gentamicin treated GM+EL = Getamicin + EL methanolic extract treated GM+VC = Getamicin + Vitamin C treated

Treatment with EL and VC were having similar effect on GSH content of mitochondrial and post-mitochondrial fractions.

SOD activity was higher in post-mitochondrial fraction as compared to mitochondrial fraction in control rats. The activity of SOD decreases by 61% in mitochondrial fraction, while decreases by 36% in post-mitochondrial fraction (Fig. 3). The EL treated group showed 78 and 66% increase in mitochondrial and post-mitochondrial SOD activity, respectively. Whereas VC treated group showed 73 and 57% increase in mitochondrial and post-mitochondrial SOD activity. EL treated rats showed little bit more increase in post-mitochondrial SOD activity as compared to VC treated rats, but having similar effect on mitochondrial SOD activity. GPx activity in the mitochondrial fraction was relatively higher than the post-mitochondrial fraction in control rats (Fig. 4). Mitochondrial GPx activity decreases by 60%, while post-mitochondrial activity decreases to 43% in GM-treated rats. The EL treated group showed increase in GPx activity by 80 and 113% in mitochondrial and post-mitochondrial fraction respectively. However, the VC treated group showed 82 and 101% increase in mitochondrial and post-mitochondrial GPx activity. Effect of EL and VC on GPx activity of mitochondrial and post-mitochondrial compartments was comparable. Catalase activity decreases 42% in post-mitochondrial fraction in GM treated group as compared to control group (Fig. 5). The EL treated group showed 95% increase in activity, while the VC treated group showed 93% increase in CAT activity. EL and VC have similar effect on post-mitochondrial CAT activity.

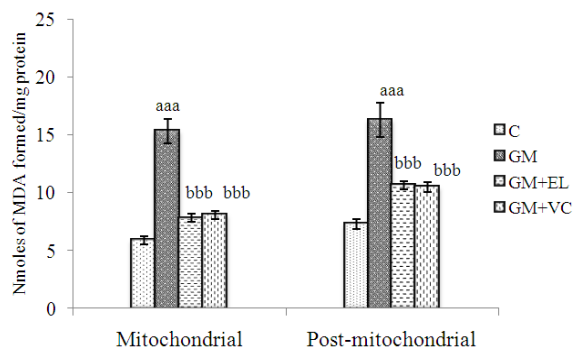


Fig. 1: Effect of EL on kidney lipid peroxidation levels in GM-induced nephrotoxicity (Values are expressed as mean ± SEM (n = 6 in each group). a, p<0.05, aa, p<0.01, aaa, p<0.001 Vs C, b, p<0.05, bb p<0.01, bbb, p<0.001 Vs GM)

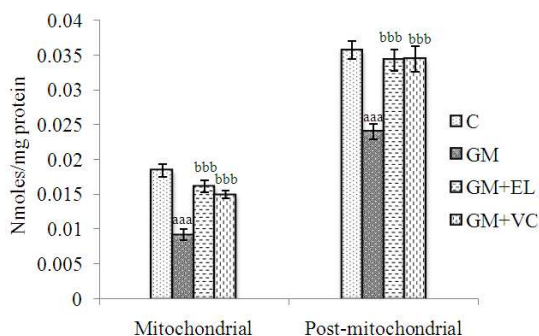


Fig. 2: Effect of EL on kidney reduced glutathione levels in GM-induced nephrotoxicity (Values are expressed as mean \pm SEM (n = 6 in each group). a, p<0.05, aa, p<0.01, aaa, p<0.001 vs C, b, p<0.05, bb p<0.01, bbb, p<0.001 Vs GM)

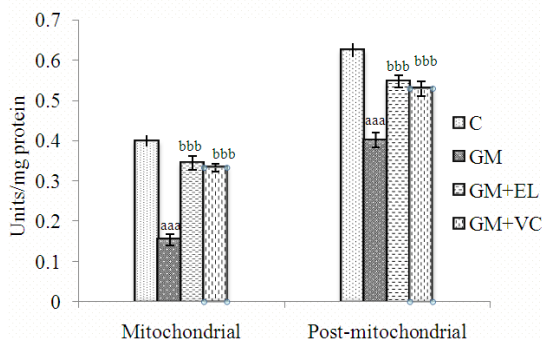


Fig. 3: Effect of EL on kidney superoxide dismutase activity in GM-induced nephrotoxicity Values are expressed as mean \pm SEM (n = 6 in each group). a, p<0.05, aa, p<0.01, aaa, p<0.001 Vs C, b, p<0.05, bb p<0.01, bbb, p<0.001 Vs GM

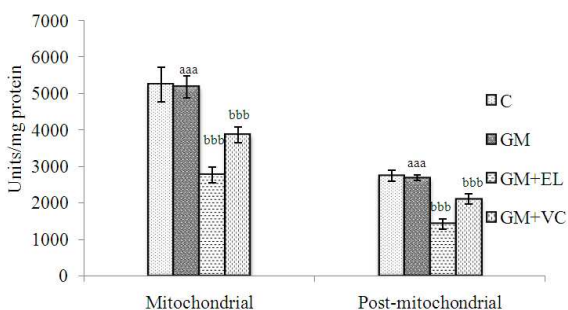


Fig. 4: Effect of EL on kidney glutathione peroxidase activity in GM-induced nephrotoxicity (Values are expressed as mean \pm SEM (n =6 in each group). a, p<0.05, aa, p<0.01, aaa, p<0.001 Vs C, b, p<0.05, bb p<0.01, bbb, p<0.001 Vs GM)

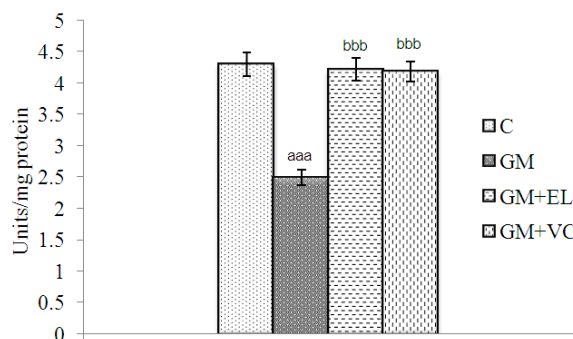


Fig. 5: Effect of EL on kidney catalase activity in GM-induced nephrotoxicity (Values are expressed as mean \pm SEM (n=6 in each group). a, p<0.05, aa, p<0.01, aaa,p<0.001 Vs C, b, p<0.05, bb p<0.01, bbb, p<0.001 Vs GM, C = Control, GM = Gentamicin treated, GM+EL = Getamicin+ EL methanolic extract treated, GM+VC = Getamicin + Vitamin C treated)

Histopathological analysis: Rats treated with GM showed sever tubular necrosis (Table 2). Treatment with GM alone caused a marked vacuolization and necrosis (+++) in proximal tubular epithelial cells. Treatment with EL (++) and VC (++) significantly decreases GM-induced tubular necrosis.

DISCUSSION

Previous studies with EL in our lab, has shown that methanolic extract of EL showed significant antioxidant activity in-vitro and in-vivo (Maroo *et al.*, 2003b). As ROS play very important role in development of pathogenic condition of many diseases, like diabetic nephropathy. We hypothesized EL extract will protect rats from GM-induced nephrotoxicity, which is mainly caused by generation of ROS.

The results of the present study indicate that GM administration brought about a significant increase in BUN and serum creatinine levels, indicating kidney dysfunction. The results are in agreement with the reports of Kumar *et al.* (2000). These alterations in biochemical parameters were well correlated with the renal histological score of GM treated rats.

Reactive oxygen species including hydroxyl radical have been implicated in the etiology of GM gentamicin-induced nephrotoxicity. Walker and Shah (1988) showed that GM enhances the generation of hydrogen peroxide by renal cortical mitochondria and that iron chelators and hydroxyl-radical scavengers protect against GM-mediated renal damage. It has been also demonstrated generation of hydrogen peroxide by rat kidney cortex and glomeruli (Guidet and Shah,

1989). Treatment with SOD or hydroxyl radical scavenger, Dimethyl-Thiourea (DMTU), two methods for reducing reactive oxygen metabolites, significantly lessened the GM-induced reduction in GFR (Nakajima *et al.*, 1994). Cuzzocrea *et al.* (2002) demonstrate that daily administration of M40403, low molecular weight synthetic manganese containing superoxide dismutase mimetic, which selectively removes superoxide to gentamicin-treated rats, resulted in an almost complete normalization of kidney functions.

Our results show that GM-induced oxidative stress as demonstrated by significant elevation in lipid peroxidation and decrease in GPx and SOD activities both in mitochondrial and post-mitochondrial fraction. Lipid peroxidation levels were higher in mitochondrial fraction as compared to post-mitochondrial fraction. Similarly, decrease in mitochondrial SOD and GPx activity was higher in mitochondrial fraction as compared to post-mitochondrial fraction. The depletion in GSH content was higher in mitochondria fraction as compared to post-mitochondrial fraction. Decreased GSH status might be responsible for the observed decrease in GPx activity. Even post-mitochondrial CAT activity decreased significantly. Decrease in GPx and CAT activity indicates decrease efficiency in H₂O₂ scavenging activity in kidney tissue of GM treated rats, which might be responsible for increase in lipid peroxidation levels in both the fractions. Hence, above results clearly indicates higher oxidative stress in mitochondrial fraction as compared to post-mitochondrial fraction in GM-induced nephrotoxicity. Histopathological changes observed in GM treated rat well correlates with GM-induced oxidative stress.

Oral administration of EL and Vit C was found to have a protective potential against GM-induced nephrotoxicity. Treatment with EL and Vit C showed increase in SOD and GPx activity, in both the fractions, along with increase in the GSH content, which is a primary antioxidant defense system of the cell. Amelioration in antioxidant defense system in both mitochondrial and post-mitochondrial fractions, leads to decrease in lipid peroxidation levels. However, the improvement in antioxidant defense system was more in mitochondrial fraction than in post-mitochondrial fraction which is a major site of ROS generation. In the present study, EL and Vit C treatment showed comparable protective effect on GM-induced nephrotoxicity. However, EL treatment showed better ameliorating effect on certain biochemical parameters as compared to Vit C treatment.

CONCLUSION

In summary, the present study provides evidence that co-administration of EL along with GM attenuates the increase in lipid peroxidative damage, restores antioxidant status, markers of renal injury and histopathological alterations. The present findings demonstrate that EL possesses significant therapeutic effects and it is a promising candidate for chemoprevention of GM-induced renal damage.

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