Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage:www.elsevier.com/locate/apjtb

Document heading doi:

Efficacy Study of *Dolichos biflorus* in the Management of Nephrotoxicity

Sarmistha Saha, Ramtej J Verma*

Department of Zoology, University School of Sciences, Gujarat University, Ahmedabad 380009, India

ARTICLE INFO

Article history:
Received 11 August 2012
Received in revised from 15 September 2012
Accepted 9 December 2012
Available online 24 December 2012

Keywords:
Antihyperuricemic activity
Dolichos biflorus
Ethylene glycol
Cystone
Creatinine
Uric acid

ABSTRACT

Objective: Ethylene glycol is widely used as a solvent and automobile antifreeze agent. Therefore, there have been a number of cases of human exposure from accidental or intentional ingestion of antifreeze, with the kidney being the most sensitive target organ. This study was designed to investigate the protective effect of seeds of *Dolichos biflorus* (Fabaceae), on ethylene glycol induced nephrotoxicity in adult female Wistar rats. **Methods:** The hydro-methanolic (30:70, v/v) extract of *D. biflorus* was orally administrated at doses of 150 and 300 mg/kg body weight/day along with ethylene glycol (0.75% v/v) for 28 days. The results were compared with a parallel study conducted with standard marketed drug cystone under identical dosage conditions. The ionic chemistry was measured in urine and serum. Statistical differences and linear regression analysis were performed using GraphPad prism 5 software. **Results:** Ethylene glycol induced a significant elevation in the creatinine, uric acid, urea levels in urine as well as in serum and urinary electrolytes (sodium and potassium) excretion levels. *D. biflorus* significantly ($P<0.001$) protected the elevated levels of urine and serum parameters. Moreover, *D. biflorus* shows higher renoprotective index than cystone at identical dose levels. **Conclusions:** These results provided an evidence of the significant protective effect of *D. biflorus* towards hyperuricemic and nephrotoxicity and thus can be used as potent dietary food.

1. Introduction

Ethylene glycol is an intermediate in the synthesis of a number of commercial chemical products, including polyethylene terephthalate resins, unsaturated polyester resins and polyester fibers and films. It is also a constituent in antifreeze, deicing fluids, surface coatings, heat transfer fluids, industrial coolants, hydraulic fluids, surfactants and emulsifiers[1]. General population, or consumer, exposure occurs primarily from the use of ethylene glycol in automotive antifreeze. There have been a number of acute human poisonings from accidental or intentional ingestion of antifreeze, with the kidney being the most sensitive target organ.

Plants are known to provide a source of inspiration for novel drug compounds and this is sequel to the fact that medicines derived from plants have made large contributions to human health and well being. *Dolichos biflorus* L. is a genus of family Fabaceae, a leguminous edible pulse crop of the subtropics, commonly known as Kulthi in the Indian Systems of Medicine and is a branched or trailing annual, with small trifoliate leaves, and very wide climbing, slender stem. When it matures, it gives narrow, flat and curved pods. The pods contain 5-6 flattened ellipsoid seeds. Traditionally its seeds are used in traditional formulations for the treatment of leucorrhoea, menstrual troubles and ulcer.

In Unani medicine, the concentrated water extract of kulthi seeds and shalgram (Brassica rapa) seeds are given for destroying stones in the kidney. The aqueous extracts of seeds of *D. biflorus* inhibited homogenous precipitation of calcium hydrogen phosphate dihydrate crystals[2]. However, the effects of these extracts have not been evaluated in animal models. The seeds of *D. biflorus* have been reported to show anti-hepatotoxic[3], anti-nephrotoxic[4], free radical scavenging activity[5,6], antioxidant[7,8] and hypolipidemic[9] activity.

Soup prepared from seeds is beneficial in enlarged liver and spleen. Analysis of seeds showing moisture 11.8%, crude protein 22.0%, fat 0.5%, mineral matter 3.1%, fibre 5.3%, carbohydrate 57.35%, calcium 0.28% and phosphorous 0.39%; iron 7.6mg, nicotinic acid 1.5 mg, carotene 119 (international vitamin unit A unit) per 100 g and rich in various enzymes.

*Corresponding author: Dr. Sarmistha Saha. Department of Zoology, University School of Sciences, Gujarat University, Ahmedabad - 380 009, (GUJ.) INDIA.
Tel. : 91-079-26302362
E-mail: sarmistha_pharmacol@yahoo.com; ramtejverma200@yahoo.com

Other chemical constituents present are streptogenin, beta-sitosterol[10], a phyto-haemagglutinin, beta-N-acetylglucosaminidase, alpha and beta galactosidases, alpha mannosides and beta glucosides[11].

In the present study the nephroprotective activity of seeds of *D. biflorus* has been investigated against ethylene glycol induced nephrotoxic model.

2. Materials and methods

2.1. Plant material and Preparation of extract

The plant material was collected from local market of Ahmedabad, India in the month of September, 2011 and was identified as seeds of *D. biflorus* by Dr. Yogesh T. Jasrai, Department of Botany, Gujarat University, India. A voucher specimen was deposited in the herbarium for future reference.

The seeds of *D. biflorus* were thoroughly cleaned, coarsely powdered and extract was prepared according to the WHO protocol CG-06. Briefly, 5 gm of powder and 100 ml of methanol: water (70:30, v/v) were mixed and stirred on a magnetic stirrer and then filtered twice with Whatman filter paper no 1. After evaporation of the solvent, the crude extract was dried under vacuum and stored in air tight container at 4℃. The dried extract (DBE) was dissolved in distilled water and used for further study.

2.2. Chemicals and apparatus

Ethylene glycol (Batch No.: SJ5S550736) was obtained from Merck Ltd., Mumbai, India. Cystone (Batch No.: A029156 B) was obtained from Himalaya Drug Company, Bangalore, India. All the chemicals used in the present experiment were of analytical reagent grade. Fully autoanalyzer (Siemens dimension xpand), cold centrifuge (Remi Instruments C-30BL), UV-spectrometer (Shimadzu Scientific Instruments, UV-3600) were used in the study.

2.3. Phytochemical studies

Qualitative analysis for determining the presence of tannins, saponins, glycosides, flavonoids, steroids and alkaloids in the plant extract were carried out using standard methods [12].

2.4. Experimental animals

Healthy female rats of Wistar strain weighing between 200-250 gm of equivalent age groups were obtained from Torrent Research Centre, Ahmedabad, India. They were acclimatized for 15 days in polypropylene cages under controlled conditions (temperature 25±2℃; relative humidity 50-55%; 12 h light/ dark cycle) in the Animal House of Zoology Department, Gujarat University, Ahmedabad, India. Animals were maintained on certified pelleted rodent feed supplied by Amrut Feeds, Pranav Agro Industries Limited, Pune, India and water ad libitum. The experimental procedures were approved by "The Committee for the Purpose of Control and Supervision of Experiment on Animals" (Reg - 167/ 1999/ CPCSEA), New Delhi, India.

2.5. Acute toxicity studies

The acute oral toxicity study[13] was carried out as per the guidelines set by Organization for Economic Cooperation and Development (OECD) received from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

2.6. Experimental design and treatment schedule

In the experiment, a total of 40 rats were randomly divided into eight groups containing five animals in each group. Group I served as untreated control and received regular rat feed and drinking water ad libitum. Group II and III served as standard and plant controls, received standard antiurolithiatic drug, cystone and DBE (300 mg/kg body weight/day) only respectively to determine any effect associated with plant extract and standard drug treatment. Ethylene glycol (0.75%, v/v) (EG) in drinking water was administered to animals of Groups IV-VIII for induction of renal calculi till 28th day. Group V and VI received oral administration of standard drug, cystone at two dose levels (150 and 300 mg/kg body weight/day) simultaneously from day 1st till 28th day along with ethylene glycol. Similarly, rats of Group VII and Group VIII received DBE at two same dose levels (150 and 300 mg/kg body weight/day) simultaneously along with EG from day 1st till 28th day and served as preventive regimen. All extracts were given once daily by oral route.

2.7. Collection and analysis of urine

The early morning urine samples were collected on 28th day of calculi induction treatment. Urinalysis was done for the estimation of uric acid, creatinine and urea using diagnostic kits by Siemens in Siemens rapid lab wet chemistry analyzer, USA. Sodium and potassium were measured by flame photometer[14].

2.8. Serum analysis

The blood was collected from the retro-orbital sinus under mild ether anaesthesia conditions on 28th day of treatment and serum was analysed for creatinine, blood urea nitrogen and uric acid using diagnostic kits by Siemens dimension

Xpand fully autoanalyzer, USA.

Saluretic index for Na^+ (Na^+ in treated group/Na^+ in control group), K^+ (K^+ in treated group/K^+ in control group) and aldosterone secretion index (Na^+/ K^+) were also calculated[15]. The kidney protecting activity of the *D. biflorus* extract was expressed as renoprotective index which was calculated using the formula:

where D is mean value of *D. biflorus* or cystone along with the ethylene glycol (Groups V-VIII), T is the mean value of ethylene glycol alone (Group IV) and C is the mean value of control animals (Group I).

2.9. Statistical analysis

The results are expressed as mean ∫ SEM. Statistical analysis and linear regression analysis were performed using GraphPad Instat, software, version 3.0. The values were analyzed by one-way Analysis of Variance (ANOVA) followed by Tukey multiple comparison test at a significance level of $P < 0.05$.

3. Results

3.1. Clinical Observations

There was no treatment related clinical signs and no animal mortality was recorded were observed during the experiment in controls and plant extract treated animals.

3.2. Phytochemical analysis

Phytochemical analysis indicated the presence of tannins, saponins, flavonoids, steroids, volatile oils and alkaloids. The hydro methanolic extract revealed the presence of cardiac and saponin glycosides and the absence of anthraquinone glycosides.

3.3. Acute toxicity studies

From the acute toxicity study, no toxic symptoms or death was observed in any animals during the 14 consecutive days of the treatment. Therefore, the acute minimum lethal dose (LD_{50}) was found to be 3 000 mg/kg body weight for the extract.

3.4. Urine analysis

In the present study, administration of 0.75% (v/v) ethylene glycol in aqueous drinking water to female Wistar rats for 28 days resulted in elevated creatinine levels. The concentration of urinary creatinine excretion was 25.76± 4.35 mg/dl in untreated control group which was increased to about 806.5% in EG induced nephrotoxic rats in Group IV ($p < 0.01$). However, DBE treatment significantly lowers the level of urinary output of creatinine with protective indices of 32 and 74% at 150 and 300 mg/kg b.w. (Table 1, $P< 0.001$). Moreover, DBE treatment shows more significant protection than standard drug cystone at both the doses selected with protective indices of 14 and 49% at 150 and 300 mg/kg b.w. (Table 1, $P< 0.01$).

The urinary excretion of uric acid was 0.96±0.04 mg/dl in untreated control group which was significantly increased to about 2.63±0.18 with 274% in EG induced nephrotoxic rats in Group IV ($P< 0.01$), which was prevented in the animals receiving a simultaneous treatment of EG and DBE at both the dose levels with protective indices of 45 and 81% at 150 and 300 mg/kg b.w. (Table 1, $p < 0.001$). Comparatively, our extract, DBE is more potent than standard drug cystone which showed protective indices of 12 and 69% at 150 and 300 mg/kg b.w.

Similarly, ethylene glycol induced nephrotoxicity caused significant increase in the excretion of urea in EG treated rats in Group IV ($p < 0.001$). However, simultaneous treatment with DBE with EG significantly prevented these changes in urinary excretion of urea with protective indices of 62% and 94% at 150 and 300 mg/kg ($P< 0.001$, Table 1). Whereas standard drug cystone shows protective indices of 19% and 78% at the same dose levels ($P< 0.01$).

Table 1

Effect of oral administration of D. biflorus on biochemical parameters in urine (mg/dl) of normal and ethylene glycol induced urolithiatic Wistar rats

Experimental groups	Creatinine	Uric Acid	Urea
I. Untreated Control	25.76 ±4.35	0.96 ±0.04	365.12 ±5.23
II. Cystone Control (SD)	25.38 ±2.82	0.97 ±0.07	363.42 ±4.08
III. D. biflorus (DBE)	25.46 ±2.16	0.90 ±0.07	363.10 ±6.40
IV. Ethylene glycol (EG)	207.78 ±2.37 a*	2.63 ±0.18 a*	1007.23 ±9.09 a*
V. EG + SD150	181.74 ±9.41 a* c*(14)	2.42 ±0.09 a* c*(12)	887.82 ±6.33 a*b*c*(19)
VI. EG + SD300	117.72 ±7.98 a* b*(49)	1.48 ±0.13 a†b*(69)	508.84 ±4.37 a*b*(78)
VII. EG + DBE150	150.04 ±6.06 a*b*c#(32)	1.88 ±0.12 a*b*(45)	609.46 ±5.84 a*b* c*(62)
VIII. EG + DBE300	73.46 ±6.04 a*b*c*(74)	1.27 ±0.04 b*(81)	406.08 ±4.28 a*b*(94)

Results are expressed as mean ±SEM; n = 5. Values shown in parenthesis indicate renoprotective percentage. a: as compared with untreated group, b: as compared with toxin-treated (group 4), c: as compared with toxin + standard-treated (group 6), No significant difference was noted between groups 1 – 3. Level of significance †$P < 0.05$; #$P< 0.01$; *$P< 0.001$.

Table 2

Effect of oral administration of D. biflorus on urinary electrolytes excretion of normal and ethylene glycol induced urolithiatic Wistar rats

Experimental groups	Urine electrolyte concentration (mEq/L)		Saluretic index		Na^+/K^+
	Na^+	K^+	Na^+	K^+	
I. Untreated Control	92.56 ±2.16	60.88 ±2.35	–	–	1.52
II. Cystone Control (SD)	92.32 ±1.23	60.48 ±0.78	–	–	1.53
III. D. biflorus (DBE)	92.10 ±2.45	60.90 ±0.77	–	––	1.51
IV. Ethylene glycol (EG)	175.38 ±6.6 a*	96.82 ±2.22a*	1.89	1.59	1.81
V. EG + SD150	146.48 ±4.76 a*b*c*	84.48 ±2.67 a*b#c†	1.58	1.39	1.73
VI. EG + SD300	115.70 ±3.62a#b*	73.70 ±2.59 a#b*	1.25	1.22	1.57
VII. EG + DBE150	130.90 ±2.68a*b*	79.60 ±3.12a*b*	1.41	1.31	1.64
VIII. EG + DBE300	105.08 ±3.70b*	67.54 ±1.55 b*	1.13	1.11	1.55

Results are expressed as mean ±SEM; n = 5. Values shown in parenthesis indicate renoprotective percentage. a: as compared with untreated group; b: as compared with toxin-treated (group 4), c: as compared with toxin + standard-treated (group 6), No significant difference was noted between groups 1 – 3. Level of significance †P< 0.05; #P < 0.01; *P< 0.001.

Table 3

Effect of oral administration of D. biflorus on biochemical parameters in serum (mg/dl) of normal and ethylene glycol induced urolithiatic Wistar rats

Experimental groups	Creatinine	Uric Acid	Blood Urea Nitrogen
I. Untreated Control	0.46 ±0.02	1.26 ±0.17	33.02 ±0.45
II. Cystone Control (SD)	0.44 ±0.02	1.34 ±0.17	33.82 ±0.90
III. D. biflorus (DBE)	0.40 ±0.04	1.28 ±0.09	33.18 ±1.29
IV. Ethylene glycol (EG)	0.83 ±0.04 a*	4.25 ±0.39 a*	47.70 ±1.65 a*
V. EG + SD150	0.78 ±0.04 a* c† (12)	4.00 ±0.32 a* c†(8)	44.74 ±1.40 a* (14)
VI. EG + SD300	0.62 ±0.04 a† b# (56)	2.98 ±0.19 a* b#(43)	40.72 ±0.95 a* b# (48)
VII. EG + DBE150	0.67 ±0.02 a#b†(43)	3.66 ±0.15 a*(20)	42.16 ±0.74 a*b† (38)
VIII. EG + DBE300	0.51 ±0.02 b*(86)	2.36 ±0.06 a† b*(63)	35.66 ±1.11 b*(82)

Results are expressed as mean ±SEM; n = 5. Values shown in parenthesis indicate renoprotective percentage. a: as compared with untreated group, b: as compared with toxin-treated (group 4), c: as compared with toxin + standard-treated (group 6), No significant difference was noted between groups 1 – 3. Level of significance †P< 0.05; #P < 0.01; *P< 0.001.

Table 2 reveals that the urinary excretion of potassium is significantly elevated in nephrotoxic rats (160%, P< 0.001) but at the end of 28th day, DBE reversed the status by significantly reducing the excretion of potassium level as compared with the cystone. Same table reveals that there was significant rise in urinary sodium excretion levels in nephrotoxic rats (189%, P< 0.001), which was significantly restored by DBE as compared to cystone (P< 0.01, Table 2).

The saluretic index and Na_+/K_+ of the ethylene glycol induced nephrotoxic rats in Group IV were significantly higher which is again significantly restored by DBE administration at both the oral doses tested (P < 0.001) (Table 2).

3.5. Serum analysis

The concentrations of creatinine was also measured in serum of all the animals on 28th day of treatment, which showed a significant increase of 180% in serum level of creatinine in EG treated rats. The simultaneous administration of DBE significantly restored the serum creatinine level with protective indices of 43 and 86% at 150 and 300 mg/kg b.w. (P< 0.001, Table 3). Results were statistically more significant as compared with standard drug cystone.

The concentration of serum uric acid level was 1.26±0.17 mg/dl in untreated control (Group) I which was significantly increased in EG treated rats (337%, P< 0.001) (Group IV). However, DBE treatment significantly decreased this elevation by 20 and 63% at 150 and 300 mg/kg b.w. showing more potent protection as compared to cystone (P< 0.001, Table 3).

Similarly, nephrotoxic induction caused significant increase (145%, P<0.001) in serum blood urea nitrogen level in Group IV (Table 3), which was dose-dependently prevented in the animals receiving a simultaneous treatment of DBE with protective indices of 38 and 82% at 150 and 300 mg/kg b.w. (P < 0.01).

A strong positive correlation was obtained between urinary and serum creatinine (r^2 = 0.9768, P< 0.0001, Figure 1), suggesting a dependence of urinary excretion of creatinine on serum concentration of creatinine. Similarly, a significant positive correlation was also obtained between urinary and serum concentrations of uric acid (r2 = 0.9674, P < 0.0001, Figure 2) and urea (r^2 = 0.9153, P< 0.0001, Fi

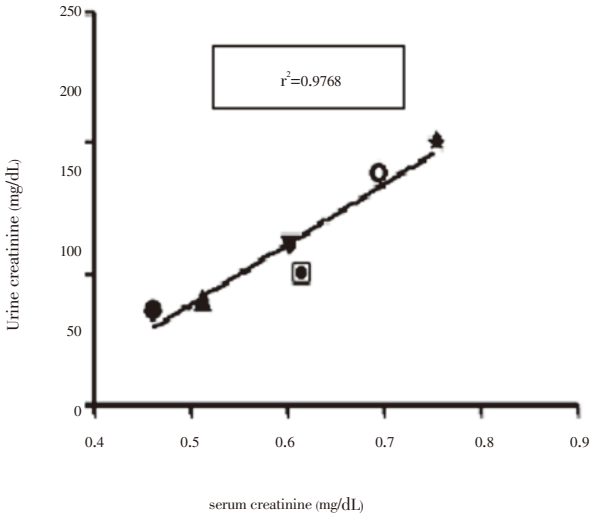

Figure 1. Correlation of urinary creatinine excretion and serum creatinine concentration.

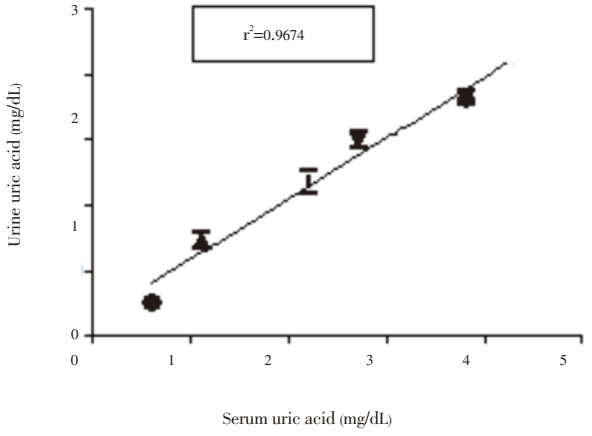

Figure 2. Correlation of urinary uric acid excretion and serum uric acid concentration.

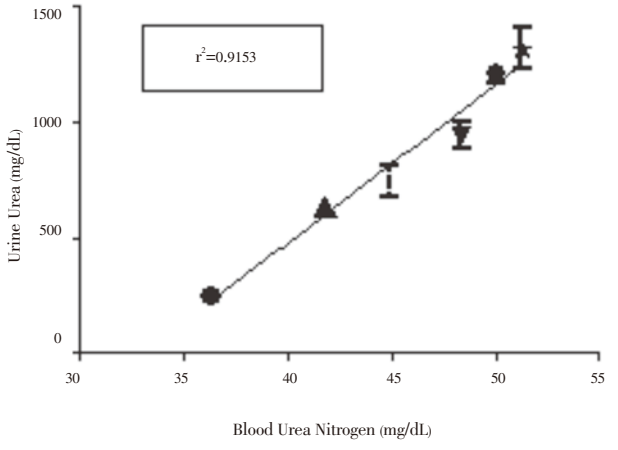

Figure 3. Correlation of urinary urea excretion and serum urea concentration. Each point represents a treatment group (●) Untreated Control, (◆) EG treated group, (□) EG + SD150 group, (■) EG + SD300, (▼) EG + DBE 150 group, (▲) EG + DBE 300 group.

4. Discussion

Pathologic studies[16] have shown that the renal failure from EG is associated with proximal tubule cell necrosis leading to production of several metabolites (glycolaldehyde, glycolate, glyoxylate and oxalate, in order) and accumulation of large calcium oxalate monohydrate crystals in tubular lumen.

An Ayurvedic compound preparation (Cystone) has been clinically used extensively for treating various renal disorders[17] and many urinary tract complications such as urolithiasis[18], burning micturition[19], nephro-ureterolithiasis[20], urinary tract complications in pregnancy[21].

In this study, the results clearly showed that the administration of ethylene glycol caused a remarkable increase in urinary output, electrolytes excretions and hyperuricemia. In response to ethylene glycol treatment, urea, creatinine and uric acid were increased in the urine, suggesting an impairment of kidney functions. These effects could also be attributed to the changes in the threshold of tubular re-absorption, renal blood flow and glomerular filtration rate[22]. However, administration of DBE along with ethylene glycol attenuated the toxicity by significantly decreasing the urinary creatinine and urea levels.

Ethylene glycol is known to decrease the glomerular filtration rate due to the obstruction to the flow of urine by stones in urinary system. Due to this, the waste product, particularly nitrogenous substances such as urea, creatinine and uric acid gets accumulated in blood. The increased serum level of creatinine can be attributed to the damaged nephron structural integrity[23]. Our studies demonstrated the restoration of urinary excretions by hydro methanolic extract of *D. biflorus* more significantly than standard drug cystone. It should be mentioned that the reducing uric acid production, a crystallization salt which may facilitate the heterogeneous nucleation of the calcium oxalate stone, will not only exert anti-gout effect but also will contribute to the anti lithiatic property and other uric acid-related diseases[24]. The role of xanthine oxidase is to catalyze the oxidation of hypoxanthine to xanthine and generates uric acid, hydrogen peroxide and superoxide anion[25]. Our results showed that DBE reduced the serum as well as urinary urate level after 28 days of treatment which might be through the inhibition of xanthine oxidase.

Sodium depletion stimulates rennin release and subsequence production of Angiotensin II, a potent vasoconstrictor[26]. Increased sodium levels inhibit rennin release from the juxtaglomerular cells and consequent withdrawal of angiotensin II[27]. When modulation of the rennin angiotensin system is pharmacologically prevented, changes in salt intake markedly affect long term levels of arterial blood pressure[28]. There is therefore a need to strike a balance in the levels of blood sodium to avoid either of the extreme of hypotension or hypertension. The excretion of sodium and potassium were significantly pronounced and predominant with ethylene glycol administration which is restored by the DBE treatment showing the natriuretic effect of the extract reflecting the tendency of *D. biflorus* to have a diuretic property with higher efficacy than that of standard drug cystone administration.

It has been reported that inhibition of sodium reabsorption in the more proximal segments will cause an increase in distal delivery and increases potassium secretion into the tubular lumen in a flow-dependent manner[29]. More investigations are desirable to identify clearly whether or

not potassium may play a role in some of the biological properties of *D. biflorus*.

The *D. biflorus* is unlikely acting as thiazide diuretic because they decrease urinary potassium level and alter urinary Na_+/K_+ output[30]. The hydro methanolic extract of *D. biflorus* consists of many alkaloids and phenolic compounds which might be responsible for this protective effect.

5. Conclusions

The overall result of the present study indicated the nephroprotective potential of *D. biflorus* against the toxic effects of ethylene glycol in kidneys of rats. This nephroprotective activity of *D. biflorus* might be due to the synergetic effect of chemical compounds present in them making them good sources for the production of a nephroprotective herbal medicine.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgement

The financial assistance to Sarmistha Saha from Lady Tata Memorial Trust (LTMT/AD/Q2/2011-2012) in the form of Senior Research Fellowship is highly acknowledged with thanks.

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