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RESEARCH ARTICLE

ANTI UROLITHIATIC ACTIVITY OF NERIUM OLEANDERON ETHYLENE GLYCOL INDUCED NEPHROLITHIASIS IN RATS

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ABSTRACT

The leaves of **Nerium oleander** (Family: Apocynaceae), ethanol fraction of ethanol extract was investigated for it'santiurolithiatic. Ethylene glycol (0.75% in water) feeding resulted in hyperoxaluria as well as increased renal excretion of calcium and phosphate. EFE extract (250 &500 mg/kg) of **Nerium oleander** was given orally in curative and preventive regimens over a period of 28 days. Supplementation with extract significantly ($P < 0.001$) lowered kidney homogenate levels of, calcium and phosphate. Furthermore, high serum levels of BUN, creatinine and uric acid were significantly ($P < 0.001$) reducedby the extract. The results were comparable with the standard drug, cystone (750 mg/kg). The reduction of stone forming constituents in kidney and their decreased kidney retention reduces the solubility product of crystallizing salts such as calcium and phosphate, which could contribute to the antiurolithiatic property ofthe extract. The extract exhibited significant antiurolithiatic activity at dose of 500 mg/kg body weight as evidenced by histopathological studies

INTRODUCTION

Urolithiasis is a common chronic disorder in humans and the most common type of renal stone is made of calcium oxlalate (Dodamatekurke *et al*, 2007). Various therapies have been used to treat this complaint. The recent remedies like surgical removal, percutaneous techniques and extracorporeal shock wave lithotripsy are costly for the common man. The recurrence is quite common with these procedures and patient has to be subjected to careful follow-up for a number of years (Prasad *et al*, 2007). Thus, there is need for more effective alternative therapy. Medicinal plants remain an important source of new drugs. These plant products are reported to be effective in decreasing the recurrence rate of renal calculi with no side effects (Diwakar *et al*, 2010).Urinary calculi are the third prevalent disorder in the urinary system. Approximately 80% of these calculi are composed of calcium oxalate and calcium phosphate. Urinary calculi may cause obstruction, hydronephrosis, infection and hemorrhage in the urinary tract system (Hadjzadeh *et al*, 2007). The majority of kidney stones are made up of calcium oxalate (CaOx) crystals in the urinary system of patients with urolithiasis. Extracorporeal shock wave lithotripsy and percutaneousnephrolithotomy techniques mainly include the surgical removal of stones. But, these techniques cause undesirable side effects such as tubular necrosis, hypertension, hemorrhage and subsequent fibrosis of the kidney leading to cell injury and recurrence of renal stone formation (Terlecki *et al*,2007, Kaur *et al, 2007*).

Nerium oleander is widely cultivated as an ornamental shrub or as an informal hedge in warm-temperate and dry subtropical regions, and as a plant for the conservatory in cooler climates (Pagen *et al*, 1987). Nerium oleander is used for treating cardiac conditions in patients who cannot tolerate digitalis. In traditional medicine, the leaves have been used for a variety of medicinal purposes, including the treatment of heart diseases, as a diuretic, antibacterial, and against snake-bite. The roots have been used externally in traditional medicine for treating cancer, ulcers and leprosy (Brukill *et al*, 1997).

MATERIAL AND METHODS

Plant material

The fresh leafs of *Nerium oleander* were collected from local areas of Kurnool, Andhra Pradesh, India and authenticated at Botanical survey of India, Coimbatore. The leaves were dried in shade and ground to get a coarse powder.

Preparation of extract

The air dried powdered drugs weighing about 50g each were taken and extracted successively in sox-let apparatus using solvents in the order of increasing polarity ,as petroleum ether, acetone, chloroform, ethanol. Each time before extracting with the next solvent, the material was dried in hot air oven at a temperature not exceeding 50°C. All the extracted were concentrated by distilling off the solvents and evirating to

dryness on the water bath. Then percentage yield of extracts were recorded.

Qualitative evaluation of successive extracts

The successive extracts were subjected to various qualitative tests to determine the presence of various phytoconstituents using reported methods (Kokate, 1999).

Preparation of selective extracts and fractions

The total ethanol extract was dissolved in ethanol and subjected for fractionation using column chromatography with help of chloroform and ethanol in an attempt to distribute the present constituents. The solvents (chloroform and ethanol extracts) were distilled in order to concentrate the extracts. Then obtained extracts were dried in desiccators and percentage yields of extracts were recorded. The resulting extracts (CFEE and EFEE) were subjected for phytochemical screening.

Animals

Healthy adult male albino rats of Wister strain weighing 150-120g were selected for the study. The animals were acclimatized to standard laboratory condition with temperature 25±2ºC and fed with standard animal pellet feed (Hindustan lever limited) and water *ad libitum.* The protocol was approved by animal ethics committee constituted for the purpose of animal experimentation as per CPCSEA guidelines.

Acute toxicity studies

Acute toxicity study was performed as per OECD guidelines 420. The animals were randomly divided into 5 groups and were orally supplemented graded doses (200, 400, 800, 1600 or 3200gm per kg body weight)(OECD, 1996) of methanol extract and were observed for behavioral changes and mortality till 72 hour and LD50 was calculated.

Ethylene glycol induced urolithiasis model

Ethylene glycol induced hyperoxaluria model (Atmani *et al*, 2003) was used to assess the antilithiatic activity in albino rats. Animals divided into six groups containing six animals in each group. Group I serve as Normal and received regular rat food and drinking water ad *libitum.* Ethylene glycol (0.75%) in drinking water was fed to groups II-V for induction of renal calculi till 28th day. Group III received standard antiurolithiatic drug, cystone (750mg/kg body weight) from 15th day till 28th day (Mitra *et al*, 1998) Group IV & V received *EFELNO* of (250mg/kg & 500mg/kg body weight). All extracts were given once daily by oral route.

Assessment of antiurolithiatic activity

Collection of urine analysis

All animals were kept in individual metabolic cages and urine samples of 24 hr were collected on 28th day. Animals had free access to drink water during the urine collection period. A drop of concentrated hydrochloride acid is added to the urine before being stored at 4ºC. Urine was analyzed for calcium, phosphate and oxalate content (Fiske *et al,* 1999).

Serum analysis

After the experimental period, blood was collected from the retro-orbital under anaesthetic conditions. Serum was separated by centrifugation at 10,000×g for 10 min and analyzed for creatinine, uric acid and blood urea nitrogen.

Kidney homogenate analysis

The abdomen was cut open to remove both kidneys from each animal. Isolated kidneys were cleaned off extraneous tissue and preserved in 10% neutral formalin. The kidneys were dried at 80ºC in a hot air oven. A sample of 100mg of the dried kidney was boiled in 10ml of 1N hydrochloric acid for 30min and homogenized.

Statistical Analysis

All the values are expressed as mean ± SEM. The data were statistically analyzed by one-way ANOVA followed Dunnet-t-test. P values < 0.05 were considered significant.

RESULTS

Preparation of extracts

The ethanol extract was subjected for simple fractionation using chloroform and ethanol solvents, pecentage yield was found to 4% & 8% respectively.

Preliminary Phytochemical Analysis

Qualitative phytochemical studies were performed on extracts using suitable chemicals and reagents to confirm the presence of alkaloids, carbohydrates, glycosides, saponins, proteins, flavonoids, steroids and triterpenoids. The results of qualitative phytochemical studies indicates that the maximum number of chemical constituents were present in the ethanol extract when compared to the other extracts [Table 1] and hence, ethanol extract was subjected for simple fractionation with chloroform and ethanol, ethanol fraction shows more number of phytoconstituents [Table 2] selected for further pharmacological screening.

Table 1 Phytochemical Analysis ofleaf extract from *Nerium oleander*

Extract	Alkaloids	Glycosides	Saponins	Carbohydrates	Tannins	Flavonoids	Sterodis	Triterpenoids
Pet.ether	-	-	-	+	-	+	+	+
Acetone	+	+	-	-	-	+	-	-
Chloroform	+	+	+	-	-	-	+	+
Ethanol	+	+	+	-	+	+	+	+

"+" indicates the presence.
"-" indicates the absence.

Table 2 Phytochemical Analysis of Chloroform and Ethanol fraction of ethanolic extract of *N.oleander*

Fraction	Alkaloids	Glycosides	Saponins	Carbohydrates	Tannins	Flavonoids	Sterodis	Triterpenoids
Chloroform	-	-	-	-	-	-	+	+
Ethanol	-	+	+	+	+	+	+	+

"+" indicates the presence.
"-" indicates the absence.

Table 3 Estimation of Serum Parameters of Normal and Urolithiatic Rats

S.No	Group & Drug Treatment	Estimation of Serum Parameters		
		BUN(mg/dl)	Creatinine(mg/dl)	Uric acid(mg/dl)
1	Normal control (Saline)	17.98±1.315	1.243±0.04	0.53±0.109
2	Disease control(0.75% EG)	29.74±2.21	3.220±0.32	1.376±0.213
3	Standard (Cystone 750 mg/kg	16.06±1.21*	1.153±0.188***	0.676±0.143**
4	T_1 (*EFELNO*250 mg/kg)	17.08±2.09*	1.334±0.09***	0.77±0.09*
5	T_2 (*EFELNO* 500 mg/kg)	20.60±5.56^{ns}	1.568±0.162***	1.08±0.10^{ns}

Acute toxicity studies

The ethanol extract (EE) was subjected to acute toxicity determinations as per OECD 420 guidelines. Extract was not showed mortality even at of 3200 mg/kg dose level and therefore considered safe.

Antiurolithiatic studies

All values are expressed as mean ±S.E.M for six rats in each group.

Comparisons made between

$^{***}p<0.001$,$^{**}p<0.01$,$^{*}p<0.05$; Standard,T_1,$T_2$$V_s$Calculi induced, One-way ANOVA followed by Dunnet's -t test.

Table 4 Estimation of Urinary Electrolytes of Normal and Urolithiatic Rats

S.No	Group &Drug Treatment	Estimation ofUrinary Electrolytes	
		Calcium(mg/dl)	Phosphate(mg/dl)
1	Normal (Saline)	1.567±0.22	3.75±0.22
2	Disease control (0.75% EG)	4.827±0.26	6.83±0.38
3	Standard (Cystone 750 mg/kg)	1.742±0.155***	3.54±0.31***
4	T_1 (*EFELNO* 250 mg/kg)	1.917±0.23***	4.02±0.13***
5	T_2 (*EFELNO* 500 mg/kg)	2.802±0.22***	4.20±0.156***

All values are expressed as mean ±S.E.M for six rats in each group.

Comparisons made between

$^{***}p<0.001$,$^{**}p<0.01$,$^{*}p<0.05$; Standard,T_1,T_2V_s Calculi induced, One-way ANOVA followed by Dunnet's -t test.

Table 5 Estimation of Kidney Homogentae electrolytes of Normal and Urolithiatic Rats

S.No	Group &Drug Treatment	Estimation of Kidney Homogenate electrolytes	
		Calcium(mg /dl)	Phosphate(mg /dl)
1	Normal (Saline)	3.69±0.23	2.34±0.22
2	Disease control (0.75% EG)	8.97±0.14	4.69±0.23
3	Standard (Cystone 750 mg/kg)	5.18±0.14***	2.26±0.14***
4	T_1 (*EFELNO* 250 mg/kg)	6.19±0.23***	3.57±0.23**
5	T_2 (*EFELNO* 500 mg/kg)	8.02±0.16**	3.095±0.25***

All values are expressed as mean ±S.E.M for six rats in each group.

Comparisons made between

$^{***}p<0.001$,$^{**}p<0.01$,$^{*}p<0.05$; Standard,T_1,T_2V_s Calculi induced, One-way ANOVA followed by Dunnet's -t test.

Histopathological studies

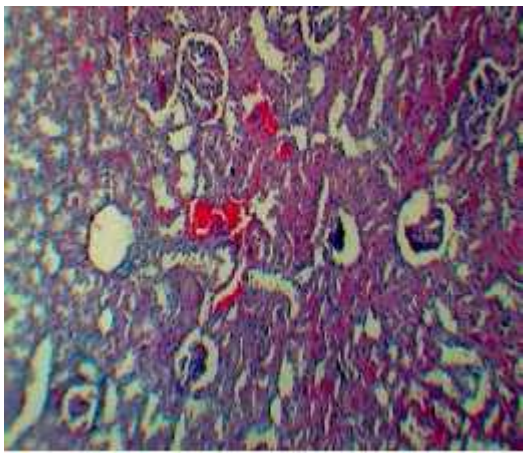

Fig 1 T.S.of Kidney of Normal Rats

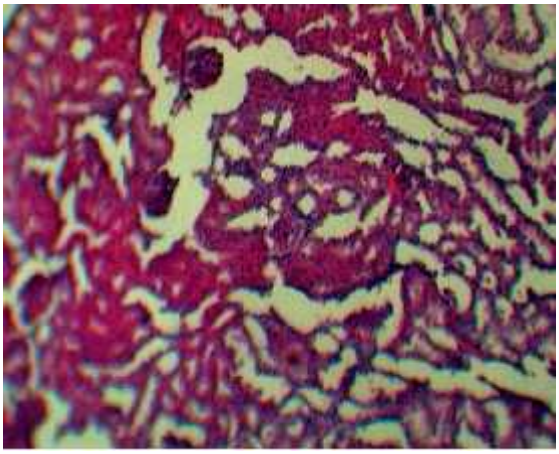

Fig 2 T.S.of Kidney of Treated With Ethylene Glycol Alone

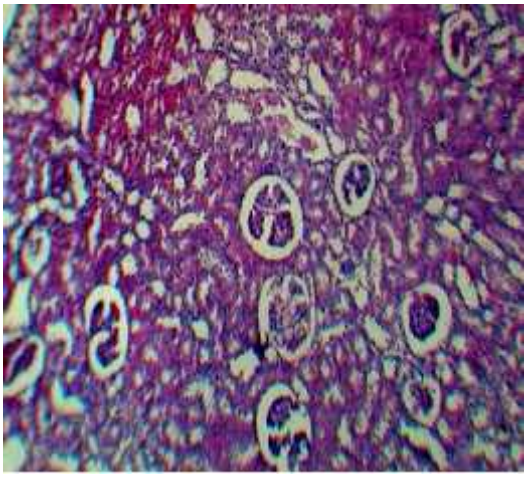

Fig 3 T.S. of kidney treated with standard cystone drug (750mg/kg)

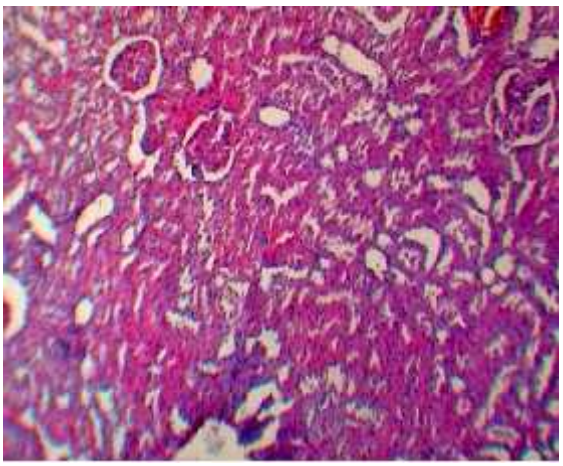

Fig 4 T. S. of Kidney Treated With *Efelno*(250mg/kg)

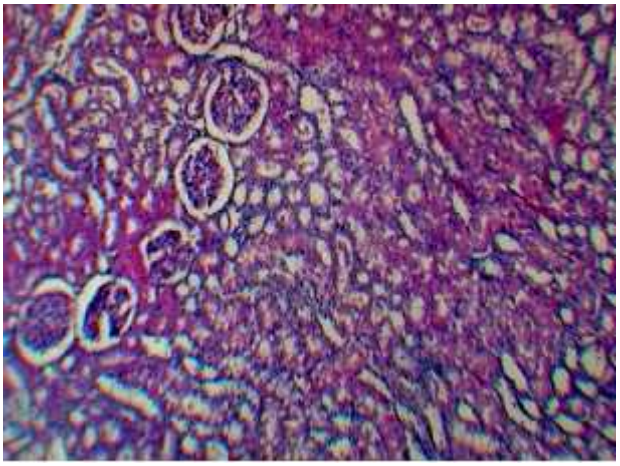

Fig 5 T.S. of Kidney Treated With *Efelno*(500mg/kg)

DISCUSSION

In the present study, chronic administration of 0.75% (v/v) ethylene glycol aqueous solution to Wistar rats resulted in hyperoxaluria. As mentioned in table 4 the urinary excretion of calcium and phosphate are significantly increased in calculi-induced rats (4.827±0.26 and 6.83±0.38 mg/dl) when compared with normal control (saline) rats (1.57±0.22 and 3.75±0.22 mg/dl). When standard drug (Cystone 750mg/kg. p.o.) administered to calculi-induced rats the excretion levels of calcium and phosphate were significantly decreased to (1.742±0.155 and 3.54±0.31mg/dl). The test drug, *EFELNO* was used in two different concentrations 250 and 500mg/kg on calculi induced rats to determine the efficacy of the test drug.

It was observed that the excretion levels of above mentioned parameters were significantly (P<0.01) decreased in test drug treated groups. It was also observed that low concentration of *EFELNO* decreased excretion levels of calcium significantly than the standard drug whereas excretion levels of phosphate were significantly less when compared with that of disease control. The results of lower dose test drug (250mg/kg) treated group is almost equal to standard drug treated group.

As mentioned in table 5 the deposition of the crystalline components in the renal tissues, namely calcium and phosphate were increased in calculi induced rats (8.97±0.14, 4.69±0.23mg/dl) as compared to normal control (saline) rats. The deposition levels of calcium and phosphate were significantly decreased (5.18±0.14, 2.26±0.14mg/dl) in Standard (Cystone-treated) group rats. However, supplementation with *EFELNO* (2500mg/kg) significantly lowered the elevated levels of calcium and phosphate (P<0.001) as compared to disease control group rats. [Table 5, Group IV].*EFELNO* (250, 500mg/kg) non-significantly lowered the elevated levels of Calcium and phosphate (P>0.05) as compared to standard (Cystone- treated) group rats.

The data presented in table 3 indicates the serum Blood urea nitrogen (BUN), Creatinine and Uric acid remarkably increased in calculi-induced rats (29.74±2.21, 3.220±0.32, 1.376±0.213). When standard drug (Cystone 750mg/kg) was used in calculi-induced rats the deposition levels of Blood urea nitrogen (BUN), Creatinine and Uric acid were significantly decreased (16.06±1.21, 1.153±0.188, 0.676±0.143 mg/dl.) indicating marked renal damage. However, *EFELNO* (250mg/kg) treatment significantly lowered the elevated levels of BUN, Creatinine and Uric acid (P<0.001) as compared to calculi induced group rats. [Table 3, Group IV].

EFELNO (500mg/kg) treatment remarkably reduced the elevated levels of BUN, Creatinine and Uric acid (P<0.001) as compared to Standard (Cystone- treated) group rats and the altered values were found to be statistically significant (P<0.001). As compared to the calculi induced group rats *EFELNO*(500mg/kg) treatment significantly minimized the BUN, Creatinine (P<0.001) and Uric acid (P<0.01) [Table 3, Group V].

Histopathology Report*(100x)*

Fig-1-Normal: Few glomerulic shows shrinkage tubules butnormal renal tissue. Interstitium appears normal.

Fig-2-Control: Shows renal tissue with focal tubular damage, interstitial inflammatory collection. Glomeruli shows epithelial proliferation.

Fig-3-Standard: The glomeruli show mild shrinkage in tubules and show cloudy glomerulus, Interstitium normal.

Fig-4-Test-I (250mg/kg): The few glomeruli show mild shrinkage, the tubules show the changes of cloudy swelling, and tubular cast but the Interstitium appear normal.

Fig-5-Test-II (500mg/kg): Tubules shows cloudy swelling glomeruli and also shows increased nuclear of cells. Interstitium and vessels appear normal.

CONCLUSION

The present investigation, *EFEL* of *Nerium oleander*was shows good anti urolithiatic activity against ethylene glycol induced nephrolithiasis in rats. The results of the present study of EFEE of 500 mg/kg dose level shows significantly decrease the development of urolithiasis in rats. Hence it may be hypothesized that flavonoids are responsible for the exhibited anti urolithiatic activity of the extract. However, further detailed study is required to explore the active principle responsible for this and also to know the exact mechanism involved in observed activity profile.

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