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ANTI UROLITHIATIC ACTIVITY OF WHOLE PLANT EXTRACT OF *PERGULARIA DEAMIA* AGAINST ETHYLENE GLYCOL INDUCED UROLITHIATIC RATS

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ABSTRACT
The whole-plant, *Pergularia daemia* (Family: Asclepediaceae), extract was investigated for it's antiurolithiatic. Ethylene glycol (0.75% in water) feeding resulted in hyperoxaluria as well as increased renal excretion of calcium and phosphate. Pet-ether extract (100 and 200mg/kg) of *P. daemia* was given orally in curative and preventive regimens over a period of 28 days. Supplementation with extract significantly ($P < 0.001$) lowered the urinary excretion and kidney retention levels of oxalate, calcium and phosphate. Furthermore, high serum levels of urea nitrogen, creatinine and uric acid were significantly ($P < 0.001$) reduced by the extract. The results were comparable with the standard drug, cystone (750 mg/kg). The reduction of stoneforming constituents in urine and their decreased kidney retention reduces the solubility product of crystallizing salts such as calcium oxalate and calcium phosphate, which could contribute to the antiurolithiatic property of the extract.

KEYWORDS: *Pergularia daemia*, Ethylene glycol, Cystone, Anti-Urolithiasis.

INTRODUCTION
Nowadays stone formation is the oldest and serious painful urologic disease with significant prevalence in the population due to change in lifestyle and dietary factors. Stone formation or lithiasis is characterized by calculi formation. It has two main types such as nephrolithiasis and urolithiasis. Calculi formation in urinary bladder, ureter or any part of urinary tract rather than kidney is known as urolithiasis while nephrolithiasis is characterized calculi formation in kidney.[1] Urolithiasis is a condition in which crystals in the urine combine to form stones, also called calculi or uroliths. These can be found anywhere in the urinary tract, where they cause irritation and secondary infection. Most end up in the bladder or urethra.

Urolithiasis refers to the solid nonmetallic minerals in the urinary tract. This is the third most common condition of the urinary tract after urinary tract infection and pathologic condition of prostate.[2] Urolithiasis is a complex process that is a consequence of an imbalance between promoters and inhibitors in the kidneys. The formation of kidney stones involves several physicochemical events beginning with crystal nucleation, aggregation, and end with retention within the urinary tract.[3] Among the several types of kidney stones, the most common are calcium oxalate stones representing up to 80% of the analyzed stones.[4]

Urolithiasis is a common disorder estimated to occur in approximately 12% of the world population, with a recurrence rate of 70-81% in males and 47-60% in females.[5] In the traditional systems of medicine including Ayurveda, most of the remedies were taken from plants and they were proved to be useful though the rationale behind their use is not well established through systematic pharmacological and clinical studies except for some composite herbal drugs and plants. These plant products are reported to be effective in decreasing the recurrence rate of renal calculi with no side effects.[6]

Pergularia daemia is a perennial twining herb, foul-smelling when bruised and with much milky juice, stem hairy. Leaves are thin, broadly ovate, heart-shaped or nearly circular, hairless above, velvety beneath. The whole plant is used as an anthelmintic, antiseptic, antivenin, emmenagogue, emetic expectorant and expectorant. Extract of this plant is taken orally for gastric ulcers, uterine and menstrual complaints. The leaves are useful in leprosy and haemorrhoids. *Pergularia daemia* is having pharmacological & traditional uses like diuretic, antioxidant, anti inflammatory and that is why it can be used in the treatment of urolithiasis. *Pergularia daemia* contains alkaloids and flavanoids like withanolide, which can be used to give anti urolithiatic effect.

MATERIALS AND METHODS

Animals: Healthy adult male albino rats of wistar strain weighing 150-120 Gms 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.

Plant material: The whole plant of *Pergularia daemia* were collected from local areas of Tirupathi, Andhra pradesh, India and authenticated by Prof.Dr. K.Madhava Chetty, Assistant Professor, Department of Botany, Sri Venkateswara University, Tirupathi The whole plant was dried in shade and ground to get a coarse powder.

Preparation of extract: The Pet ether extract of whole plant was prepared by using pet ether, by sohxlate extraction method. The extract was concentrated by simple evaporation at room temperature. A suspension of Tween 80 was prepared for oral administration.

ACUTE TOXICITY STUDIES

Acute oral toxicity study was performed as per OECD – 423 guidelines (acute toxic class method). Three animals are used for each step. The dose level to be used as the starting dose is selected from one of four fixed levels 50, 300, 500, 2000 and 5000 mg/kg body weight. The starting dose level should be that which is most likely to produce mortality in some of the dosed animals. Information suggests that mortality is unlikely at the highest starting dose level (5000 mg/kg body weight). The time interval between treatment groups is determined by the onset, duration, and severity of toxic signs. Treatment of animals at the next dose should be delayed until one is confident of survival of the previously dosed animals. Animals are observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours and daily thereafter, for a total of 14 days. It should be determined by the toxic reactions, time of onset and length of recovery period, and may thus be extended when considered necessary. All observations are systematically recorded with individual records being maintained for each animal. Additional observations will be necessary if the animals continue to display signs of toxicity.

Ethylene glycol induced urolithiasis model: Ethylene glycol induced hyperoxaluria model[7] 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[8]. Group IV & V received *PEEPD* of (100mg/kg & 200mg/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 [9] and oxalatecontent.[7]

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 Tukey multiple comparison test. P values < 0.05 were considered significant.

RESULTS AND DISCUSSION

Preparation of extracts: The preliminary extracts of *Pergularia dameia* whole plantusing water, chloroform, benzene, ethanol, methanol, pet ether and ethyl acetate were prepared by using cold maceration technique. The dried and purified extracts were weighed and stored in air tight container. The percentage yields of various extracts were calculated as 1.5, 2, 1.5, 2, 1, 2.5, and 1.8% respectively. Based on the preliminary phytochemical studies, pet ether was selected as solvent for extraction using sohxlet extraction process.

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 pet ether extract when compared to the other extracts [Table 1] and hence, pet ether extract was selected for further pharmacological screening.

Acute toxicity studies: The purifiedand completely dried yield of *PEEPD* was subjected for the acute toxicity study to determine the therapeutic dose using albino mice in controlled environment.Acute toxicity studies were performed according to the OECD 423 guidelines.The extract was administered through oral

route to different groups of mice using oral feeding needle (22gauge). No deviation from normal behavioural pattern was observed. But only few animals showed mild behavioural changes like dyspnoea and mild writhings in higher dose. Observation was done continuously for 13 days and mortality was not observed in any of the drug treated group, hence it was conformed that the test drug *PEEPD* is practically non toxic in normal mice and fall under the category of class V drug, according to[10] $1/10^{th}$ of dose was considered as therapeutic dose and to identify the dose dependent action the 50% of therapeutic dose was considered as minimum dose for further pharmacological evaluation in animal model.

Antiurolithiatic studies: In the present study, chronic administration of 0.75% (v/v) ethylene glycol aqueous solution to male Wistar rats resulted in hyperoxaluria. As mentioned in table 1 the urinary excretion ofoxalate, calcium and phosphate are significantly increased in calculi-induced rats (2.10±0.08, 8.15±0.33 and 7.2±0.06 mg/dl) when compared with normal control (saline) rats (0.34±0.02, 2.916±0.17 and 3.64±0.04 mg/dl). When standard drug (Cystone 750mg/kg. p.o.) administered to calculi-induced rats the excretion levels ofoxalate, calcium and phosphate were significantly decreased to 1±0.05, 3.916±0.25 and 3.81±0.09mg/dl. The test drug, *PEEPD* was used in three different concentrations 100 and 200mg/kg on calculi induced rats to determine the efficacy of the test drug.

It was observed that the excretion levels ofabove mentioned parameters were significantly (P<0.01) decreased in test drug treated groups. It was also observed that all the three concentrations of*PEEPD*decreased excretion levels ofoxalate and calcium significantly than the standard drug whereas excretion levels of phosphate were significantly less at lower doseof 100 and 200mg/kg of*PEEPD.* The results of higher dose test drug (1000mg/kg) treated group is almost equal to standard drug treated group.

As mentioned in table 2 the deposition of the crystalline components in the renal tissues, namely oxalate, calcium and phosphate were increased in calculi induced rats (1.650±0.06, 5.216±0.14, 3.74±0.10mg/dl) as compared to normal control (saline) rats. The deposition levels ofoxalate, calcium and phosphate were significantly decreased (0.500±0.05, 3.633±0.15, 2.52±0.07mg/dl) in Standard (Cystone-treated) group rats. However, supplementation with *PEEPD* (100mg/kg) significantly lowered the elevated levels ofoxalate (P<0.05), calcium and phosphate (P<0.001) as compared to standard (Cystone-treated) group rats. oxalate, calcium and phosphate (P<0.001) as compared to calculi induced rats. [Table 2, Group IV].*PEEPD* (100, 200mg/kg) non-significantly lowered the elevated levels of oxalate, calcium and phosphate (P>0.05) as compared to standard (Cystone- treated) group rats. Significantly lowered the elevated levels of oxalate, calcium and phosphate (P<0.001) as compared to calculi induced rats. [Table 2, Group V and VI].

The data presented in table 3 indicates the serum uric acid and blood urea nitrogen (BUN) remarkably increased in calculi-induced rats (7.866±0.25, 25.098±0.24), while serum creatinine is slightly elevated in calculi-induced rats (0.855±0.01). When standard drug (Cystone 750mg/kg) was used in calculi-induced rats the deposition levels of Uric acid, BUN and Creatinine were significantly decreased (5.033±0.08, 21.398±0.39, 0.981±0.006 mg/dl.) indicating marked renal damage. However, *PEEPD* (100mg/kg) treatment significantly lowered the elevated levels of BUN, Creatinine and Uric acid (P<0.001) as compared to Standard (Cystone-treated) and calculi induced group rats. [Table 3, Group IV].

PEEPD (200mg/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 *PEEPD* (200mg/kg) treatment significantly minimised the BUN, Creatinine (P<0.001) and Uric acid (P<0.01) [Table 3, Group V].

Marked decrease in the levels ofBUN, Creatinine (P<0.001) and Uric acid (P<0.01) were observed in *PEEPD* (100mg/kg) treated group as compared to Standarddrug (Cystone)treated group rats.When compared to calculi induced group rats, in *PEEPD* (100mg/kg) treated group rats elevated levels of BUN (P<0.05), Creatinine (P<0.001) and Uric acid (P>0.05) were significantly lowered. [Table 4, Group VI].

Table 1: Phytochemical Analysis of root extracts of *Ichnocarpus frutescens*

Extract	Alkaloids	Glycosides	Saponins	Carbohydrates	Tannins	Flavonoids	Sterodis	Triterpenoids	Lignins	Proteins	Aminoacids
Aqueous	+	+	+	+	-	+	+	+	-	-	-
Chloroform	+	-	-	-	+	+	+	+	-	-	-
Benzene	+	-	-	+	+	-	-	+	-	-	-
Pet.ether	+	+	+	+	-	+	+	+	-	+	-
Ethyl acetate	+	+	-	-	+	+	+	+	-	-	-
Ethanol	+	-	-	+	+	-	+	+	-	-	-
Methanol	+	+	+	+	-	-	+	+	-	-	-

Table 2: Estimation of Urinary Electrolytes of Normal and Urolithiatic Rats.

S.No	Group & Drug Treatment	Estimation of Urinary Electrolytes		
		Oxalate(mg/dl)	Calcium(mg/dl)	Phosphate(mg/dl)
1	Normal control (Saline)	0.34±0.02	2.916±0.170	3.64±0.04
2	Calculi induced(0.75% EG)	$2.10\pm0.08^{\dagger}$	$8.150\pm0.33^{\dagger}$	$7.29\pm0.06^{\dagger}$
3	Standard (Cystone 750 mg/kg)	1.00 ± 0.05^{x}	3.916 ± 0.250^{x}	3.81 ± 0.09^{x}
4	T_1 (*PEEPD* 100 mg/kg)	$0.616\pm0.06^{a,***}$	$2.966\pm0.128^{c,***}$	$4.24\pm0.10^{b,***}$
5	T_2 (*PEEPD* 200 mg/kg)	$0.350\pm0.04^{a,***}$	$2.983\pm0.185^{c,***}$	$4.14\pm0.09^{C,***}$

All values are expressed as mean ±S.E.M for six rats in each group.
Comparisons made between
$^{a}p<0.001$,$^{b}p<0.01$,$^{c}p<0.05$; T_1,T_2V$_s$Standard.
$^{***}p<0.001$,$^{**}p<0.01$,$^{*}p<0.05$; T_1,T_2V$_s$Calculi induced.
$^{\dagger}p<0.001$,$^{\alpha}p<0.01$,@p<0.05; Calculi induced V$_s$ normal control.
$^{x}p<0.001$,$^{y}p<0.01$,^{z}p,<0.05; Calculi induced V$_s$Standard., One-way ANOVA followed by Tukey test.

Table 3: Estimation of Kidney Homogenate Electrolytes of Normal and Urolithiatic Rats.

S.No	Group & Drug Treatment	Estimation of Kidney Homogenate Parameters		
		Oxalate(mg/dl)	Calcium(mg/dl)	Phosphate(mg/dl)
1	Normal control (Saline)	0.191±0.02	4.783±0.38	2.35±0.03
2	Calculi induced(0.75% EG)	$1.650\pm0.06^{\dagger}$	$5.216\pm0.14^{\dagger}$	$3.74\pm0.10^{\dagger}$
3	Standard (Cystone 750 mg/kg)	0.500 ± 0.05^{x}	3.633 ± 0.15^{x}	2.52 ± 0.07^{x}
4	T_1 (*PEEPD* 100 mg/kg)	$0.233\pm0.03^{b,***}$	$2.588\pm0.23^{c,***}$	$2.08\pm0.10^{a,***}$
5	T_2 (*PEEPD* 200 mg/kg)	$0.483\pm0.04^{***}$	$3.033\pm0.12^{***}$	$2.97\pm0.07^{***}$

All values are expressed as mean ±S.E.M for six rats in each group.
Comparisons made between
$^{a}p<0.001$,$^{b}p<0.01$,$^{c}p<0.05$; T_1,T_2V$_s$Standard.
$^{***}p<0.001$,$^{**}p<0.01$,$^{*}p<0.05$; T_1,T_2V$_s$ Calculi induced.
$^{\dagger}p<0.001$,$^{\alpha}p<0.01$,@p<0.05; Calculi induced V$_s$ normal control.
$^{x}p<0.001$,$^{y}p<0.01$,^{z}p,<0.05; Calculi induced V$_s$ Standard., One-way ANOVA followed by Tukey test.

Table 4: 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)	43.89±2.53	0.593±0.05	2.478±0.12
2	Calculi induced(0.75% EG)	53.69±4.36†	0.71±0.02†	3.411±0.23†
3	Standard (Cystone 750 mg/kg)	41.79±0.59^{x}	0.499±0.03^{x}	2.398±0.11^{x}
4	T_1 (*PEEPD* 100 mg/kg)	37.72±2.17^{a,***}	0.50±0.02^{a,***}	2.047±0.08^{a,***}
5	T_2 (*PEEPD* 200 mg/kg)	37.45±1.22^{a,***}	0.533±0.02^{a,***}	1.607±0.26^{a,*}

All values are expressed as mean ±S.E.M for six rats in each group.
Comparisons made between
$^{a}p<0.001$,$^{b}p<0.01$,$^{c}p<0.05$; T_1,T_2V_sStandard.
$^{***}p<0.001$,$^{**}p<0.01$,$^{*}p<0.05$; T_1,T_2V_s Calculi induced.
$^{\dagger}p<0.001$,$^{\alpha}p<0.01$,@$p<0.05$; Calculi induced V_s normal control.
$^{x}p<0.001$,$^{y}p<0.01$,^{z}p,<0.05; Calculi induced V_sStandard, One-way ANOVA followed by Tukey test.

CONCLUSION

In the present investigation *PEEPD* was used as an antiurolithiatic agent. The phytochemical investigation revealed the presence of of alkaloids, carbohydrates, glycosides, saponins, tannins, proteins, amino acids, phenolic compounds, flavonoids, triterpenoids and phytosterols. From the acute toxicity study, it was confirmed that the test drug *PEEPD* is practically nontoxic on oral administration. Rats have been a suitable species for study of anti-urolithiatic activity because of its urinary system resembling closely that of the human.[11]

Hyperoxaluria is measured by determining urinary oxalate, and crystal deposition in the kidney is confirmed by examining paraffin sections of kidney.[12] In the current investigation, Ethylene glycol (0.75% p.o. for 28 days) induced hyperoxaluria model was used for rapid screening. Experimental induction of hyperoxaluria resulted in rapid formation of calculi oxalate crystals in renal tubules of experimental animals. The major reason for this instantaneous crystal formation after ethylene glycol challenge is the rapid increase in urinary excretion of oxalate. After administration of ethylene glycol, the crystalline deposition first reaches in the cortex, then in medulla and then renal tubules.[12] Results of oxalate administration in this study confirm the histological findings of a relationship between the amounts of calcium oxalate deposition. Ethylene glycol administration in rats decrease in urine volume and pH shows the super saturation of urine and developed significant calcium oxalate crystal deposition in the kidneys. The severity of microscopic kidney crystal deposition correlated well with calculi oxalate concentration in kidney.[13]

The petroleum ether extract of *Pergularia daemia* and cystone showed significant decrease in calcium oxalate concentration in kidney. Oxalate determination in the kidney sections (histology) confirmed the relationship between the amount of calcium oxalate crystal deposition and the ethylene glycol administration. The appearance of calcium oxalate crystal in renal tubules after ethylene glycol is associated with necrosis of tubular cells, which results in exposure of tubular basal lamina and formation of luminal cellular debris. The calcium oxalate crystals do cause cytolysis of polymorph nuclear leukocytes after phagocytosis. The crystal aggregates may be destructive to renal epithelium because they are large and irregular and mechanically disrupt the epithelium.

In the current investigation, histopathological evaluation showed the maximum prevention of crystal deposition at the dose of 100mg/kg compared to 200 mg/kg, which may be due to the active compounds that are present in petroleum ether extract showing activity at lower dose. The active ingredients at higher dose may become inactive or a less active metabolite is formed, which reduced its activity. The extract treatment significantly ($p < 0.001$), lowered oxalate level in urine and kidney. Extract-treated group showing a significant increase in the creatinine clearance is indicative of improvement in kidney function.

The mechanism of antilithiatic activity of ethyl acetate extract may involve the inhibition of oxalate induced toxic manifestations and free radical production along with enhancement of the body defense system. Drug-treated group showing cytoprotection due to its effect on prevention of deposition or aggregation of calcium oxalate in tubules so the mechanical disruption of epithelium is less or protection against free radicals rearrangements.[14]

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REFERENCE

1. Colella J, Kochis E, Galli B, Munver R. Urolithiasis/nephrolithiasis: what's it all about? Urol Nurs 2005; 25(6): 427-48, 475, 449.
2. Marshall L, Stoller MD. Urinary stone disease. In: Tanagho EA, McAninch JW, editors. Smith's General Urology. 16th Ed. New York: McGrawHill Lange Medical Books; 2004; 256–91.

3. Khan SR. Interactions between stoneforming calcific crystals and macromolecules. Urol Int. 1997; 59: 59–71. [PubMed]
4. Atmani F, Slimani Y, Mimouni M, Hacht B. Prophylaxis of calcium oxalate stones by Herniaria hirsute on experimentally induced nephrolithiasis in rats. BJU Int. 2003; 92: 137–40. [PubMed]
5. Aroujo Viel T, Domingos CD, Da Silvamonteiro AP, Riggo Lima-Landman MT, Lapa AJ, Souccar C. Evaluation of the antiurolithiatic activity of the extract of Costus spiralis Roscoe in rats. Journal of Ethnopharamacology 1999; 66(2): 193-198.
6. Prasad, K., Sujatha, D., Bharathi, K., Herbal drugs in urolithiasis – a review. Phcog. Rev. 2007; 1: 175–179.
7. Atmani F., Slimani Y., Mimouni M., Hacht B., Prophylaxis of calcium oxalate stones by Herniaria hirsuta on experimentally induced nephrolithiasis in rats. British Journal of Urology International, 2003; 92: 137–140.
8. Mitra S.K., Gopumadhavan S., Venkataranganna M.V., Sundaram R., Effect of cystone, a herbal formulation, on glycolic acid-induced urolithiasis. Phytotherapy Research, 1998; 12: 372–374.
9. Fiske C.H., Subbarow Y., The Colorimetric determination of phosphate. Journal of Biological Chemistry, 1925; 66: 375–381.
10. Anupama S, Handa S.S., Hepatoprotective activity of andrographolide from Andrographis paniculata against CCl_4. Indian Journal Of Medical Research, 1990; 92: 276.
11. Anand R, Patnaik GK, Srivatsava S, Kulshreshtha DK, Dhawan DK., Evaluation of Antiurolithiatic activity of Tribulus terrestris. International Journal of Pharmacology, 1994; 32(3): 217-224.
12. Khan SR, Shevock PN, Hackett RL. Acute hyperoxaluria, renal injury and calcium oxalate urolithiasis. Journal of Urology, 1991; 147: 226–230.
13. Fan J, Michael AG, Chandhoke PS., Impact of ammonium chloride administration on a rat ethylene glycol urolithiasis model. Scanning Microscopy, 1999; 13(2–3): 299–306.
14. Malini MM, Lenin M, Varalakshmi P., Protective effect of triterpenes on calcium oxalate crystal-induced peroxidative changes in experimental urolithiasis. Pharmaceutical Research, 2000; 41: 413–418.