ANTIUROLITHIATIC ACTIVITY OF ETHANOLIC EXTRACT OF *TAXILLUS TOMENTOSUS* PLANT ON ETHYLENE GLYCOL AND AMMONIUM CHLORIDE INDUCED UROLITHIASIS IN WISTAR RATS

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ABSTRACT

The present study investigates the potential of anti-urolithiatic activity of ethanolic extract of *Taxillus tomentosus* plant (EETT). Urolithiasis was induced by feeding the 0.75% of Ethylene glycol (EG) and 2% of Ammonium chloride (AC) in drinking water along with the normal feed in male wistar rats and then the anti-urolithiatic activity of EETT was evaluated. Acute toxicity study was conducted by LD₅₀ cutoff dose of 2000mg/kg body weight (BW), indicates that the drug to be much safer and 1/10th (200mg/kg BW) and 1/5th (400mg/kg BW) of LD₅₀ doses were selected for study. Serum and urine samples were analyzed for knowing the concentration of creatinine, calcium, urea, uric acid, oxalates and during the entire study, urolithiatic animals showed significantly high concentrations than normal animals. The EETT (200mg/kg BW and 400mg/kg BW) was showed good response to antiurolithiatic activity when compared to the standard drug cystone (CST).

Keywords: anti-urolithiatic activity, *taxillus tomentosus*, ethylene glyco, ammonium chloride, cystone.

INTRODUCTION

Presently, urolithiasis is the third most common disorder of the urinary tract. It is the presence of calculi in the urinary tract with male-to-female incidence ratio of four times and more among white males than in black males. Occurrence of kidney stones is more with increasing age and among men. In more than 50% of the patients, the disease usually reoccurs within ten years. Of all kidney stones, 85% contain calcium salts as their main crystalline components (calcium oxalate and/or calcium phosphate) (Hess, 2003). In some patients, chances of reoccurrence of stone is more due to the large amount of excretion or defective forms of crystallization inhibitors, which allows the formation of large crystal aggregates as stones precursors. Crystal adhesion to urothelial surfaces might be increased in stone formers (Karadi, *et al.*, 2006). A less common type of stone is, because of infection in the urinary tract, called as struvite

or infection stone. Uric acid stone is a bit less common and cystine stones occur rarely (Rivers, *et al.*, 2000; Mitra, *et al.*, 1998).

Research on etiology and prevention of stone disease in urinary tract had been directed in the direction of abnormal increase in urinary levels of calcium, oxalate and uric acid in formation of stone and reduces urinary citrate levels (Hongshi, *et al.*, 2013). Citrate and magnesium are the main inhibitors of stone formation in the urinary tract and decreased levels or lack of these inhibitors in the urine causes stone formation (Fleisch, 1978). Cystinuria and hyperoxaluria (high concentrations of cystine and oxalate respectively) are two rare and inherited metabolic disorders that cause kidney stones (Vishal and Jack, 2011).

Taxillus tomentosus plant is one of the herbal plants belongs to the family of genus *Taxillus* was found in Maharashtra, Karnataka, Kerala, Tamilnadu, Andhra Pradesh and also in Srilanka. Previous studies reported that this

plant have different pharmacological actions like antidiabetic, neuroprotective, hepatoprotective, cardioprotective (Mohammed, *et al.*, 2015), nootropic, antistress activities (Silpa, Jamal and Jayasekhar, 2014). Now the present study was designed to evaluate the antiurolithiatic potency of EETT against EG and AC induced urolithiasis in male wistar rats as such study using the plant extract was not reported earlier.

MATERIALS AND METHODS

Collection of plant material and authentication

The plant *Taxillus tomentosus* was collected from local areas of Tirumala hills, Tirupati, India and was authenticated by Dr. M. Madhava chetty, Department of botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India (Prof. M. Madhava chetti, Department of botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India (Voucher number: 1616).

Plant extraction

The *Taxillus tomentosus* plants were collected and extraneous matter was removed, shade dried for 2 days. Then the dried material was powdered with a mechanical grinder and passed through sieve no. 40 and stored in an air tight container for further processing. The dried powdered material (250g) was extracted with 95% ethanol using soxhlet apparatus for about 72h, concentrated by distilling the solvent and evaporated to dryness. The dried extract was subjected for experimental studies (Venkateswarlu *et al.*, 2015). The percent yield was found to be 8.12g.

Phytochemical analysis

The EETT was analyzed for the presence of phytochemical constituents like tannins, flavonoids and alkaloids (Khandelwal, 2005).

Drugs and chemicals

Ethylene glycol (EG) (AR Grade) and Ammonium chloride (AC) were procured from Merck Laboratories, Mumbai, India. Cystone syrup (Cystone® syrup 100mL) was purchased from Himalaya™ herbal health care, Bangalore, India.

Experimental animals

The male Wistar rats (of 200-250g) were procured from the central animal house of Sigma Institute of Clinical Research and Administration Pvt. Ltd, Hyderabad. Animals were well housed at a temperature $22\pm3^{\circ}\text{C}$; relative humidity 30-70% and 12h light/dark cycle was followed. All animals were allowed free access to water and standard pelleted laboratory animal diet. Animals were acclimatized to laboratory conditions one week before the experiment to be performed and all the procedures were approved by the Institutional Animal Ethical Committee (Ref. No. 769/2015) which was constituted according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India (Tandon *et al.*, 2000).

Acute oral toxicity study

Acute toxicity studies were performed according to OECD Guideline No. 423 (short term toxicity). Wistar rats (n=3) of either sex were selected by random sampling technique and kept in their cages for at least 5 days prior to the dosing to allow the acclimatization for laboratory conditions. If the mortality was observed in 2/3 or 3/3 of animals, then the dose administered was considered to be as a toxic dose. However, if the mortality was observed only in one animal out of three, then the same dose was repeated again to confirm the toxic effect.

Urolithiasis induction

In 0.75% of EG and 2% of AC were mixed well and dissolved in required amount of distilled water. This solution was prepared daily basis by considering the number and weight of animals and given orally as feed. Body weight of each animal was recorded daily (Touhami *et al.*, 2007).

Assessment of antiurolithiatic activity

EG and AC induced urolithiasis model in wistar rats were divided into five groups containing 6 animals in each and kept in metabolic cages. All the experimental animals had free access to regular diet and drinking water *ad libitum* for 28 days. Normal group animals were had regular diet and drinking

water *ad libitum* for 28 days. Control, standard and treated group animals (TGI & TGII) were supplemented with 0.75% EG and 2% AC in distilled water *ad libitum* for 15 days and incase of standard group animals which were administered with CST (5mL/kg BW) from 15th day onwards to 28th day. TGI and TGII animals were administered with EETT 200mg/kg BW and EETT 400mg/kg BW respectively from 15th onwards to 28th day.

Biochemical parameters

The concentration of creatinine, calcium, uric acid, urea and oxalate was estimated in urine and serum by using diagnostic kits obtained from Robonik Diagnostics Ltd., India.

Urine analysis

Urine samples were collected on 28th day (24h) and a drop of concentrated HCl was added prior to the storage. Urinary creatinine, calcium, uric acid, urea, oxalate contents and urine pH, urine volume were estimated. Urine was centrifuged (Heraeus® Biofuse® pico, Kendro Laboratory Products GmbH, Hanau) to pool the crystals and observed under the light electron microscope at 5X for knowing the size and shape of crystals (Patel *et al.*, 2012).

Serum analysis

Blood was collected from retro orbital plexus under anesthetic condition. The blood was centrifuged at 10,000rpm for 10min to separate the serum and analyzed for creatinine, uric acid, urea and calcium oxalate contents (Parasuraman *et al.*, 2010).

Histopathology

The either kidneys of each animal were collected/removed by making a cut to the abdomen. The extraneous tissue of isolated kidneys were cleaned off and preserved in 10% neutral formalin solution. By using the conventional methods, one of the isolated kidneys was embedded in paraffin and was cut into thin sections of about 5µm. These sections were stained with H&E dye and finally mounted in diphenyl xylene. Then these sections were observed under the microscope for knowing the changes in histopathology of kidney architecture and weight of the each kidney was determined (Hodgkinson, 1970).

Statistical analysis

All the data was expressed as mean (n=6) ± SEM. Statistical significance between different groups was tested by two way ANOVA followed by Dunnett's multiple comparison test using computer based fitting program (GraphPad Prism version 6.04) and statistical significance was set at p<0.05.

RESULTS AND DISCUSSION

Acute toxicity study

Acute toxicity studies were conducted in wistar rats by dividing five groups containing six animals each shows the lethal dose and effective dose of the drug at safe levels for rats. LD₅₀ cut-off doses at 2000mg/kg body weight, indicating that the drug to be much safer and 1/10th (200mg/kg of body weight) and 1/5th (400mg/kg of body weight) of LD50 dose were selected for further studies.

Phytochemical analysis

From the phytochemical analysis studies, it was found that the EETT consists of phytochemicals like flavonoids, alkaloids, tannins and flavonoids were present in high concentration when compared to the alkaloids and tannins (Figure 1).

Urine analysis

Microscopic examination of Urine

The microscopic examination (5x) has revealed that the urine of normal group animals was devoid of any crystals or similar structure (Figure 2(A)). Urine samples from urolithiatic rats (control group) showed abundant, large peritubular crystals of CaOx with characteristic rectangular shape scattered throughout (Figure 2(B)). The standard group animals showed few or almost dissolved small crystals or remnants (Figure 2(C)). On curative treatment, the EETT showed better dissolution of the preformed crystals of CaOx (Figure 2(D&E)). However, small fragments of crystals were seen in both TGs and standard group.

Evaluation of urine, blood and physiological parameters

Effects of CST and EETT on urine and blood parameters of wistar rats (Table I and II). After urolithiasis induction, the parameters like creatinine, calcium, oxalate, urea, uric acid in urine and serum were remarkably increased in

Figure 1. nucleophilic addition of amine in *p*-aminophenol and vanillin, forming imine group in PAPVN structure

Figure 2. Microscopic observations of urine in various treatment groups.

Table I. Effect of EET'T on urine parameters in urolithiatic rats

Groups	Urine parameters				
	Creatinine (mg/dL)	Calcium (mg/dL)	Uric acid (mg/dL)	Urea (mg/dL)	Oxalate (mg/dL)
Normal group	2.09±1.40	6.79±1.13	3.83±1.86	55.78±1.95	8.15±1.19
Control group	5.36±2.07 ^b	18.93±1.45 ^a	7.97±0.90 ^a	71.51±2.13 ^a	16.08±1.40 ^a
Standard group	2.32±0.59*	12.18±1.60***	4.56±0.91**	63.99±1.66***	9.10±1.39***
TGI	2.35±1.06*	14.10±1.98***	2.50±1.46***	68.22±1.21*	10.38±1.57***
TGII	2.87±1.22*	13.15±1.43***	1.99±1.34***	62.75±1.37***	8.37±2.31***

Note: All the values of mean $\pm$ SD; n=6; Significant from control *p<0.05, **p<0.01, ***p<0.001; Significant from normal a=p<0.001 and b=p<0.01.

control group. On treatment with EET'T 400mg/kg BW and EET'T 200mg/kg BW, the elevated levels of these parameters were significantly reduced in TGs. Specifically the dose of EET'T 400mg/kg BW showed significant reduction in concentration of the parameters like calcium, uric acid, urea, oxalate

and EET'T 200mg/kg BW was effective in reducing the creatinine concentration in urine. In case of serum parameters, the dose of EET'T 400mg/kg BW showed significant reduction in the concentrations of calcium, urea, oxalate and the dose of EET'T 200mg/kg BW was effective in reducing the creatinine and uric acid levels.

Figure 3. Histopathology of kidneys in various treatment groups. Animals had regular diet, B) Animals had regular diet + Supplemented with 0.75% EG and 2% AC in distilled water, C) Animals administered with CST (5mL/kg BW), D) TGI Animals administered with EETT 200mg/kg BW, E) TGI Animals administered with 400mg/kg BW.

Table II. Effect of EETT on blood parameters in urolithiatic rats

Groups	Blood parameters				
	Creatinine (mg/dL)	Calcium (mg/dL)	Uric acid (mg/dL)	Urea (mg/dL)	Oxalate (mg/dL)
Normal group	1.0±0.68	3.86±1.61	4.05±1.47	31.15±1.98	2.87±1.02
Control group	3.38±0.72 ^a	10.48±1.82 ^a	7.09±1.53 ^c	54±1.72 ^a	6.3±1.13 ^b
Standard group	1.78±0.81*	4.12±1.63***	3.91±1.04**	44.96±1.73***	3.90±1.34*
TGI	0.55±0.16***	4.90±1.49***	1.80±1.47***	49.53±2.47**	4.09±1.43*
TGII	2.52±0.96	4.04±1.72***	2.00±1.64***	45.25±1.89***	2.83±1.52**

Note: All the values of mean ± SD; n=6; Significant from control *p < 0.05, **p < 0.01, ***p < 0.001; Significant from normal a = p < 0.001, b = p < 0.01, c = p < 0.05.

The EETT also affects the physiological parameters like urine volume, urine pH and kidney weight (Table III). There was significant reduction in urine volume observed in control group animals compared to the normal group and TG animals. However, the elevation in urine volume was observed in TG animals but the dose of EETT 400mg/kg BW was considered as effective dose in enhancing the urine volume. The pH of the urine was found to be 6.9, 5.15 and 7.93 in normal, control and standard group animals respectively. TG animals (specifically TGI) were showed the urine pH closed to that of normal group. The kidney weight of normal group was about 0.55g and it was greater in the case of control group animals. Among the TG animals, TGI showed the kidney weight closed to that of normal group.

Histopathology

The histopathological study of the kidney sections was supported the above findings. Histopathological profile of normal group animals showed normal renal peritubular interstitial architecture (Figure 3(A)). Control group animals showed inflammatory mediator infiltration, flattened epithelial cells with vacuolar degeneration, focal collecting duct cell necrosis with increased cast density in the peritubular interstitium (Figure 3(B)). Micrograph section from standard group animals showed renal epithelial cell recovery and reduced cast density indicating the effectiveness of the treatment as evident by increased urine volume (Figure 3(C)). Micrograph of TG animals showing the renal epithelial cell recovery, reduced cast density, improved renal architecture (Figure 3(D & E)) and it is

Table III. Effect of EETT on physiological parameters in urolithiatic rats

Groups	Physiological parameters		
	Urine volume (mL)	Urine pH	Kidney weight (gm)
Normal group	16.4±2.0	6.9±0.45	0.55±0.16
Control group	6.73±0.79 ^a	5.15±3.09 ^b	2.29±0.31 ^a
Standard group	13.58±1.77***	7.93±0.86***	0.65±0.16***
TGI	9.87±2.15	6.30±0.86	0.7±0.51***
TGII	10.96±2.42**	7.81±0.35***	1.03±0.53***

Note: All the values of mean $\pm$ SD; n=6; Significant from control **p < 0.01, ***p < 0.001; Significant from normal a = p < 0.001 and b = p < 0.01.

clearly evident by the biochemical parameters (Table I-III). However, the test EETT was found to be less significant compared to the standard group in producing renal tubular epithelial cell recovery.

Urinary stone formation takes place due to the changes in urinary chemistry, such as hyperoxaluria and hypercalciuria, leading to urinary super saturation, which later crystallized, aggregates and end up in stone formation (Hongshi, *et al.*, 2013; Vishal and Jack, 2011). Male wistar rats were selected due to occurrence of kidney stones is more in males than females and its urinary system resemblance to that of humans (Prasad *et al.*, 1993). Evidences in previous studies indicates that, in response to 15 days administration period of 0.75% v/v EG along with 2% w/v AC induces urinary stones in wistar rats forms renal calculi (Selvam *et al.*, 2001; Atmani *et al.*, 2003). Different components like calcium, urea, uric acid, oxalate and creatinine were increased tremendously in urolithiatic rats which were detected during pathogenesis of renal stones. Increased calcium level in urine is a factor favours the precipitation and nucleation of apatite or CaOx from urine and subsequent crystal growth. The increase in calcium deposition in kidney and its urinary excretion is due to the defective renal tubular reabsorption or an increase in absorption from the intestine as the patients with renal calcium stones were reported to have hyper absorption of calcium. (Hongshi *et al.*, 2013; Vishal and Jack, 2011). Increased urinary calcium excretion along with stress of oxalate seems to provide an environment appropriate for the formation of

stones by forming CaOx crystals, which epitaxially induces CaOx deposition (Patel *et al.*, 2011). Treatment with EETT restores urinary phosphate levels, thereby reducing the risk of stone formation. In urolithiasis, the GFR decreases, due to the obstruction of urine out flow by stone in urinary system. The waste products, predominantly nitrogenous substances such as urea, creatinine and uric acid get accumulated in blood (Patel *et al.*, 2011). In addition, increased lipid peroxidation and decreased level of potential of antioxidant had reported in kidneys of rats supplemented along with a diet which produces calculi (Sumathi *et al.*, 1993; Saravanan *et al.*, 1995). In this milieu, calcium has been reported to induce calculi and to cause renal tissue destruction by reacting with poly unsaturated fatty acids in the cell membrane (Hess, 2003). In the calculi induced rats, the increased serum levels of uric acid and creatinine indicates marked renal damage. However, antioxidant property of phytochemicals present in EETT hastens the procedure of dissolving the stones which were formed before in curative regimen and prevents the formation of new stone in excretory system on prophylactic treatment. Antiurolithiatic activity of many plants has been attributed to their high phenolic contents and particularly, flavonoids contain antiurolithiatic activity, which was evident from previous studies (Vayas *et al.*, 2011; Shashank and Pandey, 2013). EETT consists of flavonoids in high concentration when compared to the alkaloids and tannins. Hence, it indicates that the antiurolithiatic activity of EETT might be due to the presence of flavonoids in extract.

Antirolithiatic activity of EETT was also supported by histopathological observations. The markedly elevated serum levels of creatinine, urea, uric acid, oxalate, calcium in stone forming animals were indicative of prominent necrosis of renal epithelia. In the calculi induced animals, there was damage to the last part of nephron. Elevated levels of oxalate in urine and even its retention in kidneys may one of the causative factors for the peroxidative degeneration of renal epithelia. However the curative and preventive treatment with ethanolic extract of whole plant of *Taxillus tomentosus* prevented the stone formation as evident by various biochemical parameters.

CONCLUSION

It was concluded from the results that the administration of EETT whole plant into the EG and AC induced urolithiatic rats reduced the formation of urinary stones. The mechanisms underlying this effect is still unknown, but apparently related to diuresis and lowering of urinary concentration of stone forming constituents. Hence, the present study has showed the antirolithiatic potential of EETT by the mechanisms to be deciphered in future course of study.

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