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Anti-Urolithiatic Activity of *Indigofera tinctoria* by Ethylene Glycol Induced Model

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ABSTRACT

Aim: The present study was designed to investigate the effects of ethanol extract of root of *Indigofera tinctoria* (EEIT) in ethylene glycol induced urolithiasis model. Albino rats of either sex, weighing about 150-180 gm were used. Anti-urolithiasis activity was done by ethylene glycol administration for first 15 days followed by treatment with extract 200, 400 mg/kg and standard drug, cystone 750 mg/kg for 15 days. On 30th day we collected urine and serum for estimation of urine and serum variables. The extract of EEIT at doses (200 and 400 mg/Kg, p.o.) revealed the Anti-urolithiasis activity while the EEIT 200 mg shows less significant to ethylene glycol control. The anti-parkinsonism activity was confirmed by estimation of Calcium, phosphate, and oxalate from urine sample and BUN, creatinine, uric acid and calcium from serum. The extract EEIT significantly decreased the oxalate, phosphate, uric acid, creatinine, BUN and increased the urine volume and pH. The calcium level in serum significantly increased with treated group. The results of the present work suggested that the EEIT has a potent anti-urolithiatic activity.

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INTRODUCTION

Nephrolithiasis (renal stone formation) is worldwide in distribution and a common disorder estimated to occur in approximately 12% of the population, with a recurrence rate of 70-80% for males and 47-60% females [1]. The majority of stones, up to 80%, are composed mainly of calcium oxalate [2].

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 [3]. The surgical operation, lithotripsy and local calculus disruption using high-power laser are widely used to remove the calculi. However, these procedures are highly costly and with these procedures, recurrence is quite common [4]. The recurrence rate without retentive treatment is approximately 10% at 1 year, 33% at 5 years and 50% at 10 years [5].

Urolithiasis denotes stones originating anywhere in the urinary tract, including the kidneys and bladder. However, the pathophysiologic bases for the formation of kidney and bladder stones are entirely different. Kidney stones form as a result of physicochemical or genetic derangements leading to supersaturation of the urine with stone-forming salts or, less commonly, from recurrent urinary tract infection with urease producing bacteria. Stasis in the upper urinary tract due to local anatomic anomalies may also promote or enhance stone formation in susceptible individuals. In contrast, bladder stones form almost exclusively as a result of urinary stasis and/or recurrent infection due to bladder outlet obstruction or neurogenic bladder. The patient populations at risk for different locations of stones are disparate, with kidney stones occurring most often in otherwise healthy individuals.

The health care providers are learning about the positive and potentially negative effects of using herbal medicines to help treat health conditions. Some health care providers, including doctors and pharmacists, are trained in herbal medicine. They can help people create treatment plans that use herbs, conventional medications, and lifestyle changes to promote health. Many remedies have been employed during ages to treat renal stones. Most of the remedies were taken from plants and proved to be useful, though the rationale behind their use is not will be established

except for a few plants and some proprietary composite herbal drugs and they are reported to be effective with no side effects [6].

In most cases, the management of urolithiasis involves both surgical and medical approaches, i.e., Percutaneous Nephrolithotomy (PCNL), Extracorporeal Shock Wave Lithotripsy (ESWL) and antibiotics [7]. However; these treatments are relatively costly, painful and require expert hands with the availability of appropriate equipment. This has stimulated research on traditional remedies showing anti-urolithiatic activity. These plant products are reported to be effective in decreasing the recurrence rate of renal calculi with no side effects [4]. The plant *Indigofera tinctoria* Linn belongs to the Family Fabaceae and its root has a traditional claim of treatment for urinary stone [8] but there is no documented proof so the present study designed to evaluate its traditional claim.

MATERIALS AND METHOD

Preparation of extracts & Preliminary Phytochemical screening

The roots of *Indigofera tinctoria* were collected in Madanapalle and collected roots of *Indigofera tinctoria* were shade dried completely. The dried root was then coarsely powdered and was sieved (sieve # 60) to get uniform powdered. The 500 g powdered material was loaded in Soxhlet extractor and defatted with n-hexane. The marc was dried and extracted with ethanol in a Soxhlet apparatus. Extracts were concentrated by vacuum drying. The traces of the solvents were removed by keeping the dried extracts into desiccators. The ethanolic extract of the *Indigofera tinctoria* was subjected to a chemical test for identification.[9]

Experimental animal

Swiss albino mice and rats of both sex weighing 20 ± 5 g and 150-180g were used for the present study. The animals were obtained from the Venkateswara Agency, Bangalore. They were housed at room temperature of $23 \pm 1^\circ\text{C}$, relative humidity $55 \pm 5\%$ under 12 hr light/12 hr dark cycles in the animal house. Animals were fed with commercial pellet diet and water *ad libitum* freely throughout the study. The animals were transferred to the laboratory at least 1 hr before the start of the experiment. All animal procedures were performed after approval from the IAEC and in accordance with the recommendations for the proper care and use of laboratory animals.

DOSE DETERMINATION

Acute oral toxicity studies

Acute oral toxicity studies were performed as per OECD-423 guidelines with ethanol extract of roots of *Indigofera tinctoria* using albino mice of either sex, selected by random sampling for acute toxicity study. Animals fasted prior to dosing (e.g. with the rat, food but not water should be withheld overnight with the mouse, food but not water should be withheld for 3-4 hr). Following the period of fasting, the animals were weighed and the test substance was administered. After the substance has been administered, the food was withheld for a further 3-4 hr in mice. Three animals were used for each step. The dose level to be used as the starting dose was selected from one of four fixed levels 5, 50, 300 and 2000 mg/kg body weight. Animals are observed individually after dosing at least once during the first 30 min, periodically during the first 24 hr, with special attention given during the first 4 hr, except where they need to be removed from the study and humanely killed for animal welfare reasons are found dead. If mortality was observed in two out of three animals, then the dose administered was assigned as a toxic dose. If mortality was observed in an animal, then the same dose was repeated again to confirm the toxic dose if mortality was not observed, the procedure was repeated for higher doses [10].

ANTIUROLITHIATIC ACTIVITY [11]

Experimental protocol

Rats were divided randomly into 5 groups (n = 6) and were treated as follows. Animals of group I was untreated and served as normal control. Rats of group II were received 0.75% ethylene glycol in purified drinking water *ad libitum* for 15 days and purified drinking water for the next 15 days. Rats of group III, IV, and V were received 0.75% ethylene glycol in purified drinking water *ad libitum* for 15 days and fed orally with Cystone 750mg/kg, ethanolic extract of 200 and 400 mg/kg for next 15 days respectively. On the 30th day, we collected blood from retro-orbital and urine by the metabolic cage.

Estimation of Serum variables

On 30th day, animals were anesthetized with diethyl ether. Blood was collected from orbital venous plexus in non-heparinized tubes and centrifuged at 2000 rpm for 20 min to obtain serum. Serum levels of calcium, Blood Urea Nitrogen (BUN), uric acid, creatinine were evaluated using Automated Clinical biochemistry Analysis System, Analytical Nova.

Estimation of urine variables

After 30 days, animals were placed in metabolic cages and a urine sample was collected upto 24 hours measured the volume of urine and urine pH by the digital pH meter. The urine samples subjected to analysis of calcium, phosphate, oxalate, uric acid and creatinine were evaluated using Automated Clinical biochemistry Analysis System, Analytical Nova.

Statistical analysis:

Experimental data were expressed as mean±standard error of mean (SEM). Statistical analysis was performed by one-way ANOVA followed by Dunnett's method of multiple comparisons was employed using Graphpad Instat 3.0 software. Data were considered significant at $p < 0.05$.

RESULTS

Preliminary phytochemical screening

Table 1: Phytochemical screening of ethanolic extract of root of *Indigofera tinctoria* L

Extract	Steroids	Alkaloids	Glycosides	Saponin	Flavonoid	Tannin	Carbohydrates
Ethanol	+	+	+	+	+	+	+

The preliminary phytochemical analysis of roots of *Indigofera tinctoria* shows the presence of steroids, alkaloids, flavonoids, glycosides, saponins, tannin and carbohydrate. (Table 1)

Acute oral toxicity

The ethanolic extract had a good margin of safety and did not show any lethal effects on the animals up to the doses of 2000mg/kg. Hence the LD50 of ethanol extract were considered as 2000mg/kg. Studies were carried out with 1/10 of the LD50 as effective dose 200mg/kg and double the dose of ED50 (400mg/kg).

Urine volume and urine pH

Treatment with EEIT 400 mg/kg and cystone groups shows a significant increase in urine volume and pH when compared to ethylene glycol induced urolithiasis group but EEIT 200mg/kg revealed less significant changes comparable to induced control. The Group III and V showed no significant variation in normal control.

Table: Effect of *Indigofera tinctoria* on urine volume and urine pH in ethylene glycol induced urolithiasis on 30th day

Groups	Treatment	Urinary volume in ml	Urinary pH
I	Normal control	23.54±0.92	7.89±1.27
II	Ethylene glycol	12.16±0.43	5.24±1.89
III	Cystone 750mg/kg	22.34±0.21**	7.85±1.28**
IV	EEIT 200mg/kg	18.72±0.58*	7.15±0.95*
V	EEIT 400mg/kg	21.10±0.61**	7.82±1.38**

Significant difference at *P<0.05 & **p<0.01 when compared to ethylene glycol control. Values are Mean ± SEM from 6 animals in each group

Urine variables

Table 2 shows that on 30th day treatment, there was significant (P<0.01) decrease in oxalate and phosphate in Group III & V compared to Group II. Group V shows less significant than standard in Calcium level compared to group II. Group IV (p<0.05) shows fewer urine variables compared to group V (p<0.01). The both cystone (G-III) and EEIT 400mg/kg (G-V) effect were comparable to normal control (G-I).

Table 2: Effect of *Indigofera tinctoria* on urine variables in ethylene glycol induced urolithiasis on 30th day

Groups	Treatment	Calcium mg/dl	Oxalate mg/dl	Phosphate mg/dl
I	Normal control	3.09±0.98	1.36±0.45	6.39±0.25
II	Ethylene glycol	9.52±1.21	4.35±1.10	8.25±0.96
III	Cystone 750mg/kg	3.48±1.25**	1.56±0.64**	6.47±0.47**
IV	EEIT 200mg/kg	6.89±0.74*	2.14±0.96*	7.12±0.41*
V	EEIT 400mg/kg	4.24±0.52*	1.59±0.58**	6.55±0.56**

Significant difference at * $p < 0.05$ & ** $p < 0.01$ when compared to ethylene glycol control. Values are Mean \pm SEM from 6 animals in each group.

Serum variables

The table 3 shows that there is a significant increase in serum BUN, Creatinine and uric acid level from ethylene glycol induced urolithiasis group when compared with normal control. Administration of 400 mg/kg revealed that there is significant ($p < 0.01$) decrease in all serum parameters except calcium when compare to urolithiasis induced group but EEIT 200 shows less significant variation ($p < 0.05$). The calcium level in serum decreased with group II and increased with all another group the Group III and V values non-significant with normal control. But the effect of EEIT 200mg/kg shows less significant than the other treated group when compared to group I. This shows that extract acts in a dose-dependent manner.

Table 3: Effect of *Indigofera tinctoria* serum variables in ethylene glycol induced urolithiasis on 30th day

Groups	Treatment	BUN mg/dl	Creatinine mg/dl	Uric acid mg/dl	Calcium mg/dl
I	Normal control	3.09±0.98	1.36±0.45	6.39±0.25	4.51±0.25
II	Ethylene glycol	9.52±1.21	4.35±1.10	8.25±0.96	1.21±0.59
III	Cystone 750mg/kg	3.98±0.58**	1.52±1.05**	6.74±0.45**	3.98±0.12**
IV	EEIT 200mg/kg	5.89±0.74*	1.61±0.96*	7.12±0.41*	3.29±0.28*
V	EEIT 400mg/kg	4.24±0.52**	1.52±0.58**	6.85±0.56**	3.57±0.23**

Significant difference at *p<0.05 & **p<0.01 when compared to ethylene glycol control. Values are Mean ± SEM from 6 animals in each group.

DISCUSSION

This study showed that the ethanol extract of *Indigofera tinctoria* root had a preventive effect on CaOx calculus formation in the rat kidney. Demonstrated a curative effect of IT on the disruption of CaOx calculi formed in the kidney due to ethylene glycol consumption. Administration of ethylene glycol caused statistically increases in the level of calcium, oxalate, and phosphate in urine and serum calcium level decreased. But increase the nitrogenous waste product in serum was found. The decrease of serum calcium concentration indicates an increase of urinary calcium and calcium oxalate stone formation. This suggestion is in agreement with several studies like Rajagopalet *al.* [12] who reported that the level of serum calcium was decreased and urinary calcium increased in rats treated with ethylene glycol. Moreover, Soundararajan *et al.*[13] showed that calcium oxalate excretion was significantly increased in the urine of ethylene glycol induced urolithic rats. Additionally, they stated that ethylene glycol disturbs oxalate metabolism by way of increase the substrate availability that increases the activity of oxalate synthesizing enzymes in rats. Moreover, several investigations demonstrated that ethylene glycol treatment increased urinary calcium excretion significantly in lithiatic rats [14, 15]. The calcium level maintained with EEIT treated group in serum and decreased in urine calcium indicates that reduce the chance of stone formation. The increase in uric acid excretion was observed in urolithiatic induced group. Increased excretion of uric acid excretion has been reported in stone

formers and hyperoxaluric rats. Uric acid interferes with calcium oxalate solubility it contains protein that binds to calcium oxalate and modulates the crystal nature this is important in role in stone formation[16]. The treatment with EEIT rats decreases the uric acid excretion and reduces the stone formation.

In urolithiasis, GFR is decreased due to obstruction of urine outflow by the formation of stone. Due to this, a waste product, particularly nitrogenous substances like creatinine, uric acid and BUN accumulation in the blood. The result of *Indigofera tinctoria* treated groups decreases the above parameters in serum confirmed the antiurolithiatic activity. The concentration rather than the amount of the crystallizing solutes is what ultimately establishes stone formation, reduced urinary volume will amplify the saturation of all solutes and raise the risk of all stone formation and the strong evidence that urine volume increases with *Indigofera tinctoria* once again support the beneficial effect on decrease the incidence of stone formation in kidney [17].

The basis for calcium stone formation is supersaturation of urine with stone-forming calcium salts. A number of dietary factors and metabolic abnormalities can change the composition or saturation of the urine so as to enhance stone-forming propensity. Among the metabolic conditions are hypercalciuria, hypocitraturia, and hyperoxaluria[18]. However, the role of other factors like inhibitors, infection, matrix formation as well as urinary obstruction should not be ignored [19].

There is evidence that in response to ethylene glycol administration, young male albino rats form renal calculi composed mainly of calcium oxalate. Stone formation in ethylene glycol fed animals is caused by hyperoxaluria, which causes increased excretion of oxalate and its urinary concentration [20]. Therefore, this model was used to evaluate the effect of *Indigofera tinctoriaroot* extract on calcium oxalate urolithiasis. Consistent with some previous reports, stone induction by ethylene glycol caused an increase in oxalate excretion[21] and co-treatment with *Indigofera tinctoriaroot* extract reduced the rate of increase in the oxalate excretion.

The exact mechanisms involved in the effect of *Indigofera tinctoria* on CaOx calculi are not clear; however, the following mechanisms are possible. Firstly, hyperoxaluria is a major risk factor in calcium oxalate stone formation; the ethanolic extract of *Indigofera tinctoria* was able to reduce the urine oxalate in treatment groups on day 30. Thus, it seems that the preventive

effect of *Indigofera tinctoria* extract on CaOx formation can be in part attributed to alteration of urine oxalate concentration. *Indigofera tinctoria* could possibly control the levels of oxalate by inhibiting the synthesis of oxalate.

CONCLUSION

Overall, the results indicate that administration of the ethanolic root extract of *Indigofera tinctoria* to rats with ethylene glycol-induced lithiasis reduced and prevented the formation of urinary stones in a dose-dependent manner. We concluded that the 400 mg/kg dose has an anti-urolithiasis effect. The root *Indigofera tinctoria* is a good and valuable herbal medicine to prevent kidney stone formation. The mechanism and constituents underlying this effect are unknown, so further studies need to isolate and elucidate the possible mechanism.

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