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Antiurolithiatic Activity of Ethyl Acetate Root Extract of *Ichnocarpus frutescens* using Ethylene Glycol Induced Method in Rats.

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Abstract:
Aim: The inhibitory effect of the root of *Ichnocarpus frutescens* on nephrolithiasis induced in rats by feeding with ethylene glycol water (0.75%) for 28 days was summarized. Method: Ethylene glycol feeding resulted in hyperoxaluria as well as increased renal excretion of calcium and phosphate. Results: Supplementation with ethyle acetate extract of *Ichnocarpus frutescens* significantly reduced the elevated urinary oxalate, showing a regulatory action on endogenous oxalate synthesis. The increased deposition of stone forming constituents in the kidneys of calculogenic rats was also significantly lowered by the *EAREIF* treated groups. Conclusion: The result indicates that the root of *Ichnocarpus frutescens* is endowed with antiurolithiatic activity.

Key Words:
Ethylene glycol, Hyperoxaluria, *Ichnocarpus frutescens*, Nephrolithiasis,Urinary stones, Urolithiasis.

INTRODUCTION:

Urinary stone disease is a common disorder estimated to occur in approximately 12% of the population, with a recurrence rate of 70-81% in males, and 47-60% in females. Occurrence of urolithiasis requires formation of nidus, its reaction and growth in the urinary tract which may cause obstruction of the ureter [1].

Ichnocarpus frutescens (Apocynaceae) is a large, evergreen, lactiferous, woody creeper with red appearance, found almost throughout India, ascending up to an altitude of 4000 ft. The root of the plants are used in the medicine as a substitute for Indian Sarsaparilla (*Hemidesmus indicus*) and are often mixed with the latter; neither their therapeutic properties nor their suitability for use as 'Sarsaparila' substitute have been established. *Hemidesmus indicus* is commonly used in various Ayurvedic formulations and local Vaidyas also used it frequently in asthma, fever, inflammatory diseases, headache and snake bite etc [2,3]. The root portion of this plant was much more used in traditional as well as in modern era. It was showed the presence of phenylpropanoids, phenolic acids, coumarines, flavonoids, sterols and pentacyclic triterpenoids. Pharmacological study revealed hepatoprotective, antioxidant, anti-inflammatory, analgesic activity, antidiabetic and antitumor activity [4,5,6,7,8]. There not much more data was found on its leaf, stem and root phytochemical analysis. Therefore *Ichnocarpus frutescens* roots were investigated for its antiurolithiatic activity.

MATERIALS AND METHODS:

Plant material

The fresh roots of *Ichnocarpus frutescens* 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 roots were dried in shade, the bark was separated and the root was ground to get a coarse powder.

Preparation of extract

The Ethyl acetate extract (*EAREIF*) of root was prepared by using ethyl acetate (RFCL Limited), by maceration method for 72hrs at room temperature. The extract was concentrated by simple evaporation at room temperature. A suspension of *EAREIF* in 2% (w/v) Carboxy methyl cellulose (Loba chemie Pvt. Ltd) was prepared for oral administration.

Pharmacological screening for antiurolithiatic activity

Animal selection

For acute toxicity studies, Wistar albino mice of either sex weighing between 25 and 30 g were selected and healthy adult male Wistar rats weighing 150-120 gms were selected for the antiurolithiatic study. The animal obtained from the Animal house, School of pharmacy, Vels University. The animals were acclimatized to standard laboratory condition with temperature 25±2ºC and fed with standard animal pellet feed (Sai meera foods Pvt limited, Bangalore, India) and water *ad libitum*. The protocol was approved by animal ethics committee

constituted for the purpose of animal experimentation as per CPCSEA guidelines. (IAEC. Ref.No: 290/CPCSEA/ 2009-PH/PCOL-05)

Acute toxicity studies

The acute oral toxicity study was carried out as per the guidelines set by Organization for Economic Co-operation and Development-423 (OECD) received from committee for the purpose of control and supervision of experiments on animals (CPCSEA). One-tenth of the median lethal dose (LD_{50}) was taken as effective dose [9].

Ethylene glycol induced urolithiasis model

Ethylene glycol induced hyperoxaluria model [10] was used to assess the antilithiatic activity in albino rats. Animals divided into seven groups containing six animals in each group. Group I serves as control and received regular rat food and drinking water *ad libitum.* Ethylene glycol (0.75%) in drinking water was fed to groups II-VII for induction of renal calculi till 28th day. Group III and IV received standard antiurolithiatic drug, cystone (750mg/kg body weight) from 15th day till 28th day [11] Group V received *EAREIF* of (250mg/kg body weight), Group VI received *EAREIF* of (500mg/kg body weight), and Group VII received *EAREIF* of (1000mg/kg body weight) from 15th day till 28th day. 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 15th day and 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 analysed for calcium, phosphate [12] and oxalate [10]content.

Serum analysis

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

Preparation of extracts

Extracts of *Ichnocarpus frutescens* root using water, chloroform, benzene, ethanol, methanol 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 yield of various extracts was calculated as 2, 2, 1.5, 2, 1, 1.8% 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, tannins, proteins, amino acids, phenolic compounds, flavonoids, triterpenoids and phytosterols. The results of qualitative phytochemical studies indicate that the maximum numbers of chemical constituents were present in the ethyl acetate extract when compared to the other extracts and hence, ethyl acetate extract was selected for further pharmacological screening.

Acute toxicity studies

The purified and completely dried yield of *EAREIF* 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 *EAREIF* is practically non toxic in normal mice and fall under the category of class V drug, according to [9]. $1/10^{th}$ of dose was considered as therapeutic dose and to identify the dose dependent action the 50% and 200% of therapeutic dose was considered as minimum and maximum 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 of oxalate, 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 of oxalate, calcium and phosphate were significantly decreased to 1±0.05, 3.916±0.25 and 3.81±0.09mg/dl. The test drug, *EAREIF* was used in three different concentrations 250, 500 and 1000mg/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 all the three concentrations of *EAREIF* decreased excretion levels of oxalate and calcium significantly than the standard drug whereas excretion levels of phosphate were significantly less at lower dose of 250 and 500mg/kg of *EAREIF*. 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 of oxalate, 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 *EAREIF* (250mg/kg) significantly lowered the elevated levels of oxalate ($P<0.05$), 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]. *EAREIF* (500, 1000mg/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, *EAREIF* (250mg/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].

EAREIF (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 *EAREIF* (500mg/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 of BUN, Creatinine (P<0.001) and Uric acid (P<0.01) were observed in *EAREIF* (1000mg/kg) treated group as compared to Standard drug (Cystone) treated group rats. When compared to calculi induced group rats, in *EAREIF* (1000mg/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 3, Group VI].

Table 1: Estimation of Urinary Electrolytes of Normal and Urolithiatic Rats.

Group & Drug Treatment	***Estimation of Urinary Electrolytes***		
	Oxalate(mg/dl)	***Calcium(mg/dl)***	***Phosphate(mg/dl)***
Normal control (Saline)	0.34±0.02	2.916±0.170	3.64±0.04
Calculi induced(0.75% EG)	2.10±0.08†	8.150±0.33†	7.29±0.06†
Standard (Cystone 750 mg/kg)	1.00±0.05^{x}	3.916±0.250^{x}	3.81±0.09^{x}
T_1 (*EAREIF* 250 mg/kg)	0.616±0.06^{a,***}	2.966±0.128^{c,***}	4.24±0.10^{b,***}
T_2 (*EAREIF* 500 mg/kg)	0.350±0.04 ^{a,***}	2.983±0.185^{c,***}	4.14±0.09^{C,***}
T_3 (*EAREIF* 1000 mg/kg)	0.450±0.04 ^{a,***}	3.00±0.146***	4.01±0.06***

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_2,T_3V$_s$ Standard.
***p<0.001,**p<0.01,*p<0.05 ; T_1,T_2,T_3V$_s$ Calculi induced.
†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 2: Estimation of Kidney Homogenate Electrolytes of Normal And Urolithiatic Rats.

Group &Drug Treatment	***Estimation of Kidney Homogenate Parameters***		
	Oxalate(mg/dl)	***Calcium(mg/dl)***	***Phosphate(mg/dl)***
Normal (Saline)	0.191±0.02	4.783±0.38	2.35±0.03
Positive control (0.75% EG)	1.650±0.06†	5.216±0.14†	3.74±0.10†
Standard (Cystone 750 mg/kg)	0.500±0.05^{x}	3.633±0.15^{x}	2.52±0.07^{x}
T_1 (*EAREIF* 250 mg/kg)	0.233±0.03^{b,***}	2.588±0.23^{c,***}	2.08±0.10^{a,***}
T_2 (*EAREIF* 500 mg/kg)	0.483±0.04 ***	3.033±0.12***	2.97±0.07***
T_3 (*EAREIF* 1000 mg/kg)	0.616±0.04 ***	3.566±0.09***	2.69±0.08***

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_2,T_3V$_s$ Standard.
***p<0.001,**p<0.01,*p<0.05 ; T_1,T_2,T_3V$_s$ Calculi induced.
†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; Calcsuli induced V$_s$ Standard., One-way ANOVA followed by Tukey test.

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

Group & Drug Treatment	***Estimation of Serum Parameters***		
	BUN(mg/dl)	***Creatinine(mg/dl)***	***Uric acid(mg/dl)***
Normal (Saline)	16.369±0.30	0.721±0.01	5.80±0.12
Positive control (0.75% EG)	25.098±0.24†	0.855±0.01†	7.866±0.25†
Standard (Cystone 750 mg/kg)	21.398±0.39^{x}	0.981±0.006^{x}	5.033±0.08^{x}
T_1 (*EAREIF* 250 mg/kg)	33.431±0.73 ^{a,***}	1.146±0.01 ^{a,***}	6.533±0.14^{a,***}
T_2 (*EAREIF* 500 mg/kg)	28.328±0.60 ^{a,***}	1.058±0.01 ^{a,***}	7.233±0.08^{a,*}
T_3 (*EAREIF* 1000 mg/kg)	27.588±0.71 ^{a,**}	1.038±0.005^{c,***}	8.116±0.10^{a}

^{a}p<0.001,^{b}p<0.01,^{c}p,<0.05; T_1,T_2,T_3V$_s$ Standard.
***p<0.001,**p<0.01,*p<0.05 ; T_1,T_2,T_3V$_s$ Calculi induced.
†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; Calcsuli induced V$_s$ Standard., One-way ANOVA followed by Tukey test.

HISTOPATHOLOGY

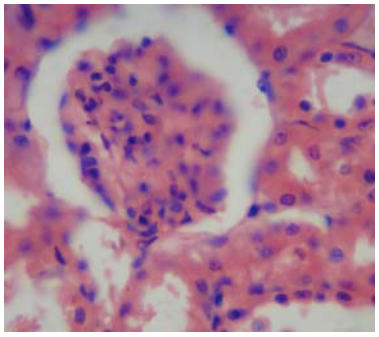


Fig:1. T.S. OF KIDNEY OF NORMAL RATS

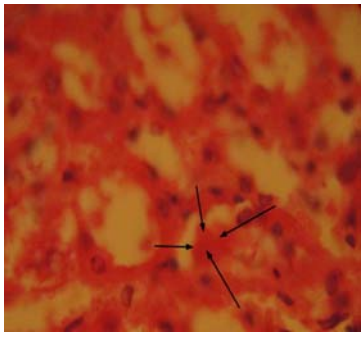


Fig:2. T.S. OF KIDNEY OF TREATED WITH ETHYLENE GLYCOL ALONE

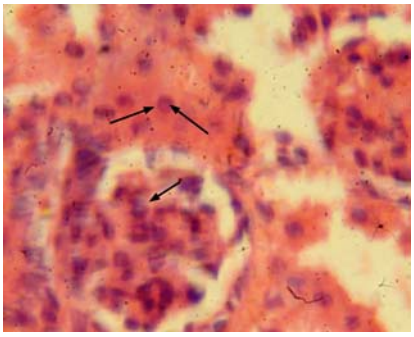


Fig:3. T.S. OF RAT KIDNEY TREATED WITH STANDARD CYSTONE DRUG (750mg/kg)

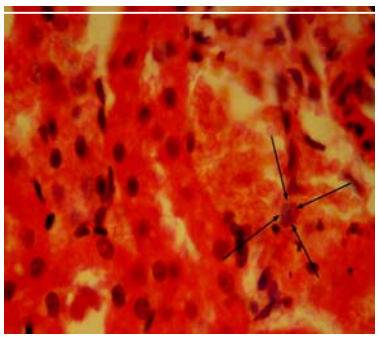


Fig:4. T. S. OF RAT KIDNEY TREATED WITH *EAREIF*(250mg/kg)

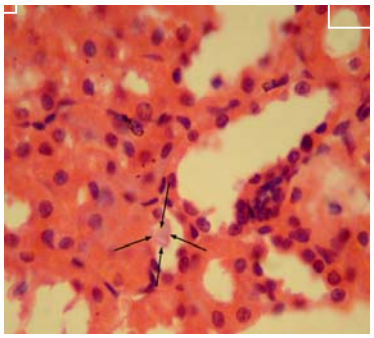


Fig:5. T.S. OF RAT KIDNEY TREATED WITH *EAREIF*(500mg/kg)

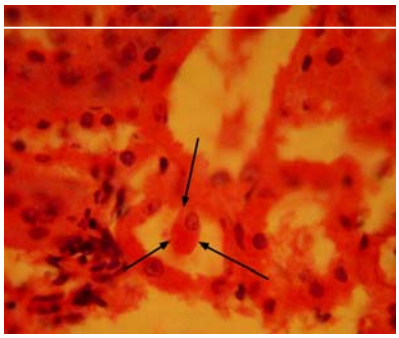


Fig:6. T.S. OF RAT KIDNEY TREATED WITH *EAREIF* (1000mg/kg)

Histopathology Report (100x)

Fig-1-Normal : Few glomerulic shows shrinkage tubules but normal 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 shows mild shrinkage in tubules and shows 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.

Fig-6-Test-III (1000mg/kg) **:** Shows renal tissue with tubular epithelial damage. RBC with in the tubules.

DISCUSSION

Preliminary phytochemical studies revealed the presence of alkaloids, flavonoids, tannins, triterpenoids and steroids in *EAREIF*. Previous studies on triterpenoids have been shown to possess antiurolithiatic effect [13] the lowering of oxalate excretion on treatment with the triterpene indicates the synthesis of oxalic acid from the glycollic acid is some how inhibited. The increased calcium excretion in lithiatic animals were reported by [14] in sodium glycollate induced urolithiasis in rats. The supersaturation of oxalate and calcium in urine leads to calcium oxalate crystal deposition eventually. The treatment with triterpenes increased by urine output thus reducing the risk of calcium oxalate supersaturation in the urine [15].

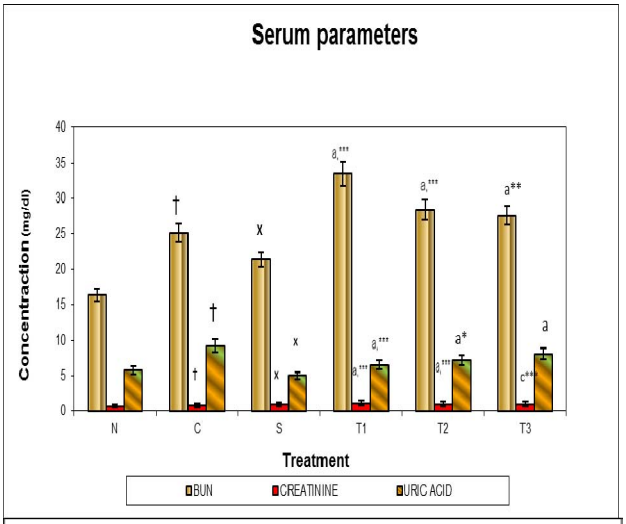
The purified and completely dried yield of *EAREIF* was subjected for the acute toxicity study to determine the therapeutic dose using albino mice in controlled environment. Acute

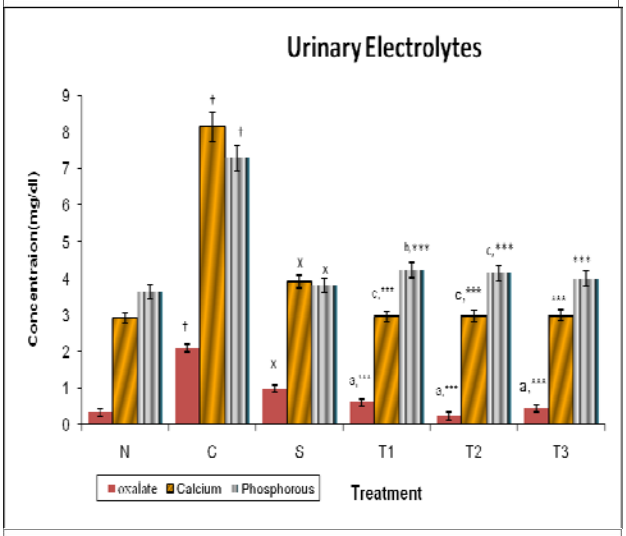
toxicity studies were performed according to the OECD 423 guidelines. The extract was administered through oral route to different groups of mice using oral feeding needle (22gauge). No deviation from normal behavioural pattern was observed. But only few animals showed mild behavioural changes like dyspnoea and mild writhings in higher dose. Observation was done continuously for 13days and mortality was not observed in any of the drug treated group, hence it was confirmed that the test drug *EAREIF* is practically non toxic in normal mice and fall under the category of class V drug, according to [9]. $1/10^{th}$ of dose was considered as therapeutic dose and to identify the dose dependent action the 50% and 200% of therapeutic dose was considered as minimum and maximum dose for further pharmacological evaluation in animal model.

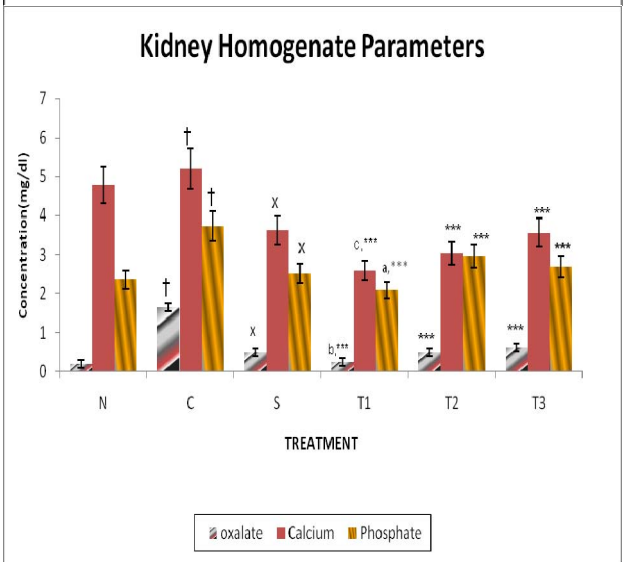
In the present study, male rats are selected to induce urolithiasis because the urinary system of male rats resembles that of humans [16], and earlier studies showed that the amount of stone deposition in female rats was significantly less [17].

Urinary supersaturation with respect to stone-forming constituents is generally considered to be one of the causative factors in calculogenesis. Urinary stone formation takes place due to a change in urinary chemistry such as hypercalciuria and hyperoxaluria, leading to urinary super saturation which later crystallizes, aggregates and ends up in stone formation. Evidences in previous studies indicated that in response to 14days period of ethylene glycol (0.755, v/v) administration, young male albino rats form renal calculi composed mainly of Calcium oxalate [10,18,19]. The biochemical mechanisms for this process are related to an increase in the urinary concentration of oxalate. Stone formation in ethylene glycol fed animals is caused by hyperoxaluria, which causes increased renal retention and excretion of oxalate.

In the present study, oxalate and calcium excretion are progressively increased in calculi-induced animal (GroupII). Since it is accepted that hyperoxaluria is a far more significant risk factor in the pathogenesis of renal stones than hypercalciuria [20], the changes in urinary oxalate levels are relatively much more important than those of calcium.







Increased urinary calcium is a factor favouring the nucleation and precipitation of calcium oxalate or calcium phosphate from urine and subsequent crystal growth [21]. However, *EAREIF* lower the levels of oxalate as well as calcium excretion.

An increase in urinary phosphate is observed in calculi-induced rats (Groups II). Increased urinary phosphate excretion along with oxalate stress seems to provide an environment appropriate for stone formation by forming calcium phosphate crystals, which epitaxially induces calcium oxalate deposition [22]. Treatment of *EAREIF* restores phosphate level, thus reducing the risk of stone formation.

In urolithiasis, the glomerular filtration rate (GRF) decreases due to the obstruction to the outflow of urine by stones in urinary system. Due to this, the waste products, particularly nitrogenous substances such as urea, creatinine and uric acid get accumulated in blood [23]. Also, increased lipid peroxidation and decreased levels of antioxidant potential have been reported in the kidneys of rats supplemented with a calculi-producing diet [24,25]. In this context, oxalate has been reported to induce lipid peroxidation and to cause renal tissue damage by reacting with polyunsaturated fatty acids in cell membrane. In calculi-induced rats (Group II), marked renal damage was seen as indicated by the elevated serum levels of creatinine and uric acid and BUN.

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